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Preface

The constantly changing landscape surrounding modern pharmaceutical science makes it challenging for experts and practitioners to stay informed of the field's most up-to-date research. That is why IGI Global is pleased to offer this two-volume comprehensive reference collection that will empower students, researchers, and academicians with a strong understanding of these critical issues by providing both broad and detailed perspectives on cutting-edge theories and developments. This compilation is designed to act as a single reference source on conceptual, methodological, and technical aspects, as well as to provide insight into emerging trends and future opportunities within the discipline.

Pharmaceutical Sciences: Breakthroughs in Research and Practice is organized into six sections that provide comprehensive coverage of important topics. The sections are:

1. Clinical Trials, Education, and Information Access
2. Drug Delivery and Patient Care
3. Functional Foods
4. Molecular Modeling and Drug Design
5. Nanostructures and Technology
6. Toxicology

The following paragraphs provide a summary of what to expect from this invaluable reference source:

Section 1, "Clinical Trials, Education, and Information Access," opens this extensive reference source by highlighting the pivotal nature of clinical studies and educational initiatives in contemporary medical practice. Through perspectives on software innovations, health policies, and information management, this section demonstrates the importance of research and education in the pharmaceutical industry. The presented research facilitates a better understanding of the continuous developments in learning tools and clinical studies for medical professionals.

Section 2, "Drug Delivery and Patient Care," includes chapters on emerging tools, innovations, and approaches for improved drug delivery techniques. Including discussions on nanoparticles, medical informatics, and personalized medicine, this section presents research on the impact of therapeutic innovations in patient wellness and care. This inclusive information assists in advancing current practices in various therapeutic applications.

Section 3, "Functional Foods," presents extensive coverage on utilizing foods for enhanced nutrition, health, and disease reduction. By identifying components such as protein structure, phytonutrients, and

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health effects, this section highlights the effects of food intake on overall health. These inclusive perspectives contribute to the available knowledge on health promotion through nutraceuticals.

Section 4, "Molecular Modeling and Drug Design," discusses coverage and research perspectives on innovative approaches to drug design and development techniques. Through analyses on molecular docking, computer-aided methodologies, and QSAR studies, this section contains pivotal information on the shifting landscape of modeling and design techniques for pharmaceuticals. The presented research facilitates a comprehensive understanding of various methodologies for drug design and molecular modeling.

Section 5, "Nanostructures and Technology," includes scholarly perspectives on the use of nanomaterials in the medical industry. Highlighting trends in therapeutic techniques, nanomedicine, and nanotechnology, this section presents critical information on the implementation of nanostructures for improved medical care and practice. These innovative perspectives highlight the growing presence of nanomedicine in the pharmaceutical industry.

Section 6, "Toxicology," concludes this reference book with the presentation of research on the dangers and impacts of toxins and poisonous substances on human health. Through perspectives on engineered particles, water contaminants, and nanoparticles, this section illustrates the vital importance of understanding potential toxicity and its dangerous effects. This section provides a comprehensive examination of toxicology in medical contexts.

Although the primary organization of the contents in this work is based on its six sections, offering a progression of coverage of the important concepts, methodologies, technologies, applications, social issues, and emerging trends, the reader can also identify specific contents by utilizing the extensive indexing system listed at the end.

As a comprehensive collection of research on the latest findings related to *Pharmaceutical Sciences: Breakthroughs in Research and Practice,* this publication provides researchers, practitioners, and all audiences with a complete understanding of the development of applications and concepts surrounding these critical issues.

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Section 1

Clinical Trials, Education, and Information Access

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Chapter 1
Overview of Clinical Trial and Pharmacovigilance Process and Areas of Application of Computer System

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ABSTRACT

The overall process of getting a drug to the market is a long one and takes 10-15 years and costing close to a billion dollar. The success rate as the compound travels from the initial discovery phase to clinical and then through to the market is about 1 in 10,000. The two key phases which together contribute the most to the cost and timeline are clinical development and pharmacovigilance. These two phases together also account for the maximum number of failures. In this chapter, we will look in detail at these two phases with a focus on the business process and process areas which have application of computer systems. The chapter will focus on looking at the various phases of clinical development and their endpoints. Clinical development is the process of testing a drug for safety and efficacy in human subjects. Clinical trial is conducted in 3 phases with the 4th phase which is ongoing post approval which forms an important part of the pharmacovigilance process. These phases will be elaborated in detail.

INTRODUCTION AND BACKGROUND

The overall process of getting a drug to the market is a long one and takes 10-15 years and costing close to a billion dollar. The success rate as the compound travels from the initial discovery phase to clinical and then through to the market is about 1 in 10,000. The two key phases which together contribute the most to the cost and timeline are clinical development and pharmacovigilance. These two phases together also account for the maximum number of failures. In this chapter, we will look in detail at these two phases with a focus on the business process and process areas which have application of computer systems.

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Figure 1 illustrates the value chain of bringing a drug to the market starting from understanding of disease to commercialization of the drug for the disease. (R&D Pipeline Management, University of Wisconsin, n.d)

The entire process of drug discovery and development starts *in silico* (in the computer), moves to *in vitro* (in the laboratories) and then finally *in vivo* (inside living beings like animals and humans). This is keeping with the spirit of ensuring safety to all living beings. As the molecule progresses to becoming a drug it graduates from computer to laboratory to living beings.

The initial phase of drug discovery is focused on understanding the disease better and potential drug targets that are relevant for the disease which is largely driven by biology. This part primarily focuses on:

- Understanding the mechanism of diseases;
- Identifying potential targets for therapeutic intervention;
- Evaluating potential drug candidates.

Once the disease is better understood and potential targets identified, the next phase focuses on various aspects of the molecule that can become potential drugs and is largely driven by chemistry. This part addresses the following needs:

- Inventing or identifying safe & effective chemical entities that will become potential drug candidates.
- These drug candidates will act as leads to act on the target disease

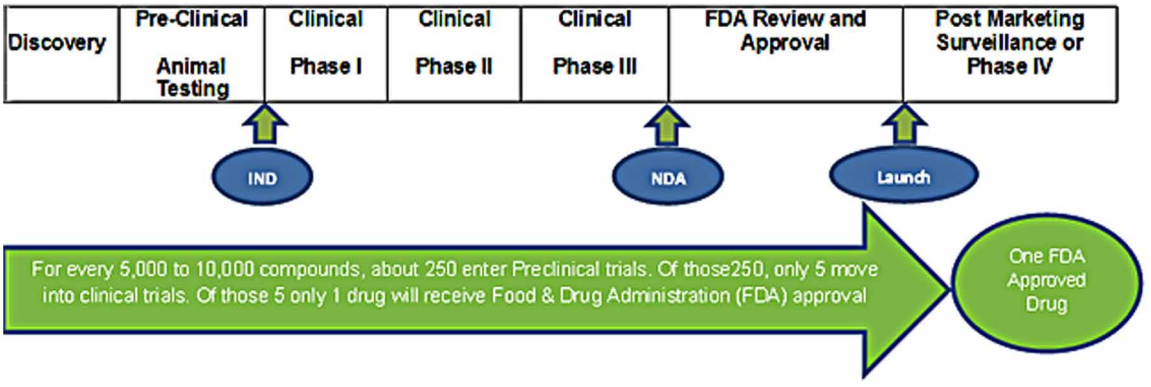
These two initial phases of biology and chemistry are largely *in silico* and *in vitro*.

One of the key qualifying parameter for a molecule to become a drug is its medicinal properties. This science is called medicinal chemistry and this characterizes the molecule before it is used in any living being.

Medicinal chemistry is a scientific discipline involved with designing, synthesizing and developing pharmaceutical drugs.

Medicinal chemistry involves the identification, synthesis and development of new chemical entities suitable for therapeutic use. It also includes the study of existing drugs, their biological properties, and

Figure 1.



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their quantitative structure-activity relationships (QSAR). One of the key set of parameters that defines the molecule is referred to as ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity).

- **Absorption:** Refers to movement of drug into the body from the site of administration. Before a compound can exert a pharmacological effect in tissues, it has to be taken in to the bloodstream — usually via mucous surfaces like the digestive tract (intestinal absorption). Uptake into the target organs or cells needs to be ensured, too.
- **Distribution:** Refers to movement of drug from intravascular to extravascular compartment. The compound needs to be carried to its effector site, most often via the bloodstream. From there, the compound may distribute into tissues and organs, usually to differing extents.
- **Metabolism:** Refers to chemical transformation of drug. Compounds begin to be broken down as soon as they enter the body. The majority of small-molecule drug metabolism is carried out in the liver by redox enzymes, termed cytochrome P450 enzymes.
- **Excretion:** Refers to removal of drug from the body. Compounds and their metabolites need to be removed from the body via excretion, usually through the kidneys (urine) or in the feces. Unless excretion is complete, accumulation of foreign substances can adversely affect normal metabolism.
- **Toxicity:** Adverse effects occurring as a result of repeated daily dosing of a drug compound, or exposure to the drug compound, for part of an organism's lifespan. With experimental animals, the period of exposure may range from a few days to 6 months. (Clinical Pharmacology I, MCCQE Review Notes, 2002)

ADMET is an extremely important set of parameter since about 50% of the drug candidates fail due to unacceptable side effects and toxicities (ADMET). Some of these failures occur much later in the value chain resulting in additional spend that is avoidable. So it is critical that a compound needs to have a favorable ADMET profile for it to become a drug

Due to this very important nature of ADMET life sciences companies have started monitoring drugs for ADMET across the value chain. ADMET is measured all the way from *in silico* to *in vitro* to *in vivo*. The tests and the end points measured may vary. *in silico* ADMET is also popularly called Predictive ADMET (Gombar VK[1], Silver IS, Zhao Z., 2003).

Once the medicinal property and safety of the drug is established the molecule becomes a potential drug and is taken up for testing in animals and this phase is called preclinical.

A preclinical study is done to test promising compounds in animals. Companies conduct extensive toxicological tests and animal studies to determine whether the drug is likely to be safe and effective in humans.

If data from the above preclinical study prove the drug's safety and effectiveness, the pharmaceutical research organization submits all the results and files an Investigational New Drug Application (IND) in Food and Drug Administration (FDA) for US and other relevant regulatory authorities for other countries to test drug candidates in humans.

Primary Objectives of Preclinical Studies

- Support for human pharmacology
- Prediction of human pharmacokinetics

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- Support for human toxicology
- Support in screening new dosage forms and formulations

Once a drug is determined to have a therapeutic use and safety has been tested in animals the drug becomes a candidate for development. Since drug development is so expensive, compound are carefully considered based on their market potential, availability of other treatments, and difficulty of bringing the drug to market. This phase is called clinical trials and is conducted over four phases as described below.

Clinical Phase 1 Trials

- In phase I, researchers test an experimental drug or treatment in a small group of people (20-80) for the first time to evaluate its safety, determine a safe dosage range, and identify side effects.
- Phase 1 studies are designed to determine:
  - Metabolic and pharmacologic actions of the drug in humans,
  - Side effects associated with increasing doses,
  - Early evidence on effectiveness.
- Phase 1 studies evaluate:
  - Drug metabolism,
  - Structure-activity relationships,
  - Mechanism of action in humans.

Clinical Phase 2 Trials

- In phase II trials, the experimental study drug or treatment is given to a larger group of people (100-300) to see if it is effective and to further evaluate its safety.
- Helps to determine the common short-term side effects and risks associated with the drug and to determine optimal dosing (dose and regimen). This is done by subjecting the volunteers/patients through various strengths of dose and arriving at the optimal dose.
- The trials done are typically well controlled and closely monitored.

Clinical Phase 3 Trials

- In phase III trials, the experimental study drug or treatment is given to large groups of people (1,000-3,000) to confirm its effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow the experimental drug or treatment to be used safely.
- Intended to gather the additional information about effectiveness and safety that is needed to evaluate the overall benefit-risk relationship of the drug.
- Helps in establishing that the current drug works better than other drugs currently available.
- Provide an adequate basis for extrapolating the results to the general population and transmitting that information in the physician labeling.

The three phases of clinical trials are followed by submissions and approvals. There are designated organizations in various countries that review and approve drugs in various countries.

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An NDA is filed following the successful testing on humans. A New Drug Application is the submission of documentation of safety and efficacy of the new drug to seek approval for marketing. It is also known as a "Marketing Application". The FDA in US reviews the NDA and approves the product for manufacturing and launch.

Table 1 summarizes the typical activities and success ratio across the 3 phases of the clinical trials

In the last several years with drugs showing toxic side effects post approval, a new phase called Phase 4 studies or Post marketing surveillance has been introduced. Post-marketing surveillance ensures that drugs once launched commercially, the safety of a drug is monitored closely. Both regulatory authorities and life sciences companies share responsibility for post-marketing surveillance. Here are some additional details on this phase.

- Post-marketing surveillance (also known as Phase IV trial) is the practice of monitoring the safety of a pharmaceutical drug or device after it has been launched in the market and forms an important part in retrospective or observational studies. Post-marketing surveillance is a part of Pharmacovigilance, an area that we will cover in subsequent sections. All Post-marketing surveillance studies are phase IV trials; however, the converse is not true.
- Since clinical trials are conducted in a comparatively controlled environment on fewer patient subjects, there is quite a chance to miss out studying the impact of the drug on medical conditions that might prevail in general populations, other than the participating subjects; hence, post-marketing surveillance can further refine, or confirm or deny, the safety of a drug after it is used in the general population by large numbers of people who have a wide variety of medical conditions in a neutralized environment.
- Post-marketing surveillance is a mandate imposed by the FDA for drug safety. Various approaches are used to monitor the safety of licensed drugs such as spontaneous reporting databases, prescription event monitoring, electronic health records, patient registries and record linkage between health databases. These data are reviewed to highlight potential safety concerns in a process known as data mining.
- In certain instances, a licensed drug's labeling might need to get updated, its indication may need to be limited to particular patient groups, or in rare cases, the drug might need to be withdrawn from the market completely.

The entire process of drug discovery and development and the associated phases can be summarized in Table 2. (Michigan Institute for Clinical and Health Research, n.d; Cern Foundation, n.d; ClinicalTrials.gov, n.d)

Table 1.

	Number of subjects (approx.)	**Duration (approx.)**	**Purpose**	**Percentage drugs successfully tested**
Phase I	20 to 80	Up to several months	Primarily safety dose (based on tolerance)	70%
Phase II	100 to 300	Up to 2 years	Effective dosage, short term safety/ side effects	33%
Phase III	1000 to 3,000	Up to 4 years	Efficacy and comparison to commonly used treatments, long term safety	25 – 30%

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Table 2.

Process	Typical Activities
New Entity	1. Molecular modification 2. Organic synthesis 3. Medicinal chemistry
Pre-Clinical Studies	1. Pharmacokinetics 2. Pharmacology/Toxicology 3. ADMET 4. Pre-Formulation/Formulation 5. Manufacturing control
IND	1. Submission 2. FDA review and approval
Clinical Trials	1. Phase 1 2. Phase 2 3. Phase 3 4. Formulation 5. Manufacturing controls
NDA	1. Submission 2. Review, Inspection and Approval
Post Marketing Surveillance	1. Product defect reporting 2. Adverse event reporting

Regulations Involved in Drug Development

The process of clinical trials and pharmacovigilance is highly regulated and governed by FDA in the US, EMEA in Europe and MHRA in the UK and other such authorities in different countries.

Here are some reasons why regulations and regulatory authorities are critical for success.

- Ensuring Good practices during the Laboratory research phase, Clinical phase and the Manufacturing phase.
- Ensuring that the consumer (patients, clinical research subjects) are informed and their consent is obtained.
- Ensuring that the identity of patients / clinical research subjects are protected.
- Ensuring Security and Authenticity of e-data submitted to the FDA (21 CFR Part 11).
- Ensuring that all the Life Sciences processes and applications perform their functions "as intended" (Computer Systems Validation).
- Ensuring Safety, Efficacy and Quality of the end-product.

Relevance of Computer Systems in Clinical Trials

Clinical trials are structured and conducted in a very scientific manner to ensure hypothesis generation and validation using statistical techniques. The clinical trials generate data over a period of time which needs to be stored, processed and submitted in pre-defined formats and these are the areas where computer systems are playing a big enabling role to make the overall process efficient and reliable.

Most common experimental designs implemented in clinical trials across phases are randomized controlled trails (RCT) but there is a new emerging area called adaptive design that is being used fre-

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quently nowadays. Adaptive design is to make the design flexible to account for findings from previous stage of clinical trials. Adaptive design will be explained later in this chapter. The key aspects of RCT are as follows.

- **Randomization:** Subjects are assigned to intervention vs. Control treatment in random process to overcome confounding factors and any imbalance in the assignment.
- **Blinding:** None of the participants or researchers knows about the specific treatment details. This is done to reduce bias and unwarranted special care to any patients as that may manipulate the results
- **Stratification:** Patients are stratified to create balance among the stratifying variables such as gender, age and so on. This is done to achieve balance with respect to stratifying variables and also gain knowledge about sub groups in the patient population.

The various activities within the clinical trials process can be outlined as in Table 3. Each trial has one of more studies as part of the trial process. Each study involves enrolment of sites typically hospitals or clinics and a principal investigator in the site who in turn recruits patients along with his supporting staff within each of these identified sites.

Each of these activities involves set of start and end points that are inter related and come together to ensure a successful trial.

There are several computer systems that support the activities outlined above. Some examples of such systems are as defined below.

- **Electronic Data Capture Systems (EDC):** These systems help capture all the patient information in a structured format so that they can be easily extracted and analyzed at various points during the conduct of the studies. This is set up for every study.
- **Clinical Trial Management Systems (CTMS):** These systems help capture trial related information like sites, investigators and other non-patient related information which are important to the conduct of the trials.
- **Clinical Supplies Management Systems (CSMS):** These systems capture information related to supply of materials used in the conduct of the trials.
- **Clinical Data Repository (CDR):** These systems help bring together information from multiple studies/trials and make them available for analysis to arrive at insights and conclusions that are then used to file for approval with the regulatory authorities.

Table 3.

Study design and planning	Site initiation	Patient enrollment and recruitment	Study conduct	Analysis, reporting and study close out
1. Study planning 2. Protocol development 3. Investigator selection	1. Site selection 2.. Site initiation 3. Contract management	1. Patient screening 2. Patient recruitment 3. Informed consent 4. Patient enrollment for specific studies	1. Subject registration and randomization 2. Data entry in case report forms (CRFs) 3. Data validation and cleansing 4. Query generation and resolution	1. Data analysis, reports for submission 2. Database lock 3. Data retention and archiving

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Usually these systems do not operate in isolation. The systems are integrated and data flows across them thereby enabling easy understanding of the status of trials and insights on the progress of the trial and the ability to meet the expected end point.

Relevance of Computer Systems in Pharmacovigilance

Pharmacovigilance is the science of collecting, monitoring, researching, assessing and evaluating information from life sciences companies, healthcare providers and patients on the adverse effect of medications, biological products, etc. with a view to:

- Identifying new information about hazards associated with medicines
- Preventing harm to patients

Why Do We Need Pharmacovigilance

- Unexpected adverse reactions.
- Drug-Drug interactions.
- Long term efficacy.
- Quantify and recognise risk factors.
- Adverse drug Reactions (ADRs).
- Limitations of pre-marketing data:
    - Pre-Clinical data: insufficient predictive value.
    - Clinical Trial data provide provisional evidence of safety but: Too small (patient number limited), limited duration, do not represent the real world.

The Pharmacovigilance process can be outlined as in the Table 4. The process starts from the receipt of an adverse event (AE) and moves from the intermediate processing steps to determine the nature of the event eventually leading to a time bound submission to the health authorities. (Uppsala, n.d)

The above processes are supported by a set of computer systems that enable proactive monitoring and reporting. Some such systems are explained below.

- **Adverse Events Reporting Systems (AERS):** These systems help capture adverse events and have workflows that enable processing and eventual submission to health authorities
- **Safety Datawarehouse:** These systems bring adverse events information from various different sources and store it in a single location to enable reporting. Various health authorities have different requirements and frequencies for reporting safety adverse events.

One of the key benefits of a safety warehouse is also the ability to run signal detection. Signal detection is a pro-active approach to identifying potential adverse events and correlations.

- **Signal Detection:** Signal detection aims at finding causality between intervention(s) to adverse event(s) that have not been earlier found or refuted. These findings which can be beneficial or

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Table 4.

Process	Activities
Adverse event case receipt	1. Receive AE case 2. Document receipt 3. Index, file source documents
Adverse event case triage	1. Identify duplicate adverse event cases 2. Assign priority 3. Enter other case data into adverse event reporting system (AERS) 4. Perform preliminary quality assessment of data entered
Event assessment	1. Prepare company narrative for review 2. Assess case from medical perspective 3. Perform final review of case for report ability
Processing follow up information	1. Identify additional information required to analyze and report the case 2. Follow up to obtain additional information 3. Update additional information in AERS
Risk/Benefit Analysis	1. Perform risk/benefit analysis based on AERS data and data available with regulatory agencies 2. Prepare analysis reports
Regulatory submission	1. Prepare safety report 2. Facilitate final review by regulatory affairs 3. Submit report to regulatory agency 4. Track submission date of report and follow up, if any

harmful require\deserve immediate attention from the concerned groups. The findings deviate from what is expected from the drug or medical entity. Signal detection is traditionally done by examining the medical evaluation of case reports. But a more comprehensive and practical way of analyzing is through data mining. Data mining allows for a systematic evaluation of the adverse events. Generally a score is assigned based on statistical computation and the higher magnitude of the score reflects stronger association between the adverse event and the drug. Ultimately these scores act as a guideline for providing safety alerts. Based on previous knowledge and understanding of the current data, a threshold is chosen for these scores. Scores exceeding this threshold requires attention. The thresholds for detecting safety signals are a trade-off between sensitivity and specificity. Too stringent threshold results in missing significant signals and thereby effecting sensitivity and too relaxed threshold results in too many false positives impacting the specificity. (Uppsala, n.d)

FUTURE RESEARCH DIRECTIONS

Innovations in Clinical Development

Clinical development is also evolving with technology as new techniques like simulation, predictive modeling, and process automation mature and is available to be used in the design of better trials. The concept of personalized medicine and diagnostics is the new focus as safety events make it evident that one drug does not suit all patients.

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Adaptive Trial Design

An adaptive clinical trial is a clinical trial that evaluates patients' reactions to a drug beginning early in a clinical trial and modifies the trial in accordance with those findings. The adaptation process continues throughout the trial. Modifications may include dosage, sample size, drug undergoing trial, patient selection criteria and "cocktail" mix. In some cases, trials have become an ongoing process that regularly adds and drops therapies and patient groups as more information is gained. The aim is to more quickly identify drugs that have a therapeutic effect and to zero in on patient populations for whom the drug is appropriate. A key modification is to adjust dosing levels. Traditionally, non-adverse patient reactions are not considered until a trial is completed.

The FDA issued draft guidance on adaptive trial design in 2010. In 2012, the President's Council of Advisors on Science and Technology (PCAST) recommended that FDA "run pilot projects to explore adaptive approval mechanisms to generate evidence across the lifecycle of a drug from the premarket through the post market phase." While not specifically related to clinical trials, the Council also recommended that FDA "make full use of accelerated approval for all drugs meeting the statutory standard of addressing an unmet need for a serious or life threatening disease, and demonstrating an impact on a clinical endpoint other than survival or irreversible morbidity, or on a surrogate endpoint, likely to predict clinical benefit."

Adaptive trials have resulted in accelerated trials and are supportive of personalized medicine and sub population strategies explained in the next section below. (FDA, n.d)

Translation Medicine and Personalized Medicine

Translational Medicines: Translating Laboratory Discoveries to New Therapies

Translational research is typically a three step process where studies performed in a lab are translated into treatments and/or therapies that are effectively integrated into medical practice.

The first step of translational research is investigating a topic such as the cause of a medical condition or a specific treatment. Researchers then look for new ways to approach some of medical science's most confounding issues.

Discoveries made in the laboratory are used to prompt clinical trials where research findings are given a practical application. During this phase, study volunteers and participants are exposed to the findings from the lab and data about participants' responses, feelings and reactions is recorded. If a positive outcome is achieved, the third step is to put the finding into practice. Doctors implement the researcher's findings, supported by the results from the clinical trials, and patients receive a new form of treatment or therapy.

This three step circular process is often described as taking a "bench to bedside" approach where researchers take what they learned at the research bench and apply it to patients at their bedside.

The process being circular starts at different points and applies the techniques to find effective drugs. (Clinical and Translational Science Institute, University of California Los Angeles n.d)

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Personalized Medicine

Personalized medicine refers to tailoring the drugs based on the genetic profile of the patients. The focus is on how patients with different genetic variations respond to the therapeutic treatment and thereby design the drugs accordingly. The advent of genomics technologies like next generation sequencing technologies eases and facilitates personalized medicine. Most of the clinical trials fail at later stages of trials such as Phase III. The main reason is the inappropriate selection of targets. This can remedy pharmacogenomics which is a major tool for personalized medicine. This ensures whether variations in molecular makeup of patients attributes to inefficacy and it can be proved that the intervention can be beneficial to patient populations as determined by the pharmacogenomics.

Centers conducting clinical trials should use real world clinical data obtained from medical institutions, hospitals and academic centers and use them as basis in clinical trial design as most of the clinical trials are controlled designs gathering information from real world data does help. Genomes or exomes of subjects participating in clinical trials needs to be sequenced and subjected to therapeutic intervention to undermine risk benefits of drugs based on genetic profiles. Personalized medicine also helps determine optimal does for the patients. (FDA, n.d)

Simulations and Human Disease Models

Computer simulation of clinical trials has evolved over the past two decades from a simple instructive approach to complete simulation models yielding pharmacologically sound, realistic trial outcomes. The need to make drug development more efficient and informative and the awareness that many industries make extensive use of simulation in product development have advanced considerably the use of simulation of clinical trials in pharmaceutical product development over the past decade.

The availability of data through various open industry initiatives and the reducing research productivity is driving significant adoption of simulation models and making it an integral part of the drug development process.

The other aspect of simulations is complete disease models. These are human models built with the understanding of biology and replicate the behavior of the disease in the computer human model built using experimental and derived data. These models provide researchers with a tool to both understand the mechanisms driving untreated disease progression and to predict physiological responses to therapeutic interventions, including variability across different patient phenotypes. (FDA, n.d)

Innovations in Pharmacovigilance

Pharmacovigilance is moving from a reactive reporting focus to a proactive model where monitoring of safety events start very early and multiple channels including social media are being effectively leveraged to track and report such incidents.

Increasing Use of Social Media to Capture Safety Data

The healthcare industry uses digital media for a number of purposes, including raising disease and treatment awareness, safety issues of drugs, clinical trial enrolment, recruitment and patient support.

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With people engaging on health issues more and more online, it is essential that companies track this information and derive important insights out of it to have a better vigilance on patient safety

CONCLUSION

Viewpoint: Future of Drug Development

Drug development is a very old process and over the period as the complexity of diseases has increased, most of the known diseases have been addressed, is facing increasing challenges around innovation and more importantly productivity.

There are several late stage failures of drugs and this attrition is causing an increase in the cost of every successful drug that comes to the market. Clearly there is a need to re-look at the approaches being used in drug development and very rapidly bring in changes that allow for more focused way to improve the techniques used and increase innovation.

As explained in the new innovations in this chapter, there are several new techniques and technologies that have emerged over the last decade which raise hopes of a dramatic change in the way drug development is done. Also the move from one drug fits all to a more focused targeted approach also enables bringing new therapies to the market faster.

One other key trend that will continue to evolve is the use of computer systems across the drug discovery and development processes to increase chances of success as the drug moves through the value chain. This is now supported by increasing availability of data which is a key for success of any computer based approach.

In summary computer systems are becoming increasingly important for drug development and their use will increase steadily. We will study more details in the subsequent chapters more details on their usage and applications.

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KEY TERMS AND DEFINITIONS

Clinical Study: A research study using human subjects to evaluate the effect of interventions or exposures on biomedical or health-related outcomes. Two types of clinical studies are interventional studies (or clinical trials) and observational studies.

IND: Investigational New Drug.

NDA: New Drug Application.

Pharmacovigilance (PV): The science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other drug-related problem.

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Chapter 2
Pharmacogenomics Genome Wise Association Clinical Studies

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ABSTRACT

Pharmacogenomics deals with drug responses in individual based on genetic variation in genome. Based on genetic variations, drugs may produce more or less therapeutic effect, and same way in side effects also. Physicians can use information about your genetic makeup to choose those drugs and drug doses to get better therapy. Optimizing drug therapy and rational dose adjustment with respect to genetic makeup will maximize drug efficacy and minimal adverse effects. This broken traditional 'trial and error' method of 'one drug fits all', and 'one dose fits all' which contributing to 25–50% of drug toxicity or treatment failures. This will contribute to improve the ways in which existing drugs are used, genomic research will lead to drug development to produce new drugs that are highly effective without serious side effects. This approach to bring personalized medicine more practice and drug combinations are optimized for each individual' genetic makeup.

INTRODUCTION

Human genetic profiling can be done by microarray expression, human SNP arrays and whole genome variants determination by using next generation sequencing technology. Whole genome wide screening approach enables better understanding about genetic profile, and provides possibility to check efficacy design for any animal experimental design. These technologies are made available to simultaneously monitor many cellular transcripts in parallel allows that result in more complete analysis of complex disease states and prediction of therapeutic response. Applications ranging from expression profiling to SNP genotyping, microarray gene expression in oncology are revolutionized with more clinical support to ensure better analysis of role of genetics in drug response.

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Allelic variation in individual genotype also provides phenotypic relation, like intermediate, ultra-rapid, non-response categories of CYPgenes metabolizer etc. Several genetic events influence a same phenotypic trait, therefore, establishing genotype-to-phenotype relationships can be far from consensual with many enzymatic patterns. Many genes involved in pharmacokinetics process have been described as being highly polymorphic. Genes likes DPD, UGT1A1, TPMT, CDA involved in the pharmacokinetics of 5-FU/capecitabine, irinotecan, 6-mercaptopurine and gemcitabine/cytarabine, respectively. Patients affected by these genetic polymorphisms will experience severe/lethal toxicities upon drug intake, and that pre-therapeutic screening does help to reduce the risk of treatment-related toxicities through adaptive dosing strategies (Evans & Relling, 1999).

Drug adverse effects vary from patient to patient as well as disease to disease. Drug response characteristics are very complex and depends on profound gene-drug interactions. For drugs that have a narrow therapeutic index caused by inactivation of certain polymorphic drug-metabolizing enzyme and expected to have an increased risk of adverse drug reactions. Some drugs require a metabolic activation by polymorphic drug- metabolizing enzyme (for example, codeine) low therapeutic efficacy or treatment failure. For many drugs that have a broad therapeutic window caused by genetic variants which leads to impaired drug metabolism.

Pharmacogenomics researchers have already identified many genes whose variations affect drug responses. They also know where to look for the numerous others they are bound to discover in the future. Pharmacogenomics tests are used to identify the patients are most likely to respond to certain cancer drugs, tests provide tools for physicians to better manage medication selection and side effect amelioration. Pharmacogenomics is also known as companion diagnostics, meaning tests being bundled with drugs. Examples include KRAS test with cetuximab and EGFR test with gefitinib. Beside efficacy, germline pharmacogenetics can help to identify patients likely to undergo severe toxicities when given cytotoxics showing impaired detoxification in relation with genetic polymorphism like canonical 5-FU.

All drugs cannot be personalized, clinical significance in tailored medicine for prodrugs, drugs with a narrow therapeutic index and drugs that target a key molecule or a critical pathway. Drug safety is the first arena in which patients can benefit from pharmacogenetics and pharmacogenomics. Tumor responses to the inhibitors of oncogenic tyrosine kinases are associated with the presence of activating mutations within the genes encoding the target kinases, targeted cancer therapy is thus a promising individualized drug therapy. The U.S. Food and Drug Administration (FDA) recommends genetic testing for certain chemotherapy drugs like mercaptopurine (Purinethol) to patients with acute lymphoblastic leukemia. Some people have a genetic variant that interferes with their ability to process the drug. This processing problem can cause severe side effects and increase risk of infection, unless the standard dose is adjusted according to the patient's genetic makeup.

Using microarray, cellular transcripts expression in disease states and after treatment to identify drug response at molecular level. Microarray is either silicon chip or glass slide contain series of immobilized complementary DNA molecules or oligonucleotide probes. Targeted DNA labelled with a fluorescent, transcripts abundance based on amount of hybridized labelled dye on each microarray. Each gene represented by a probe set with 11-12 pairs of oligos composed of 25 nucleotides. This approach would make it possible to consider the measured intensities as a proxy for actual mRNA concentration. The scanner produce DAT file is the intensity for each pixel on the array and CEL file, which is the summary of probe intensity for every probe on the array. An expression file, which is the Affymetrix Suite compilation of the probes into gene expression values. An intensity of each probes are corrected

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for background noise normalize combined into probeset expression values. Normalization will remove systematic measurement errors, both within and between arrays. These can be seen in the scatterplot matrices as deviations from linearity between arrays. This should improve our power to detect differential expression. Transcriptional profiling has use to identify class of tumors, response to therapy etc. Many studies used in cases like acute leukemias, diffuse large B-cell lymphomas, and breast tumors (Evans & Johnson, 2001; McLeod & Evans, 2001; Phillips,Veenstra,Oren,Lee & Sadee, 2001).

BACKGROUND

DNA variation in human genome occurs at every 100 to 300 bases leads to amino acid change in protein coding and noncoding part of the genome. Some of these variations may not produce any effect in cell but few can cause pathogenic effect and can also change influence in drug response etc. Most of the population showing monogenic (single-gene) or mendelian inherited pharmacological traits are caused by genetic polymorphisms. Phenotype differences caused by homozygosity of an SNP with 1% has a frequency in the population of one in 10000. Plasma concentration versus time curve (AUC) or the mean steady-state concentration of the drug is a very specific marker of drug elimination. Low concentration leads to rapid elimination and a high plasma concentration will slow down elimination. If the elimination of drug depends on one enzyme, then the drug level is a very specific in vivo marker of the enzyme in question.

Pharmacogenetics of drug metabolizing enzymes and particular the cytochrome P450 (CYP) enzymes has been in focus for almost 30 years. Remainder of this chapter deals with in vivo methodologies and strategies in CYP pharmacogenetics. Drug metabolism takes place in liver which transforms drug into active molecule called as biotransformation, metabolism typically inactivates drugs, some drug metabolites are pharmacologically active sometimes even more so than the parent compound. Metabolism processing by oxidation, reduction, hydrolysis, hydration, conjugation, condensation, or isomerization; whatever the process, the goal is to make the drug easier to excrete. The enzymes involved in metabolism are present in many tissues but generally are more concentrated in the liver. Drug metabolism rates vary among patients. The intensity and duration of pharmacological action of most lipophilic drugs are determined by the rate they are metabolized to inactive products. Cytochrome P450 monooxygenase system is the most important pathway in this regard. This depends on phenotype & genotype of the patients. However, in cases where an enzyme is responsible for metabolizing a pro-drug into a drug, enzyme induction can speed up this conversion and increase drug levels, potentially causing toxicity. Some patients metabolize a drug so rapidly that therapeutically effective blood and tissue concentrations are not reached; in others, metabolism may be so slow that usual doses have toxic effects. Individual drug metabolism rates are influenced by genetic factors, coexisting disorders, and drug interactions. For many drugs, metabolism occurs in 2 phases. Phase I reactions involve formation of a new or modified functional group or cleavage, these reactions are non-synthetic. Phase II reactions involve conjugation with an endogenous substance reactions are synthetic. Metabolites formed in synthetic reactions are more polar and thus more readily excreted by the kidneys and the liver than those formed in non-synthetic reactions. The most important enzyme system of phase I metabolism is cytochrome P-450 (CYP450), a microsomal superfamily of isoenzymes that catalyzes the oxidation of many drugs. The electrons are supplied by NADPH–CYP450 reductase, a flavoprotein that transfers electrons from NADPH to CYP450. CYP450 enzymes can be

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induced or inhibited by many drugs and substances resulting in drug interactions in which one drug enhances the toxicity or reduces the therapeutic effect of another drug (Evans & Relling, 1999; Collins, 2010, pp. 2–3).

Genetic variation accounts for some of the variability in the effect of drugs with N-acetyltransferases, individual variation creates a group of people who acetylate slowly and those who acetylate quickly, split roughly 50:50 in the population of Canada. This variation may have dramatic consequences, as the slow acetylators are more prone to dose-dependent toxicity. Cytochrome P450 monooxygenase system enzymes can also vary across individuals, with deficiencies occurring in 1 – 30% of people, depending on their ethnic background. Some drugs metabolized by single CYP but not same for all drugs, if the drug has a low therapeutic index and if clinical dose titration is not feasible. For CYP2D6 the possible candidates include tricyclic antidepressants, some antipsychotics, and some antiarrhythmics and for CYP2C9 the possible candidates could be warfarin and phenytoin. However pheno or genotyping for CYP enzymes has never achieved widespread use in clinical practice because, as explained above, the response is not determined alone by a single enzyme or gene.

Individual differences in drug metabolism and therapeutic response based on genetic makeup, cytochrome P450 (CYP) genes, which encode enzymes that influence the metabolism of the most drugs. Various metabolic phenotypes like normal, intermediate, ultra-rapid, and poor is based upon the allelic variation within the individual genotype. However, several genetic events can influence a same phenotypic trait, and establishing genotype-to-phenotype relationships can thus be far from consensual with many enzymatic patterns. Number of evidence suggests that patients affected by these genetic polymorphisms will experience severe/lethal toxicities upon drug intake, and that pre-therapeutic screening does help to reduce the risk of treatment-related toxicities through adaptive dosing strategies. Depending on the type of CYP variation present, the patient's metabolizer phenotype and the type of drug, therapeutic drug response is often suboptimal. Poor metabolizers are unable to metabolize certain drugs efficiently, resulting in a potentially toxic build-up of an active drug or the lack of conversion of a prodrug into an active metabolite. In contrast, in ultra-rapid metabolizers, an active drug is inactivated quickly, leading to a sub therapeutic response, while a prodrug is quickly metabolized, leading to rapid onset of therapeutic effect. Sometimes drugs inhibit metabolizing enzyme like quinidine inhibit CYP2D6 which leads to poor metabolizer (Belle & Singh, 2008; Ermak, 2013, pp. 2).

The CYP2C19 enzyme contributes to the metabolism of a large number of clinically relevant drugs and drug classes such as antidepressants, proton pump inhibitors (PPIs), and the antiplatelet prodrug clopidogrel. Like other CYP450 genes, inherited genetic variation in CYP2C19 and its variable hepatic expression contributes to interindividual phenotypic variability in CYP2C19-substrate metabolism. The CYP2C19 "poor metabolism" phenotype was initially discovered by studies on impaired mephenytoin metabolism and the major molecular defect responsible for the trait is the CYP2C19*2 (c.681G>A; rs4244285) loss-of-function allele. CYP2C19 genotype has since been shown to affect the metabolism of several drugs and clinical CYP2C19 genetic testing is currently available. CYP2C19 is predominantly expressed in the liver and, to a lesser extent, in the small intestine. Constitutive expression of CYP2C19 is largely mediated by hepatic nuclear factors 4 alpha (HNF4alpha, HNF4A) and 3 gamma, and transcriptional activation is mediated by the drug responsive nuclear receptors CAR, PXR, and GRalpha, suggesting regulation by endogenous hormones and by drugs such as rifampicin. In vitro expression studies have recently shown that the GATA-4 transcription factor also upregulates CYP2C19 transcriptional activity by binding to two predicted GATA-specific promoter elements. Reduced CYP2C19 activity among women using steroid oral contraceptives results from transcriptional down-regulation of CYP2C19 expression

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through binding of ligand-activated estrogen receptor alpha to a specific ERE consensus half-site in the CYP2C19 promoter. Certain selective serotonin reuptake inhibitors have an inhibitory effect on CYP2C19, which may cause drug-drug interactions with co-administered CYP2C19-metabolized drugs. For example, early studies suggested that omeprazole diminished the pharmacodynamic antiplatelet effects of clopidogrel and increased corresponding cardiovascular risks. However, it is currently not clear if identified changes in ex vivo platelet aggregation due to concomitant omeprazole and clopidogrel administration translates into clinically meaningful outcome differences. Although a number of genotyping technologies can be used to interrogate variant CYP2C19 alleles in Clinical Laboratory Improvement Amendments (CLIA)-approved laboratories, two genotyping platforms have been approved by the U.S. Food and Drug Administration (FDA) (Mohd Zaki Salleh, 2013).

CYP2C9 is a phase I drug-metabolizing cytochrome P450 (CYP450) enzyme isoform that plays a major role in the oxidation of both xenobiotic and endogenous compounds. Also identified CYP2C9 as one of several CYP2C genes clustered in four gene group CYP2C8-CYP2C9-CYP2C19-CYP2C18 size of 500kb region on chromosome 10q24. Single nucleotide polymorphism (SNP) identified between the CYP2C8 and CYP2C9 genes, primarily expressed in the liver, and the expression level is reported to be the second highest among CYP isoforms. Only the CYP enzyme CYP3A4 is quantitatively more highly expressed in human liver. It has been estimated that CYP2C9 is responsible for the metabolic clearance of up to 15%-20% of all drugs undergoing Phase I metabolism. CYP2C9 is induced by rifampicin which has been shown consistently to increase the clearance of drugs eliminated by CYP2C9. The clearance of losartan, phenytoin, tolbutamide and S-warfarin is approximately doubled in healthy volunteers or patients treated with rifampicin. CYP2C9 is inhibited by amiodarone, fluconazole, and sulphaphenazole among other drugs. Dangerous drug-drug interaction can arise when an inhibitor such as one of these is added to a therapeutic regime that include drugs with a low therapeutic index, such as S-warfarin, tolbutamine, and phenytoin. For example, there are numerous studies documenting potentiation of the anticoagulant effect of warfarin in patients coadministered with amiodarone. CYP2C9 is the enzyme responsible for the metabolism of the S-isomer of warfarin that is principally responsible for the anticoagulant effect of the drug. The crystal structure of both human CYP2C9 in complex with warfarin and unliganded CYP2C9 showed unanticipated interactions between CYP2C9 and warfarin, revealing a new binding pocket, suggesting that CYP2C9 may simultaneously accommodate multiple ligands during its biologic function. Structural analysis suggested that CYP2C9 may undergo an allosteric change when binding warfarin. The gene coding for the CYP2C9 enzyme is highly polymorphic, including functional variants of major pharmacogenetic importance. Changes in metabolic activity caused by genetic variants in CYP2C9 play a major role in pathogenesis due to ADRs. Patients with low enzyme activity are at risk of ADR, especially for CYP2C9 substrates with a narrow therapeutic window, such as S-warfarin, phenytoin, glipizide, and tolbutamide. A large body of literature investigates two common non-synonymous variants within CYP2C9, leading to poor metabolism phenotypes. Individuals with these variants are at risk of prolonged bleeding time and increased incidence of severe bleeding in warfarin therapy, higher possibility of low blood sugar levels during glipizide and tolbutamide therapy, and more frequent symptoms of overdose in phenytoin therapy (Mohd Zaki Salleh, 2013, pp. 1371; Desta, 2002, pp. 913-58).

The cytochrome P450 2D6 (CYP2D6) is an enzyme of great historical importance for pharmacogenetics and is now thought to be involved in the metabolism of up to 25% of the drugs that are in common use in the clinic. Several years before the gene was cloned, researchers observed that Caucasian subjects responded in a bimodal pattern to certain drugs such as debrisoquine and sparteine, with most patients exhibiting “normal” pharmacokinetics, whereas others seemed to have great difficulty in metabolizing

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debrisoquine or sparteine. Debrisoquine and sparteine became examples of so-called probe drugs, and were used to phenotype patients. This finding led researchers to conclude that there were common polymorphisms in an as yet unidentified metabolic gene that contributed to the variable pharmacokinetics of these drugs. The protein responsible for the altered metabolism was later purified from human liver microsomes. The gene encoding this protein was initially localized to chromosome 22 and cDNA was cloned from human liver cDNA libraries using an antibody against the rat ortholog. The deduced human protein revealed 73% sequence similarity with the rat protein and by use of human-rodent somatic cell hybrids the gene was localized to human chromosome 22, confirming the earlier study. This gene came to be called CYP2D6, and is part of the cytochrome P450 gene family a group of enzymes that is responsible for Phase I metabolism and elimination of numerous endogenous substrates and a diverse array of drugs. Among the drug-metabolizing CYPs, CYP2D6 is the only non-inducible enzyme, which results in a large contribution of genetic variation to the interindividual variation in enzyme activity. CYP2D6 is highly polymorphic, with over 90 known allelic variants (Tranah, 2005).

CYP2D6 became an object of intense research following its identification as the gene responsible for the altered activity observed with debrisoquine and other drugs. It soon became apparent that there were many different polymorphisms in all parts of the world that impacted CYP2D6 activity. There were alleles that led to a complete loss of CYP2D6 activity, which were common in the initially studied Caucasian populations; however, studies in populations of other ethnic origins revealed reduced function and even hyperfunctional CYP2D6 alleles. A system of assigned patients into four categories based on their ability to metabolize CYP2D6 substrates began to emerge. They are, listed in order of highest functioning to lowest: ultrarapid metabolizers (UM), extensive metabolizers (EM), intermediate metabolizers (IM), and poor metabolizers (PM). An individual's highest functioning CYP2D6 allele predicts his/her phenotypic activity. EMs possess at least one fully functional CYP2D6 allele, and are thought of as phenotypically normal. IMs and PMs are not able to metabolize CYP2D6 substrates as well as their EM counterparts, and may be at increased risk for adverse effects resulting from higher plasma levels of the parent drug, or lack of efficacy resulting from an inability to form an active metabolite. UMs, or ultrarapid metabolizers, possess multiple functional copies of a single CYP2D6 gene. The CYP2D6 copy number has been found to be from 2-13. Each functional copy of CYP2D6 that is present increases the rate of metabolism of CYP2D6 substrates significantly. CYP2D6 allele distributions exhibit significant interethnic differences.

Personalized medicine is to achieve maximum therapeutic efficacy with minimum the risk of drug toxicity for an individuals. Inter-individual genetic profile significant to diseases susceptibility, and response to drugs. After the completion of the Human Genome Project, an explosion of genetic susceptibility to complex diseases and genetic variability will cause differences in drug responses between the individuals. Genomics has become an integral part of modern drug development, and a large number of pharmaceutical companies are using this information to identify novel drug targets, identify patient subpopulations that are likely to benefit from the therapy under development, or for other screening purposes (Collins, 2010, pp. 2–3; Crawley, 2007, pp. 821-822).

Genetic Basis of Variable Drug Response

Drug response involves various phenotypic factors, gene drug interactions is clinically important to dose determining factor. Based on the response, it will be inefficacy, efficacy, resistance, and toxicity. The

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drug-metabolizing enzymes, three clinically important isoforms cytochrome P450 (CYP)2D6, CYP2C9, and CYP2C19 responsible for major drug metabolism which possess a large number of functionally significant genetic polymorphisms. Drugs those produce a broad therapeutic window possible for carrying genetic variant or variants may exhibit impaired drug metabolism and disposition,but these may be of limited clinical relevance (Takahashi & Echizen,2003).

MAIN FOCUS OF THE CHAPTER

Affymetrix

The GeneChip arrays are chemically attractive between DNA nucleotides adenine (A), guanine (G), thymine (T) and cytosine (C). In this C bond with G and T bond with A. DNA strand matches a complementary strand of RNA, the two strands are complementary and will stick to each other. Also a single base that doesn't match its partner able to make one single strand from sticking to another single strand. Hybridization is use for base pairing attraction to identify each SNP genotype. Human Genome Project made DNA sequence around any SNP can genotype with sequencing methods. The surface of the Affymetrix arrays look like square checkerboard. The 10K array, which is a piece of glass about the size of a thumbnail, has over 400,000 squares. These squares are called features. The features on Affymetrix arrays are incredibly small about 8 microns across. Probe is a unique DNA strand which fit in this square. One square would hold the ATTCATG probe we built, and another square would hold the ATTTATG probe. But, while each square holds one type of probe, there isn't just one probe in each square, but millions of identical copies of the same. These probes one layer at a time, using the same type of manufacturing technology that is used to build computer semiconductors. Multiple probes are synthesized in parallel.

A probe designed to genotype a SNP for extracted the DNA from our sample, DNA can be taken from any biological sample such as blood or saliva. The DNA ready to be genotyped can analyze the SNPs from the DNA you extracted, need to make millions of copies of each piece of DNA containing a SNP. Copying the sequence containing the SNP allows the DNA to be more easily detected on the array and the SNP to be more easily genotyped. The copied of all the SNPs that will be analyzed on the microarray, chemical process to fragment the DNA chains up into millions of short pieces. Then chemical called biotin are attached to each strand and this will act as a molecular glue for fluorescent molecules that will later be washed over the array. Passing the laser on the array will get fluorescent on molecule where the sample RNA has stuck to the DNA probes on the array.

The prepared DNA sample is washed over the array for 14 to 16 hours. The number of molecules involved in this wash are staggering. There are millions of copies of each DNA probe in every square on the chip, and there are also millions upon millions of pieces of tagged DNA from the sample and match will be made. If the sequence of bases in the sample DNA matches that of a DNA probe, then there will be a perfect match and the sample will stick to the probe. The hybridized RNA is tagged with molecular glue (biotin), it's as if each hybridized square on the array has been coated with sticky glue. The glow in the dark molecules (red ball) stick to the biotin glue, the fluorescent molecules are shaken away and the stain only sticks to those places on the array where DNA has bound. After all of this, researchers shine a laser light on the array, causing the stain to fluoresce (Apidianakis, et al., 2005; Boerma, et al., 2002).

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Illumina

BeadArray technology is the use of 3μM beads contains a large number of a specific oligonucleotide and a 23 bases that randomly assemble in either a fibre optic bundle substrate or a silica slide substrate. This available on two types, 96-sample array matrix format based on fibre optic bundle and the BeadChip format based on the silica slide. The randomly assembled beads are then decoded through a series of sequential hybridization processes. Illumina SNP genotyping includes the GoldenGate, iSelect, Infinium II and Infinium HD assays. The GoldenGate assay is based on allele specific extension and ligation used to interrogate 384 - 1536 SNPs simultaneously with multiplex also. The Infinium II assay, coupled with BeadChips, allows large scale interrogation of variations in the human genome at unlimited levels of loci multiplexing. Whole genome BeadChips covering SNP content ranging from 240,000 to 1 million and iSelect Infinium for custom Genotyping Panels.

Golden Gate

The activated DNA used for binding parametric particles by robust proces, depending upon the multiplex level, this equates to only 160pg of DNA per SNP genotype call. Assay oligonucleotides, hybridization buffer, and paramagnetic particles are then combined with the activated DNA in the hybridization. Three oligonucleotides are use to sequence for each SNP locus. Two oligos are specific to each allele of the SNP site, called the Allele-Specific Oligos(ASOs). A third oligo that hybridizes several bases downstream from the SNP site is the Locus-Specific Oligo (LSO). All three oligonucleotide sequences contain regions of genomic complementarity and universal PCR primer sites;the LSO also contains a unique address sequence that targets a particular bead type. Up to 1,536 SNPs may be interrogated simultaneously in this manner using GoldenGate technology. During the primer hybridization process, the assay oligonucleotides hybridize to the genomic DNA sample bound to paramagnetic particles, then washing performed to reduce noise by removing excess and mis-hybridised oligonucleotides. These joined, fulllength products provide a template for PCR using universal PCR primers P1, P2, and P3.

Universal PCR primers P1 and P2 are Cy3 and Cy5 labeled. After downstream-processing the single-stranded, dye-labeled DNAs are hybridized to their complement bead type through their unique address sequences. Hybridization of the GoldenGate Assay products onto the Array Matrix or BeadChip allows for the separation of the assay products in solution, onto a solid surface for individual SNP genotype readout. After hybridization, the BeadArray Reader is used to analyze fluorescence signal on the Sentrix Array Matrix or BeadChip, which is in turn analyzed using software for automated genotype clustering and calling (Mohd Zaki Salleh, 2013). Overall workflow shown in figure1.

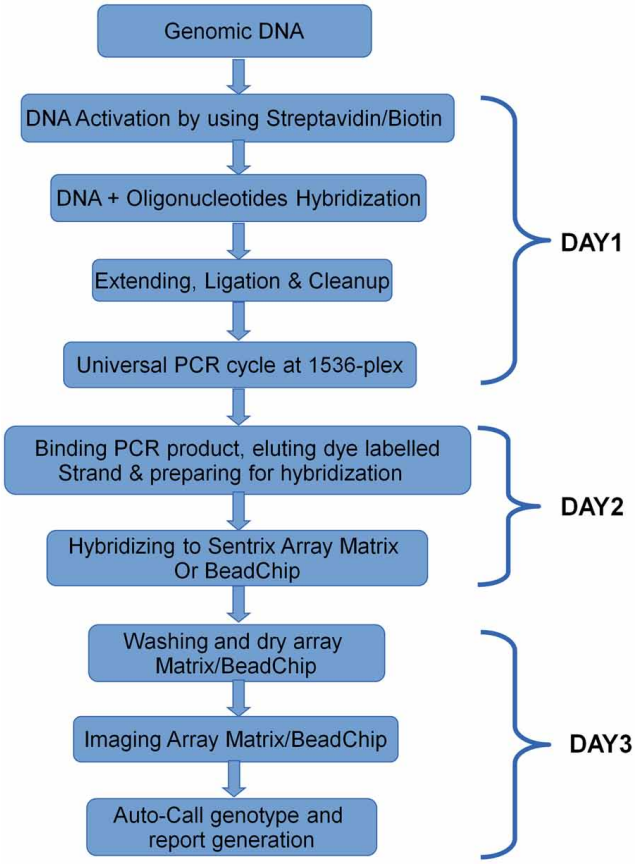
Infinium II Assay Workflow

Genotyping Assay is designed to interrogate a large number of SNPs at unlimited levels of loci multiplexing. Using a single bead type and dual color channel approach, the Infinium II Assay scales genotyping from 10,000 to hundreds of thousands of SNPs per sample. Illumina's optional Laboratory Information Management System (LIMS) and automation ensure positive sample tracking while reducing time required and labor costs.

This amplification has no appreciable allelic partiality. Additionally, a relatively low DNA sample requirement of 750 ng is sufficient to assay over 500,000 SNP loci. The amplified product is then

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Figure 1. Illumina GoldenGate Workflow



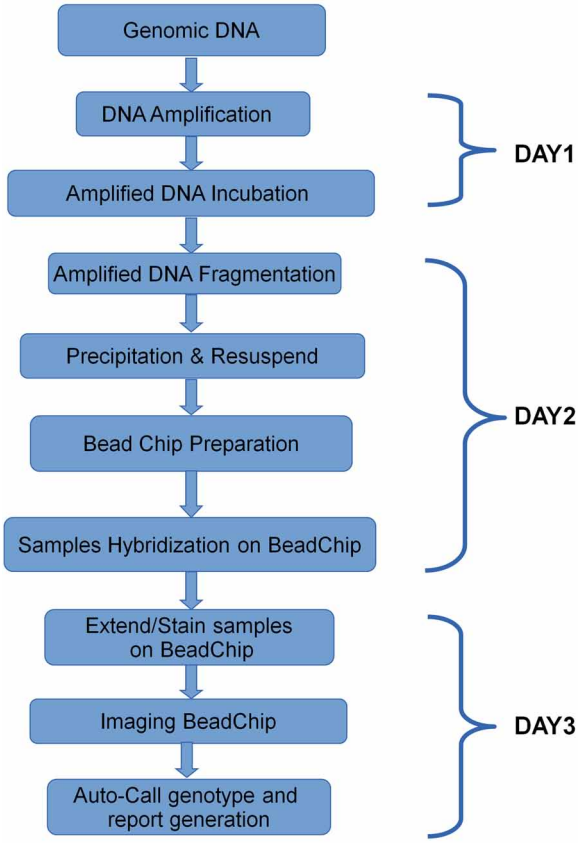
fragmented by a controlled enzymatic process that does not require gel electrophoresis. After alcohol precipitation and resuspension of the DNA, the BeadChip is prepared for hybridization in the capillary flow-through chamber; samples are applied to BeadChips and incubated overnight. The amplified and fragmented DNA samples anneal to locus-specific 50-mers during the hybridization step. One bead type corresponds to each allele per SNP locus. After hybridization, allelic specificity is conferred by enzymatic base extension. Products are subsequently fluorescently stained. The intensities of the beads' fluorescence are detected by the Illumina. BeadArray Reader, and are in turn analyzed using Illumina's software for automated genotype calling. With Illumina's optional Laboratory Information Management System (LIMS) to ensure positive sample tracking, the Infinium II Assay is a robust protocol with a straight forward workflow that can be automated or processed manually (Armstrong, et al., 2002; Mohd Zaki Salleh, 2013). Overall workflow shown in figure2.

Data Processing and Software Tools

Genotyping of specific gene or whole genome by using either Affymetrix or Illumina can be perform to get personal genetic profile of the patient. Individualizing therapy can be provide either through diagnosis of risk factors or markers which present in the patient. Overall process should be genotyping, markers

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Figure 2. Illumina Infinium Workflow



identification and pathogenicity or clinical impacts to predict drug response. Each genotyping platform providing their own genotype call software like genotype console from Affymetrix, Genome Studio from Illumina etc. Apart from those software, some commercial software supporting multiple platforms for data analysis. CLC genomics workbench, Partek genomics suite, DNA STAR, GeneSpring GX from Agilent etc. PharmGKB is pharmacogenetics knowledge base of genomic, phenotype and clinical information curated from pharmacogenetic studies. This data base can use to browse, query, download, submit, edit and process the pharmacogenetic information for registered members and subset is publicly available. Retrieving markers with associated clinical information will provide diagnostic level annotation to use personal medication [23,24].

ISSUES AND SUGGESTED RECOMMENDATIONS

This arrays are high intensive labour requirement for synthesizing, purifying, and storing DNA solutions before microarray fabrication is more expensive. Also during genotyping experiments in the laboratory, sequence homologies between clones representing different closely related members of the same gene family may result in a failure to specifically detect individual genes and instead may hybridize to spot designed gene to happen cross hybridization. In situ oligonucleotide array formats are expensive spe-

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cialized equipments to carry out the hybridization, staining of label, washing, and quantitation process. Ready made in situ oligonucleotide array format are still expensive, although there has been reductions in cost as the market of microarrays has expanded. Short-sequences used on the array confer high specificity, they may have decreased sensitivity/binding compared with glass cDNA microarrays. Such low sensitivity however is compensated for by using multiple probes. Array designs are not flexible, there are occasions when the production of the array, hybridization and detection equipment are restricted to centralized manufacturer facilities, thus limiting the researcher's flexibility. Similarly, the cost and time needed to manufacture the in situ oligonucleotide array format makes it uneconomical for an average laboratory to synthesize its own chips. Multiplexing can reduce per individual cost, integrating data analysis up to secondary level may reduce complexity of the analysis, drug specific markers arrays or disease specific markers may useful to focus more [20,21].

For example anticoagulant drug Warfarin is one of the most commonly using via oral treatment for a range of thrombotic conditions, including deep vein thrombosis, pulmonary embolus and atrial fibrillation. The aim of treatment with Warfarin is to prevent arterial and venous thromboembolism by thinning the blood to a safe and effective level. Ideally patients recieving Warfarin treatment should have stable INR's within a specified and disease specific range: for example, in order to prevent recurrent myocardial infarction patients should have INR's of between 2.5 to 3.5. Despite the longstanding and widespread use of Warfarin, both loading and maintainance dose, for any particular patient, displays wide variability. While the majority of such variation is thought to be due to lifestyle and environmental factors, Warfarin action and metabolism also has a pharmacogenetic component. CYP P450 genes are important in drug response. However, in this case, it is variation in the CYP2C9 gene that is important as well Single Nucleotide Polymorphism variation in the VKORC1 gene. As in the case of thiopurine metabolism, mutations of the CYP2C9 gene can create phenotypes that are extremely sensitive to Warfarin and, hence, are at an increased risk of ADR. Thus, in a similar way to thiopurine treatment, Warfarin pharmacogenetics can be used to improve safety in drug prescription. However, in this case, another gene is also implicated in drug metabolism at the other end of the spectrum: here SNP variation in the VKORC1 gene appears to mediate the efficacy of Warfarin treatment. As noted above Warfarin has a narrow theraputic index. That CYP2C9 variants mediate the safety of Warfarin treatment, and VKORC1 independently mediate its efficacy, are not the only complications for clinicians in making both, inital, and long-term, prescribing decisions. The effect of Warfarin is heavily infulenced by a number of lifestyle and environmental factors, phenotypic factors like age, weight, alcohol consumption, drug reigeme compliance, and diet. These account for around 80% of variation in Warfarin patient INR and, given the serious consequences of both over and under anticoagulation, require that this drug therapy is accompanied by intensive and costly pharmacovigilance regimes [33, 34, 35].

FUTURE DISCUSSION AND CONCLUSION

Based on the Pharmacogenomics research on CYP enzymes states the role of genetic factors for variation in drug response can predict. Genetic profile will help to determine some extent of drug response but a single gene or by a group of genes not enough to get predict fully. With full human genome it is possible for the treatment to the individual patient on the basis of the patient genetic profile. Genotyping as an aid to select the right dose of the right drug in the individual patient would be of theoretical use if the response is mainly determined by a single gene or a limited group of genes, and if all of the

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environmental and constitutional influences have a more limited influence, and besides are known in detail and can be measured in the individual patient. Coming years in the CYP field studying pharmacogenetics or genomics will lead to new important insights and discoveries that will ultimately lead to the development of new and better drugs and to the rational use of drugs that are already on the market. At present, after not reached in patients & physician may be lack of pharmacogenomics knowledge and gap between sequencing & data analysis. In future, integrating knowledge data base with software tools will provide on-spot clinical significance of markers after genotype call.

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KEY TERMS AND DEFINITIONS

Acetylators: An organism capable for metabolic acetylation.

Acute Lymphoblastic Leukemia: Also called acute lymphoblastic leukemia, is a cancer that starts from white blood cells called lymphocytes in the bone marrow (the soft inner part of the bones, where new blood cells are made).

Allele: (short for allelomorph) A variant of a gene were the DNA sequence differs between two or more variants.

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Allelic Variation: The presence or number of different allele forms at a particular locus (locus or loci = place) on a chromosome (allelic variation is sometimes used more loosely to describe the overall diversity present).

Antiarrhythmics: Drugs that are used to treat abnormal heart rhythms resulting from irregular electrical activity of the heart.

Antidepressant: A substance that is used in the treatment of mood disorders, as characterized by various manic or depressive affects.

Antipsychotics: A class of medicines used to treat psychosis and other mental and emotional conditions.

Cellular Transcription: The process in a cell by which genetic material is copied from a strand of DNA to a complementary strand of RNA (called messenger RNA).

Chemotherapy: A category of cancer treatment that uses chemical substances, especially one or more anti-cancer drugs.

Complementary DNA: (c-DNA) DNA synthesized from a messenger RNA (mRNA) template in a reaction catalysed by the enzymes reverse transcriptase.

Conjugation: A process by which one bacterium transfers genetic material to another through direct contact.

Cytotoxics: The quality of being toxic to cells.

Detoxification: The physiological or medicinal removal of toxic substances from a living organism, including, but not limited to, the human body and additionally can refer to the period of withdrawal during which an organism returns to homeostasis after long-term use of an addictive substance.

Electrophoresis: Separations technique that is based on the mobility of ions in an electric field.

Endogenous: Those that originate from within an organism, tissue, or cell.

Genetic Makeup: The genes that determine what you look like and what physical characteristics you have.

Genetic Polymorphism: The occurrence in the same population of two or more alleles at one locus, each with appreciable frequency, where the minimum frequency is typically taken as 1%.

Genetic Profiling: Science of identifying individual genetic characteristics by way of DNA analysis.

Genetic Variation: Variation in alleles of genes, occurs both within and among populations.

Genome: The complete set of genes or genetic material present in a cell or organism.

Genome Variants: Variations of genomes between members of species, or between groups of species thriving in different parts of the world as a result of genetic mutation.

Genotype: Genetic makeup of a cell, an organism, or an individual usually with reference to a specific characteristic under consideration.

Genotyping: Process of determining the genetic constitution – the genotype – of an individual by examining their DNA sequence.

Germline: The cellular lineage of a sexually reproducing organism from which eggs and sperm are derived; also - the genetic material contained in this cellular lineage which can be passed to the next generation.

Homozygosity: A cell is said to be homozygous for a particular gene when identical alleles of the gene are present on both homologous chromosomes. The cell or organism in question is called a homozygote.

In Situ: In the normal location. An in situ tumor is one that is confined to its site of origin and has not invaded neighboring tissue or gone elsewhere in the body.

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International Normalized Ratio (INR): A state of achieving anticoagulation for prothromboin time.

Lipophilic Drugs: Drugs that are able to dissolve much more easily in lipid (a class of oily organic compounds) than in water.

Metabolites: The products of enzyme-catalyzed reactions that occur naturally within cells.

Microarray Expression: Provides a comprehensive portrait of the transcriptional world enabling us to view the organism as a 'system' that is more than the sum of its parts.

Monogenic: Involving or controlled by a single gene.

Next Generation Sequencing: Method for sequencing genomes at high speed, at low cost and with great accuracy.

Oligonucleotide: Short, single-stranded DNA or RNA molecules that have a wide range of applications in genetic testing, research, and forensics.

Oncogenic: Causing development of a tumour or tumours.

PCR: Polymerase chain reaction, or PCR, is a laboratory technique used to make multiple copies of a segment of DNA. PCR is very precise and can be used to amplify, or copy, a specific DNA target from a mixture of DNA molecules.

Pharmacodynamics: The study of what a drug does to the body, whereas pharmacokinetics is the study of what the body does to a drug.

Pharmacogenetics: The branch of pharmacology concerned with the effect of genetic factors on reactions to drugs.

Pharmacokinetics: The branch of pharmacology concerned with the movement of drugs within the body.

Phenotype: The set of observable characteristics of an individual resulting from the interaction of its genotype with the environment.

Phenotypic Trait: An obvious and observable trait; it is the expression of genes in an observable way. An example of a phenotypic trait is hair color, there are underlying genes that control the hair color, which make up the genotype, but the actual hair color, the part we see, is the phenotype.

Polymorphisms: The presence of genetic variation within a population, upon which natural selection can operate.

Probe: A fragment of DNA or RNA of variable length (usually 100-1000 bases long) which is used in DNA or RNA samples to detect the presence of nucleotide sequences (the DNA target) that are complementary to the sequence in the probe.

Scatterplot Matrices: A great way to roughly determine if you have a linear correlation between multiple variables. This is particularly helpful in pinpointing specific variables that might have similar correlations to your genomic or proteomic data.

SNP: A Single Nucleotide Polymorphism (SNP, pronounced snip; plural snips) is a DNA sequence variation occurring commonly within a population (e.g. 1%) in which a single nucleotide — A, T, C or G — in the genome (or other shared sequence) differs between members of a biological species or paired chromosomes.

SNP Arrays: A type of DNA microarray which is used to detect polymorphisms within a population.

Therapeutic Index: A comparison of the amount of a therapeutic agent that causes the therapeutic effect to the amount that causes death (in animal studies) or toxicity (in human studies).

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Thrombosis: Local coagulation or clotting of the blood in a part of the circulatory system.

Thromboembolism: Obstruction of blood vessel by a blood clot that has become dislodged from another site in the circulation.

Tumors: A swelling of a part of the body, generally without inflammation, caused by an abnormal growth of tissue, whether benign or malignant.

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Chapter 3
Normalizing Cross-Border Healthcare in Europe via New E-Prescription Paradigms

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ABSTRACT

The 21st century started with some significant efforts globally in the e-health sector. This was mainly pushed as a generic strategy from many nations and international organizations in order to cope with issues such as ageing population, demographic shift, social security limitations, and financial instability. A second reason was the introduction of new technologies such as cloud computing, Web interoperability standards, mobile health, and social media that are steadily changing the way healthcare has been seen in the last decades. In addition to that, globalization, commuting, immigration, and increased mobility raised the issue of cross-border healthcare and the right to access normalized healthcare services anywhere, anytime. In that context, the authors analyze the technological offerings and result of the epSOS (European Patient Smart Open Services) framework and how it has affected strategic decisions in electronic prescription in Greece, thus creating a new useful e-health national application. They prove that by rethinking healthcare, reusing established standards such as HL7 CDA (Health Level Seven Clinical Document Architecture) and IHE (Integrating the Healthcare Enterprise) profiles, it is possible to propose a new innovative system that is in fact based upon new technological propositions such as REST (Representational State Transfer) architecture and cloud computing.

INTRODUCTION

The healthcare delivery landscape will change significantly in the future to master changes of society, such as constant population ageing and demographic rise (Mirkin, 2001). Those changes have already started and demand that e-health plays a significant role within a reformed healthcare system (European

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Commission, 2012). It is commonly agreed that e-health is a benefit for the patients, the healthcare practitioners, the healthcare provider organizations and the nations as a whole.

Eysenbach states that:

E-health is an emerging field in the intersection of medical informatics, public health and business, referring to health services and information delivered or enhanced through the Internet and related technologies. In a broader sense, the term characterizes not only a technical development, but also a state-of-mind, a way of thinking, an attitude, and a commitment for networked, global thinking, to improve health care locally, regionally, and worldwide by using information and communication technology. (Eysenbach, 2001)

The future of healthcare is to be patient-centric and with a focus on health promotion and health maintenance. Patient centred healthcare implies that patients and their relatives have a more active role in the design and enforcement of new healthcare treatments. So, patient should be the centre of care and all related information and decisions should incorporate each patient's individual options for better treatment. Patients and practitioners should operate in partnership so that medical decisions respect patient's need, beliefs and preferences. Practitioners should focus on making sure that patients have substantial information and support so that they can make appropriate decisions and participate in their own care (Institute of Medicine, 2001). New e-health services with the use of ICT (Information and Communication Technologies) such as Internet, cloud computing, social media are reshaping day by day the healthcare delivery systems (Apostolakis et al, 2012; Gwee, 2011; Cambria et al, 2010).

Appropriate and viable use of interoperable e-Health solutions has already demonstrated its ability to propose a strategy for reshaping the system towards a patient centred system. In other words, e-health seems to have the ability to enable and establish patient centred healthcare systems, where information systems interoperate and exchange patient information seamlessly on behalf of patient's welfare and social rights. However, the more complex and the more fragmented the healthcare provision and the insurance systems are the more complex and fragmented will the e-Health services be. Both positive as negative lessons learned from the past should influence future decisions (Calliope, 2010b).

On the demand side, citizens and patients want to be empowered and are increasingly well informed with the use of social media. Life expectancy steadily rises (Leon, 2011) and so is the prevalence of chronic conditions that are more often diagnosed at early stages. At the moment, the vast majority of national healthcare budgets concerns chronic disease management (Kaiser, 2012). Medical treatments, diagnostics tools and procedures, as well as the extensive use of new technologies in equipment and pharmaceutical industry increase the cost of healthcare. The use of new ICT technologies can and should automate and optimize healthcare delivery and related costs to the extent possible. Patient societies and other stakeholders demand and expect that no geographical borders exist in healthcare delivery, that seamless and adequate continuum of care prevails. Today's citizens are far more mobile for leisure or work than before, and this is well studied and documented (Rosenmöller et al, 2006; Glinos, 2012). Early epidemiological surveillance via warning and alert systems (WHO, 2005), as well as research and continuous professional training will require enhanced secondary use of aggregated health data of individuals to support the effectiveness, efficiency and sustainability of the health systems.

On the supply side, the demographic shift and population ageing has puts increased pressure on healthcare delivery systems (Mirkin, 2001, Bloom, 2011). In addition, the increase of patients' numbers creates a shortage of skilled healthcare labour resources and medical expertise. As a consequence

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healthcare systems and process should be re-invented to deal with those pressures. In addition, the increased complexity of healthcare delivery and the need for increasingly shared information demand new working procedures and workflows.

The World Health Organization (WHO) has issued an important report in 2002 (WHO, 2002), that focused on that year, on the amount of disease, disability and death in the world that is attributed to a specific selection of the most significant risks to human health. The same document also calculates how much of this burden could be avoided in the upcoming decades, if the same risk factors are reduced from now and onwards. One way to reduce risk is to establish monitoring facilities (AHRQ, 2003; NHMRC, 2010). They would then control, benchmark and assess risk factors at a national and a global level. In that sense, Health cannot be restricted within national borders. In the way the planet, with the significant intervention of technology, has moved towards globalized structures, people, goods and services are constantly moving between regions and nations. As health is concerned, it is obvious that cross border healthcare has become an inevitable matter to deal with in all its instances: legal, social, technical, medical. Extensive information exist in the reference section of this chapter (WHO 2002b; WHO 2008b; Salgado, 2010, Samb et al, 2009). Globalization is putting the social cohesion of many countries under stress, and health systems, as key constituents of contemporary societies, are not performing as well as they should (WHO, 2008).

European Union has foreseen the aforementioned needs and open issues and has created policy papers and directives to respond to them (European Commission, 2004 & 2012), especially noting the need for standardization and cross border healthcare regulations in an attempt to provide adequate care for all citizens independently of their location within a unified Europe. As a consequence, the Large Scale Pilot Project on cross border e-Health interoperability, epSOS (European Patient Smart Open Services), and the thematic network of CALLIOPE (CALL for InterOPErability) until 2011 and now the e-Health Governance Initiative are the new drivers for creating, initially, new working paradigms in e-Prescription and Patient Summary use cases across Europe (eHGI, 2012).

EpSOS has created a series of new technical and functional requirements that enable the safe routing of medical information across regional and national healthcare systems, based on the efficient reuse of existing standards and integration profiles. After more than three years of hard work to develop feasible cross-border e-Health services, the efforts of the epSOS project team have now culminated in the large scale pilot entering into operational mode on the 13th of April 2012. In this piloting phase, the developed solutions are tested in a real-life environment during a one-year period (epSOS, 2008).

The technical and functional requirements already have a significant acceptance within EU member states (20 of which participate in epSOS) and not only (Norway, Switzerland and Turkey have Joined epSOS). In that sense, a significant, common ground has been introduced, assessed, disseminated and openly accepted concerning interoperability issues, codification and nomenclature reuse, transcoding, security, data privacy and many more.

EpSOS has introduced concepts such as the National Contact Point (NCP) which is, in fact, an enterprise information bus that securely, seamlessly transport medical information across healthcare systems of any type and complexity. This enables a democratic regulation of health IT market allowing common testing procedures (by reusing profiles and test tools provided by IHE), common interoperability frameworks (by accepting common HL7 CDA r2 type documents for the electronic prescription) and certification procedures (by deploying national certification and accreditation bodies and reusing IHE, EuroRec Institute or HL7 EHR-FS (Electronic Healthcare Record Functional System) standards and methods).

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In this chapter we propose to demonstrate the flexibility and reuse of the epSOS framework at a national and regional level by depicting the Greek e-Prescription case. It will explain how new and innovative e-services were introduced, in order to shape the new paradigm shift in e-Health in the 21st Century. From a methodological point of view, the chapter will follow a top down approach starting from presenting in the Background section; the high level European strategic decisions and mandates will then proceed to a thorough presentation and analysis of the epSOS case and analyze the current situation regarding e-Prescription. The next paragraphs present a detailed analysis of specific adoption of the EU approach at a country level with emphasis on the New Greek e-Prescription case, which introduces a new business paradigm in e-Prescription. In the "future research direction" section, we will propose the relative areas where future research is dominant in relation with the topics of this chapter. Finally, the author with deploy a synthesis of the chapter and state their opinions and beliefs in the conclusion section.

BACKGROUND

The Need for Cross Border Healthcare

WHO in its 2010 report (WHO, 2010) states the need for finding financial tools to achieve universal health coverage for patients and citizens. The literature on universal health coverage mostly focuses on five main themes such as accessibility to healthcare resources, population coverage capacity, point of care geography, rights to healthcare access and population protection from social and economic consequences of the lack of health. In that sense WHO is seeking ways to find ways to make universal health coverage financially sustainable especially in more underserved countries (Stuckler et al, 2010)

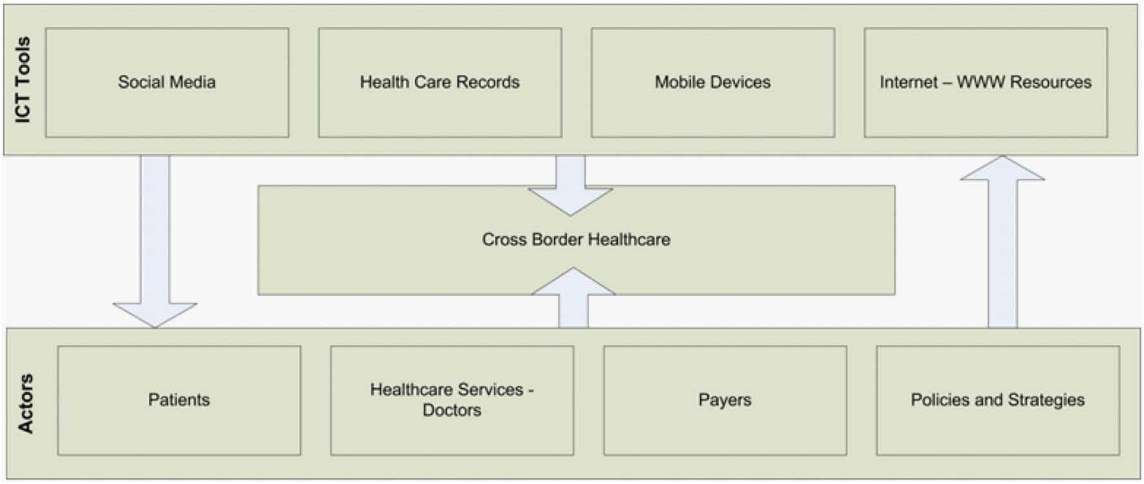
People are increasingly impatient with the inability of health systems to deliver services that meet stated demands and new needs. So many European patients, for example, seek healthcare services on the global scale, either because their country is, unfortunately, unable to serve them, or because they seek high expertise and skills at the point of delivery. In the latter case, information and communication technologies (ICT) have proven again to be a driver for fast information dissemination via the Internet and social media (Biermann et al, 2006; DeVries, 2012; Chou et al, 2009; Eysenbach 2002; Eysenbach, 2008).

As a consequence, Europe for many years know has focused on the subject and tried to find ways to include the need for cross border healthcare and adequate quality of healthcare delivery for all European citizens. Cross-border health care has simply become a more prominent phenomenon in the European Union (E.U.). When in need of medical treatment, patients supported by social media, for example, act as informed consumers who claim the right to choose their own provider beyond their national borders. Cross-border health care is also not restricted to patients. Medical doctors and nurses go abroad for training, to provide services temporarily or to establish them in another Member State. Increasingly, individual doctors and hospitals in different Member States cooperate with each other (Wismar et al, 2011). The above mentioned relations between actors and tools for cross border healthcare are depicted in Figure 1.

In order to deal with the cross border issue Europe has envisaged three alternatives to reduce legal burden. The first alternative focuses on the law of each country. So, when an action or service is performed in a different country that the country of establishment, then the applicable law is the one of the country of establishment. This is a basic principle taken focusing of the free movement of services and goods. The second alternative is the introduction of specific ruling in the framework of coordination

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Figure 1. Cross border healthcare actors and new tools



of social systems, which resulted in the establishment of the council regulation No 1408/71 of 14 June 1971 that focused in building a common European labor market. The third alternative was the most complex and time consuming that ended recently with the adoption of the new "Directive 2011/24/EU of the European parliament and the council of 9 March 2011on the application of patients' rights in cross-border healthcare" (OJEU, 2011). In fact, this was an open discussion and need noted more than a decade ago (Busse & Wismar, 2002).

European Strategy for E-Health

The European Union provides a common framework, policies, and service across all Member States. In many areas, there are common policies, but healthcare remains a national prerogative. There are substantial variations between the respective national healthcare systems, although, many similar issues exist – i.e. ageing population, chronic diseases prevalence, financial pressures. However, the capital right for mobility, both for patients and healthcare workers, has intensified the need for sharing of health records across national boundaries and the use of standards, processes and technologies to support them. In this ambiguous environment, most Member State Governments assume that they have full responsibility and control over their own health services, on the basis of Article 152 of the Amsterdam Treaty, but in fact, it is apparent that the Single European Market (SEM) has a substantial impact on health services (Busse & Wismar, 2002). The SEM relies on a set of principles such as free movement of goods and services and freedom of establishment. Those influence health services since patient often move for business, leisure of better care selection, thus demanding to receive equal care with Europe based upon SEM principles (Wismar et al, 2011).

The "Bolkestein Directive on services in the internal market", introduced in December 2006 (OJEU, 2006), kept the delivery of healthcare out of scope. A number of official rulings of the European Court of Justice (ECJ), regarding the application of the internal market principles to health, clearly state, that internal market regulation have an influence on the delivery of health services. Consequently, a legal document was called for, to settle the ECJ's open issues, concerning patients' rights to get reimbursement

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for healthcare services done in another Member State. This resulted in the directive 2011/24/EU as a response to the raised concerns, in the context of cross-border healthcare, with a focus on e-Prescription.

This Directive states that patients are allowed to receive healthcare in another Member State and get reimbursement up to the costs accepted in the country of origin, as if this treatment had been provided inland. For reasons of general interest, a country may restrain the application of cross-border healthcare rules on reimbursement. In order to monitor incoming flows of patients and to ensure efficient access to healthcare, the country of delivery of care is allowed to adopt measures concerning access to treatment where this is justified. This means that the Directive targets European citizens and protect their rights to equal care within E.U. borders, but it is not effective for other foreigners where other official documents and bilateral agreements are legally binding healthcare processes. Finally, Member States need to establish national contact points in order to provide patients with information concerning their rights and practical aspects of getting cross border healthcare.

In parallel, the EU has also matured in terms of introducing ICT in the healthcare sector in order to deal with issues such as those aforementioned. In the past decades a series of important studies, reports and communications, issued by the European Commission, urge Member States to take immediate action in that domain. Willing not to make extensive description of EU strategic goals and decisions, it is nevertheless important to state at least the most important steps that lead us to today's situation and future promising prospects. Those are duly noted and analyzed in CALLIOPE project documentation (Calliope, 2010a, 2010b) with the focus on e-Health.

The vision for health is for a smart, citizen-centered health system paradigm in which each citizen's needs are at the centre of redesigned health and social care services, but there are a number of challenges to address. Some of those issues have been technically addressed at a regional level (Berler, 2009). Europe's Member States are continually attempting to respond to demand for quality, safety and access. They are also fighting for healthcare systems sustainability in an unfriendly environment led by of demographic changes, ageing population reactions, increasing incidence of chronic diseases, rising demand for personalized healthcare and financial pressures.

The 2010 OECD (Organisation for Economic Co-operation and Development) report (OECD, 2010) provides clear evidence of the benefits that can result from ICT implementation into four domains: increasing quality of care and efficiency, reducing operating costs of clinical services, reducing administrative costs and enabling new care modalities. European Union's current initiatives focus on implementing the Europe 2020 strategy (European Commission, 2010a), while keeping up on the recommendation made in the Communication of the European Commission in 2004 (European Commission, 2004). This chapter is also proposing an analytical listing of further reading in this subject.

In short, "Europe 2020" proposes three priorities: Smart growth, Sustainable growth and Inclusive growth. The Digital Agenda (European Commission, 2010b), as part of the Europe 2020 strategy, places e-Health in a cross-sectoral framework with the following action points:

- **Key Action 13:** Undertake pilot actions to provide Europeans with secure online access to their personal health data by 2015 and to achieve by 2020 extensive deployment of telemedicine and telecare services.
- **Key Action 14:** Define a minimum common data set for interoperability of healthcare records to be accessed or exchanged electronically by 2025.

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Major reports and studies (Stroetmann et al, 2011; Valeri, 2010; Warden, 2012) conclude that there is a significant healthcare improvement potential using e-Health as a catalyst. Examples of quantified potentials include (Gartner, 2009):

- 5 million outpatient prescription errors per year could be avoided through e-Prescriptions.
- 100,000 inpatient adverse drug events per year could be avoided through Clinical Decision Support and Computerized Physician Order Entry Systems. This would free up 700,000 bed-days per year, corresponding to a value of almost €300 million.
- 9 million bed-days per year could be released, through the use of Electronic Patient Records, thus increasing output or decreasing waiting times, corresponding to a value of approximately €3,7 billion.

Project epSOS

In order to support health policies at a European Level, standardization effort in healthcare, e-health, to promote and to establish the enforcement of the new EU Directive on cross border healthcare, the European Commission, via the Information and Communication Technologies Policy Support Program (ICT PSP) as part of the Competitiveness and Innovation Framework Program, is co-funding the Large Scale Pilot (LSP) "European Patient Smart Open Service" or epSOS in short.

EpSOS mandate is to design, build and evaluate a service oriented infrastructure that demonstrates cross-border interoperability between electronic health record systems in Europe (epSOS, 2008). EpSOS is a large scale pilot involving more than 105 healthcare provider point of care (Hospitals / General Practitioner / Medical Specialist offices) and 168 Pharmacies all spread across 23 countries in Europe. The whole extend and description of the pilot settings can be reached electronically via epSOS central access point at www.epsos.eu. The project features are shortly described in Table 1.

EpSOS goal is to offer seamless healthcare to European citizens and a technical and functional framework for cross border healthcare. Key goals are to enhance the quality and safety of healthcare services for people travelling or commuting to another country. Moreover in Europe, it concentrates on developing a practical e-Health framework and ICT infrastructure that enables secure access to patient health information among different European healthcare systems. EpSOS aspiration is to makes a significant contribution to patient safety by reducing the frequency of medical errors when providing quick access to patient summary documentation. In emergency situations, this documentation may provide

Table 1. epSOS in figures

Time period	1st July 2008 - 31st December 2013
Duration	66 months (5 1/2 years)
Volume	€ 36,5 Million: co-funded by the European Commission Competitiveness and Innovation Programme (CIP) within the ICT Policy Support Programme
Number of Beneficiaries	47 Beneficiaries (national ministries of health, national/regional competence centers, a consortium of industry and the Project Management Team):
Number of countries	23 different European countries: 20 EU member states and 3 non-EU member states.

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the medical personnel with important life-saving information and minimize the repetition of diagnostic procedures that are both costly and unnecessary (Abba, 2009; Forni, 2010).

The project also tests for more than one full year the technical, legal and organizational concepts developed during the project. EpSOS today tests cross-border services in the following areas concerning patient summary and cross-border use of e-prescriptions. In a later phase (epSOS has been extended until end of 2013) the project envisions the following new scenarios: Integration of the 112 emergency services at each nation, Integration of the European Health Insurance Card (EHIC), patient access to their own data, the latter being the most important for all as noted in the new e-health action plan released by the European Commission in December 2012 (European Commission 2012).

epSOS intention is to prove that cross-border healthcare services are possible within Europe. Patients will have the opportunity when seeking healthcare in participating epSOS pilot countries to have access to epSOS cross border healthcare services in many different scenarios whether as tourists, business travelers, commuters or exchange students. All epSOS material is freely available to reuse and distribute via its Web portal.

Rethinking E-Health and Reusing What's Worth

Eysenbach described the term e-health as emerging in 2001. This is not the case anymore. As described in previous paragraphs, designing e-health services is not new, almost two decades have passed at a global level with extensive business planning, business process reorganization activities, brainstorming, piloting, standardization activities, and many more (Eder, 2000; Harmoni, 2002; Shortliffe et al, 2001; Stegwee, 2001; Black, 2011). We are now on the process of rethinking e-health's definition, focusing more on issue such as patient centered healthcare (Finkelstein, 2012), patient empowerment (Wald, 2011) and social media in healthcare (Neuhauser, 2003; Gwee, 2011; DeVries, 2012).

During this process, a lot of effort was dedicated to consensus building and stakeholder's managements. In fact, this is the right thing to do since in healthcare everyone is a stakeholder (Yasnoff, 2004). One of the most crucial issues that all involved technocrats learned is that what is essential is the user satisfaction, where a user is practically anybody. In that sense, building successful application is not about using the best technology nor the more innovative one, it is about understanding what is needed to be done (Iakovidis 1998; Iakovidis, 2000).

So, what is actually needed to make the e-health story a success? After many years of globally successful and unsuccessful projects, the most noteworthy thing relies in the three pillars of quality in healthcare as described by Avedis Donabedian many years ago (Donabedian, 1980, 1982, 1985, 1993, 2003): a healthcare organization is a system formed by the interaction of structures, processes, and outcomes. Structures establish processes that create healthcare outcomes. Those have a back effect on structures which then need to change or adjust new processes to meet the new required outcomes. Healthcare outcomes should be more influential than financial outcomes in any healthcare system. As such, intangible assets are more valuable than tangible assets. (Cleverley, 2007; Berler 2005; Berler, 2008). Redirecting the Donabedian approach to the case of creating sustainable e-health systems since outcomes are mostly located in the Medical domain, we need to focus on the processes (thus functional specifications) and structures (thus standards and semantics).

It becomes then quite clear that what is needed is to focus on semantics and interoperability. In fact, the epSOS project did exactly that. This multi-million international co-investment spent most of its

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financial and human resources on decrypting those two sides of the Donabedian triangle by building cross border consensus, by focusing on semantic interoperability and by sticking to existing and viable international healthcare standards.

Using ICT for Health Policy and Management

Nations face similar challenges of meeting the increased need for care that are caused by the rapid aging of populations and the respective increase in patients with chronic and expensive diseases. In publicly financed health systems such as the ones existing in Europe (each one with its variations), these challenges are transformed into demand for care and a strain on shrinking healthcare budgets (OECD, 2011). The historical origins of national healthcare systems across the EU often vary. This is reflected in all aspects of a healthcare system: the structure, the manpower and its skills, the financing models. Nevertheless, all systems converge towards common future visions concerning healthcare delivery models and reforms needed to manage common epidemiological and financial pressures. ICT technologies can help in addressing key health policy and management issues (Calliope, 2010b) such as:

- **Patient Centered Care and Personalized Care:** The individual—patient, citizen—is a fundamental part of the care group who cooperates with healthcare practitioners to reach safe health related decisions. A healthcare system must respect cultural traditions, personal preferences and values, family situations, and lifestyles. The empowered individual is thus an active participant in the regional, national, and possibly international health system network, a trend that is highly supported by social media and their related applications. Healthcare in the near future will be a personalized care totally independent and focused to each one of us, driven by our own biological markers. DNA analysis will make possible new feature of personalized care such as health promotion, disease prediction, prevention, and individualized therapy
- **Continuity of Care Integration:** Patient-centered care demands that providers, departments, and healthcare settings are coordinated and operate efficiently. Care integration provides an effective means to reducing waste and to balancing health needs against demands for care. It aims to realize a new model of cross-sectoral, person-centered service delivery that will break down boundaries between different organizations' missions and resources. Semantics and Interoperability are the cornerstones of care integration. Languages and processes must be ported to each stakeholder individually. The seamless care environment should be one where the ratio of effectiveness to cost reaches its highest value while avoiding duplicating information.
- **Information Management:** As stated earlier the seamless interchange of information is key to the success of any electronic service in the healthcare sector. Information management means to know what the needed information are, at what time, and whom to address it to. In fact, it is all about semantic interoperability.
- **Rethinking Health Policy:** Generally, much of the health of populations is dependent on social and environmental factors. Thus, healthcare systems have a remarkable opportunity to control costs by extending influence outside their borders, supporting WHO policies for international health regulations with the support of new technologies for early epidemiology warning and crisis management.

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- **Open Collaboration of Stakeholders:** Consensus building and strategic partnerships for healthcare restructuring can manage healthcare complex processes, foresee different type of risks and establish common values. Open and transparent collaboration between major stakeholders is of tremendous importance to set up a viable mechanism that will handle the needed changes.
- **Incentives:** creating effective and operable e-health processes must be rewarded at all levels. As a consequence, regulatory bodies at a national and international level need to establish adequate incentives.
- **Legal Aspects:** Legislation should be a facilitator by legally supporting innovation and its change dynamics by means of full protection and legal certainly, rather than inserting constant burden and insecurity as it is often, unfortunately, the case. This can be achieved in synergy with other enablers including standardization bodies, clinical governance and through fostering security and quality cultures under an integrated framework of trust that is enforced and protected by law.

E-Services for the Healthcare Sector: It's an Interoperability Issue

Taking into account all the above issues, a successful electronic service, e-Prescription, for example, should focus on solving, or at least propose a series of tools of dealing with semantic sustainability and interoperability. In fact, semantic sustainability is an interoperability issue; it is one of the pillars of an interoperability framework that historically had three distinct dimensions: organizational, semantic, and technical. The European Interoperability Framework—EIF (ISA 2011)—extends this by adding political and legal dimension, resulting in Political – Legal – Organizational – Semantic –Technical (see Table 2).

All the dimensions of the proposed EIF model interact with each other. Each dimension though is extremely valuable to achieve an end to end interoperability. Especially in healthcare the Political and Legal dimension are of extreme importance due to the domain's complexity (eHGI, 2012). The other three dimensions are somehow known and dealt with many years ago, unfortunately, in a separate manner and from different stakeholders. In 2009, the United States' branch of the Institute of Electrical and Electronic Engineers (IEEE) revised its initial definition of interoperability for the healthcare sector stated in 1990 as being too vague:

In healthcare, the ability "to use the information that has been exchanged" means not only that healthcare systems must be able to communicate with one another, but also that they must employ shared

Table 2. European interoperability framework dimensions

Political Context	Legal Interoperability	Organisational Interoperability	Semantic Interoperability	Technical Interoperability
Political mandates and statements	Legal foundations	Business process alignment	Ability to share information	Apply Open International Standards
Policies	Data privacy	Organisational interconnectivity	Reuse of coded data and International Standards and Nomenclatures	Ability of Systems to exchange information
Business needs - Social Needs	Access to information	Interaction between two or more distinct organisations	Use commonly agreed data sets	Collaborative data processing and transaction synchronizations

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terminology and definitions. This latter emphasis places a much greater burden upon system designers and electronic engineers to make the information truly usable in the distributed clinical setting of our healthcare environment" (IEEE-USA, 2009, p 1).

Historically, technical interoperability in the healthcare started in the mid 80s via a group of visionaries under the umbrella of what is today known as Health Level Seven – HL7 (Datta, 2010). Semantic interoperability and the creation of commonly understood data set started way behind the era of computing since the need for medical practitioners to interact existed before with the use of nomenclature and codifications such as the International Classification for Diseases – ICD, Systematized Nomenclature of Medicine – SNOMED, and many others (Stroetmann, 2009). Organizational Interoperability is also often seen as process interoperability, where the correct organization and management of processes and workflows between distinct and independent organization is the key issue (Gibbons, 2007). In healthcare, for example, the creation and maintenance of a master patient index is such an issue, the exchange of medical information as part of a nationwide healthcare record is another. It is clear that coded data and data exchange in not enough to achieve interoperability, processes need to be there to integrate and orchestrate the information (Ducrou 2009).

The European Commission introduced the concepts of Political Context and Legal interoperability in order to reach a consensus and manage business and legal complexity amongst the European nations. The classical three-dimension model for interoperability did not result in to practical success since legal constraints and the associated policies tampered the good technical and scientific results.

This "five dimension model" for interoperability is now the key for the successful completion of large scale implementation projects in the healthcare sector. It is the basis for an Interoperability Framework (Berler, 2004; Mykkänen, 2006; Grilo, 2010) that is necessary to achieve consistent and interoperable healthcare scenarios, such as needed for cross border healthcare. As a consequence, project epSOS embraced this approach and created its own interoperability Framework. This document analyses all the steps taken in each of the five aforementioned interoperability dimensions (epSOS, 2010).

Europe (and epSOS) decided to opt for the reuse of existing standards in the fields, the collaboration with existing standard organization bodies and interaction with industry vendors and communities to enhance sustainability of the final result. Project epSOS collaborated with official standardization bodies such as International Standards Organization (ISO), International Electro-technical Commission (IEC) and International Telecommunication Union (ITU). It also collaborated with the United Nations Centre for Trade Facilitation and Electronic Business (UN/CEFACT), the European Telecommunications Standards Institute (ETSI), Health Level Seven (HL7), the European Committee for standardization (CEN) and the International Health Terminology Standards Development (IHTSD). The established interoperability framework also included standards and processes from the Organization for the Advancement of Structured Information Standards (OASIS), and the World Wide Web Consortium (W3C). This framework has also references to the Internet Engineering Task Force (IETF), the European Computer Manufacturers Association (ECMA), the Object Management Group (OMG), the Web Services Interoperability Organization (WS-I) and Integrating the Healthcare Enterprise (IHE).

From all the above HL7 and IHE do have a leading role. HL7 is the Standards Development Organizations that has created the CDA (Clinical Document Architecture) Release 2.0 used to create and semantically exchange documents in the form of XML (eXtensible Markup Language) documents while IHE is the Profile Enforcement Organization that have created the needed analytical technical frameworks and integration profiles. Those XML Medical documents must be valid according to CDA.xds

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schema. A CDA document has in two sections a "header" and a "body". The aim of this chapter is not to expand on technical analysis of both standards that can be located in their respective Web portals (www.hl7.org and www.ihe.net) and further readings of this chapter. It is necessary though to understand that epSOS specifications substantially reuse concepts and processes from those two principal international independent organizations.

Of course, other standards need to be used to create the adequate interoperability framework with all its pillars. SAML 2.0 (Security Assertion Markup Language), for example is an adequate choice for encoding security tokens (OASIS, 2005b). Legal interoperability usually is about handling data privacy and confidentiality issues, thus creating a need for a strong security and legal framework. XACML (eXtensible Access Control Markup Language) deals with identity management issues and handles complex rules and policies related to patient consent mechanisms needed to give or revoke access to personal and sensitive medical information (OASIS, 2005a). Other standards such as WS-security and WS-Trust from the World Wide Web Consortium (W3C) complete a full security framework related to Web Services technologies. Since the goal of Web service technology is to provide an interoperable platform that facilitates distributed computing based on the messaging paradigm, a standardized messaging platform is an essential requirement. The foundation for the standards is the SOAP (Simple Object Access Protocol) Messaging Framework which defines an XML based message format and rules for message processing. IPSec (Internet Protocol Security) and SSL (Secure Sockets Layer) provide additional security by networking protocols in order to secure the communication channel based on encryption and public key infrastructure technologies.

Semantic interoperability is of foremost importance. Data elements should be coded, for example, with UCUM (Unified Code for Units of Measures) and HL7 code system. Adequate terminology must be used to control, transcode and translate the vocabulary. The most dominant terminology standards are EDQM (Standard Terms of European Directorate of Quality in Medicine), LOINC (Logical Observation Identifiers names and codes), ATC (Anatomical Therapeutic Chemical classification system), ICD (International Statistical Classification of Diseases and Related Health Problems) and SNOMED CT (Systematized Nomenclature of Medicine-Clinical Terms).

E-Prescription

E- Prescribing is the use of computing devices to create, modify, review, and/or transmit medication prescriptions from a healthcare provider to a pharmacy (Center for Health Transformation 2008). The Institute of Medicine published a report on medication errors, revealing startling statistics on the dangers patients face in the healthcare system in the US, where 1.5 million medication errors happened every year, up to 98,000 Americans are killed every year by preventable medical errors, and more than 7,000 Americans are killed every year from preventable medication errors (Kohn, 2000).

In general, an electronic Prescription Service (e-Prescription) consists of electronic prescribing and electronic dispensing. e Prescribing is defined as the electronic prescribing of medicine with the use of software by a legally authorized health professional and the electronic transmission of said prescription data to a pharmacy where the medicine can then be dispensed (Hider, 2002; Hale, 2007). E-Dispensing is defined as the electronic retrieval of a prescription and the dispensing of medicine to the patient as indicated in the corresponding e-Prescription. Once the medicine has been dispensed, the dispenser is to report the dispensation information using the e-Prescription software (epSOS, 2008).

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E- Prescribing has been the subject of extensive discussions, white papers, policy papers, strategic plan and e-Health Roadmap (Center for Health Transformation, 2008; Teich, 2004). By itself, it should be a simple process: the use of ICT to make a medicine prescription at a healthcare provider organization and dispense this prescription at a pharmacy. In reality it is a complex service that encompasses many stakeholders, involve fully or partially a lot of people with conflicting interests, thus making process story boarding a puzzle in multiple dimensions. This complexity can be explored in the literature where many nations, profiling and standardization bodies has released extensive functional and technical documentation. To the Authors' knowledge, the most complete sets of specification can be reached, most of them as open data (creative commons rules or similar), via the Web. Table 3 is a non-exhaustive list of similar specifications.

In the United States, e-Prescription started as pilots and standardization activities in 1995 and became an increasing necessity due to the Medicare modernization act established in 2003 (Hale, 2007). As stated in the national progress report on e-prescribing and interoperable Healthcare for 2011, (Surecripts, 2011), the number of e-prescription issued increased from 54 Million in 2008 up to 789 Million in 2011 which an incremental increase of 15 times in four years time. In the same period, the active prescribers have risen from 74.000 to 390.000 users. Certified software systems were 43 in 2008 and are 157 in 2011. By the end of 2011, 58 percent of office based physicians in the United States had adopted electronic prescribing. This figure was 10 percent in 2008.

Nowadays, only some European countries have managed to create an operational National e-Prescription service for the primary care sector. Nevertheless, most of the EU Member States mention e-Prescription in their e-Health strategy and implementation plan since 2006 (European Commission 2007), a number which reached 22 in 2010 (Stroetmann et al, 2011). Denmark, England, Estonia, Iceland, Scotland, Croatia, Greece, Belgium, and Sweden are nations that have implemented or are in the process of implementing

Table 3. Freely available eprescription documentation

epSOS D3.1.2 Final definition of functional service requirements – ePrescription	http://www.epsos.eu/uploads/tx_epsosfileshare/D3.1.2_Final_Definition_of_Functional_Service_Requirements_ePrescription_01.pdf
IHE Common Parts Document	http://www.ihe.net/Technical_Framework/upload/IHE_Pharmacy_Suppl_Common_Rev1-1_TI_2010-12-30.pdf
IHE Community Medication Prescription and Dispense (CMPD)	http://www.ihe.net/Technical_Framework/upload/IHE_Pharmacy_Suppl_CMPD_Rev1-2_TI_2011-12-31.pdf
IHE Hospital Medication Workflow (HMW)	http://www.ihe.net/Technical_Framework/upload/IHE_Pharmacy_Suppl_HMW_Rev1-2_TI_2011-12-31.pdf
IHE Pharmacy Dispense (DIS)	http://www.ihe.net/Technical_Framework/upload/IHE_Pharmacy_Suppl_DIS_Rev1-2_TI_2011-12-31.pdf
IHE Pharmacy Pharmaceutical Advice (PADV)	http://www.ihe.net/Technical_Framework/upload/IHE_Pharmacy_Suppl_PADV_Rev1-2_TI_2011-12-31.pdf
IHE Pharmacy Prescription (PRE)	http://www.ihe.net/Technical_Framework/upload/IHE_Pharmacy_Suppl_PRE_Rev1-2_TI_2011-12-31.pdf
Healthcare Information Technology Standards Panel (HITSP) IS 07 - Medication Management	http://www.hitsp.org/Handlers/HitspFileServer.aspx?FileGuid=ffc182f4-2e73-44dc-b00d-810f2f9e86de
NEHTA - National E-Health Transition Authority e-Medication Management	http://www.nehta.gov.au/e-communications-in-practice/emedication-management
HL7 version 3 & CDA	http://www.hl7.org/implement/standards/index.cfm?ref=nav

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nationwide e-Prescription systems. The Netherlands have established and are using e-Prescription in some regions, with different maturity levels, depending on the GP or hospital environment. In Spain, the region of Andalucía has implemented a fully functional regional e-Prescription service. However, this services in not applicable for the other Spanish regions, which are engaged in different regional strategies. Norway, the Czech Republic, Austria, Finland, Italy, France, Portugal and Poland are currently piloting different solution at a regional or national level.

In Estonia, the digital prescription (e-prescribing) was launched in 2010. This countrywide project lasted 5 years and its aim was to make e-prescribing of drugs possible in every doctors' office and filling in digital prescriptions in every pharmacy of Estonia. In May 2011, just 15 months after the launch, 84% of prescriptions are issued digitally. More than 95% of pharmacies are ready to process e-prescriptions. According to a survey "Citizens' satisfaction with health and healthcare" 91%, of users of digital prescription are satisfied with the service (EIPA, 2011).

In Denmark (Purves 2002) reported ten years ago that Medcom has effectively rolled out e-Prescription in Denmark with on average 63% of all prescriptions items transferred electronically in 2002—this varies across Counties. It has taken 8 years to get to this point, where 3500 GP's in about 2000 general practice with 14 different IT-systems—85% of the GP's are able to send electronic prescriptions and 332 pharmacies with 4 different IT-systems—100% are able to receive electronic prescriptions. Today 85% of Danish prescriptions are electronically transferred (MEDCOM, 2012).

After Denmark, Sweden is the most e-health oriented nation in Europe. In December 2007, 68% of all new prescriptions were transferred electronically in Sweden (Åstrand, 2009). In another study, most respondent physicians believed they were able to provide the patients better service by e-Prescribing (92%), and regarded e-Prescriptions to be time saving (91%) and to be safer (83%), compared to handwritten prescriptions (Hellström, 2009). Finally, a nationwide survey showed that a vast majority of Swedish patients had positive attitudes towards e-prescriptions and electronic storing of prescriptions (Hammar, 2011).

Iceland has a national e-Prescription system, is based on one technical solution and it has the approval of the Data Protection Ombudsman. Medical Practitioners enter the prescription through a portal which is part of the national Healthnet framework. All healthcare facilities are connected to a single administrative entity and make use of special data sets (Doupi, 2010). In England, the program "connecting for Health" responsible for computerizing the National Healthcare System (NHS), started designing the Electronic Prescribing System (EPS) in the 1990s. The EPS project has been divided into two "releases": In Release One (R1) (which has now been completed), a mechanism was developed whereby General Practitioners (GP) systems can download drug data automatically from the core network, but the system still uses a paper prescription infrastructure. In Release Two (R2), the main feature is the switch to an electronic encrypted signature instead of the traditional paper signature. The only physical item remaining will be a numeric or barcode token, which the patient can present to the pharmacist when picking up his or her prescription. Currently R2 is in the deployment phase with only 1.5 Millions of prescriptions have been dispensed via this system (Van Dijk, 2011).

In the Netherlands, the first computer at a Dutch GP office was installed in 1978. By 1990, 23 percent of GPs were using a computer. Today, 97 percent of GPs use a computer-based GP Information System (Van Dijk, 2011). The electronic prescribing procedure through e-Prescription between GPs and pharmacists has been regional routine in the Netherlands for many years now via the regional OZIS network, a regionally accessible electronic medication record communication protocol layer responsible for medication data exchange. Up till now, no national standard has been defined yet. The take up for

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the regional transmission of e-Prescription lies between 20 and 50%, whereas a distinction has to be made between general GPs and specialists: GPs have an estimated take up of 50% and specialists below 10% (Flim, 2010).

There are multiple reports and documents on how different nations are coping with the introduction of e-Prescription systems and related electronic services. The authors provide a short list of further readings where extensive information can be extracted. The Greek case and its innovative approach are described in more details in the next sections of this chapter.

DESIGNING A NATIONAL E-HEALTH INFRASTRUCTURE THAT IS CROSS BORDER HEALTHCARE READY: A PARADIGM SHIFT AND A SUCCESS STORY

Creating Sustainability with ICT in Healthcare

EpSOS has introduced concepts such as the National Contact Point (NCP) which is, in fact, an enterprise information bus that securely, seamlessly transport medical information across healthcare systems of any type and complexity. This enables a democratic regulation of the health IT market allowing common testing procedures (by reusing profiles and test tools provided by IHE), common interoperability frameworks (by accepting common HL7 CDA r2 type documents for the electronic prescription) and certification procedures (by deploying national certification and accreditation bodies and reusing IHE, Eurorec or HL7 EHR-FS standards and methods).

Semantic interoperability is one of the keys towards sustainable and interoperable information systems. EpSOS invested a lot on securing the semantic interoperability layer, due to its dramatic importance and enhanced by the cultural and linguistic puzzle that comprises the European Union. As a consequence, across epSOS participating nations, there are different languages, different standards and different coding schemes, and so the challenge was how to address this. The solution was to produce two master files: the Master Value Sets Catalogue (MVC), which applies across all members and the epSOS Master Translation / Transcoding Catalogue (MTC). The content of the MVC is in English; the terms are based on criteria defined by the use-cases identified by the Participating Nations. Each nation has then translated the terms into their language and transcoded into them in their national coding system, thus creating the epSOS Master Translation / Transcoding Catalogue (MTC). The value sets in the MVC have been selected from many Standards Development Organizations (SDOs) as noted earlier in this chapter. The MVC and MTC are supported by the epSOS Central Reference Terminology Server; each nation has its own Local Terminology Repository as a copy of its MTC. If an update is made to the epSOS Central Reference Terminology Server, the Local Terminology Repositories are notified and updated.

At a European Level, the European Commission is preparing itself for the large scale deployment of e-Health services under the Connecting Europe Facility (CEF) which will run in the timeframe between 2014 and 2020. The need for ensuring cross-border connectivity through optimizing functional, semantic and technical interoperability is necessary, including enabling cross-organizational & cross-border information flows to support further clinical needs such as chronic disease management. This leads to the second fundamental need for sustainability: create the Market. Thus, in addition to semantic interoperability, which requires institutions and procedures from the buyer side, the provider side need to adhere, support and enhance the needed technical viable solutions that encompass all technical and functional specifications needed additionally for cross border healthcare. In order to support that the European Com-

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mission via its EIF program is supporting the establishment of open source reference implementations to boost the economic market and reduce investment risks for the Market. EpSOS also focused a lot on this instance, by initially creating a reference implementation of the needed components that all together create the aforementioned NCP. This reference implementation is to be strategically created and ported into open source software built, supported by participating nations, as well as the industry team of the project, OpenNCP as it is named, should be ready by the end of the project in 2013. All material is and will be made publicly available on the EU open source repository at the Join-up (www.joinup.eu) portal.

Use of epSOS Specifications: The Case of Greece

Project epSOS selected W3C SOAP as a top level messaging protocol considering the project's tight time frame for implementation and piloting, the strong demand for well known security measures and the existing experiences in industry (epSOS, 2010). An alternative is to use the REST (Representational State Transfer) architectural style, which relies on HTTP (Hypertext Transfer Protocol) commands and URIs (Uniform Resource Identifier). IHE and HL7 experts, task forces and working groups commonly agree on those technological approaches, and define healthcare actors as Web services and actions as REST commands.

Finally, coming to the Greek case, Greece is participating in epSOS from the beginning of the project with significant participation at all levels. Recent development in Greece and the introduction of a new central e-Prescription system that operated for the first time in October 2010 changed the importance of the epSOS project at a National level. The Greek pilot in epSOS focuses only in a simple scenario of dispensing EU citizen existing prescription in a number of pharmacies in Greece. At the design phase of the project, Greece had no existing e-Health infrastructure in place. This lead to a paradigm shift: while all countries organised themselves in order to connect existing systems with the proposed NCP structure of epSOS, Greece decided to do the opposite and expand its initial small pilot system so that it can be immediately "epSOS ready", thus being able to handle both national and cross border prescriptions. This has been depicted in the official tender documents of the National e-Prescription system initially released in August 2011 and heavily supported by the European Commission (IDIKA, 2011). The aforementioned issues are the reasons that are positioning the Greek case as a different approach in e-prescription implementation at a global scale.

In parallel the initial pilot system, which is a Web-based system, grew substantially covering basic prescription scenarios, solving initial user interface issues, creating and training the user community. In August 2012, the system is able to handle more than 590.000 prescription transactions per day (IDIKA 2012a). The pilot Web system is able to cover classic prescription transactions but was unable to provide the rich environment needed by the end users both at the pharmacies (dispensing the prescriptions) and at the physicians (creators of the prescriptions), creating an ongoing debate on the quality and capabilities of the system as a whole. It is at that point that it was decided to invest in the epSOS specification and mostly in its interoperability framework. This resulted in the creation of an Enterprise Service Bus, a simplified approach of the epSOS NCP than proposed to exchange all prescription (and future documents) in the CDA R2 format proposed by epSOS (IDIKA, 2012b).

The proposed overall architecture relies on the REST architectural style on the end user application layer for two main reasons: the existing system is based on Web technologies to which the REST archi-

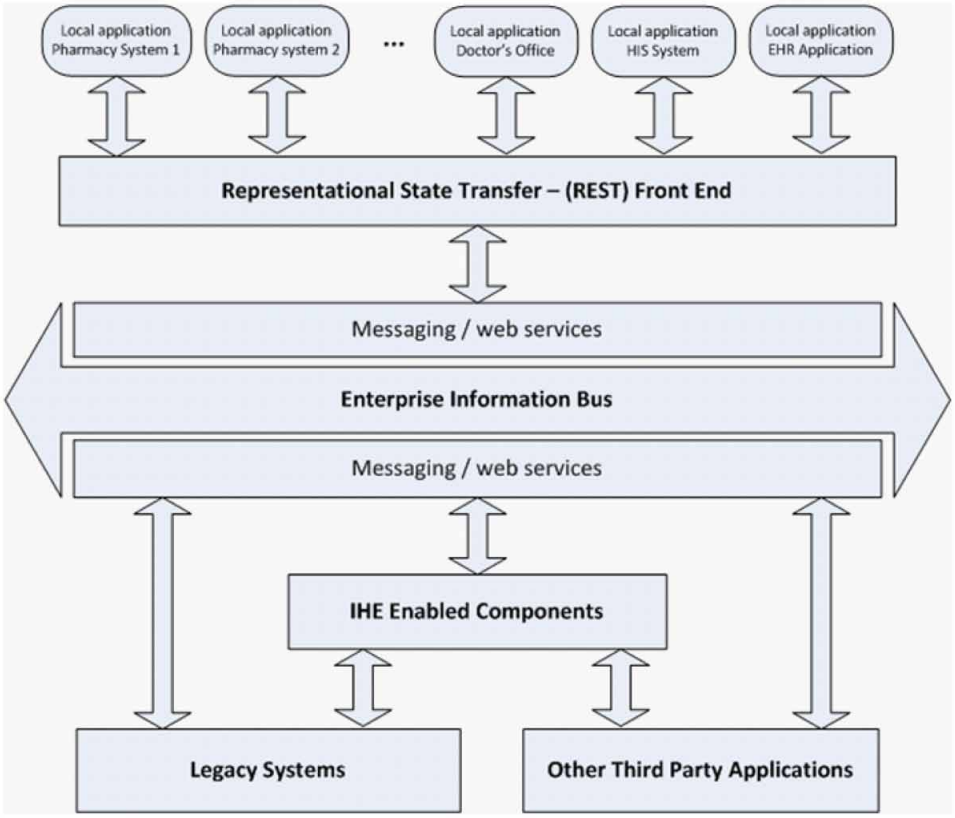
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tecture is faster to implement and secondly the existing system did not support CDA type documents nor had the ability to create such high level WS to implement a full WS SOAP approach. This architecture is depicted in Figure 2.

This architecture implies the creation of a RESTful API that is called by the third party end user applications. Information is transported via WS/Messaging services and an enterprise service bus to ensure and monitor document delivery to the main eP system. The use of additional IHE compliant tools enables the system to be compliant with epSOS scenarios and specifications while enabling the possibility of more enhanced cross border or cross enterprise scenarios between multiple data repositories

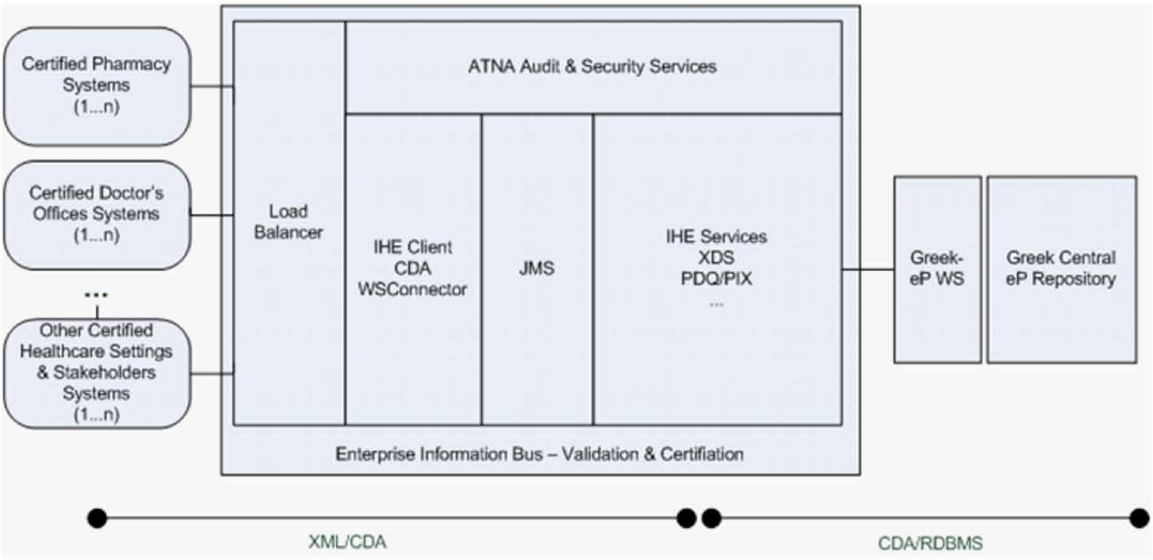
Figure 3 describes the Enterprise Information Bus (EIB) application layer from a functional point of view. In that sense, the Greek eP System has created a series of WS to handle information exchange. Those are connected to the IHE service components that transform and validate the information in HL7 CDA format. All messages are queued via a Java Messaging Service (JMS) and sent to the appropriate recipient ensuring safe delivery (will keep message history until safe delivery). The IHE client is the RESTful structure that via a set of commands makes the information available to the end users applications passing through a Load balancing mechanism to ensure timely delivery and workload distribution (a series of virtual machines). On top, the ATNA (Audit Trail and Node Authentication) Audit and security service logs and monitor the whole process ensuring data security and privacy according to the respective IHE ATNA integration profile. All messages are encrypted, and users are strongly authenticated

Figure 2. REST type architecture of the Greek eP system



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Figure 3. Enterprise information bus logical architecture



(use of API KEY and hashtags conformant to RFC 2104 specifications). The whole system is epSOS compatible since it can handle SAML messaging and Public Key Infrastructure (PKI) tokens.

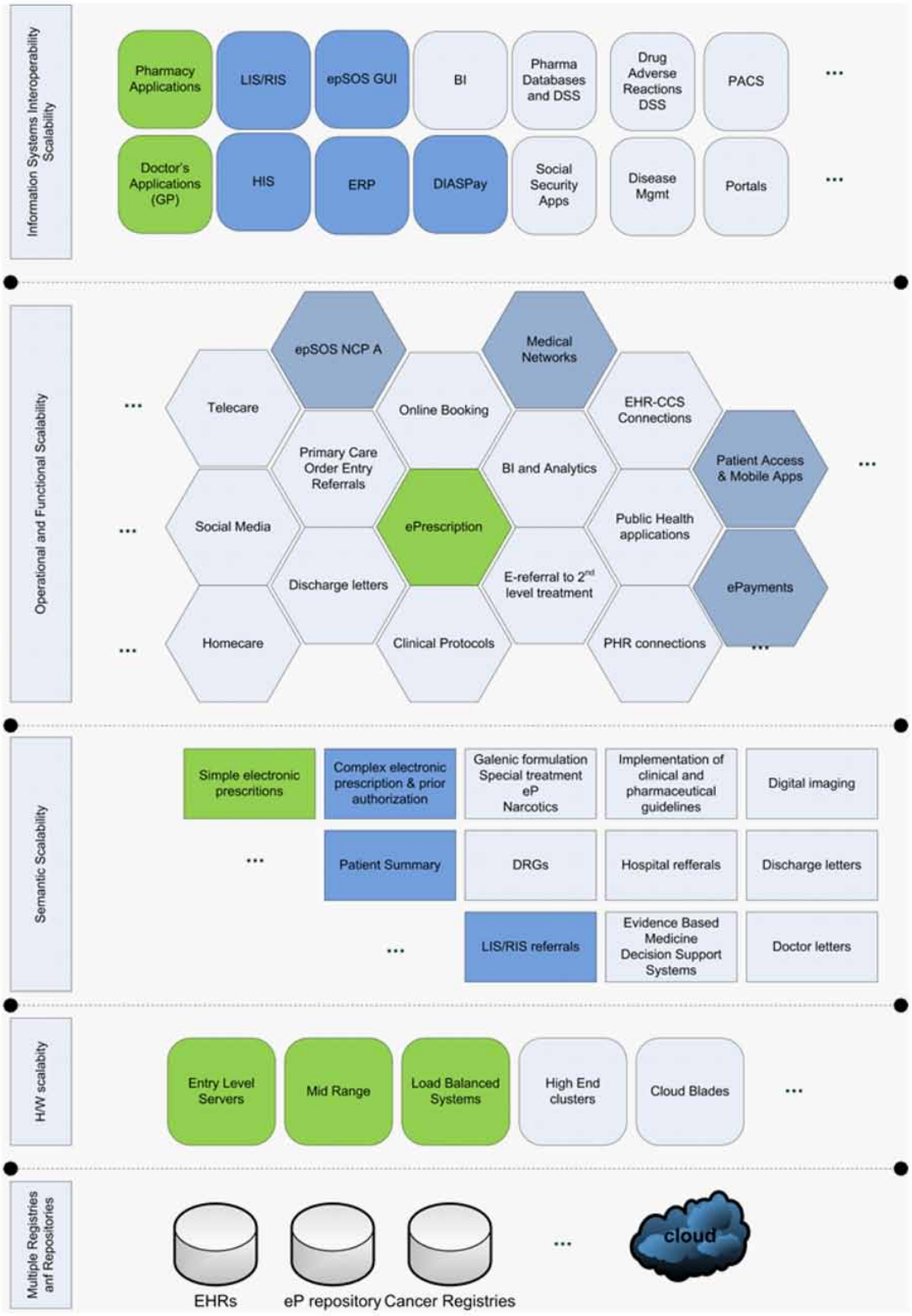
The propose architecture has a series of significant benefits and advantages, namely:

- The system is epSOS Ready and will allow the Greek Government to implement its obligations under the EU Directive 2011/24/EC on time before the deadline of October the 25th, 2013.
- The system is HL7 compliant allowing for efficient functional scalability and interoperability.
- The system can implement both cross border healthcare scenarios and national prescription scenarios.
- The system is technologically up to date with the current technological trends.
- The system is service oriented and compatible with both REST and WS/SOAP technology.
- The system is "cloudable" and thus can operate in various business scenarios in the future both as a governmental cloud or a private cloud or both.
- The system has a strong and internationally approved interoperability framework.
- The system is based on current profiling approached and profile standards (namely IHE) enabling a democratic involvement of the market and reducing the "vendor lock in effect."
- The system allows third party software to be certified to specific use cased and scenarios enhancing market competition and quality of services.

As shown in Figure 4 the system is high scalable at many levels. It can support the integration with multiple repositories (IHE Cross-Enterprise Document Sharing-XDS compliant structures), it is scalable on a physical architecture point of view enabling complex, and up to date virtualization techniques, and it is scalable at the semantic interoperability level, at the service provision level and the information systems interoperability.

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Figure 4. Proposed system scalability and sustainability



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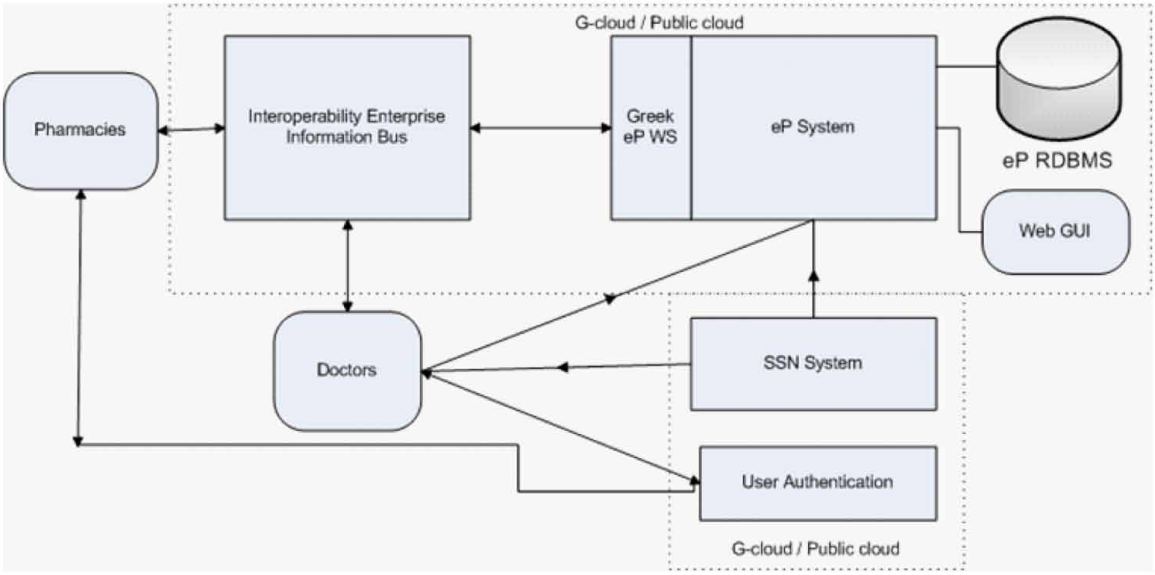
Solutions and Recommendations

The case of Greece is in our opinion a good focal point were European strategies, cross border healthcare needs, e-prescription implementation and e-health standards all collide to create a new strategy that could be the mirror of Europe's future. Greece was for many years a lager in e-health issues and is probably still is in some features. This case proves that by focusing on European policy, major steps can be made in a quite reasonable time frame. Of course, Greece has the need and the opportunity to rely on Europe to restructure its entire government and national strategy, willing to come closer to Europe mindset. As a conclusion, one could say that in the e-prescription case the reuse of European standards and methods can conclude to success and a European best practice. Europe has vastly invested in e-health. Those investments seem to have quite good acceptance overseas and reach out to decision makers in the United States, since the cross border scenarios used with epSOS for Europe present little discrepancy to be effectively implemented at a global scale. The recent Memorandum of Understanding that has been signed between Europe and the US clearly marks that success story (MoU, 2010).

Coming back to the proposed solution in Greece, one of the main benefits of the proposed architecture is its flexibility to operate in different business modalities. In Figures 5, 6, 7, and 8 we shortly present four different mode of operation.

Figure 5 describes the logic of a central EIB facility strongly bound to the existing eP system. This proposition has the fastest implementation time since the EIB is located in the same physical structure of the current eP system. By the use of virtualisation methods, one can efficiently use physical devices in the best possible manner. In this case since the eP system is operated as Governmental cloud setup, the EIB facility can only operate as a G-cloud structure. Otherwise, the whole system has to be given as managed service or business service provision in order to work in another business mode. In this case, providing control of sensible data out of a public setting may under some circumstances created data protection related risks.

Figure 5. Central EIB facility



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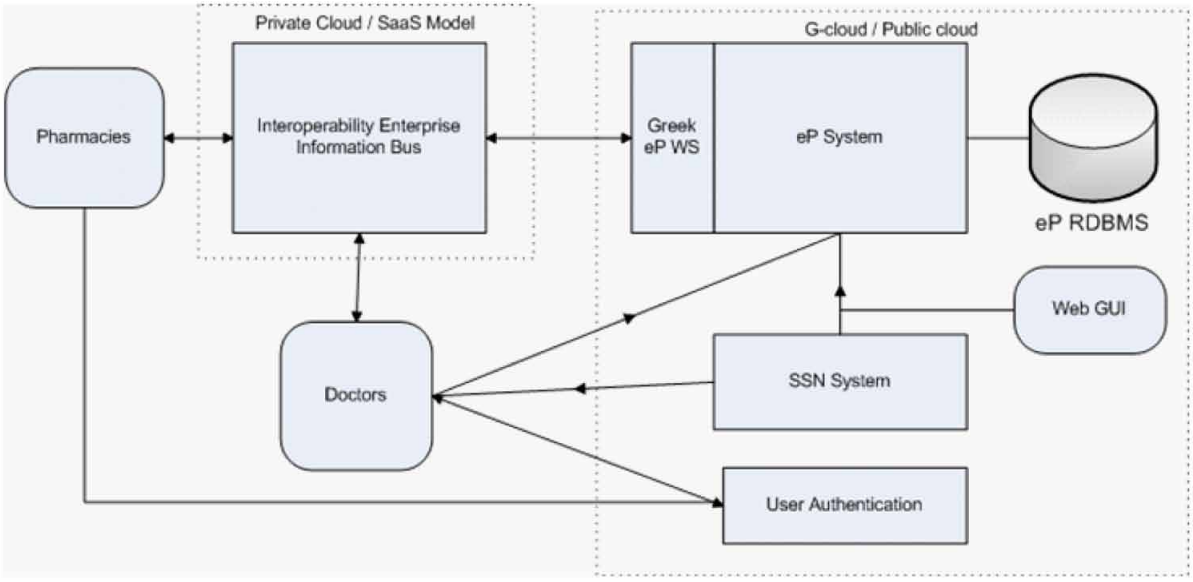
In Figure 6 we propose to operate the EIB service as a separate service that can be located in another physical location, other that the location of the eP system. This scenarios increases flexibility and enable to operate the EIB in a private cloud model in a Software as a Service mode enabling new and more viable financial models for sustainability.

Figure 7 correspond to an EIB that has the capacity of handling multiple repositories enabling in that sense the possibility to have more complex business scenarios. Such a setting can serve with the same infrastructure more than one e-health scenarios and multiple healthcare stakeholders. For example the same EIB could handle discharge letters and e-referral documents and link the primary and secondary healthcare facilities. The system can both operate in a G-cloud or a private cloud setting, and thus does not create any business barriers. In fact, both operation modes could be combined in a hybrid cloud approach where the EIB could run in a SaaS mode in a private cloud while the eP system and/or any other system can be in the G-cloud.

Finally, Figure 8 points out a variation of the system where we can have multiple, federated EIB structures connected to the central eP system (or other systems and repositories) by enhancing the reuse of IHE enabled components that are XDS compliant (multiple repositories). This scenario is extremely flexible allowing, for example, the EIB structure to be managed by relevant stakeholder group and collectively connect their software system uniformly to central government systems. This would allow for faster implementation of regional settings and architecture versus a more rigid and inflexible central system. This scenario has of course to be compliant with national strategies concerning healthcare data handling. It has though the advantages that there is no single point of failure and in case of unexpected events the system can partially operated serving specific geographical locations or specific stakeholders groups (i.e. pharmacy associations, etc).

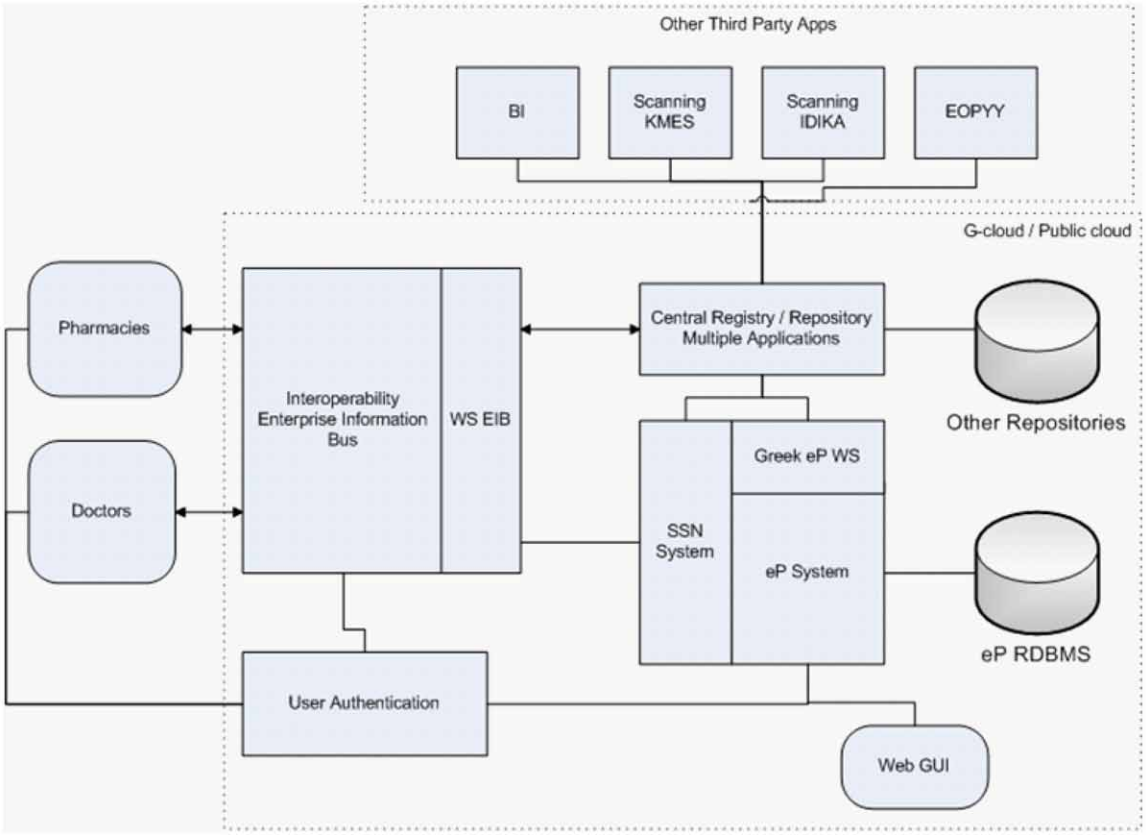
The State Authority that manages the e-Prescription (IDIKA) system decided to implement the architecture of the independent EIB mostly because of its increasing role in Greece as a de facto e-health

Figure 6. Software as a service EIB facility



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Figure 7. Independent EIB facility

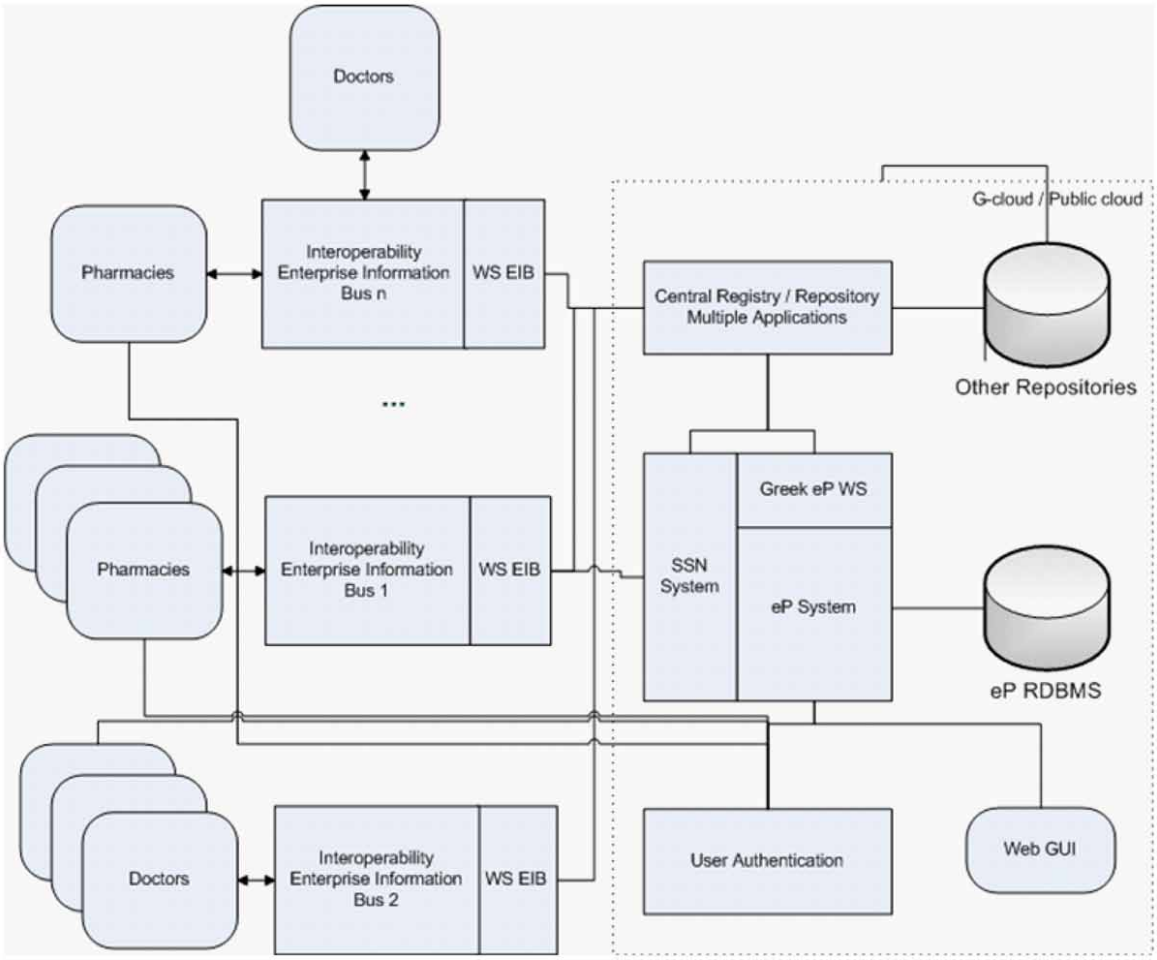


competence authority and the need to incorporate and reuse IEB components as much as possible for the seamless integration of other information systems in place. Other systems that need to be integrated for example are existing legacy systems such as the prescription antifraud and control system (KMES) located at the National organization for the provision of healthcare services (EOPYY in Greek). Up to the date of finalization of this chapter, the EIB structure is handling transaction for the community pharmacies, whose stakeholder's organization (pan-Hellenic society of pharmacies) heavily supported the integration of the existing pharmacy information systems with the e-Prescription system. All 12 software providers have been able to interoperate via the EIB and certify their respective products. IDIKA has setup a thorough roadmap to complete the integration process of all interested stakeholders and existing public information systems that need to transparently interoperate with the e-Prescription system. A significant next step of the system is to allow physicians to electronically prescribe not only medicines but also send referrals and order entries for examinations and treatments.

In order for the above to happen, some fundamental issues need to be strongly kept in mind and continuously dealt with. It is essential to always keep in mind that e-Health can only grow and expand if all stakeholders are informed and participate in decision making process via a well established consensus building mechanism. Such a mechanism can be installed with the assistance of organizations that have built their success and results based upon consensus. HL7 and its affiliates as well as IHE are organization capable of supporting central e-Health competence centre in deploying such mechanisms.

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Figure 8. Federated EIB facility



A second point of importance is certification. There is no serious and robust interoperability framework without rules and code of ethics. This is a point that was immediately foreseen by the European Commission which has established the necessary structures in all interoperability related project. In epSOS, this role has been dedicated to IHE. IHE Europe is a member of the consortium and is providing testing and certification services to the Commission. As a consequence, epSOS has periodically undergone the so called "projectathons" that are exact replicas of the connectathon process that certifies adherence of software products to IHE integration profiles. We believe that is in the best interest of the Greek government to incorporate such practices as soon as possible. The exact process of IHE is well described on its international Web portal.

A third point is installing an open mechanism for data privacy and security. All European countries have to adhere to particularly strong framework that protects personal data for misuse or abuse. The fundamental rights and the principles recognized in particular by the European Convention of Human Rights and Fundamental Freedoms (1950) have to be respected and duly protected. The fundamental right of privacy is of key importance (Art 8). Also, rights to healthcare have been recognised in the Charter of Fundamental Rights of the EU in 2000. The right to healthcare during a temporary stay in another

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member state (MS) was foreseen in Regulation 1408/71, for all persons insured under the legislation of a MS. The common EU framework on data protection is provided by "Directive 95/46/EC of the European Parliament and of the Council of 24 October 1995 on the protection of individuals with regard to the processing of personal data and on the free movement of such data". It is the most prestigious European legally binding document governing protection of personal data. The EU Article 29 Working Party (hereinafter "the WP29") has played a key role promoting harmonization of data protection in order to achieve a high level of data protection in the EU, fostering compliance with the data protection standards set up by Directive 95/46/EC and providing guidance and advice to the different actors in the data protection arena (WP29, 2004). Its Working document on the processing of personal data relating to health in electronic health records (WP 131) provides guidance on a uniform interpretation of the Directive 95/46/EC. Although documents of WP29 are not legally binding this document is one of those which must be taken into consideration. We recommend that the Greek e-Prescription system adhere to the strong policies for data privacy of epSOS (epSOS, 2009).

A fourth point of recommendation is to always keep in mind the patient itself. The European Directive on data protection and its Greek exact translation (Law 2472/1997) clearly states that all personal and sensitive data belong to the patient, and as a consequence he has a right to know on how his personal information is processed. He has the right to revoke access to his data (totally or partially) and can grant access to specific third parties. For this to happen, a series of processes and regulations must be in place. In addition, this is a must as cross border healthcare is concerned since in the country of treatment the rules that are valid are the rules and policies of the country of establishment. EpSOS has established extremely strict rules for handling patient consent and is now moving to the next step which is to grant direct patient access. We recommend that the Greek e-Prescription systems incorporate those rules. From a technical point of view XACML, is an open protocol that enable to handle direct rules based upon central e-Identity structure, thus personalising access rights to information.

FUTURE RESEARCH DIRECTIONS

Cross border healthcare is an issue that has been foreseen from a public health point of view from the World Health Organization at least since 2002 in order to reduce health risks and cross border dissemination of diseases. Information technology is now being seen a key driver to this. EpSOS is as such by itself a living proof of the importance of normalizing healthcare processes across nations. Another fact is the newly installed collaboration at a high level on this issue between the United States and the European Union. In fact, a Memorandum of Understanding (MoU, 2010) has been signed in mid December 2010. This MoU opens the agenda for joint working groups in creating commonly accepted policies in the region of health IT. Thus, this is an area of research that surpasses the health IT domain and promotes research in areas such as health economics, interoperability, health policy and many more. In that sense, cross border healthcare is not a European term but a global one. The same concepts that apply to Europe certainly apply to the borders between states and provinces in the United States, Canada and elsewhere. The recent MoU between the US and the European Commission clearly mark the interest of the American policy makers to import from Europe all the best practices that are possible.

Another important area of research is the direct implication of the end user. At a global level, it is already proven that new technologies such as mobile health and social media are already altering the patient – physician equation (Apostolakis et al, 2012). Social media in healthcare have introduced the

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term Social Health. Gwee (2011) describes the 6 Ps' of social health which reflect the leading role of social media in health as being personalization, participatory (online patient communities and e-Patients), preventative medicine, Peer-to-Peer patients, portability and passion. As a consequence, in a world of constant changes where social media and mobile applications are changing the way we know the delivery of healthcare services, it obvious to say that those two areas by themselves or combined are valid area of research. E-Health services are already hugely improved and will be even more affected in the near future. Patients are already demanding to have direct access to their information that is being handled via national or international e-health settings. For example epSOS has foreseen this need adding a patient access feature to the epSOS services (epSOS, 2010). On the same path in the United States, the Blue Button Project is expanding, giving the opportunity to the end user to control and manage the access to his medical information created during the various episodes of care (Chopra, 2010).

When expanding the end user involvement one cannot omit the Patient oriented outcomes domain which is our third relevant discussion and future research direction. It seems that information technology do have some impact on healthcare outcomes by reducing medical errors and empowering the patient (Mitchell, 2010). Clinical outcome measures examine discrete, patient-focused endpoints such as readmission, length of stay, morbidity and mortality. Those are easily monitored by information systems (ACEP, 2010). An alternative approach is to start from the patient's needs and select a goal oriented approach to monitor healthcare outcomes for each individual alone (Reuben, 2012). Going a few steps further patient oriented outcomes are often monitored easily by patients via the support of patient advocates and patient societies. Both are handling extremely well the new social media and tools on behalf of the patients. Thanks to social media, patient advocacy is moving from focusing on lobbying government officials towards working more with individuals online to create local change in communities (Brandt, 2011).

A fourth relevant area of discussion a continuous improvement that will not leave the healthcare sector unaffected is cloud computing. We already described an architecture that enables both cross border healthcare and allow establishing different cloud computing scenarios. One thing is certain, in an era of global financial instability where healthcare costs are redesigned and restructured cloud computing and its different business model will play a key role. This has been clearly depicted in a recent study for the Greek market (Danchev, 2011). Cloud computing is becoming a trend of the IT landscape. Each year, millions of users add up and remotely use data centers and Web-based applications in order to exchange various types of information.

Finally, the domain of interoperability itself is a driver for further research. For example, HL7 International has done, within 2012, two extremely significant announcements that will change once again the shape of interoperability. Initially, it has officially announced its decision to make much of its intellectual property (IP), including standards, freely available under licensing terms. This landmark decision represents HL7's commitment to the betterment of healthcare worldwide by ensuring that all stakeholders have equal access to its healthcare information technology standards. The new policy is expected to take effect in the first quarter of 2013 (HL7, 2012a). The second turning point is the release of Fast Healthcare Interoperability Resources (FHIR) Initiative (http://HL7.org/fhir). FHIR is a new HL7 draft standard for data exchange in healthcare that is based on current industry principles, including the cloud, Web 2.0 and RESTful principles. It defines a set of "resources" representing granular clinical concepts that can be managed in isolation, or aggregated into complex documents (HL7, 2012b). This flexibility offers coherent solutions for a range of interoperability problems. As described in this chapter, the proposed architecture is a RESTful implementation, and as such, is already in line with upcoming future developments.

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In parallel, IHE has focused a lot in the integration process of devices and mobile devices. It has created a new technical framework called IHE Patient Care Device and has also implemented a new integration profile for Mobile Health Devices (MHD) that also reuses RESTful approaches. This is a booming area of research and innovation not so much in the technical background but mostly in creating, new innovative, healthcare scenarios that implicate the use of connected medical devices and mobile applications. It is thus not surprising that high tech evangelist envision a world of 50 billion interconnected devices by 2020 in a machine to machine (M2M) mode (Ericsson, 2011).

CONCLUSION: DISCUSSION

In this chapter we made an attempt to analyze as thoroughly as possible the need of cross border healthcare and how the European Union, which indeed needs to solve existing issues in that domain, is proposing to deal this theme. As stated earlier, people are increasingly impatient with the inability of health services to deliver services that meet stated demands and new needs. So many patients seek healthcare services on the global scale, either because their country is, unfortunately, unable to serve them, or because they seek high expertise and skills at the point of delivery. As a consequence, Europe for many years know has focused on the subject and tried to find ways to include the need for cross border healthcare and adequate quality of healthcare delivery for all European citizens. Cross-border health care has simply become a more prominent phenomenon in the European Union (E.U.). Those issues have been taken into account in the directive 2011/24/EU with a focus on the need for e-health. As a consequence, the European Commission has established an e-health network (article 14 of the Directive).

Part of the solution relies on creating ICT facilities that will enable to interchange medical information between nations is a seamless and secure fashion. The epSOS project is operating since 2008 as a facilitator and a test bed to gradually get to the needed result. All participating nations gave their best of themselves in this endeavor and have manager to reach consensus in many issues. As it is stressed many times in this chapter, e-Health success does not rely solely on technological excellence but mostly on the way consensus and common decision are made. Until now the results are promising since for the initially designated scenarios of cross border healthcare an extensive set of technical and functional specification focusing on e-prescription and patient summary, were openly and publicly released.

This effort is closely monitored both by the EU Commission which expects to reach to a solution that will make the implementation of the new Directive on cross border healthcare a reality, and by the nation themselves. Each nation seems to act differently to the new prospect. In general, smaller countries such as Greece, Slovakia, and Portugal for example tend to seek a way to transfer this knowledge back home and do the best possible action with the local stakeholders to adhere and implement the new technologies. Other countries, the more mature ones in the e-health domain, such as Sweden, Denmark, Finland, Norway do their best to connect their existing infrastructure to the new need pointed out at a European level. All the other rely in between and act in various flavors according to stakeholders needs and taking into account that ongoing strategic plan are in the process of deployment.

It is important to distinguish the policy layer from the technological layer when trying to introduce new paradigms in a domain so complex as healthcare. The policy layer is needed in order to set the goals and agree on actions and plans. The technological layer has its complexities but the recent developments

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in healthcare interoperability have made the solutions to look more feasible. HL7 is a global leader in this domain and will grant free access to its standards starting from 2013. IHE is another key driver in this domain, having cleared out successfully an important series of barriers by proposing and continuously improving its technical frameworks and integration profiles.

The case of Greece, has proven until now that efficient reuse of best practices and adherence to new technological solutions of the 21st century, namely interoperability standard reuse, cloud computing business models, and semantic clarity via CDA document, can make the deployment of user friendly new e-services in the healthcare sector. The Greek e-Prescription case has incorporated epSOS specifications and other innovative technological features. The final success remains to be seen, analyzed and assessed in the forthcoming years. Until now though one cannot deny the huge success it already has since a nonexistent application in October 2010 is now able to handle almost 600.000 transactions per day. Of course many steps have still to be made and many battles to be won.

From the technical point of view, CDA specifications seem to work fine and cope with the complexity of the healthcare sector. The proposed introduction of RESTful technologies has proven to operate correctly. The recent trend toward this technology both by HL7 with the FHIR Initiative and IHE with its new integration profiles prove the validity and technical excellence of the proposed solution. In fact a recent connectathon in Baltimore, US was conducted by 16 large IT companies under the supervision of HL7 International proved that FHIR will very soon be the new version of the HL7 family of standards. From our side we feel lucky to have had the opportunity to monitor closely those new developments and participate in their application and test beds in the European Health IT world. A lot has still to be done and all hope for a soon adoption of e-Health technologies so that we deal more efficiently with global issues such as cross border healthcare, ageing population, healthcare systems restructuring, financial difficulties and many more.

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KEY TERMS AND DEFINITIONS

CDA: The HL7 Clinical Document Architecture (CDA) is an XML-based markup standard intended to specify the encoding, structure and semantics of clinical documents for exchange. CDA is part of the HL7 version 3 standard. Akin to other parts of the HL7 version 3 standard it was developed using the HL7 Development Framework (HDF) and it is based on the HL7 Reference Information Model (RIM) and the HL7 Version 3 Data Types. CDA documents are persistent in nature. The CDA specifies that the content of the document consists of a mandatory textual part (which ensures human interpretation of the document contents) and optional structured parts (for software processing). The structured part relies on coding systems (such as from SNOMED and LOINC) to represent concepts. CDA Release 2 has been adopted as an ISO standard, ISO/HL7 27932:2009.

Cloud Computing: Is the use of computing resources (hardware and software) that are delivered as a service over a network (typically the Internet). The name comes from the use of a cloud-shaped symbol as an abstraction for the complex infrastructure it contains in system diagrams. Cloud computing entrusts remote services with a user's data, software and computation.

Cross-Border Healthcare: Means healthcare provided or prescribed in a Member State other than the Member State of affiliation.

eHealth Interoperability: Is a characteristic of an ICT enabled system or service in the healthcare domain that allows its user to exchange, understand and act on citizens/patients and other health-related information and knowledge in a commonly interpreted way. In other words, it is a means of crossing linguistic, cultural, professional, jurisdictional and geographical border in eHealth.

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Electronic Prescribing or E-Prescribing (e-Rx): Is the computer-based electronic generation, transmission and filling of a medical prescription, taking the place of paper and faxed prescriptions. E-prescribing allows a physician, nurse practitioner, or physician assistant to electronically transmit a new prescription or renewal authorization to a community or mail-order pharmacy. It outlines the ability to send error-free, accurate, and understandable prescriptions electronically from the healthcare provider to the pharmacy. E-prescribing is meant to reduce the risks associated with traditional prescription script writing. It is also one of the major reasons for the push for electronic medical records. By sharing medical prescription information, e-prescribing seeks to connect the patients team of healthcare providers to facilitate knowledgeable decision making.

E-Services: Are all electronic services together comprise integrated ICT supported health services to citizens. Examples of such services are electronic identification, authentication and authorisation services, telemonitoring, access to electronic health records, ePrescribing, e-dispensation, and e-reimbursement.

Health Level Seven (HL7): Is a non-profit organization involved in the development of international healthcare informatics interoperability standards."HL7" also refers to some of the specific standards created by the organization (e.g., HL7 v2.x, v3.0, HL7 RIM). HL7 and its members provide a framework (and related standards) for the exchange, integration, sharing, and retrieval of electronic health information. The 2.x versions of the standards, which support clinical practice and the management, delivery, and evaluation of health services, are the most commonly used in the world.

Healthcare: Means health services provided by health professionals to patients to assess, maintain or restore their state of health, including the prescription, dispensation and provision of medicinal products and medical devices.

Integrating the Healthcare Enterprise (IHE): Is an initiative by healthcare professionals and industry to improve the way computer systems in healthcare share information. IHE promotes the coordinated use of established standards such as DICOM and HL7 to address specific clinical need in support of optimal patient care. Systems developed in accordance with IHE communicate with one another better, are easier to implement, and enable care providers to use information more effectively.

Prescription: Means a prescription for a medicinal product or for a medical device issued by a member of a regulated health profession within the meaning of Article 3(1)(a) of Directive 2005/36/EC who is legally entitled to do so in the Member State in which the prescription is issued.

Representational State Transfer (REST): Is a style of software architecture for distributed systems such as the World Wide Web. REST has emerged as a predominant Web service design model. The term representational state transfer was introduced and defined in 2000 by Roy Fielding in his doctoral dissertation. Fielding is one of the principal authors of the Hypertext Transfer Protocol (HTTP) specification versions 1.0 and 1.1. Conforming to the REST constraints is generally referred to as being "RESTful."

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Chapter 4
Web 2.0 Tools in Biomedical and Pharmaceutical Education: Updated Review and Commentary

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ABSTRACT

Web 2.0 technologies are being rapidly integrated in higher education, which dramatically influences the ways learners approach and use information. Knowledge transfer has evolved into a two-way process. Users no longer simply consume and download information from the web; they create and interact with it. Several theoretical works were developed in order to discuss the possibilities of integration of Web 2.0 tools in Pharmacy, Medicine, Allied Health, Nursing and many other Biomedical Areas. Other works have started gathering qualitative and quantitative evidence of the importance of Web 2.0 tools in the learning process. By performing this integrative review, this paper will provide an overview of what is being done in biomedical and pharmaceutical education, and elaborate some of the potential opportunities and challenges that these applications present. With this updated review we hope to give our contribution to consolidate research in this promising area.

INTRODUCTION

Internet social applications, normally referred as Web 2.0 tools, are making their way in the new teaching paradigms of higher education. Since their early development, primarily for entertainment and social communication within the general population, applications such as blogs, social video sites, and virtual worlds (Barsky, 2006) are being adopted by higher education institutions in a vast range of scientific areas (Boulos, Maramba, & Wheeler, 2006). It has been argued that Web 2.0 technologies have the potential to change the education of healthcare professionals, from a didactic one way process to a collaborative and participative process, empowering the student to be an equal participant in the learning

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process(Ward, Moule, & Lockyer, 2009). Web 2.0 applications appear to offer exciting new ways to teach, however, research into the use and evaluation of Web 2.0 tools in Biomedical and Pharmaceutical Education is still in its infancy, and the current pedagogic evidence about these tools is still lacking (Boulos et al., 2006)(Cain & Fox, 2009). In specific areas such has Pharmacy and Allied Health education, the examples are not wide, but the increasing use of teaching methodologies such as case based learning (Jesus, Cruz, & Gomes, 2011), might contribute to the implementation and dissemination of these tools. Wikis, blogs, podcasts and other tools have already been mentioned in some papers regarding higher education (Poonawalla & Wagner, 2006) (Kamel Boulos & Wheeler, 2007). These Web 2.0 technologies are in fact emerging as platforms to enable or encourage students to collaboratively create and share their own insights into current and emerging themes within their education. The number of tools and users are increasing and finding a place in healthcare management, education and practice (Ward et al., 2009). While it seems Web 2.0 might offer the potential for online learning to support pedagogy, in higher education there is little understanding of how and where it is being used to support biomedical education (Ward et al, 2009). This study is aimed to explore what specific Web 2.0 tools are being used, with what purposes and in what contexts.

In this updated review we will also focus on the most recent evidence about the use o Web 2.0 tools in pharmaceutical education, and share our own experiences on this topic.

METHODOLOGY

The study design is descriptive (MacMillan & Shumaker, 1997) and adopted the format of an integrative review (Cooper, 1984) since the objective was to make a synthesis of results (secondary analysis) from previous studies (primary analysis), in order to respond to new questions, new hypotheses and to verify or establish new relationships (Fortin, 2009). It is well documented that research reviews are considered research of research, and therefore should meet the same standards as primary research in methodological accuracy (Cooper, 1998). Cooper (1998) has delineated the process of conducting a research review as encompassing a problem formulation stage, a literature search stage, a data evaluation stage, a data analysis stage, and a presentation stage.

Problem Formulation Stage

This study primarily aims to characterize the use of Web 2.0 tools in biomedical and pharmaceutical education between the years of 2004 to 2014, and gather data to assess the potential educational values and hurdles to overcome. To constitute the categories of analysis we kept in mind the specificity of the object. Accordingly, the following variables were considered:

1. Year of publication,
2. Type of web 2.0 tool,
3. Biomedical area,
4. Type of publication,
5. Type of article and, finally,
6. Type of empirical study.

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For the variable "biomedical area" we started with 5 main categories – Medicine and Dental Medicine; Nursing; Pharmacy and Pharmacology, Allied Health and Other Topics. For the variable "type of publication" we considered three categories – journal article, proceedings and other. For the variable "type of article," we adapted the proposals of Coutinho (2008) and Poirier et al (2009), and considered four main categories – theoretical/ reflection, empirical, instructional design and assessment studies (IDEAS)[1], and study protocol descriptions. Finally, in the variable "type of empirical study" we started with an initial range of six categories adapted from the proposal of Gomes & Coutinho (2008): quasi - experimental, survey, case study, mixed study, action research and qualitative study. During the data analysis we didn't found any paper that falls in the categories "case studies" and "action research". Brief research design descriptions of retrieved studies are summarized in Table 1. Classification and definition are based in the proposals of O'Brien (2001), Poirier et al., (2009) and Coutinho (2011).

Literature Search Stage

Well-defined literature search strategies are critical for enhancing the accuracy of any type of review because incomplete and biased searches result in an inadequate database and the potential for inaccurate results (Cooper, 1998). Ideally, all of the relevant literature on the problem or topic of interest is included in the review; yet obtaining this literature can be challenging and costly (Jadad, Moher, & Klassen, 1998). Our literature search consisted mainly on electronic search of published articles and congress proceedings, in international educational databases and university repositories. Published journal articles and conference proceedings, between 2004 and 2014, relating to the use of Web 2.0 tool in Biomedical and Pharmaceutical Higher Education were searched in the databases ERIC; EBSCO Academic Search

Table 1. Brief research design descriptions of retrieved studies

Type of Article		Brief Description
Empirical Study	Quasi-experimental	It is similar to an experimental design, where strict control and randomization of treatment conditions are not possible.Usually the groups of subjects are already created by some previous criteria whic are independent from the aims of the research study.This is typical in educational settings
	Survey	Structured questions given to participants, by means of interviews or questionnaires.
	Qualitative Study	Family of methods involving an interpretive, naturalistic approach of the subject.
	Mixed Study	Applies principles of Qualitative and Quantitative research
	Case Study	A form of qualitative research, the case study looks intensely at an individual or small group, drawing conclusions only about that participant or group and only in that specific context.
	Action Research	Action research involves utilizing a systematic cyclical method of planning, taking action, observing, evaluating (including self-evaluation) and critical reflection prior to planning the next cycle.
Theoretical/Reflection		Include expert opinions, reflections and theoretical aspects on the topic.
Instructional Design and Assessment Studies (IDEAS)		Includes articles that describe new courses, parts of courses, integration of selected competencies across the curriculum, assessment of instructional outcomes,the use of technologies and new delivery methods
Study Protocol		Described, proposed or ongoing research, providing a detailed account of the hypothesis, rationale, and methodology of the study

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Complete, MEDLINE, PubMed, JSTOR, CINAHL, Science Citation Index Expanded, Social Sciences Citation Index and Conference Proceedings Citation Index. Keywords, utilized in the search, consisted in: "blog", podcast", "virtual worlds", "Wiki", "Docs"," Web 2.0", " Health Education"," Biomedical Education", "Medicine Education", "Nurse Education" and "Pharmacy Education" in several combinations. The titles, abstracts, and keywords of each of these articles were reviewed by two members of the research team, to assess their relevance to the research at hand. The references of the selected articles were also surveyed for appropriate articles. Computerized databases are efficient and effective; however, limitations associated with inconsistent search terminology and indexing problems may yield only about 50% of eligible studies (Whittemore & Knafl, 2005). Thus, and following the recommendations of Conn, Valentine, Cooper, & Rantz (2003), other approaches to searching the literature were included, such as, journal hand searching and searching research registries. A total of 55 articles were selected for further analysis.

Data Evaluation

The final sample for this integrative review included empirical studies, theoretical reports instructional design and assessment studies, and study protocols. Empirical reports included a wide variety of methods. Due to the diverse representation of primary sources, reports were coded according to two criteria relevant to this review: methodological or theoretical accuracy and data relevance on a 2-point scale (high or low). Since there are limited studies on this topic, an in order not to compromise the review, no report was excluded, based on this data evaluation rating system; however, the score was included as a variable in the data analysis stage. High relevance was only attributed to empirical studies.

Data Analysis

Data analysis required that the data from primary sources was ordered, coded, categorized, and summarized into a unified and integrated conclusion about the research problem (Cooper, 1998). Data analysis comprised of data coding from primary sources to simplify, abstract, focus, and organize data into a manageable framework (Whittemore & Knafl, 2005). Relevant data of each subgroup classification was extracted from all primary data sources and compiled into a matrix. The matrix was created according to the assumptions and categories mentioned earlier. This approach provides succinct organization of the literature, which facilitates the ability to systematically compare primary sources on specific issues, variables, or sample characteristics.

Presentation

Conclusions are reported in table form. Explicit details from primary sources and evidence to support conclusions will be provided to demonstrate a logical chain of evidence, allowing the reader of the review to ascertain that the conclusions do not exceed the evidence (Oxman, 1994).

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PROSPECTS

By performing this integrative review, this chapter will provide an overview of what is being done in biomedical and pharmaceutical education, and elaborate on some of the potential opportunities and challenges that these applications present. We hope to give our contribution to consolidate research in this promising area.

RESULTS PRESENTATION AND DISCUSSION:

Year of Publication

The documents retrieved were published over a time period between the end of 2004 and the third quarter of 2014 (see Table 2). The year which shows the highest number of publications, was 2009 with 13 documents, followed by the years 2010 and 2011 with ten to eleven records.

Table 2. Array of literature

Autor	Year of Publication	Web 2.0 Tool	Biomedical Area	Type of Publication	Type of Article	Type of Empirical Study
(Bouldin, Holmes, & Fortenberry, 2006)	2006	Blog	Pharmacy	Article	Instructional Design and Assessement	
(Boulos, Maramba, & Wheeler, 2006.)	2006	Wiki+Blog+Podcast	Other Topics	Article	Theoretical/Reflection	
(Sandars, 2006)	2006	Wikis+Blogs	Medicine	Article	Theoretical/Reflection	
(Boulos, Hetherington, & Wheeler, 2007)	2007	Virtual Worlds	Medicine	Article	Theoretical/Reflection	
(Mathieu, 2007)	2007	Wiki+Blog+Podcast	Nursing	Article	Theoretical/Reflection	
(Oomen-Early & Burke, 2007)	2007	Blog	Other Topics	Article	Empirical	Survey
(Chretien, Goldman, & Faselis, 2008)	2008	Blog	Medicine	Article	Instructional Design and Assessement	
(Forbes & Hickey, 2008)	2008	Podcast	Nursing	Article	Empirical	Survey
(Geyer, Beylefeld, & Alwyn, 2008)	2008	Podcast	Medicine	Proceedings	Empirical	Survey
(Goldman, Cohen, & Sheahan, 2008)	2008	Blog	Other Topics	Article	Empirical	Survey
(Kaldoudi, Bamidis, Papaioakeim, & Vargemezis, 2008)	2008	Web 2.0	Medicine	Proceedings	Theoretical/Reflection	
(Pilarski, Alan Johnstone, Pettepher, & Osheroff, 2008)	2008	Podcast	Medicine	Article	Empirical	Survey
(Sandars, Homer, Pell, & Croker, 2008)	2008	Web 2.0	Medicine	Article	Empirical	Survey
(Skiba, 2008)	2008	Microblogging	Nursing	Article	Theoretical/Reflection	
(Sumer, 2008)	2008	Web 2.0	Medicine	Article	Theoretical/Reflection	
(Billings, 2009)	2009	Wikis+Blogs	Nursing	Article	Theoretical/Reflection	
(Bratsas, Kapsas, Konstantinidis, Koutsouridis, & Bamidis, 2009)	2009	Web 2.0	Medicine	Proceedings	Theoretical/Reflection	

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Table 2. Continued

Autor	Year of Publication	Web 2.0 Tool	Biomedical Area	Type of Publication	Type of Article	Type of Empirical Study
(Cain & Fox, 2009)	2009	Web 2.0	Pharmacy	Article	Theoretical/Reflection	
(Grassley & Bartoletti, 2009)	2009	Wikis+Blogs	Nursing	Article	Theoretical/Reflection	
(Hansen, Murray, & Erdley, 2009)	2009	Virtual Worlds	Nursing	Article	Theoretical/Reflection	
(Honey, Diener, Connor, Veltman, & Bodily, 2009)	2009	Virtual Worlds	Nursing	Proceedings	Instructional Design and Assessement	
(Meade, Bowskill, & Lymn, 2009)	2009	Podcast	Pharmacology	Article	Empirical	Survey
(Miller, Bookstaver, & Norris, 2009)	2009	Wikis	Pharmacy	Article	Instructional Design and Assessement	
(Phadtare, Bahmani, Shah, & Pietrobon, 2009)	2009	Docs	Allied Health and Nursing	Article	Empirical	Quasi-Experimental
(Rogers, 2009)	2009	Virtual Worlds	Nursing	Proceedings	Instructional design and assessement	
(Rosh, Jones, & Wahl, 2009)	2009	Web 2.0	Medicine	Article	Experiment Report	
(Shantikumar, 2009)	2009	Podcast	Medicine	Article	Empirical	Survey
(Woulfe, Williams, & Ryan, 2009)	2009	Wikis	Pharmacy	Proceedings	Empirical	
(Archambault et al., 2010)	2010	Wikis	Allied Health and Medicine	Article	Study Protocol	
(Bristol, 2010)	2010	Microblogging	Nursing	Article	Theoretical/Reflection	
(Chu, Young, Zamora, Kurup, & Macario, 2010)	2010	Web 2.0	Medicine	Article	Theoretical/Reflection	
(Melissa Tan, K. Ladyshewsky, & Gardner, 2010)	2010	Blog	Allied Health	Article	Empirical	Qualitative Study
(O'Sullivan & McGlynn, 2010)	2010	Web 2.0	Allied Health	Proceedings	Instructional design and assessement	
(Mattheos, Schoonheim, Klein, Walmsley, & Chapple, 2010)	2010	Web 2.0	Dental Medicine	Article	Theoretical/Reflection	
(Varga-Atkins, Dangerfield, & Brigden, 2010)	2010	Wikis	Medicine	Article	Empirical	Mixed Study
(Younger, 2010)	2010	Wikis	Nursing	Article	Theoretical/Reflection	
(Schreiber, Fukuta, & Gordon, 2010)	2010	Vodcast	Medicine	Article	Empirical	Experimental
(Wiecha, Heyden, Sternthal, & Merialdi, 2010)	2010	Virtual Worlds	Medicine	Article	Empirical	Survey
(Mirk, Burkiewicz, & Komperda, 2010)	2010	Wikis	Pharmacy	Article	Empirical	Survey
(Boutin & Therriault, 2011)	2011	Wiki	Allied Health	Proceedings	Instructional Design and Assessement	
(Fischer, Haley, Saarinen, & Chretien, 2011)	2011	Blog	Medicine	Article	Empirical	Quasi-Experimental
(Fox & Varadarajan, 2011)	2011	Microblogging	Pharmacy	Article	Empirical	Survey
(George & Dellasega, 2011)	2011	Web 2.0	Medicine	Article	Empirical	Mixed Study
(Llambí et al., 2011)	2011	Wikis	Medicine	Article	Empirical	Quasi-Experimental

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Table 2. Continued

Autor	Year of Publication	Web 2.0 Tool	Biomedical Area	Type of Publication	Type of Article	Type of Empirical Study
(Meade, Bowskill, & Lymn, 2011)	2011	Podcast	Pharmacology	Article	Empirical	Qualitative
(Ros-Rodriguez et al., 2011)	2011	Wiki	Pharmacy	Proceedings	Instructional Design and Assessement	
(Sparks, O'Seaghdha, Sethi, & Jhaveri, 2011)	2011	Web 2.0	Medicine	Article	Theoretical/Reflection	
(Stiffler, Stoten, & Cullen, 2011)	2011	Podcast	Nursing	Article	Empirical	Survey
(Zanussi, Paget, Tworek, & McLaughlin, 2011)	2011	Podcast	Medicine	Article	Theoretical/Reflection	
(Pierce & Fox, 2012)	2012	Podcast	Pharmacy	Article	Instructional Design and Assessment	
(Bussières, Métras, & Leclerc, 2012)	2012	Microblogging	Pharmacy	Article	Instructional Design and Assessment	
(Miller, Norris, & Bookstaver, 2012)	2012	Wikis	Pharmacy	Article	Empirical	Survey
(Stewart, Panus, & Hagemeier, 2013)	2013	Podcast	Pharmacy	Article	Instructional Design and Assessement	
(Camiel, Goldman-Levine, Kostka-Rokosz, & McCloskey, 2014a)	2014	Microblogging	Pharmacy	Article	Instructional Design and Assessement	
(Camiel, Goldman-Levine, Kostka-Rokosz, & McCloskey, 2014b)	2014	Microblogging	Pharmacy	Article	Empirical	Survey

Web 2.0 Tools

One of the main aspects to consider in the literature search, and data evaluation, was the Web 2.0 tool utilized in the studies (Table 3). Taking into account the diversity of tools available, and the possible combinations, our option was not to previously set categories, but to categorize, according to the data evaluation. During the data analysis process we accounted for 9 categories: "Web 2.0"; "Podcast/Vodcast"; "Wikis"; "Blogs"; "Second Life"; "Twitter®"; "Wikis + Blog", "Wikis + Blog + Podcast" and finally "Google Apps® and Similar". The "Web 2.0" category was considered due to the number of theoretical and empirical works being published, regarding the general application of Web 2.0 tools in biomedical sciences, or the application of more than three specific tools in the same context.

Biomedical Area

Medicine is the largest biomedical area represented in this study. This result may be explained by the large literature being published by medical professionals, the large number of medical faculties, and also because other biomedical areas, like Allied Health and Pharmacy, represent a younger existence when compared with traditional Medical Education. During the development of the study we coded two new variables – "Allied Health and Nursing"; "Allied Health and Medicine"- which are related to experiments conducted with both kind of professionals (Table 4). In this updated review we focused in newer studies regarding pharmaceutical education.

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Table 3. Number of articles according with each Web 2.0 tool

Web Tool	Total Number of Articles	Type of Articles			
		Empirical	Theoretical/ Reflection	Instructional Design / Assessment Studies	Study Protocol
Web 2.0	11	2	7	2	0
Podcast /Vodcast	11	8	1	2	0
Wikis	10	5	1	3	1
Blogs	6	4	0	2	0
Virtual Worlds	5	1	2	2	0
Twitter/Microblogging	6	2	2	2	0
Wikis + Blog	3	0	3	0	0
Wikis + Blog + Podcast	2	0	2	0	0
Google Apps/Similars	1	1	0	0	0

Table 4. Number of articles according to biomedical area

Biomedical Area	Total Studies Retrivied
Medicine and Dental Medicine	21
Pharmacy and Pharmacology	15
Nursing	11
Allied Health	3
Allied Health and Medicine	1
Allied Health and Nursing	1
Other Topics	3
Total number of Articles	55

Type of Publication

Regarding the variable type of publication, journal articles are the most common source and medium of dissemination of work being done in Biomedical Education with Web 2.0 tools, consisting on a total of 40. This may be due to two major factors. Firstly, conference proceedings, by themselves, do not constitute a characteristic and homogeneous group of scientific work that can be easily accessed through the web, mainly because only a few organizations with long-standing organization history of scientific conferences have indexing. Additionally, situations where proceedings are published (full text) in supplements of related Journals, we choose to categorize them as Journal Articles.

Type of Article and Type of Empirical Studies

As previously stated, four main categories were considered, for the variable type of article: theoretical/ reflection; empirical study; instructional design and assessment study, and study protocol (Table 5). The empirical studies relating to the topic in question, were categorized accordingly with the followed methodology. The following table summarizes the results. Surveys are the most common type of empirical study.

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Table 5. Number of articles according with type of article and type of empirical study

Type of Article	Number of Articles	Type of Empirical Study			
		Quasi-Experimental	Survey	Qualitative	Mixed Study
Empirical	24	4	15	2	3
Theoretical/ Reflection	18				
Instructional Design and Assessment Studies	12				
Study protocol	1				

RESULTS BY WEB 2.0 TOOL USED

Web 2.0

Web 2.0 technologies are being rapidly integrated in higher education, which dramatically influences the ways learners approach and use information. Knowledge transfer has become a two-way process. Users no longer simply consume and download information from the web; they create and interact with it (Rosh et al., 2009). Several theoretical works were developed in order to discuss the possibilities of integration of Web 2.0 tools in Pharmacy (Cain & Fox, 2009), Medicine (Chu et al., 2010), Allied Health (O'Sullivan & McGlynn, 2010) and many other Biomedical Areas. Other works have started gathering evidence of the importance of Web 2.0 tools for students and even show some quantitative and qualitative results. Initial works like the one from Sandars et al., (2008) propose a very straightforward approach. With a survey type study, they aimed to determine the nature and extent of the use of social software by first year medical students. From a sample of 212 students, they discovered that over 90% used instant messaging and 70% social networking sites. There was no significant difference between males and females. Blogs were read by about a fifth of the students and 8% wrote their own blogs. A fifth of males stated that they were users of media sharing and contributed to wikis. Social bookmarking was rarely used by either sex. On a more complex approach George & Dellasega (2011) used and evaluated the integration of new social media tools into the curricula of two graduate-level medical humanities electives offered to 4th year students at Penn State College of Medicine. Five social media tools were selected - Twitter®, YouTube®, Flickr®, Blogger® and Skype® - to promote student learning. At the conclusion of each course, students provided quantitative and qualitative course evaluation. Results demonstrate that students gave high favorability ratings to both courses, and expressed that the integration of social media into coursework augmented learning and collaboration. Others identified challenges such has demands on time, concerns about privacy and lack of familiarization with technology. These reports represent an integrated approach to the use of Web 2.0 technologies in Biomedical Higher Education. Reports relating to the use of specific tools are presented below.

Podcast and Vodcast

Much that is written about podcasting refers to its ability to enhance convenience, flexibility and accessibility to learning (Nathan & Chan, 2007). It appears that when one examines the purpose behind the use of podcasting, it falls into three broad categories: enhancing the flexibility of learning, increasing

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accessibility to learning (particularly in relation to enabling mobile access) and enhancing the student's learning experience (particularly in on campus courses through the use of more blended learning experiences) (McGarr, 2009). We found 10 articles relating to the use of podcast/vodcast in biomedical education. Six of them were surveys. Meade et al. (2009) tried to provide an evaluation of the usefulness of pharmacology podcasts as a supplementary learning tool for Non Medical Prescribing Students. The focus of this evaluation was on student use of the podcasts, perceptions of the usefulness and any potential impact on students' pharmacological knowledge. The podcasts were made available to students through WebCT® and the authors tracked two successive cohorts of nurse prescribing students (n = 69). Survey data, investigated reasons for podcast use and perceived usefulness of podcasts as a learning tool. Their results showed that not only 91% of students accessed at least one podcast but also that 93% of students used the podcasts to revisit a lecture. Only 22% used the podcasts because they had missed a pharmacology session. Most students (81%) generally listened to the entire podcast rather than specific sections and most (73%) used them while referring to their lecture handouts. More than 90% of students found the podcasts helpful as a learning tool, as a revision aid and in promoting their understanding of the subject. Of the 69 students, 64 completed the pharmacology exam. In order to examine any impact of podcasts on student knowledge, their exam results were compared with those of two historical cohorts who did not have access to podcasts (n = 70). Evaluation of range of marks obtained, mode mark and mean mark suggested improved knowledge in students with access to podcasts compared to historical cohorts of students who did not have access to pharmacology podcasts(Meade et al., 2009). Also in the UK, but with medical students, Shantikumar (2009) aimed to investigate medical students' perceptions of a series of enhanced podcasts for revision of contents. Thirteen audiovisual podcasts covering general surgery were developed, consisting of a PowerPoint slideshow with a voiceover narrative. A questionnaire was distributed to 211 final year medical students, two months after the podcast became available. Results showed that students who used the resource felt that the enhanced podcasts were straightforward to access, were a useful learning supplement, and felt that similar resources for the remainder of the undergraduate medical syllabus would be useful for revision purposes (Shantikumar, 2009). Similar results were described by Pilarski et al (2008) in first year medical students. According to their results, the availability of lecture recordings aided medical students in their studies and reduced stress and anxiety. Student response to the recordings was positive, and no negative outcomes were noted by students or faculty (Pilarski et al., 2008).

Analogous methods were used in Nursing Education (Forbes & Hickey, 2008), (Stiffler et al., 2011). In both cases, the authors provided an overview of the potential uses and techniques for implementing podcasting in nursing education, and described students perceptions and attitudes. The results are in consonance with the above-mentioned studies. Although these are promising results we must not be drawn to generalize their conclusions. First of all, the survey type study does not provide all relevant information about context on the application of podcasts. Secondly, student performance in these cases, except Meade et al (2009), is not categorized or even measured in a consistent way. However, student's perceptions and responses to these implementations are crucial for their success, but more detailed, in-depth studies are required in order to truly account for an impact in biomedical and pharmaceutical education. This will imply bigger samples and longer studies.

We only came across with one qualitative study exploring the use of podcast in biomedical education. Meade et al. (2011) conducted semi-structured interviews with seven non-medical prescribing students. Their results determined that students used podcasts for revisiting lectures, preparing for exams, to clarify or revise specific topics and, to a lesser extent, to catch up on a missed lecture. Barriers to

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podcast use were identified, mainly around technological issues. Lack of experience and lack of access to suitable technology to access podcasts proved a barrier for some students. Students found that using podcasts allowed them to have greater control over their learning and to gauge their learning needs, as well as helping them build their understanding of a complex topic (Meade et al., 2011). The qualitative methods provide a depth of understanding of issues that is not possible through the use of statistically based, quantitative investigations. The qualitative approach can be a useful tool since it centralizes and places primary value on how students understand experience and operate with web 2.0 tools. The results of qualitative studies can be of interest to educators and policy makers when design a strategy for the implementation of such tools in their specific academic context.

Initially we did not consider a category for vodcasts, however, in the array of literature, one study referring to vodcast in medical education was found (Schreiber et al., 2010) and one in Pharmacy (Pierce & Fox, 2012). In the first study the authors compared information recall and student experience and preference after live lectures and video podcasts in undergraduate medical education. A crossover trial was performed with a total of 100 students. The first group attended a live lecture on arthritis and then a video podcast on vasculitis, while the second group attended a live lecture on vasculitis and then a video podcast on arthritis. The study hypothesis was that knowledge after vodcast would not be significantly different from knowledge after live lectures. The primary outcome was the score on the multiple-choice questionnaire after each intervention. This study found that students showed similar information recall after video podcasts and live lectures, but preferred live lectures. Students appreciated the convenience and control over vodcasts, but found them, generally, less engaging. Design strengths in this study included testing the students only after the interventions, to avoid influencing the educational intervention with the test itself and keeping the two interventions as similar as possible using the same speaker, the same PowerPoint® slides, and the same verbal information. Authors, point out however, that selection bias might have been present, since participation was out of hours and voluntary and also a significant dropout rate occurred.

Pierce and Fox (2012), promoted vodcast of lectures prior to the scheduled class and then discussed interactive cases of patients with end-stage renal disease in class. Students' performance on the final examination significantly improved compared to performance of students the previous year who completed the same module in a traditional classroom setting. Althought these results are very promissing, we must consider taht this experiment was based in a "Flipped Classroom" model, which may also contribute to the performance improvement.

These studies open possibilities for future interventions, and although conclusions were not always favorable to the Web 2.0 tool (Stewart et al., 2013), several factors were identified as confounding factors. We suggest that any pursue in this type of designs take in consideration the duration of the intervention, the possible selection bias and of course a better isolation of threats to the internal validity. The incorporation of podcasts in biomedical and pharmaceutical education, namely in support of a mobile form of learning, may have potential, but the reason given to justify its use should be student led, and not technology led. Educators need to be cautious of the claims made in relation to new and emerging technologies, particularly in the assumptions made by proponents of the technologies (McGarr, 2009). The development of future policies in institutions should be cognisant of the limitations of the technology in supporting a more mobile form of learning. If used to provide access to records of previous lectures, or to provide summaries and syntheses of course material, careful consideration should be given to how the students should use the material. If seen as the primary source of material and learning by

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the students, this type of technology may not contribute to the students' learning experience. So it is important to ensure that students possess the study skills needed to use the support material effectively.

Blogs

Blogs may serve as a potential teaching tool, as well as an important channel for Biomedical Education. Previous research has shown that blogging, integrated into curriculum, may increase student's motivation, critical thinking, class interaction, and student's course satisfaction (Beldarrain, 2006; Olofsson, Lindberg, & Hauge, 2011). Blogging may even propel students to take social and political actions outside of the virtual environment (Oomen-Early & Burke, 2007). Concerning the use of blogs alone, in biomedical and pharmaceutical education, a total of 6 articles were retrieved. Regarding instructional design and assessment, two examples were found, in Pharmacy (Bouldin et al., 2006) and Medicine (Chretien et al., 2008), respectively. Bouldin et al., (2006) applied blogs to a reflective journaling exercise in a communication course during the second-professional year at the University of Mississippi School of Pharmacy, in order to encourage students to reflect on course concepts and apply them to environments outside the classroom, and to assess their communication performance. Seventy-eight students enrolled in the study. Two blog entries per week were required for full credit. Blogs were evaluated at three points during the entire term, and at the end of the course, students evaluated the assignment using a survey. Results showed that the assignment contributed to student learning and increased awareness level for approximately 40% of the class, however, there were some limitations. Because of the proximity to the end of the semester, student responses may have been influenced by the total curricular workload; moreover this evaluation was only taken at one point in time.

Chretien et al.(2008) performed a similar study with 91 medical students during a basic medicine clerkship rotation at an academic institution over a one-year period. Students were asked to contribute with reflective postings to the class blog during their rotation (each rotation was four weeks long). They were able to read each other's postings and leave feedback in a comment section. An instructor provided feedback on entries, aimed to stimulate further reflection. Students could choose anonymous names if desired. One hundred and seventy seven posts were written, and only a minority was not reflective. The instructor's feedback stimulated additional reflection and one-third of students left feedback comments. Both studies show that it is possible to foster reflective thinking by the use of blogs in such different biomedical areas as Pharmacy and Medicine and even provide opportunities to support student professional development. It is notable that the number of students in each study is quite considerable, a quality not often seen in these types of studies. However, the number of tutors /instructor wasn't proportional, and this caused an incomplete and not as frequent feedback. Additionally, both studies were conducted in only one institution, which may limit generalization. Nevertheless, as instructional design and assessment studies, these studies show and describe thoroughly the process by which any educator can implement and assess the opportunities promoted by blogs in their classrooms, or even during clinical practice.

Regarding the survey type study, Goldman et al (2008) evaluated whether "seminar blogs" enhanced learning in a graduate-level introductory public health school class. An online survey was conducted and the main questions were about blog use, perceived impact on learning, and interactions with classmates. The survey also asked about the "ease of blogging" compared with speaking in class, the development of group dynamics, potential usefulness of blogging in student's work, and advantages and disadvantages of seminar blogs. Results generally favor the study objective, however several limitations are addressed. First of all, the study was conducted in a post-graduate setting, where subject's age and maturity may (or

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may not) contribute for the study objectives. As referred by the authors, the short time frame (8 weeks) may have been too brief to fully determine the developing of group dynamics or social presence. And finally we should keep in mind that this a descriptive study based on student self-report of effects on learning and participation and only with a comparison group would we be able to assess the impact of the blogs on specific measurable educational outcomes.

Oomen-Early and Burke (2007) also conducted a survey to explore faculty and students' perceptions of blogging as an effective teaching and learning tool within the online health education classroom. The researchers measured if blogging increased students' interaction, engagement, and feelings of connectedness with peers. Results are favorable to the study objective, but again we are dealing with a small sample of students. This study, shows however a great deal of care in developing a survey (validated by expert and tested in a group of students), and points out several limitations and challenges regarding the use of online free blog hosting services. Using a much more robust study design Fischer et al. (2011), conducted a comparative study of student reflective writings, produced using either a blog format or a traditional essay to assess differences in content, depth of reflection and student preference. Students were quasi-experimentally assigned to one of the two groups, and although no significant difference was determined in themes addressed, or levels of reflection, the authors showed that it's possible, and quite achievable to promote the use of blogs in biomedical teaching. The study suggests that the use of blogs, in no way diminishes the ability for reflective writing. Moreover the fact that blogs support features such as, image, sound and video, can in fact enhance the participation and may even encourage those who feel greater reluctance in participate in these reflective activities. Nevertheless one should not draw hasty conclusions, since more studies in different knowledge areas, with a greater number of students and over a longer period than are necessary.

We also came across with a qualitative study, conducted by Melissa Tan et al. (2010) who aimed to determine the impact of blogging on the clinical reasoning of physiotherapy students. With a sample of 83 students, the authors divided them randomly in 8 groups. Each group had a tutor/moderator (faculty member), and all students were required to contribute to and participate in reflective practice discussions within their blog group for the duration of their clinical program with at least one post and comments on two of their group members' original posts per week. Data was retrieved and analyzed using NVivo®. About 475 blog posts were analyzed. Results demonstrated that the students applied a range of clinical reasoning skills categorized as collaborative; diagnostic; ethical; interactive; and procedural reasoning. Of these, 90% were procedural, ethical and interactive reasoning. The authors concluded, even though the students were dispersed across facilities and changed locations every four-five weeks, that blogging provided these physiotherapy students with opportunities to explore clinical reasoning dilemmas, in a safe space. The blog enabled the students to maintain connections with their peers and supported them in their fieldwork practice. This apparently promoted the development of metacognition at both an individual and collective level. This research indicates a promising result, that blogging can be used to promote learner self-discovery and to assist self-regulated learning in biomedical science. Evidence suggests that blogs help to create connections between students with diverse opinions and interests. This encourages critical thinking and teaches the value of respect towards other students' points of view. Discussions in blogs may also promote higher levels of thinking, because people can think before answering back. They can be used for individual students, or for groups, fostering collaborative learning. Some limitations were however addressed by the authors, of the studies reviewed, but they were attained as ways to improve the experience, and none of them could theoretically diminish the main conclusions.

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Wikis

Wikis are increasingly being used as means of sharing and communicating medical knowledge. The Medpedia Project, is a good example of an up-to-date, unbiased medical information repository, maintained by health experts from all around the world, and of free access (Park, Crocker, Nussey, Springate, & Hutchings, 2010). The use of wikis is quite common in other educational levels (K-12). But their potential is already being discovered by faculty members. Wikis (as tools for group authoring and collaboration) have substantial applications. Often group members collaborate on a document by emailing to each member of the group a file that each person edits on their computer, and some attempt is then made to coordinate the edits so that everyone's work is equally represented; using a wiki pulls the group members together and enables them to build and edit the document on a single, wiki page (Parker & Chao, 2007). Students can even use a wiki to develop research projects, with the wiki serving as ongoing documentation of their work, build a collaborative annotated bibliography or develop map concepts. Wikis can also be useful for brainstorming, and editing a given topic, to produce a linked network of resources. Teachers can also use wikis as a knowledge base, enabling them to share reflections and thoughts regarding teaching practices, documentation or presentation tool in place of conventional software.

The use of wikis alone, is one of the most documented, with a total of 10 articles retrieved, ranging from theoretical works (Younger, 2010), to instructional design and assessments' (Miller et al., 2009; Ros-Rodriguez et al., 2011; Boutin & Therriault, 2011), surveys (Mirk et al., 2010; Miller et al., 2012) study protocols (Archambault et al., 2010) and mixed studies (Varga-Atkins et al., 2010). There are even studies associating Wikis to Blogs (Billings, 2009; Sandars et al., 2008) and Podcast (Mathieu, 2007; Boulos et al., 2006) however, all of them are theoretical works, in several biomedical areas. Even teachers are discussing the use of wikis to promote and update study materials for their students (Rodríguez, Carrillo, & Alvarez, 2010). Focusing on instructional design and assessments', Miller et al. (2009) described their use of wikis in Advanced Pharmacy Practice, with an overall student answers with more 90% of respondents either strongly agreeing or agreeing that wikis helped reinforce concepts and ideas and that they enjoyed working with wikis. Within the same study type, Ros-Rodriguez et al. (2011) recently published their experience in the development of a Virtual Classroom of Pharmacology. Using wiki utilities the students had to transform an initial pdf outline to a wiki format. The students had to create or edit one wiki for each lesson outline and to include hyperlinks for the main concepts to additional material located in the "Virtual Classroom of Pharmacology" and had also to create a glossary with those terms. The student involvement in the task led them to relate, integrate and assimilate the knowledge about those particular aspects of pharmacology. Besides, the authors conclude that the generation of these materials reinforced the learning and promoted work as a team. Also in instructional design and assessments, Boutin & Therriault (2011) presented a pedagogical experience in an Occupational Therapy program that used a wiki to enable the learner to become a" knowledge producer and actor of hi/her learning"(Boutin & Therriault, 2011). The authors targeted three courses, each with different objectives intended for the wiki. Following application of the wiki, the degree of satisfaction of students and professors and the impact thereof on the acquisition of skills was evaluated through an online survey. The published results, from both student and faculty, seem to indicate that using a wiki as a learning tool contributes to the acquisition of knowledge and the development of skills in this new competency-based program of occupational therapy.

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On a different approach, Llambí et al. (2011) developed a quasi-experimental model in which they designed and implemented a blended-learning course on tobacco cessation. Three hundred thirty-five health professionals participated in the course. Of these, 145 attended the on-site workshop, 216 participated in the online activities, and 109 completed both phases. When comparing pre and post test scores, the authors found a statistically significant difference among those who completed both phases compared with those who only did the online phase ($p = .003$ and $p = .009$, respectively), demonstrating that wikis, could be a useful tool in promoting continuing education activities for health professionals. We must however mention that tests were completed by a minority of the participants, since fewer than half performed the pretest and fewer than a third completed both and allowed paired analysis. The fact that tests were not compulsory became a weakness of this study. Using a mixed approach, Varga-Atkins et al. (2010) tried to discover whether the use of wikis could enhance medical students' development of professionalism. Using both survey and interviews, 32 students were questioned. The authors described that the wikis helped students finding, compiling, evaluating and sharing resources related to their learning objectives on professionalism. Secondly, it was demonstrated by the study that the online wiki spaces could have a role in enhancing students' development of "professionalism in a wider sense". Although, using quite a small sample and limited time span, this is a pioneer study, which can be a launch base for more broad research in several subjects of biomedical education. More outcomes are expected as Archambault et al. (2010) develops also a mixed study on the use of wikis. The authors published the study protocol for the mixed study, that aims to develop and test the metrological qualities of a questionnaire for the assessment of healthcare professionals' intentions and the determinants of those intentions to use wiki-based reminders that promote best practices in trauma care. This promising work may contribute even further for the use of wikis in biomedical and health education.

The only survey type study regarding wikis in biomedical education at this point is the research conducted by Woulfe et al. (2009). The authors applied a "wiki approach" to Master of Pharmacy students at The University of Sydney. Normally these students were required to submit a series of group-generated PBL cases. However faculty members often reported a fragmented approach and a lacking in "patient-focus". After a successful pilot study in 2007, the authors introduced a wiki format, with a view to encourage a whole patient approach to the task. A total of 38 students were divided into 6 tutorial groups in each semester, producing one PBL case summary per group per week for a total of 10 weeks. In the first semester, students were required to submit their case summaries on paper. In the second Semester, they were required to write their case summaries in a wiki. The results are not favorable to the study objective. The technical difficulties reported by students appear to have greatly affected the quality of their case summaries, as well as influenced their perceptions of the wiki. This increases the awareness of educators for the necessity for training in the use of the wikis, before instructional changes.

We also introduced a "wiki iniciative" in our own pharmacotherapy course (Jesus, Gomes, & Cruz, 2012, 2013), mainly as an e-portfolio for the students, in Wikispaces. The choice of platform was based on the capabilities provided by the same level of editing (integrating text, images, links, videos, audio, documents, presentations and other features embed), the issue of privacy and access and log edits, and you can follow in detail the contributions of each group member. Students should produce a new page for each case would be presented with only one case per week. In total 20 were developed e-portfolios with different characteristics. A systematic analysis of the contributions made by the students allowed the observation of commitment and motivation from them and formed the basis of a quantitative evaluation.

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Microblogging

Microblogging is the practice of posting small pieces of digital content on the Internet (EDUCAUSE, 2009). Microblogging has become popular among groups of friends and even professional colleagues who frequently update content and follow each other's posts, creating a sense of online community (EDUCAUSE, 2009). Although literature about this Web 2.0 tool is still in its infancy, we uncovered two theoretical studies in nursing education(Skiba, 2008; Bristol, 2010), and four studies relating the use of Twitter® in Pharmacy Education (Fox & Varadarajan, 2011; Bussières et al., 2012; Camiel et al., 2014b, 2014a). The first study describes implementation and effectiveness assessment of Twitter® use, in encouraging interaction between faculty members, guests, and students in a pharmacy course taught simultaneously on 2 campuses. Students were required to tweet a minimum of 10 times over several class sessions. The course instructor and guest professionals also participated but no limit of tweets was established for them. One hundred forty-three students were enrolled in the course: 119 in one campus and 24 at the satellite campus. Course requirements were the same across campuses. The Twitter® activity, was worth 2% of the grade. Students were also given an opportunity to earn 5 bonus points by completing an online, anonymous evaluation of the Twitter® activity. The results presented by Fox and Varadarajan (2011), indicate that more than eighteen hundred tweets were made by students, guests and the instructor. Students tweeted most frequently with each other and found value in reading each other's tweets. From the 131 students that completed the optional evaluation survey 71% percent indicated that Twitter® was distracting, 69% believed it prevented note taking, and more than 80% indicated that it facilitated class participation and allowed an opportunity to voice opinions. These results led the researchers to conclude that the use of Twitter® in pharmacy courses (or any other biomedical course) balanced the potentially positive aspects of the technology, such as increased interaction among students, with potentially negative aspects, such as the interruptive nature of Twitter® use and the large volume of tweets generated by assignment. Far from extolling the benefits of Twitter®, this study shows clearly and objectively the results obtained, even if they do not translate into a steadfast discovery the potential of microblogging. The study design, the number of professionals involved and the methodological description shows a high robustness, which can definitely be a starting point for other researches or experiments in the biomedical classroom.

In 2012, Bussières and colleagues (2012), examined the use of Twitter® in a first-year pharmacy law course, as a backchannel (an electronic discussion that occurs simultaneously in real time during a lecture or conference where students may post questions, comments, or respond to other posts). The authors compared the frequency of questions either through Twitter® or hand-raising. Of 200 students, only 8 asked questions using Twitter® vs 30 questions with hand-raising. Despite the authors' feelings that the use of Twitter® was a failure, students reported that backchannel allowed those students to express themselves who otherwise might not have. The idea of using Twitter® as a backchannel was also pursued by Camiel and colleagues (2014a). In their paper, the authors describe the use of Twitter® in a "large required, lecture-based, team-taught nonprescription drugs/self-care products course". The students were remineded students at the beginning of each lecture that backchannel was being used. The feed was continuasly monitored, and after class, responses to unanswered questions were posted on the course learning-management system discussion board. The author promoted a survey at the end of the year, where 266 students completed an anonymous voluntary survey, where thirty-nine percent (and 40% neutral) felt that being anonymous to their classmates was important to them. In their written

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comments, a number of students commented positively about not needing to raise their hand to ask a question in front of the entire class.

In more recent article, the authors (Camiel et al., 2014b) explore the use of Twitter® for the building of a personal learning network. Although the initial results showed a high percentage of students (36%) that were not confortable using Twitter, and even considering that the use of a personal learning network was a new concept, the final results were very promising, with more than 50% students planning to continue using Twitter® as a social network.

One must consider that as diverse as microblogging may be, it has some shortcomings. Although microblogging promotes writing and editing as fun activities, and therefore may be used in informal learning (Ebner, Lienhardt, Rohs, & Meyer, 2010) there is the possibility that students get distracted and carried away during ongoing lessons due to microblogging with their mobile phones. Not only is this addiction harmful to their education but it is also disrespectful to the teachers or lectures. Further, educators insist that due to microblogs being limited to adopting 140 characters for message updates, it could potentially lead to bad grammar usage by students (Grosseck & Holotesku, 2008).

Google Apps and Similars

The use of Google Apps or other similar applications isn't particularly widespread. Only one study (Phadtare et al., 2009), using GoogleDocs® was retrieved. With a heterogeneous sample of 48 participants (from a medical, nursing and physiotherapy background from US and Brazil) randomly assigned to two groups, the authors proposed to compare on-line vs. traditional classroom-based methods for teaching scientific writing. In the on-line group, participants used virtual communication, GoogleDocs® and standard writing templates, and in the standard group participants received standard instruction without the aid of virtual communication and writing templates. Outcome variables consisted of manuscript quality and self-reported participant satisfaction. The quality of each manuscript was evaluated according to well-defined parameters using a scale. The obtained results suggest that the on-line scientific writing group performed significantly better than the standard writing guidance group in terms of writing quality. They also reported greater overall satisfaction and a greater number of participant-mentor communication events. The authors agree that the use of GoogleDocs® enhanced participants' familiarity with this method of collaboration, as well as improved the mentors' efficiency. Although being a study with several statistical procedures and high strength study design, Phadtare et al. (2009) also address the problems associated with using imperfect measurement scales, which can be prone to subjective bias. The authors also reflected on the lack of tools to objectively evaluate manuscript quality and participants' self-assessment, which makes it difficult to interpret the results of this type of study. Several other factors are also pointed out, as possible influences, including participants' previous experience with on-line courses, their grades, computer competency, and interaction with the system in question, as well as the instructor skills and the presence or absence of supervision.

Virtual Worlds

Virtual worlds, such as Second Life®, are described as online computer-based simulations where the user is given the impression of being in another place/location through replications of real life objects(Honey et al., 2009). Second Life® enables learners to manipulate information and synchronously interact with other people, via a digital representation, also known as an avatar (Rogers, 2009). Virtual worlds, like

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Second Life®, have the ability to create an artificial social structure where problem-based scenarios can be created and developed, allowing students to actively (co) construct mental models of technical and interpersonal skills (Dickey, 2005). While some literature discusses the uses of Second Life® in positive terms (Boulos et al., 2007; Kirriemuir, 2008, Hansen et al., 2009) citing its ability to support interaction and collaborative learning there is little research about actual projects. Our research retrieved one instructional design and assessment study, one qualitative study and one survey type study. Rogers, (2009) proposed to discover how virtual world simulations can assist nursing students developing characteristics and skills essential to future roles as healthcare professionals. In order to accomplish his goal, the author compiled a series of investigative interviews that researched the attitudes and experiences of a sample of sixteen nursing students enrolled in a Bachelor of Nursing Program, who were previously exposed to six simulated clinical scenarios created in Second Life®. Students were placed into groups based only on their year level and exposed to the simulation in separate locations to replicate the intended purpose of the simulation (Rogers, 2009). The students agreed that the simulation in Second Life® assisted them in the development of technical and non-technical skills and in most cases the students found the technology quite easy to use. Also, the online simulation enabled the students to collaborate and solve the scenarios as a team (even though they were isolated in the real world), allowing students to actively co-construct technical and interpersonal skills (Rogers, 2009).

Also in nursing education Honey et al. (2009) conducted a pilot study based around a simulation of a woman having a postpartum hemorrhage. The focus of the scenario was on recognizing abnormal presentation in a postpartum assessment, nursing interventions for hemorrhage, and communication among health care professionals. Although the project led to significant publication on technical issues and simulation development in Second Life, results about student's participation and assessment are scarce and insufficient for a detailed analysis. With a more structured approach, but with a different objective Wiecha et al (2010), proposed, designed and delivered a pilot postgraduate medical education program in Second Life. They enrolled and trained 14 primary care physicians in an hour-long, highly interactive event in the virtual world on the topic of type 2 diabetes. Participants completed surveys to measure change in confidence and performance on test cases to assess learning. The post survey also assessed participants' attitudes toward the virtual learning environment. On a seven-point Likert scale, participants' mean reported confidence increased from pre to post online event with respect to insulin selection, initiation and dose adjustment on test cases, the percent of participants providing a correct insulin initiation plan increased from 60% pre to 90% post. All participants agreed that this experience in Second Life® was an effective method of medical education, that the virtual world approach was superior to other methods of online education. Although these last conclusions are in fact favorable, most of the results retrieved show that insufficient evidence is still available about the potential of Virtual Worlds in Biomedical education.

There are also potential problems with using a virtual world as an educational tool. It's not intuitive, it contains many types of environments, some of which are inappropriate for educational and professional uses. Non-student avatars may appear in student locations, and some of those avatars can manipulate and even act aggressively toward others. Nevertheless, where problems arise, the teacher must see ways to overcome them. The careful planning of activities, and an on-demand technical support, are undoubtedly two features to keep in mind while developing an online educational experience in virtual worlds. Further research is also necessary to investigate the attitudes of students towards technology-supported learning and to identify and further explain links between the students' personal online practices and

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their experiences in Virtual Worlds. Moreover it is advisable to demonstrate real, quantifiable outcomes (with large number of students) of these types of interventions in order to objectively assess their impact on student learning.

FUTURE RESEARCH DIRECTIONS

Despite the great deal of opportunities for the implementation of Web 2.0 applications within higher education, there are several challenges that are not permitting a widespread use. These include finding faculty members who are willing to accept change and adopt new pedagogical approaches. Increasing faculty members' acceptance of these new tools may require efforts at improving the perceived usefulness and benefits of the applications (Cain & Fox, 2009). Institutions have great deal of power on these challenges, and the role of leadership and management is crucial in promoting short-term workshops/seminars to increase informatics literacy and promote pedagogical innovations/adaptations. Another aspect to consider, especially in survey studies is standardization. Standardization provides survey research with some of its great strengths, because standardization makes it possible to seriously consider surveys as measurement tools (Beatty, 2001). Studying students is always difficult and always involves subjectivity, but the potential for measurement gives survey research great scientific credibility. It also allows for efficiency in handling data, provides for clear quality control criteria, and minimizes random error introduced by the interviewer (Beatty, 2001). Validating these instruments can take long periods of time but it will benefit outcomes and the validity of the conclusions.

All the conclusions and results presented in this chapter are about professionals who are trained in several types of methodologies and study designs. They must know how to perform and assess studies like clinical trials and other experimental studies that have strong design strength. Similarly, studies performed in order to assess the impact of Web 2.0 tools in biomedical and pharmaceutical education need to follow the same principle. We are aware that we cannot be so straightforward in Educational Sciences, but there are options and ways to better control and predict threats to the study conclusions, giving more strength to our results. IDEAS studies and surveys have their role in creating evidence, but quasi-experimental studies and qualitative studies can provide a stronger, wider and more reliable research. The privacy issue has also arisen during the use of Web 2.0 tools, with focus in wikis and blogs. The issue can be easily controlled, if blogs are password protected, or accessed by invitation only, and if wikis are developed inside LMS Systems, like Moodle, and accessible only to members. This will also avoid vandalism and promote student participation (Boulos et al., 2006). Nevertheless an active tutor role is required in order to readily access, edit or comment on new materials posted by students. Last but not least, the technical difficulties felt by students in some of the studies referred are an alert that, although we live in a society of information, maybe we need a little more care in educating our students about these technologies in order to truly benefit from them.

Indeed, Web 2.0 tools and applications have a powerful place in instruction and in the classroom, but caution is warranted regarding the strategies used. Instead of beginning instructional planning with these tools in mind, the learning objectives and instructional strategies should guide the adoption process. An awareness of the different tools, gives faculty members additional mechanisms from which to choose, but selecting an appropriate instructional strategy is even more important.

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KEY TERMS AND DEFINITIONS

Blog: A kind of journal published on the World Wide Web consisting of posts typically displayed in reverse chronological order so the most recent post appears first. Blogs are usually the work of a single individual, occasionally of a small group, and often are themed on a single subject.

Microblogging: Microblogging is the practice of posting small pieces of digital content on the Internet.

Podcast: A type of digital media consisting of an episodic series of audio files subscribed to and downloaded through web syndication or streamed online to a computer or mobile device.

Virtual Worlds: Described as online computer-based simulations where the user is given the impression of being in another place/location through replications of real life objects.

Vodcast: A type of digital media consisting of an episodic series of video files subscribed to and downloaded through web syndication or streamed online to a computer or mobile device.

Web 2.0: Web 2.0 is a loosely defined intersection of web application features that facilitate participatory information sharing, interoperability, and user-centered design.

Wiki: A website whose users can add, modify, or delete its content via a web browser using a simplified markup language or a rich-text editor. Most are created collaboratively.

ENDNOTE

[1] Coutinho (2008) proposes four categories: theoretical/ reflection, empirical, reports of educational experiences and study protocols. Poirier et al. (2009) presents a more structured approach to reports of educational experiences – called Instructional Design and Assessment Studies (IDEAS).

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Chapter 5
Open Access Initiatives in Medical Biology: A Study of Institutional Repositories in India

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ABSTRACT

Open Access (OA) is reshaping the world by redefining the scholarly communication methods with focus on building a knowledge society. It has the power to democratize the knowledge by removing hurdles from free access to scholarly works while encouraging knowledge sharing. Institutional Repositories (IRs) play a vital role in the OA movement by facilitating the Green Route to Open Access. In India, some elite educational and research institutes such as the Indian Statistical Institute, some CSIR Laboratories, IITs, and IIMs have taken significant initiatives in building IRs. In addition to theses, a few Universities have also taken the initiative and now have their own IRs as their proudest possession. However, many Indian Irs, once functional are in bad shape and a few have been closed. This paper deals with IR initiatives in the discipline of Medicine and Biology including. The paper discusses their scope, collection strength, growth rate and their current status.

1. INTRODUCTION

The journey of man from a nomadic society to knowledge society is very interesting and perhaps never ending. Free flow of information has played a pivotal role in this journey. But the journey is still incomplete as there exits a thirst – thirst for knowledge and is still unquenched. To facilitate a free flow of information, especially scholarly information is very crucial. At the same time it is a challenge as financial aspects are also involved in this. Scholarly communication has the potential to make this world a better place to live in as it is based upon the philosophy of equal access to knowledge. Open Access is augmenting and enriching the scholarly communication cycle and making the world a global village.

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Scholarly communication can be understood as the system through which research and other scholarly works are created, evaluated, disseminated to the scholarly community, and preserved for future use. It is the cyclical process It is a cyclical process in which content is generated, reviewed, disseminated, acquired, preserved, discovered, accessed, and assimilated for the advancement of scholarship. The assimilation can potentially lead to generation of new content and thus start a new iteration of the process (or life cycle).

2. OPEN ACCESS AND THE TWINS

Open access (OA) means unrestricted online access to peer-reviewed scholarly research. Open access is primarily intended for scholarly journal articles, but is also provided for a growing number of theses, book chapters, and scholarly monographs. Open access comes in two degrees: gratis OA, which is free online access, and libre OA, which is free online access plus some additional usage rights.

The Budapest statement defined open access as - "There are many degrees and kinds of wider and easier access to this literature. By 'open access' to this literature, we mean its free availability on the public internet, permitting any users to read, download, copy, distribute, print, search, or link to the full texts of these articles, crawl them for indexing, pass them as data to software, or use them for any other lawful purpose, without financial, legal, or technical barriers other than those inseparable from gaining access to the internet itself. The only constraint on reproduction and distribution, and the only role for copyright in this domain, should be to give authors control over the integrity of their work and the right to be properly acknowledged and cited."

The Bethesda and Berlin statements add that for a work to be open access, users must be able to "copy, use, distribute, transmit and display the work publicly and to make and distribute derivative works, in any digital medium for any responsible purpose, subject to proper attribution of authorship."

The two ways authors can provide OA are Green and Golden Routes/Roads. In green OA, authors self-archive their journal articles in an OA repository (IR). In the Golden OA approach, the author decides to submit the research papers in a selected repository, making it freely available over the world wide web. In Gold Open Access, the end users i.e. The readers are not supposed to pay any subscription cost, but the author has to pay the Article Processing Charges (APCs). The third approach, which is also becoming an accepted model is Hybrid OA which is most commonly associated with Gold Open Access. In this OA model is a mix of subscription charges and publication fees. In this model, some articles are available only to subscribers, while others are made available at no charge to anyone searching the web. Authors pay an additional fee for the open access option. They may do this because open access is a requirement of their research funding agency. Or they may do it so that non-subscribers can access their article for free. In this model, only the articles for which the authors have covered APCs are available for free.

According to Bo-Christer "in just over two years [2009-2012] the number of journals from major publishers offering hybrid Open Access has more than doubled, from approximately 2,000 to over 4,400. Since the overall numbers of journals from these publishers has remained on the same level, the hybrid share has risen from 25% to around 50% of all eligible journals."

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3. PHARMACOLOGY

Pharmacology is the branch of medicine and biology concerned with the study of drug action, (Vallance P & Smart TG, January 2006) where a drug can be broadly defined as any man-made, natural, or endogenous (within the body) molecule which exerts a biochemical and/or physiological effect on the cell, tissue, organ, or organism. More specifically, it is the study of the interactions that occur between a living organism and chemicals that affect normal or abnormal biochemical function. If substances have medicinal properties, they are considered pharmaceuticals. IUPAC defines pharmacology as Science of drugs, including their origin, composition, pharmacokinetics, pharmacodynamics, therapeutic use, and toxicology.

4. RESEARCH PROBLEM

New technologies, specifically ICT, and innovative models are enhancing the shared model of scholarly information. The shared literature may include research papers, primary data and other evidence, creative activity and other products of research and scholarship including across institutions and audiences. However, on account of the exponential price rise in journal subscription, libraries, especially in underdeveloped and developing countries were finding it difficult to serve their readers adequately and qualitatively. They are still finding their budget either shrinking or stagnant. The problem is further aggravated as the funders and taxpayers do not have access to the research they pay for. Producing a research paper may require time and money. Scholars are devoid of scholarly content and open access to scholarly literature is the only solution to this problem. Also, there is need of conducting research in the discipline of Medicine and Biology repositories in India.

Figure 1. France circa 1913: R. Dubois anesthetizing machine
(Photo by Boyer/Roger Viollet/Getty Images).



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5. LITERATURE REVIEW

Halliday (2001) in his paper titled "Scholarly Communication, Scholarly Publication and the Status of Emerging Formats," explored the role of scholarly communication and provided a working definition of scholarly publication consisting of a list of criteria, which may be used to analyse the degree to which emerging formats can be categorized as scholarly publications. The paper also helped to identify the means by which they may be supplemented so that their status may be promoted to that of the 'scholarly publication.'

Ghosh (2006) in his paper "Open Access and Institutional Repositories: A Developing Country Perspective – A Case Study of India," discussed the concept of scholarly communication as a method wherein the author facilitates the availability and distribution of scholarly communication freely, as a means and effort to solve the problem of inaccessibility, primarily due to financial constraints, particularly in the developing countries. He mentioned that in India there has been a gradual realization of the usefulness of open access among various institutions. Various Open Access Initiatives have been undertaken and are operational. Many are in the developmental stage. Some Initiatives have also been taken in the area of metadata harvesting services, particularly publicly funded ones. He claimed that the future of open access in India is dependent upon a proper policy and developing a proper framework: in the implementation of open access, LIS Professionals should play a proactive role in the growth of collections in Institutional Repositories. The paper provides an overview about the present state of Open Access Initiatives by various institutions of the country.

Koganuramath (2006) discussed the future plans towards implementation of Institutional Repositories in JNU Central Library and also described some of the available Institutional Repository (IR) software. He stressed that IR is the one and only way to make the whole world know the esteem of a university. Being a premier university in teaching and research programmes, IR aims to manage networked information services, research work for benefits of faculty, students, and research scholars.

Chakravarty (2006) in the paper titled "Institutional Repositories: A Perspective for the Universities," discussed the current situation of scholarly communication. According to him, the world is witnessing a sea change in the area of scholarly communication. Perhaps the control over scholarly communication has started a gradual shift from commercial publishers to academic organizations and many authors initiatives in the area of Open Access. OA is perhaps opening up the major barriers that higher education institutes and libraries face, especially when it comes to escalating journal prices and shrinking budgets. Institutional Repositories are one of the two most powerful tools to empower and strengthen the open access movement. Universities and other academic institutions of the developed countries are already reaping the rich benefits of IRs. He emphasizes that the technology is free, the software is available free of cost, and the universities are also having the necessary infrastructure for implementing institutional repositories at their premises. The only required link that is missing is the awareness and willingness. He argues that it is high time that Indian universities make a decision and a strong commitment to develop institutional repositories and convince the faculty members and research scholars to deposit papers in digital archives. He also considered that IR may also contain learning objects in digital formats, thus facilitating IT-enabled pedagogy in Indian Universities.

Rao (2007) in the paper "Institutional Repository: A Key Role for Libraries," mentioned that institutional repository is a digital archive, owned and maintained at institutional level. It is a tool for collecting, storing, and disseminating information to advance scholarly communication. This paper also mentioned essential elements of institutional repositories. He also discussed the challenges for creating

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institutional repositories at a national level. The key role of the libraries in successfully implementing the institutional repositories is discussed.

Vishala (2007) in the article "Building Institutional Repository: Role of the Library" emphasized in an intellectual life and output of an institution. Institutional repositories are being recognized as an essential infrastructure for scholarship in the digital world. The paper seeks to provide an overview of institutional repositories and their benefits to the institutions and also describes the role of library in building institutional repositories.

Singh (2007) in this article titled "Open Source Software: A Comparative Study of Greenstone and DSpace," provided the information that software is available free of cost with source code for anyone to use. Open source software reduces the cost of creating digitization. Among open source software, Greenstone and DSpace are becoming more popular in India. This paper deals with comparisons of these two popular open source softwares. This paper helps the professionals who are planning to create and help the professionals who are planning to create an institutional repository.

Potel (2007) in his paper titled "Institutional Digital Repositories/e-Archives: INFLIBNET Initiatives in India," the author describes the technological advances, storing knowledge in less-expensive ways to create institutional preservations. INFLIBNET (Information and Library Network centre) decided to opt for DSpace for institutional repository and archive its publications, conference proceedings, and lecture notes. Tghe paer narrated the practical experiences and provides an overview of INFLIBNET's Institutional Repository and Digital Archive-India developed for the Indian academic and research community to archive their intellectual work.

Gyatri (2007) in her paper titled "Sharing Digital Resources with an Institutional Repository," emphasised on the need for storing, reusing, and sharing these digital information resources for knowledge sharing and conservation of time and effort. She discussed the concept of IR as "collection of digital objects" is where digital content assets are stored, searched, and retrieved for later use. Institutional repositories are being developed by academic institutions for the purpose of knowledge sharing, reuse, and preservation. This paper discusses the DSpace open source digital repository software, implemented at ICFAI Business School, Ahmadabad, and highlighted the need for information sharing.

Khan (2008) in his paper titled "An Assessment on Present Situation of Institutional Digital Repositories in India: A Study," highlighted the present status of Institutional Repository (IR) in India by its collection type, subject coverage, and total number of digital repository collection available to the academic community as open sources. For this purpose, the directory of Open Access Repositories (Open DOAR) and the Registry of Open Access Repositories (ROAR) have been consulted. This paper focuses to develop humanities and social sciences, institutional repositories in India. Results have been shown by collection type and subject coverage.

Nazim (2008) in his paper titled "Open Access Journals and Institutional Repositories: Practical Need and Present Trends in India," described trends in open access publishing in India. Data was collected from directors of Open Access Journals and Institutional Repositories. The URL of each Institutional Repository and Open Access Journal was visited to collect relevant data and included information from earlier studies. Case study method was used to know the trends of open access publishing in India. The data is analysed based on certain parameters, such as number of Institutional Repositories and Open Access Journals, number of documents, software used, types of documents, etc. Among the top 25 Open Access publishing countries, India ranks 12th for the overall number of journals, but drops to 18th for journals with online content. However, its positioning in the list of open access journals is fifth. At

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present, India ranks 12th in the list of countries with registered interoperable archives in the Registry of Open Access Repositories (ROAR).

Kamila (2009) in her paper titled "Institutional Repository Project in India," discussed the concept of the Institutional Repository, its relevance, merits, software requirements, and current trends in India, with special reference to the initiatives at Burdwan University, Burdwan.

Antelman (2010) in his article titled "Do Open-Access Article Have a Greater Research Impact?," Focussed at four disciplines at varying stages of adoption of open access—philosophy, political science, electrical and electronic engineering, and mathematics—to see whether they have a greater impact as measured by citations in the ISI web of science database when their authors make them freely available on the Internet. The finding revealed that, across all four disciplines, freely available articles do have a greater research impact. Shedding light on this category of open-access reveals that scholars in diverse disciplines are adopting open-access practices and being rewarded for it.

Dev (2010) in his article titled "Institutional Repository," urged to create a way for organizations to provide users with a method for self-service preservation and dissemination of digital users with a method for self-service preservation and dissemination of digital information. He found that, many organizations have reported difficulty convincing users to voluntarily deposit material into their repositories. The paper discussed the experience of The University of Rochester and carried out studies of its faculty and students to determine the reasons for their lack of interest in using the repository. Driven by these user needs, the University of Rochester created institutional repository which offers tools for authoring, collaboration, portfolios, and analytics—providing value throughout the authoring and academic lifestyles.

Bhat (2010) in his paper titled "Open Access Repositories in Computer Science and Information Technology: An Evaluation," the author explained evaluation of nine Open Access Repositories in the field of computer science IT. The repositories were identified from the open DOAR, Directory of Open Access Repositories (http://www.opendoar.org/), the findings revealed that most of the repositions are maintained by 1-2 faculty members on a part-time basis. Most of the repositories follow policies for selection of content and submission of documents have provision for withdrawal of content by the authors and voluntary faculty deposit policies. Backups are taken for short-term preservation of the content, but still no strategies for long-term preservation of the content. He observed that authors are responsible for ensuring copyright compliance of their articles. Most repositories have feedback from users. A few repositories provide access statistics. The paper provided recommendations for the establishment and management of open access repositories.

Nazim, M., and Mukherjee, B. (2011). The article discusses the status of institutional repositories (IRs) in Asia, with information on the effect of information communication technologies (ICT) on academic publishing. Topics include the efforts to launch an Open Access (OA) model that utilizes the Internet to broaden access to information resources and publications, the definition of IRs as a platform through which the research output of a specific university or research organization is available, and the growth and distribution of IRs throughout Asian countries based upon the survey results.

Jain, P. (2011). The paper reviewed the recent literature about institutional repositories (IRs) including the benefits and possible obstacles of setting up an IR. It discussed librarians' and authors' participative roles and open access. The findings reveal that in spite of all the obstacles to successful implementation, including associated negative perceptions, IRs have been increasingly recognised as a vital tool for scholarly communication and an important source of institutional visibility and a viable source of institutional knowledge management. The paper aimed at institutions with low-use repositories and at institutions considering initial development of an IR. The paper outlines the implications for IR practice

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for different groups, namely authors, librarians and academic administrative staff. It could, therefore, be used to persuade and influence different sets of stakeholders at institutions with under-populated or embryonic IRs, about the value of open access, the importance of depositing material and the potential functionality afforded by IR packages.

Krishnamurthy, M. M., and Kemparaju, T. D. (2011). The purpose aimed to study the IRs in use in Indian universities and research institutes. Repositories in various institutions in India were accessed and described in a standardised way. The 20 repositories studied covered collections of diverse types. Most of these collections have unique content. Originality/value - The goal of this study is to study the IR software and data based on the content type, metadata and characteristics. The paper also describes the collections and some important observations from this study.

Sawant, S. (2012). The study aimed to investigate various issues concerning the management of institutional repositories (IRs) developed in India. The survey method was used with the help of a web questionnaire. The questionnaire was e-mailed to the entire population i.e., all IRs identified in India. It was observed that 79 per cent of the institutions had used the DSpace IR software package. The respondents considered end-user interface to be the top-ranking IR-system feature. It was found that all IRs supported text (HTML, Postscript, PDF, Spreadsheet etc.) file formats. Half of the respondents marked bitstream copying as a long-term preservation strategy. Almost all institutional repositories were OAI-PMH compliant.. The study identified the existence of 16 functional IRs, some of which were not registered in any of the directories such as ROAR or Open DOAR.

Roy, B., Mukhopadhyay, P., and Biswas, S. (2012). The article presents the results of an analytical study investigating institutional digital repositories (IDRs) in India. An overview of the goals of IDRs and their association with the open access movement has been discussed. It was observed that the IRs are developing at a reasonably rapid pace in India and that they have the potential to be a very useful resource is commented on. Detailed information on the structure, policies, and capabilities of the 60 Indian IDRs studies is also offered and commented on.

Nagra, K. A. (2012). The author observed that IRs are increasingly becoming an essential component of academic institutions. An institutional repository showcase scholarly and research output to the wider community, and significantly helps in institutional advancement and outreach. It has the benefit of sharing and marketing research with others. It also provides an opportunity to raise the profile and brand awareness of an institution, faculty and students to the global community. Building an effective and successful institutional repository for an academic institution requires careful planning and enthusiasm from the institution's community. This article describes the detailed steps based on the review of the literature and the author's experience in creating institutional repositories. The libraries need to make critical decisions and choices before designing or redesigning institutional repositories. Among them are the funding, staffing, technology, metadata, content and operations to build, manage and run the institutional repositories successfully.

(Johnson, 2002; Nagahban, 2010). A study of nine libraries in the National Capital Region of India was conducted to: identify the benefits of IRs, learn the satisfaction level of users with respect to IR facilities, identify the incentives for publication in IRs, and identify the appropriate policies to be adopted by institutions for implementing IRs. The results of the responses from the 496 respondents indicate that: 1) Most Faculty members and Research scholars in this study indicated that they do not publish their research in IRs. 2) Opinions about the benefits of IRs are user-specific. For instance, a substantially larger percentage of those Faculty members and Research scholars in the study who do use IRs are Most satisfied with potential benefits of IR while a relatively higher percentage of Students fall in the Satisfied

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category. 3) The benefits of IRs ranked higher, although this varied by the different user groups was wider readership. Students ranked this and "quality aspect" – the opportunity to improve the quality of one's work through the provision of feedback from other researchers. 4) A serious concern about publishing in IRs is the potential for plagiarism and overall lower control over one's work. A second concern is the potential loss of content in IRs, which are often not archived as well as scholarly journals are. The findings suggest that institutions need to give due attention to policies related to two aspects of publication, especially those related to quality and copyright issues and to the academic value of research output. Other policy-related topics include a citation in another publication with due acknowledgement, inclusion in indexing systems for retrieval, interoperability with other IRs, and Permanent storage.

Haridasan, S., and Rani, K. (2012). The article offers information on the research conducted to evaluate the Institutional Repositories (IRs) initiatives in Indian universities. It mentions that the Electronic Thesis or Dissertation (ETDs) is the most commonly deposited material in IRs. It revealed that Dspace is the commonly used software and English is the most prominent language used by all IRs and the popularity about IRs can be increased through postings in list serves, web search engines and metadata harvesting services.

Fralinger, L., and Bull, J. (2013). The authors attempted to identify factors that might affect the international usage of US IRs as part of assessment efforts to determine an IR's return-on-investment. A survey was disseminated to IR administrators asking for demographic information, international usage counts for website hits and downloads, and any internationalization efforts connected to the IR in order to determine any influencing factors on an IR's international usage. While many IRs reported various rates of international usage, the largest group of respondents did not report an international usage rate for both page hits and downloads, despite overwhelmingly expressing an importance of international traffic to their IR and parent institution. Research limitations/implications – It is not clear if this non-reporting of international usage could be due to ignorance, apathy, or lack of technological support on the part of the IR administrators. Practical implications – Determining international usage as a part of an IR assessment might be problematic or even impossible for many US IRs. Originality/value – This study suggests that many IR administrators either do not know, do not care, and/or cannot record international usage data for their respective IRs, which could hinder determining an international return-on-investment for the IR.

Inefuku, H. W. (2013). In the interest of providing access to and preserving scholarship produced by faculty, students and staff, many universities have developed and implemented institutional repositories. Repositories are often organized into communities that correspond to campus units, including departments, research centres and institutes, and administrative offices. Universities, however, frequently undergo academic restructuring, which can make repository organization out of sync with university organization. This article addresses the impact of academic restructuring on repositories and looks to practices from archival arrangement and description to create repository organizational structures and community descriptions flexible enough to reflect the university organization, despite changes arising from academic restructuring.

Ruiz-Conde, E., and Calderón-Martínez, A. (2014). Are institutional repositories mere warehouses for digital documents or are they in fact establishing themselves as a rigorous option for the spread of scientific knowledge? This study analyses the competitive environment of the Top100 university repositories, defined as leaders in terms of market participation and penetration. The study also analyses the basic functionalities of preservation and diffusion of academic production through factors related to the prestige of the repositories and of the institutions that operate them. The results show that repositories with a larger digital academic supply are associated with the production of demonstrated scientific rigour.

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Nwadiuto Igwe, K. (2014). The paper is a blueprint designed for the development of the country, with emphasis on making it one of the top developed economies by the year 2020. The study showed that knowledge/information (its generation, acquisition, utilization and possible application) was not accorded the due attention as a tool for the vision's success. Added to the above statement is the fact that the research findings and intellectual output of higher education institutions (HEIs) and research-based institutes in the country mostly in the form of grey literature (i.e. Unpublished technical/research reports, manuals, theses and dissertations, monographs, etc.) gather dust in their institutions of origin, with little or no visibility, thereby affecting accessibility, utilization and application. This paper advocated for a national policy for the development and management of open access repositories in institutions, where the research findings and intellectual outputs of these institutions will be domiciled, which will be easily accessible online for utilization and possible application in various sectors of the economy for the realization of vision 20: 2020.

6. INSTITUTIONAL REPOSITORY (IR): AN INTRODUCTION

Clifford Lynch defines IR as " a university-based Institutional Repository is a set of services that a university offers to its members of its community for the management and dissemination of digital materials created by the institution and its community members. It is most essentially an organizational commitment to the stewardship of these digital materials, including long-term preservation where appropriate, as well as organization and access or distribution." Foster and Gibbons describe IR as "an electronic system that captures, preserves, and provides access to the digital work products of a community." Wikipedia defines IR as "an online locus for collecting and preserving in digital form the intellectual output of an institution, particularly a research institution. For a university, this would include materials such as research journals, articles before (pre-prints) and after (post-prints) undergoing peer review and digital versions of theses and dissertations, but it might also include other course notes, or learning objects."

6.1 Types of Material

Types of resource material include blue print, pre-print, post-print, working paper, thesis/dissertation, technical report, conference papers, progress reports, research articles, A-V chips, teaching material, diversity of contents and language used in full texts undergoing peer review and digital versions of course notes. The contents can be classified, restricted, or unrestricted/open.

6.2 Characteristics of Institutional Repositories

- Promote self-archiving.
- Are institutionally defined rather than subject based.
- Have wider distribution.
- Increases dissemination, collect content in a single location.
- Are supported by Scholarly Communication.

The following characteristics of Institutional Repository (IRs) are outlined by Crow (2002) Shearer (2003) and Ware (2004):

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- **Digital:** IR collect mainly digital information. This is different to the mandate of the university archives which is to collect all types of content related to the university
- **Institutionally Defined:** Unlike the traditional discipline-specific and digital libraries, institutional repositories capture the research of the entire population of the institution
- Institutional repositories collect scholarly material exclusively. In this context, scholarship includes (preprints, post prints, technical reports, etc.)
- **Cumulative and Perpetual:** This refers to the long-term nature of data preservation and accessibility through IR
- **Open Access:** A key defining feature of IR is the free and open access to their content
- **Interoperability:** This refers to the fact that IRs can be used by both data providers (who expose data in various forms) and service providers (who process and add value to the data)
- **Self-Archiving:** Most IRs requires the author or someone associated with the author to deposit the content directly.

6.3 Need and Advantage

Institutional Repositories facilitate innovation, wider access, visibility to research output, preserve institutional heritage, reduce publication delay, faster communication, increase citation, strength research, easy access to grey literature, persistent URL, research work archive. While the most popular open source and hosted applications share the advantages that institutional repositories bring to institutions, such as the increased visibility and impact of research output, interoperability and availability of technical support, institutional repository advocates tend to favour open source solutions for the reason that they are by their nature more compatible with the ideology of the freedom and independence of the internet from commercial interests. On the other hand, some institutions opt for outsourced commercial solutions.

In her briefing paper on open access repositories, advocate Alma Swan lists (Swan, Alma. "Open Access institutional repositories: A Briefing Paper". *Open Scholarship*. Retrieved 24 September 2013.) The following as the benefits that repositories bring to institutions:

- Opening up outputs of the institution to a worldwide audience;
- Maximizing the visibility and impact of these outputs as a result;
- Showcasing the institution to interested constituencies – prospective staff, prospective students and other stakeholders;
- Collecting and curating digital output;
- Managing and measuring research and teaching activities;
- Providing a workspace for work-in-progress, and for collaborative or large-scale projects;
- Enabling and encouraging interdisciplinary approaches to research;
- Facilitating the development and sharing of digital teaching materials and aids, and
- Supporting student endeavours, providing access to theses and dissertations and a location for the development of e-portfolios.

The Institutional Repository will:

- Help to develop National Research Repository infrastructure by setting up, populating, and linking individual repositories.

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- Stimulate development of services that draw on research information made available through the repository infrastructure.
- Provide a window that gives open access to improve the sponsoring institution visibility and status.
- Support the Open Access model of publication.

Institutional Repositories are an online locus for collecting, preserving, disseminating in digital form. Institutional Repositories are intellectual research output of an institution. It manages and disseminates its research material created by community members. Librarian plays a role of a leader in planning and building these repositories. They act as experts in the collection, preserve for document and providing digital information.

7. RESEARCH METHODOLOGY

The present study uses *Open*DOAR for generating a list of IRs in the field of Medicine and Biology. *Open*DOAR is a project to provide a comprehensive and authoritative list of repositories categorised by several criteria like country, software, subject, etc. It has two subject categories related to the scope of the present study. One is Biology and Biochemistry and the other is Health and Medicine. Indian IRs under these two subject categories were explored for further study. Eight IRs was listed under each subject category respectively, with minor overlapping. The total number of relevant IRs after combining the results of the two lists and removing the overlapping comes out to be 12. For the purpose of data analysis last five year data were collected from individual repositories.

Table 1. List of Indian IRs in medical biology

SN	Repository Name	No. of Records	Software	Status
1	CMFRI Digital Repository	9661	EPrints	Operational
2	Digital Knowledge Repository of Central Drug Research Institute (CDRI)	796	DSpace	Operational
3	ePrints@NII	10	EPrints	Broken
4	EPrints@NIRT	830	EPrints	Operational
5	Eprints@SBT MKU	89	EPrints	Broken (data till October 2011)
6	Indian Institute of Petroleum (IIP) Institutional Repository	454	DSpace	Broken
7	Institutional Repository@CSIO	311	EPrints	Operational
8	IR@NPL	996	EPrints	Operational
9	Eprints @MDRF	676	Eprints	Operational
10	Open Access Repository: Indian Academy of Sciences	91745	Eprints	Operational
11	KNOOR: Knowledge Repository Open Network	911	DSpace	Operational
12	OpenMED@NIC	2904	Eprints	Operational
Total		**108502**		

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8. DATA ANALYSIS

Out of these twelve repositories, 3 were found to be broken, i.e. non-functional. The Open Access Repository of Indian Academy of Sciences (publications of Fellows) has maximum number of records – 91745. Table 2 also reveals that the total research output under OA by these IRs in Medicine and Biology including Pharmacology is 108502.

Indian IRs has a share of 5.84% of the total IRs in Biology and Biochemistry whereas its contribution is 3.2% in the discipline of Health and Medicine. Table 2 also reveals that Indian IRs in Medicine and Biology at global level is 4.12%.

Figure 2 reveals that 15% Indian IRs are dedicated to Medicine and Biology and 85% are dedicated to other disciplines.

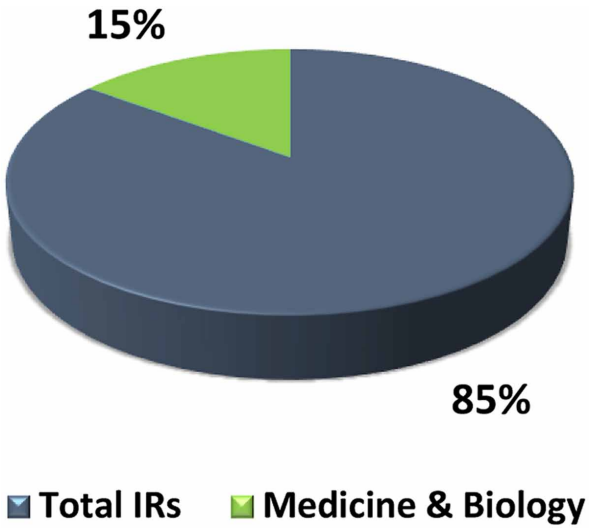
Figure 3 depicts that The Open Access Repository of Indian Academy of Sciences has the highest share of scholarly outputs in Medicine and Biology. While the CMFRI Digital Repository has 9661 records followed by OpenMED@NIC which has only 2904 records, although it specifically deals in Medical Sciences.

DSpace is an open-source digital asset management system originally created by developers from MIT and HP Labs in 2002. EPrints is a free and open-source software package originally developed by researchers at the University of Southampton School of Electronics and Computer Science in 2000 (making it the oldest of the platforms in this report). It was designed specifically for archiving research

Table 2. Share of Indian IRs in medicine and biology at global level

SN	Discipline	World	India
1	Biology and Biochemistry	137	8 (5.84%)
2	Health and Medicine	251	8 (3.2%)
	Total	388	16 (4.12%)

Figure 2. Medicine and biology IRs of India



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Figure 3. Share of scholarly literature by Indian IRs

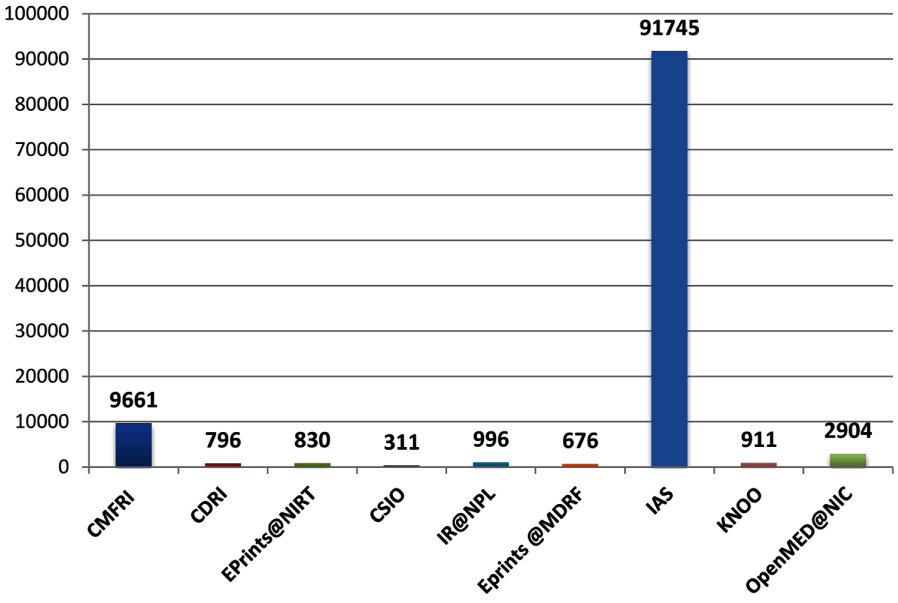
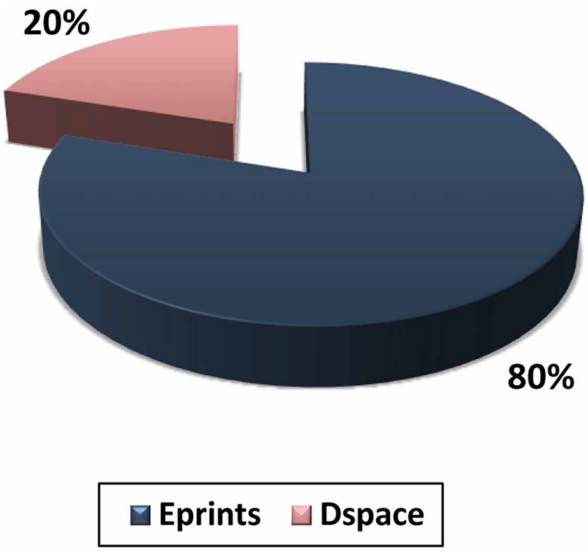


Figure 4. IR software preference



papers, theses and teaching materials, though it can accept any content. The other popular IR software includes Fedora, Figure 4 reveals that 80% of the Indian IRs on selected discipline are running on Eprints software whereas only 20% of the IRs are based on Dspace.

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9. INDIAN IR IN MEDICINE AND BIOLOGY

9.1 Eprints@CMFRI

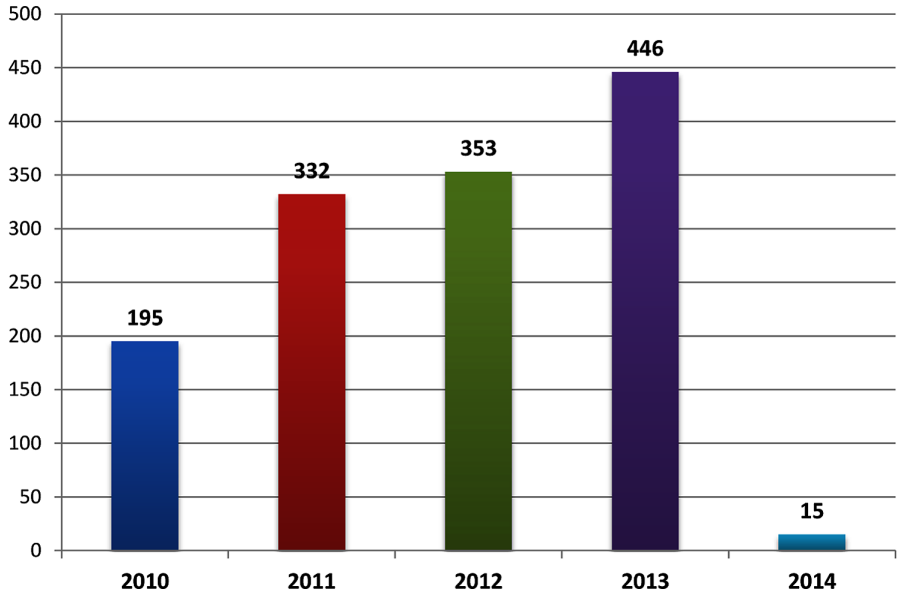
Eprints@CMFRI is the Open Access Institutional Repository of Central Marine Fisheries Research Institute. The research outputs of CMFRI include: journal papers, conference papers, reports, theses, patents etc. - are uploaded/self-archived by CMFRI scientists who do research on fisheries and related areas. Interested users can freely download and use documents as most of them are directly accessible and full-text downloadable. 'Request Copy' forms can be used for documents to which direct full-text download is restricted due to publisher embargo.

Figures 5 and 6 reveal that there is a significant rise in the number of scholarly content from 2011 which continued up to 2012. After July 2012 it has maintained a constant growth rate with the minor upward trend. The figure also reveals that the overall growth rate is over 1737 items per year.

9.2 Digital Knowledge Repository of Central Drug Research Institute (DKR@CDRI)

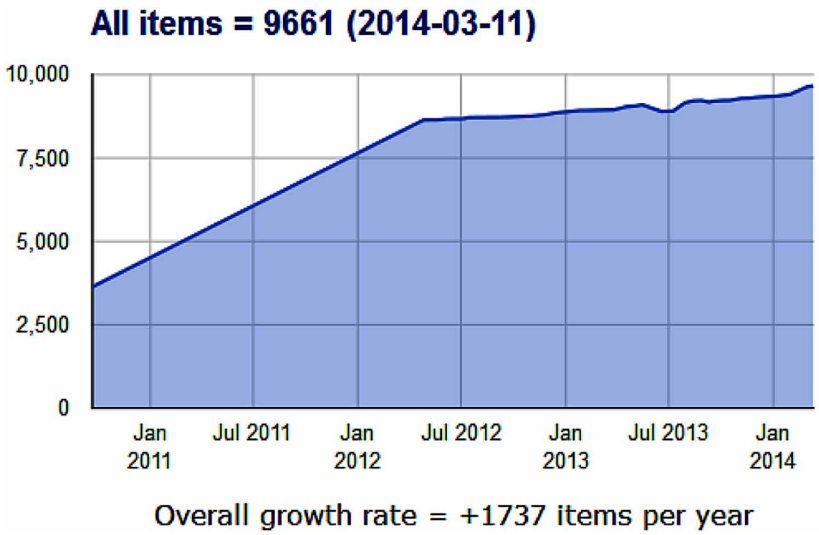
CDRI is considered to be a pioneer multidisciplinary research organization in the field of biomedical research where all the infrastructure and expertise are available to develop a drug right from its concept to market. The very latest techniques and methodologies are employed for developing drugs, diagnostics and vaccines to combat diseases prevalent among mankind in general and Indian population in particular. For administrative and scientific purposes the Institute's manpower has been grouped into 17 R&D divisions and few divisions providing technical and scientific support. In order to carry out work in cohesion and in a focused environment, the research works have been grouped into following six major

Figure 5. Growth rate of CMFRI IR



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Figure 6. Growth statistics of CMFRI IR 2011 onwards

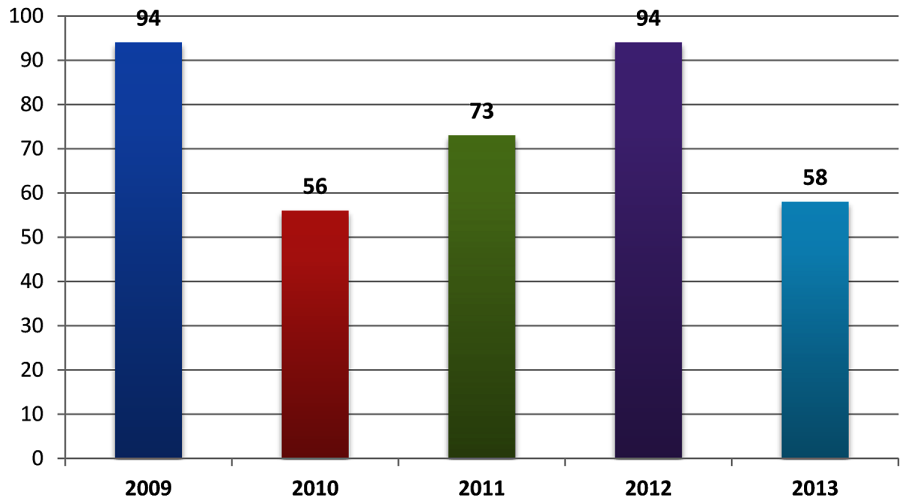


research areas. DKR@CDRI strives to collect, preserve and disseminate different institutional publications (journal articles, conference proceeding articles, technical reports, thesis, dissertations, etc.). Users can search, browse and access publications of CDRI from this collection.

Figure 7 indicates that the highest number of e-prints were archived in the year 2009 and 2012 (94 each). However, there is a downfall in the number of records in the year 2013 as only 58 records were added during this year.

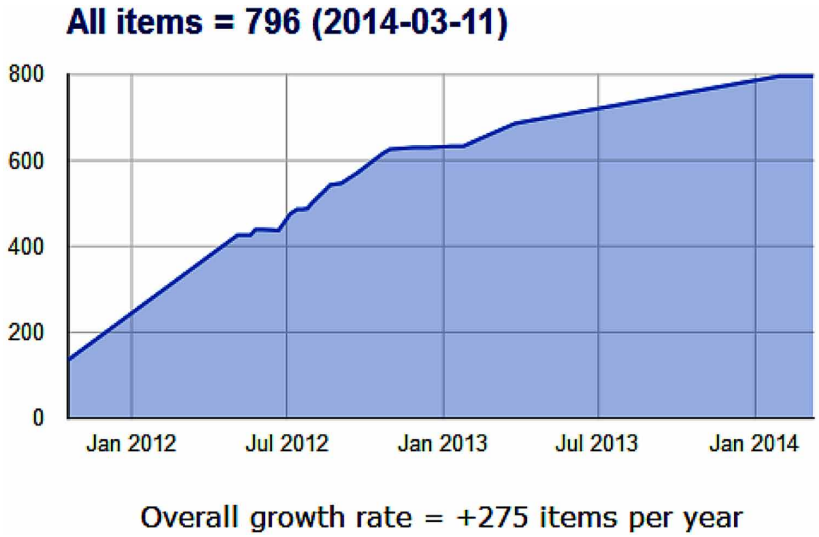
Figure 8 reveals that there is a rise in self-archiving from 2012 and is mentioned upto January 2014. This is a positive sign as more scholarly work is now available under open access. The figure also reveals that the overall growth rate is over 275 items per year.

Figure 7. Growth rate of DKR@CDRI



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Figure 8. Growth statistics of DKR@CDRI 2012 onwards



9.3 EPrints@NIRT

The National INstitute for Research in Tuberculosis (Formerly Tuberculosis Research Centre (TRC)), a permanent institute, under the Indian Council of Medical Research (ICMR), is an internationally recognized institution for Tuberculosis (TB) research. It is a Supranational Reference Laboratory and a WHO Collaborating Centre for TB Research and Training. Recently, an International Centre for Excellence in Research (ICER) in collaboration with NIH was established at the Centre. The centre evolved supervised intermittent chemotherapy, especially in urban areas and large cities subsequently. Later, treatment for approximately 6 months with powerful drugs has been shown to be equally effective and these regimens were evaluated under field conditions.

This is the Institutional Repository of the National Institute for Research in Tuberculosis (NIRTIR) Chennai, India. A scholarly archiving facility for NIRT scientific community, where the institute's intellectual output is preserved, searched and shared. The archive runs on EPrints, an open source software package. We expect the service to facilitate long-term preservation of our research output and provide easy access to these publications, as well as improve their impact. Further, by making our research findings freely available, we hope to contribute to the expanding knowledge base in the global fight against tuberculosis.

The data collected from EPrints@NIRT presents a very sad picture as we can see that after the year 2007 there is a steep fall in the deposit rate (Figure 9). To get the idea of post 2012, the IR growth chart from OpenDOAR was consulted which is reproduced in Figure 10.

Figure 10 reveals that the self-archiving momentum picked up from August 2013 and continued aupto December 2013 after which it become steady.

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Figure 9. Growth rate of EPrints@NIRT

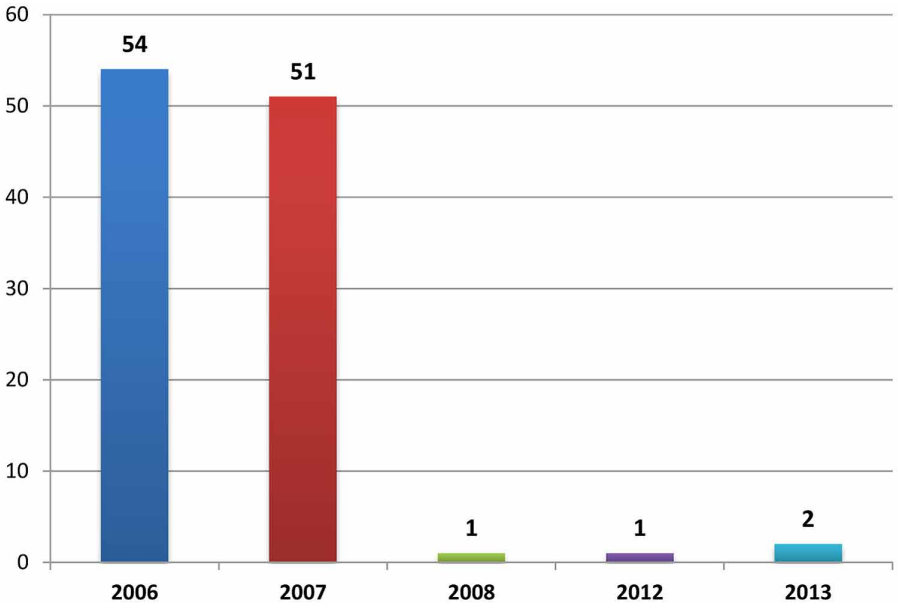
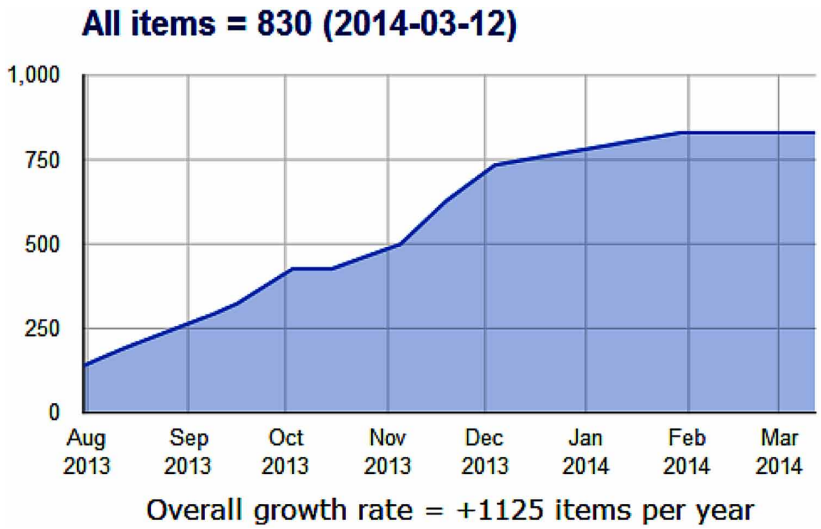


Figure 10. Growth statistics of EPrints@NIRT 2013 onwards



9.4 EPrints@CSIO

Central Scientific Instruments Organisation (CSIO), a constituent unit of Council of Scientific & Industrial Research (CSIR), is a premier national laboratory dedicated to research, design and development of scientific and industrial instruments. It is a multi-disciplinary and multi-dimensional apex industrial research & development organisation in the country to stimulate growth of Instrument Industry in India covers wide range and applications.

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CSIO's Institutional Repository is the digital archive of the research output of our scientists. This knowledge base covers journal articles, conference papers, technical reports, presentation/lectures, pre-prints, Thesis, images etc. One can browse the documents by author, division, subject, date and document type. Both simple and advanced search facilities have been provided. CSIO scientists are welcome to submit their publications on their own by registering.

Figure 11 reveals that the highest deposit ware made in the year 2012 whereas only 27 submissions were made in 2010. In the year 2013, the IR has observed lower self-archiving rate. For post 2012 self-archiving rate the OpenDOAR date is presented below.

Figure 12 reveals that the archiving rate is almost constant from September 2013 to March 2014 and the overall growth rate is above 45 per year.

Figure 11. Growth rate of EPrints@CSIO

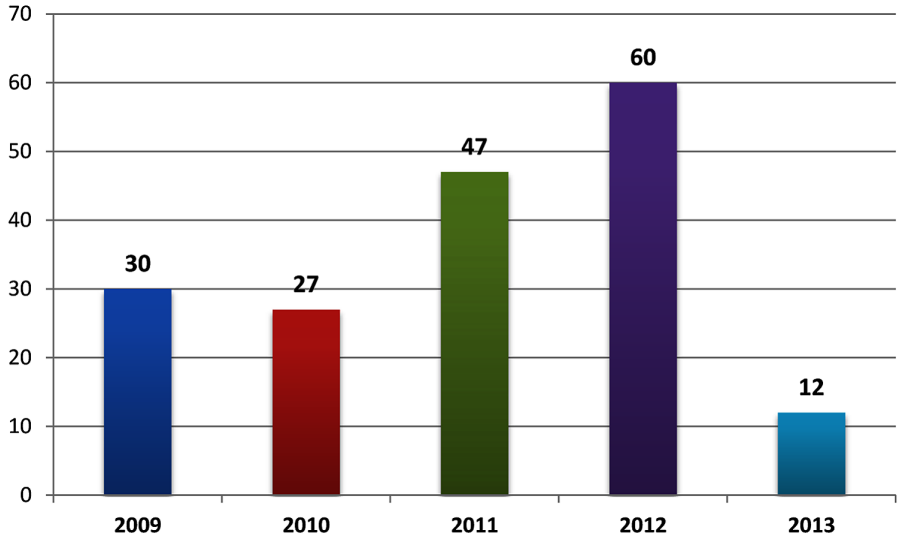
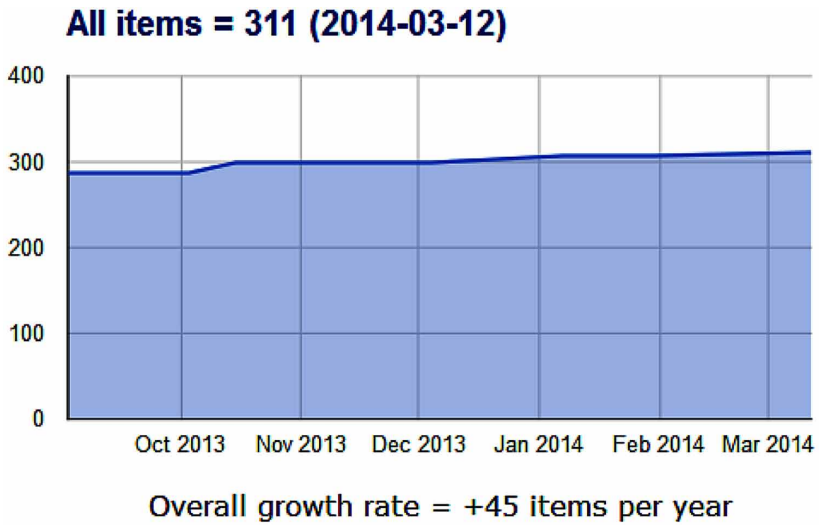


Figure 12. Growth statistics of EPrints@CSIO 2013 onwards



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9.5 Eprints@SBT MKU

This is official website of School of Biotechnology (SBT), Madurai Kamraj University (MKU). This repository provides access to the research output of the institute. Users may set up Atom or RSS feeds to be alerted to new content. The software used is EPrints, the subjects covered are biology and biochemistry, content covered are articles and language used is English. As the repository is now in broken stage data is available till 2011 only. Figure 13 shows the growth rate of collection in SBT MKU EPrint@ NML from 2006 to 2011.

9.6 IR@NPL (National Physical Laboratory)

IR@NPL has been established/maintained by the National Physical Laboratory (NPL), New Delhi, India. It is an online digital archive of research outputs of Scientists/ Researchers of the NPL. The purpose of IR@NPL is to capture, preserve and provide free worldwide access to the intellectual outputs of the NPL in the form of journal articles, research papers, conference papers, technical reports, preprints, technical bulletin, annual reports, golden jubilee document, sameeksha etc. IR@NPL can be accessed globally, but submission of documents to this repository is reserved only for the NPL community. Deposits in this repository can be browsed by author, subject, year and document type, also have simple and advance search facility.

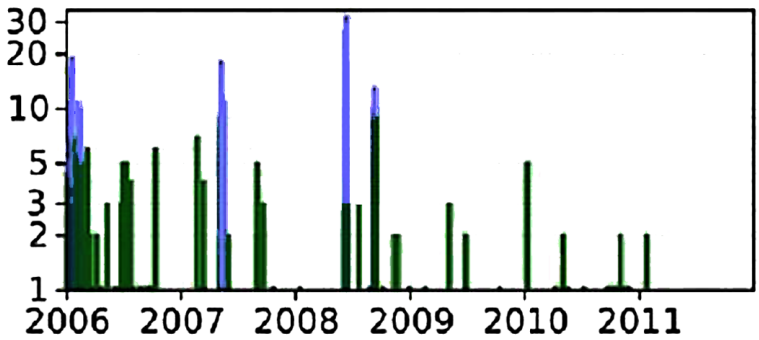
Figure 14 reveals that the highest archiving was done in the year 2010 (343) followed by the year 2009 (312), 2011 (165) and 162 in the year 2008. Lowest submission was observed in the year 2012.

Figure 15 reveals that there has been a constant submission rate after August 2013 and is maintained till March 2014. According to the above graph, the overall growth rate is more than 74 items per year.

9.7 Eprints @MDRF

The prevalence of Diabetes Mellitus is growing in epidemic proportions all over the world, particularly in India. It is now known that India has the highest number of diabetic subjects, in the world even higher than China and USA. India has the highest prevalence rates of diabetes i.e. about 20% of the total diabetic population in the world. In 1996, Dr. V. Mohan and Dr. M. Rema felt the need for an exclusive

Figure 13. Growth rate of EPrints@SBT MKU



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Figure 14. Growth rate of IR@NPL

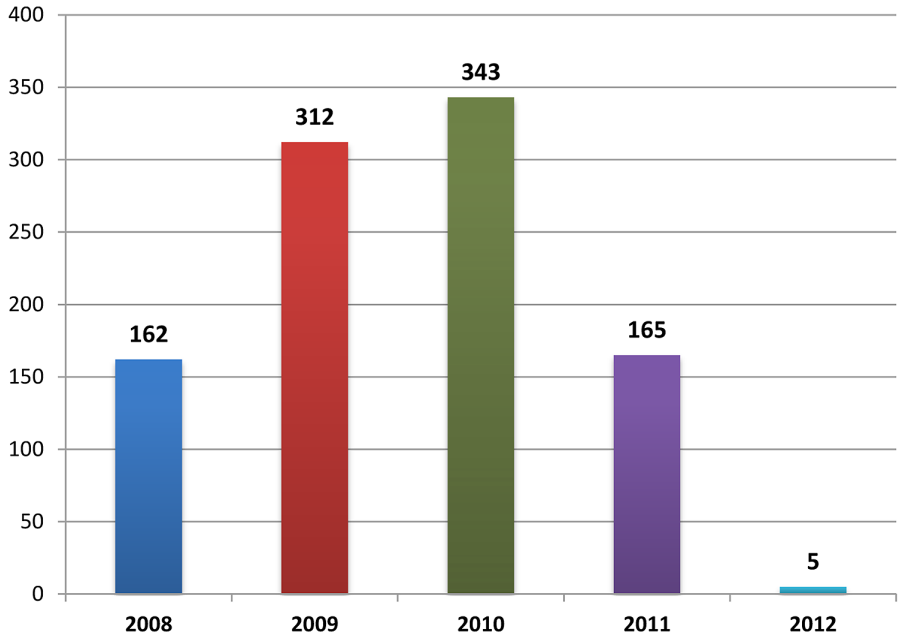
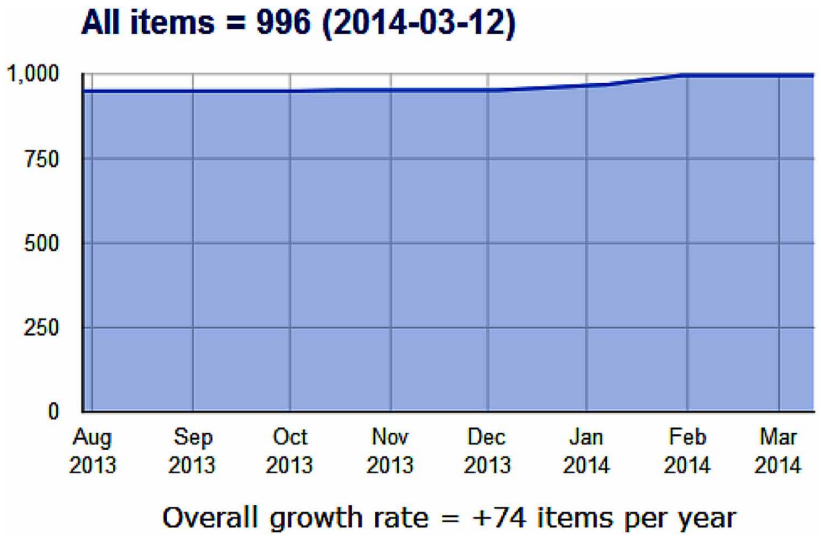


Figure 15. Growth statistics of IR@NPL 2013 onwards



Diabetes Research Foundation to do original research work with the excellent infrastructural facilities. This resulted in the setting up of the Madras Diabetes Research Foundation (MDRF)

MDRF scientists do research on Diabetes and related fields and publish the outputs in professional journals and conferences. With a view to eliminating barriers to access our local research outputs, this open access repository has been set up. Here MDRF scientists archive post-prints of the research papers soon after it is accepted by journal publishers. In some cases published version is archived with “Request

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Copy" facility. Users can send "article request" through this facility. Dr. MGR Medical University and University of Madras have recognized MDRF as a research centre for the PhD program. Eprints@MDRF holds all the theses guided by MDRF scientists

Figure 16 displays the deposit rate of pre and post prints in the MDRF repository during the year 2010 to 2014. The figure reveals that the submission rate has neither witnessed a steep rise or downfall in the submission rate. However, only three submissions were being made at the time of writing the paper (March 2014).

Figure 17 depicts the growth rate of research publications after October 2013. The overall growth rate is above 90 items per year.

Figure 16. Growth rate of Eprints @MDRF

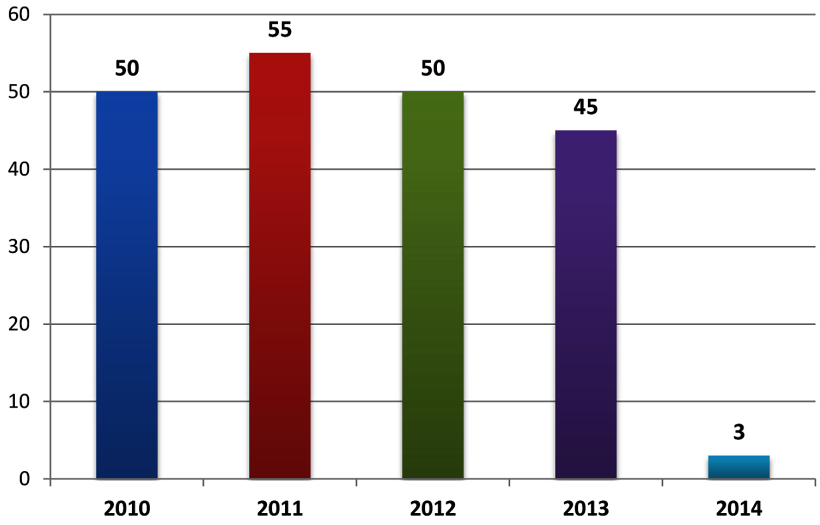
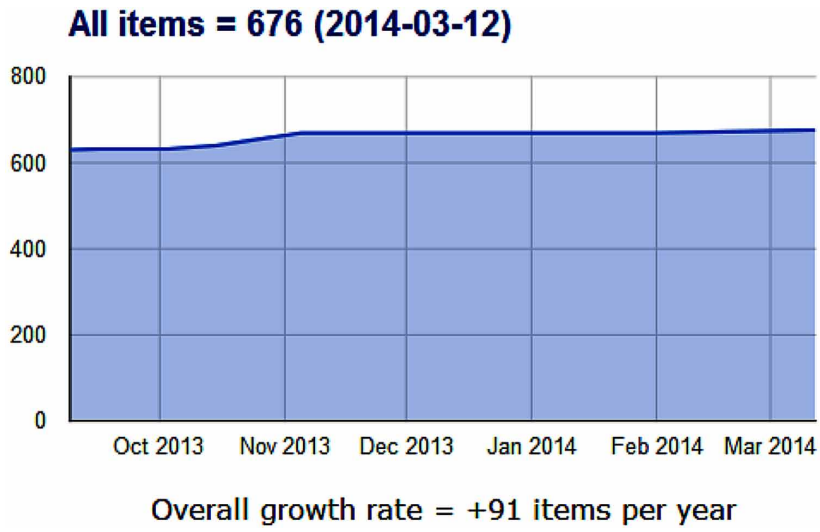


Figure 17. Growth statistics of Eprints @MDRF 2013 onwards



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9.8 Open Access Repository: Indian Academy of Sciences (IAS)

The Indian Academy of Sciences was founded and registered as a society in 1934 with the aim to promote the progress and uphold the cause of science, both in pure and applied branches. We strive to meet our objectives through original research and dissemination of scientific knowledge to the community via our meetings, discussions, seminars, symposia and publications. The academy as on date has around 1,500+ Fellows, out of which, past members account for 600-700. It is estimated that the total number of articles published so far by all fellows in various national and international publications could be around 100,000.

Publications of the IAS fellows repository collects, preserves and disseminates in digital format the research output created by the fellows of the Indian Academy of Sciences. It enables the Academy community to deposit their preprints, postprints and other scholarly publications, and organizes these publications for easy retrieval. While Publications of the IAS fellows can be accessed by anybody, submission of documents to this repository is limited to the fellows of the Academy only. Publications of the IAS fellows repository are running on EPrints open archive software, a freely distributable archive system available from eprints.org. Publications of the IAS fellows complies with the Open Archives Initiative (OAI) framework allowing publications to be easily indexed by web search engines and other indexing services.

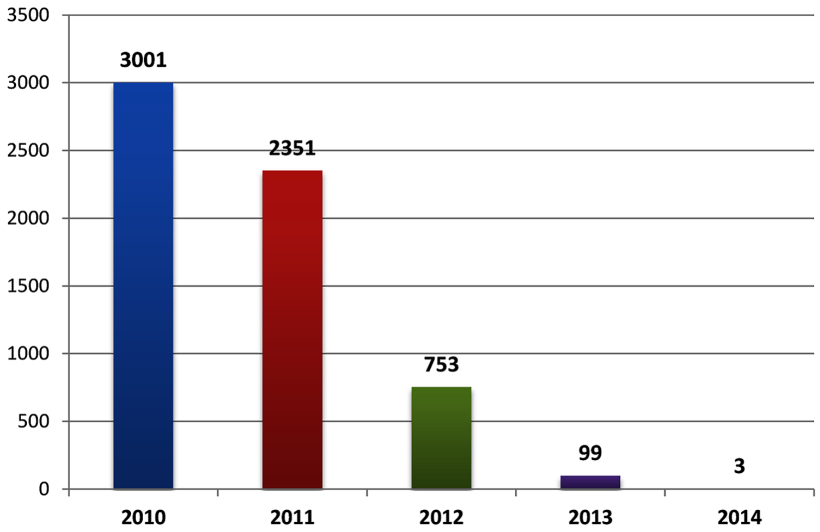
Figure 18 depicts that there is a sharp decline in the growth of IR in terms of archived papers. Number of records submitted in the year 2010 were 3001 which decreased to 2351 in the year 2011 and this trend continued in 2012 (753 records) and 2013 (99 records).

Figure 19 reveals that the overall growth rate of the IR is about 19500 items per year.

9.9 KNoor: Knowledge Repository Open Network

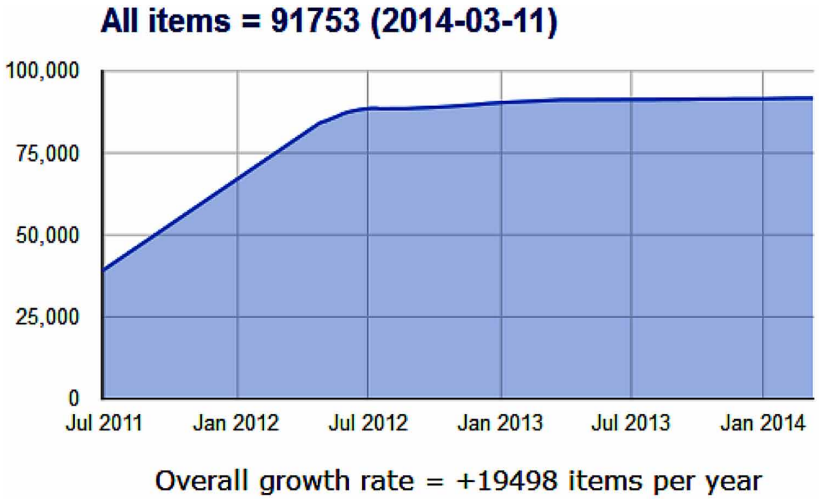
Knoor - Knowledge Repository Open Network is a joint Initiative of Premier Institutes of Jammu and Kashmir (J&K)". This repository is funded by University Grants Commission (UGC), the apex body of

Figure 18. Growth rate of IAS repository



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Figure 19. Growth statistics of Indian Academy of Sciences repository



the Indian Higher Education system under the UGC-Major Project "E-repository for J&K". It provides access to the research output from research institutes of the J&K (viz. S.K Institute of Medical Sciences, S.K University of Agricultural Science & Technology and University of Kashmir). The interface is available in English. Many items are available as abstracts only. Users may set up RSS feeds to be alerted to new content. It provides an interface for the Faculty members and researchers, for creating their own collections in the IR. It also maintains multilingual Phd Theses. Authors are supposed to mail their contributions for uploading in the Repository (peer reviewed papers only OR pre-prints) for their free, open access and high visibility. Authors are also required to check whether concerned publisher permits self-archiving at SHERPA/RoMEO's publisher copyright policies. The service is designed, maintained and regulated by the Department of Library and Information Science (University of Kashmir) with infrastructure support from Directorate of IT&SS, University of Kashmir.

Figure 20 reveals that the growth in the number of IT records has been consistent from 2011 onwards. It decreased from 82 (2009) to 30 (2010) but after that it gained momentum and there has been a constant growth in archiving by the researchers and scientists.

Figure 21 reveals that the self-archiving rate has increased from 2012 to 2014 and the overall growth rate is over 170 items per year.

9.10 OpenMED@NIC

OpenMED is an Open Access Archive in the area of Medical and Allied Sciences, including biomedical, Medical Informatics, Dental, Nursing and Pharmaceutical Sciences. It is international in scope and includes both published (post-prints) and unpublished (pre-prints) peer-reviewed documents having relevance to research in these disciplines. It is hosted by Bibliographic Informatics Division, National Informatics Centre (India) using the GNU EPrints Archive Software. The major objective of OpenMED is to provide a free facility for authors to self-archive their publications for making them available under open access and improve their impact. It encourages authors to make their work visible, searchable and usable by potential users. The metadata (core descriptive information about the documents) is expected to

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Figure 20. Growth rate of KNoor

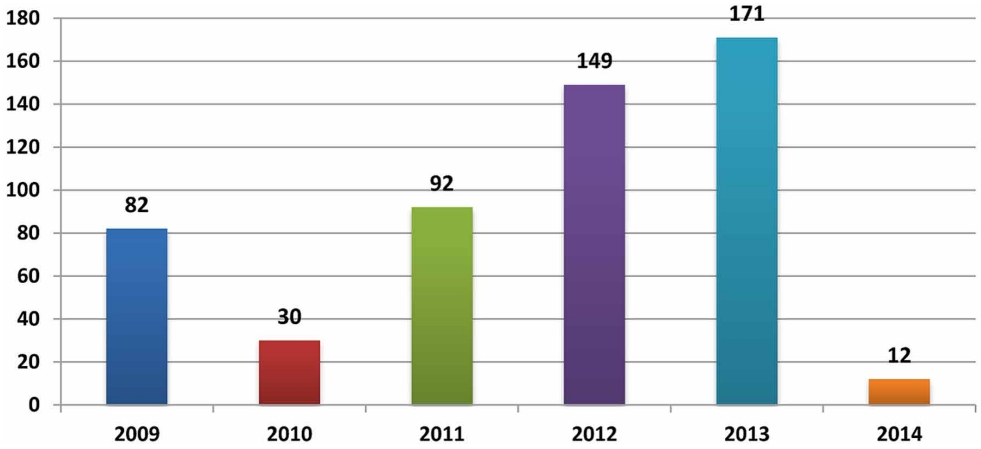
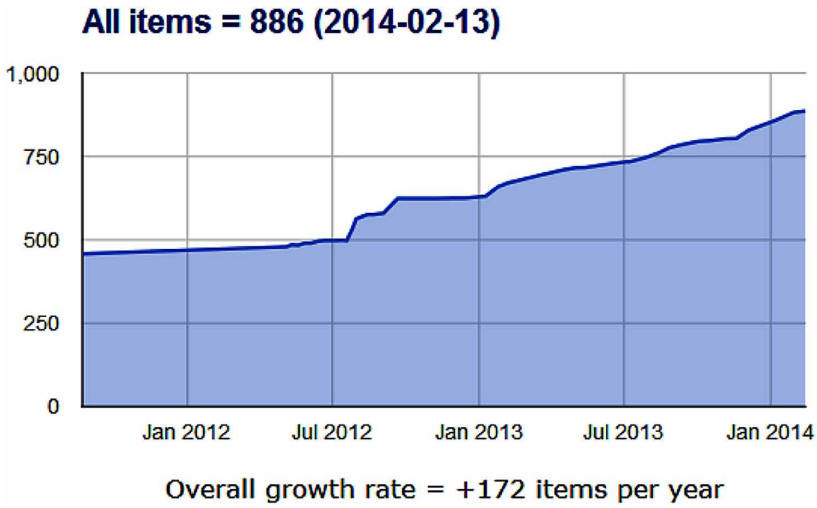


Figure 21. Growth statistics of KNoor 2012 onwards



be aggregated by various service providers and search engines compliant with OAI-PMH (Open Archives Initiative Protocol for Metadata Harvesting). It would help providing the authors the maximum exposure as well as easy and free access to the users. Submitted documents are placed into the submission buffer and becomes part of the OpenMED@NIC archive on their acceptance.

Figure 22 reveals that there is continuing decline in paper archiving as it was 188 in the year 2008 which came down to 38 in the year 2012.

Figure 23 reveals that the overall growth rate for OpenMED repository is 218 items per year.

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Figure 22. Growth rate of OpenMED@NIC IR

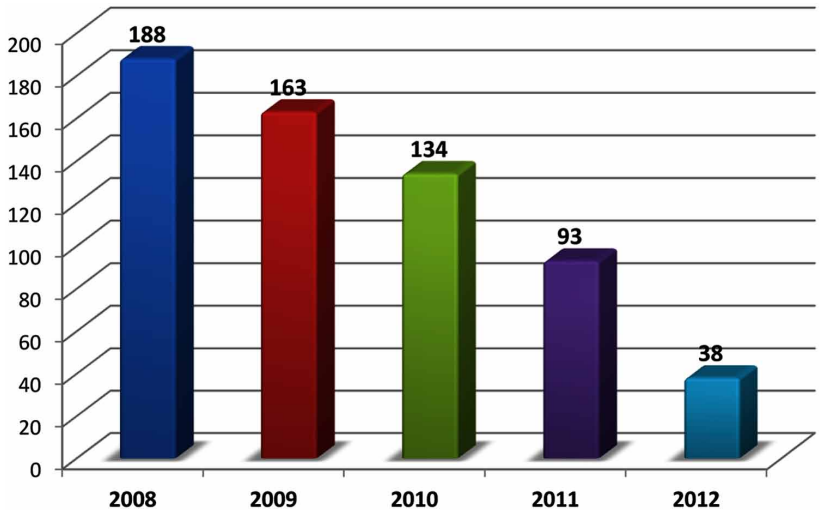
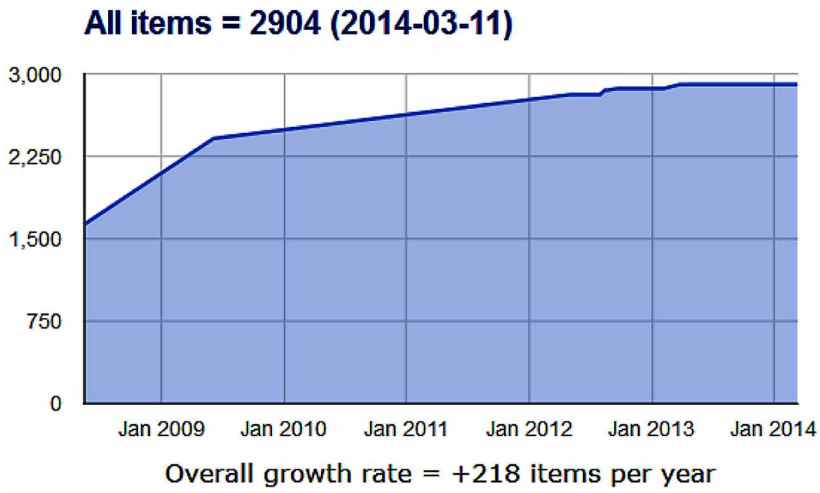


Figure 23. Growth statistics of OpenMED@NIC 2009 onwards



10. CONCLUSION

An Institutional repository is gradually becoming the life and blood of a research organization as it provides the appropriate platform to showcase the research productivity. At the same time it also serves the humanity by facilitating open access to quality literature. It protects and preserves the intellectual capital of its research scientists.

Number of IRs in the field of medicine and biology specially covering Pharmacology as a subject is very low. Although there are many premier institutions undertaking R&D work in this domain but not all of them are having dedicated Irs. Moreover, the existing IRs lack in collection, excluding a few of them.

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In such a scenario, the authors/scientists should be motivated for motivated for self- archiving. If self-archiving does not attract their attention, the approach of mediated-archiving should be facilitated. Other factors which need to be taken care of include quality, adequate budget for establishing new IRs as well as maintaining existing IRs, proper marketing and promotion of IRs, HR training for metadata creation and metadata harvesting, etc. More IRs in universities should be created, as it will enhance research and growth of the Nation's Research output. IRs should be designed to support and achieve the needs and goals of the institution, as it is the tangible way for the institution to communicate with the rest of the world.

Open Access is very important in the discipline of Pharmacology as humans, howsoever developed and advanced can't win immortality and will continue to suffer from diseases. There is an urgent requirement of free scholarly communication in this field so as to provide state-of-the-art solutions for well being of the society. It should be free from all barriers and should explore optimum benefits of Open Access either by following the golden route (OA Journals) or the green route (self-archiving in IRs).

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Section 2
Drug Delivery and Patient Care

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Chapter 6
Application of Nanoparticles as a Drug Delivery System

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ABSTRACT

Small colloidal particles having their diameter in the range of 50 to 500nm are defined as Nanoparticles. These are usually prepared either by using biodegradable or non-biodegradable polymers and are usually classified in two broad categories: (1) Nanocapsules: a type of reservoir system in which an oil or aqueous core is surrounded by a polymeric membrane. (2) Nanospheres: a type of matrix system. Preparation of nanoparticle as a drug delivery system is one of the most widely accepted approach since the prepration of nanoparticle were easy and convenient to scale up. Their high stability and conveniently easy to freeze-dried their preparations provide some additional advantages to choose Nanoparticles as a good drug delivery system. Inspite of them Nanoparticles were were able to achieve with success tissue targeting of many drugs (antibiotics, cytostatics, peptides and proteins, nucleic acids, etc.).

1. INTRODUCTION

In microcapsules drug is encapsulated as small particles or as a drug solution in a polymer fi lm or coat, whereas microspheres are solid polymeric spheres which entrap drug. Equivalent structures with particle diameters ranging from 50 to 500 nm are referred to as nanocapsules and nanoparticles. Nanoparticles (NPs) of different size and physicochemical properties have been introduced to many fields of life and biomedical sciences over the last decade (Oberdörster et al., 2005). It is worth noting that nanoparticles can be made from a fully variety of bulk materials and that they can explicate their actions depending on both the chemical composition and on the size and/or shape of the particles (Brunner et al., 2006). The literature on the ecotoxicity of nanoparticles and nanomaterials as well as the chemistry of both manufactured and natural NSPs is summarized in recent reports (Handy et al. 2008, Yu-Nam & Lead

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2008). Medical therapies have become more tailored to specific diseases and patients in recent years. Most pharmaceutical agents have primary targets within cells and tissues; ideally, these agents may be preferentially delivered to these sites of action within the cell. Selective subcellular delivery is likely to have greater therapeutic benefits. Cytosolic delivery, for instance, is desirable for drugs that undergo extensive exportation from the cell via efflux transporters such as multi-drug resistance proteins and P-glycoproteins (Faraji & Wipf, 2009).

2. PREPARATION OF NANOPARTICLES

Polymer nanoparticles including nanospheres and nanocapsules (Figure 1) can be prepared according to numerous methods that have been developed over the last 30 years. The development of these methods occurred in several steps. Historically, the first nanoparticles proposed as carriers for therapeutic applications were made of gelatin and cross-linked albumin (Scheffel et al., 1972; Marty et al., 1978). Then, to avoid the use of proteins that may stimulate the immune system and to limit the toxicity of the cross-linking agents, nanoparticles made from synthetic polymers were developed. At first, the nanoparticles were made by emulsion polymerization of acrylamide and by dispersion polymerization of methylmethacrylate (Birrenbach, Speiser 1976; Kreuter, 1976). These nanoparticles were proposed as adjuvants for vaccines. However, since they were made of non-biodegradable polymers, these nanoparticles were rapidly substituted by particles made of biodegradable synthetic polymers. Couvreur et al., proposed to make nanoparticles by polymerization of monomers from the family of alkylcyanoacrylates already used in vivo as surgical glue. They succeeded in making nanoparticles by polymerization of the monomers in oil-in-water type emulsions prepared with an acidified aqueous phase (Couvreur et al., 1979).

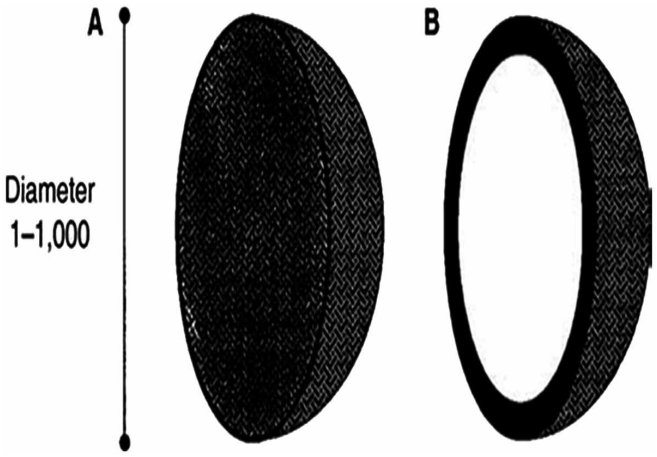
During the same period of time, Gurny et al., proposed a method based on the use of another biodegradable polymer consisting of poly(lactic acid) used as surgical sutures in humans (Gurny et al., 1981). In this method, nanoparticles were formed directly from the polymer. Based on these initial investigations, several groups improved and modified the original processes mainly by reducing the amount of surfactant and organic solvents. At that time, the methods developed were only able to produce nanospheres (Figure 1) (Legrand et. al. 1999). A breakthrough in the development of nanoparticles occurred in 1986 with the development of methods allowing the preparation of nanocapsules corresponding to particles displaying a core-shell structure with a liquid core surrounded by a polymer shell. From 1986, there was also an acceleration in the development of new methodologies for the preparation of all types of nanoparticles.

The nanoprecipitation technique was proposed (Fessi et al., 1986) as well as the first method of interfacial polymerization in inverse microemulsion (Gasco & Trotta, 1986). In the following years, the methods based on salting-out, (Alle´man, 1992) emulsion–diffusion, (Quintanar & Guerrero, 1998) and double emulsion (Zambaux, 1998) were described. Finally, during the last decade, new approaches were considered to develop nanoparticles made from polysaccharides based on the gelation properties of these natural macromolecules (Vauthier & Couvreur, 2000a). These nanoparticles were developed for peptides and nucleic acid delivery. Another goal was the development of surface modified nanoparticles to produce long circulating particles able to avoid the capture by the macrophages of the mononuclear phagocyte system after intravenous administration (Gref, 1997).

All the methods can be classified into two groups depending on whether the nanoparticles are formed at the same time than the polymer itself requiring a polymerization reaction or are directly obtained

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Figure 1. Schematic representation of a nanosphere (A) and of a nanocapsule (B). In nanospheres, the whole particle consists of a continuous polymer network. Nanocapsules present a core-shell structure with a liquid core surrounded by a polymer shell.



from a polymer. There are numerous valuable reviews on the subject (Quintanar & Guerrero, 1998b). The general principles of the methods leading to nanoparticle preparation are described and details of the most representative over are given.

Preparation of Nanoparticles by Polymerization

Nanospheres (Kucuk & Edirisinghe 2014) are mostly prepared by emulsion polymerization whereas nanocapsules are obtained by interfacial polymerization performed in emulsion or in microemulsion. In emulsion polymerization, the monomer itself, if liquid, is dispersed under agitation in a continuous phase in which it is non-miscible.

The polymerization is usually initiated by the reaction of the initiators with the monomer molecules that are dissolved in the continuous phase of the emulsion.

The polymerization continues by further addition of monomer molecules that diffuse toward the growing polymer chain through the continuous phase. The growing polymer chain remains soluble until it reaches a certain molecular weight for which it becomes insoluble. Therefore, phase separation occurs leading to the nucleation of the polymer particles and the formation of the tyndall scattering effect. Further growth of the nucleated particles occurs according to a mechanism that depends on the stability conditions of the whole system. This includes capture of new growing polymer chains, fusion or collision between nucleated particles (Lowell, 1997). Throughout the polymerization, the monomer input in the continuous phase of the emulsion takes place by diffusion from the monomer droplets, which play the role of monomer reservoirs.

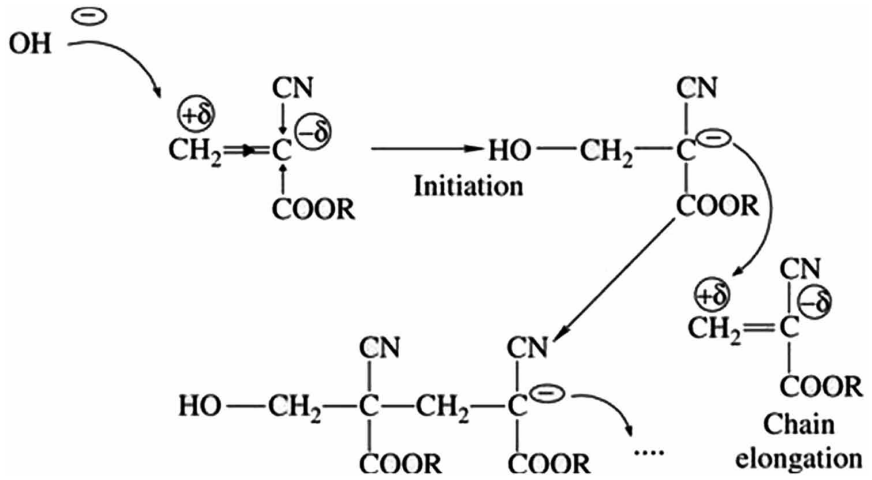
When the reaction is completed, the particles formed contains a large number of polymer chains (Lowell, 1997). Emulsion polymerization can be performed in emulsifier free systems and in both oil-in-water and waterin-oil emulsions. The poly(alkylcyanoacrylate) nanospheres, widely used as drug carriers, are prepared by emulsion polymerization according to a method initially introduced by Couvreur et al. The monomers (isobutylcyanoacrylate, isohexylcyanoacrylate, n-butylcyanoacrylate) are dispersed in

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a continuous acidified aqueous phase under magnetic agitation. The anionic polymerization of the alkylcyanoacrylate is rapidly and spontaneously initiated by the remaining OH_ ions of the acidified water and is completed within 3–4 h depending on the monomer type (Figure 2). The preparation is performed at low pH (pH _ 2.5) to slow down the anionic polymerization of the alkylcyanoacrylate, therefore allowing the polymer to arrange as colloidal particles. Dextran 70 or Pluronic_ F68 are usually dissolved in the aqueous phase to ensure the stability of the polymer particles. The size of the nanospheres can be controlled by the amount of Pluronic_ F68 from a diameter of 40–250nm for concentrations ranging from 3 to 0%, respectively. (Seijo, et. al. 1990) Numerous drugs have been associated with these nanospheres including doxorubicin, an anticancer agent, a peptide growth hormone, and several antibiotics. Antisense oligonucleotides were adsorbed on the nanosphere surface via the formation of an ion-pair with a cationic surfactant, cethyltrimethylammonium bromide. Finally, it should be mentioned that some drugs can lose their biological activity during the preparation of poly(alkylcyanoacrylate) nanospheres. Generally, these molecules contain chemical functions that are able to initiate the polymerization of alkylcyanoacrylates and be covalently attached to the polymer constituting the nanospheres. The mode of such an interaction has been elucidated for two molecules including phenylbutazone (an anti-inflammatory drug) and vidarabine (an antiviral molecule). In contrast, the side reactions can be used to achieve the covalent linkage of defined compounds to give specific properties to the nanospheres. This has been used with poly(ethylene glycol) that initiated the polymerization of isobutylcyanoacrylate to give poly(ethylene glycol)-coated nanospheres, therefore presenting a more hydrophilic surface than those prepared according to the original method (Seijo, 1990). Methylidene malonates are other monomers that give biodegradable polymers and polymerize according to a similar mechanism than alkylcyanoacrylates. These monomers were also used to make nanospheres by emulsion polymerization for drug delivery (De Keyser, 1991).

Nanocapsules can be prepared by interfacial polymerization of alkylcyanoacrylates. The main advantage of using these monomers is their very fast polymerization rate when they come into contact with water. Oil containing nanocapsules were prepared by the rapid dispersion of an ethanol phase including ethanol, the oil, the monomer, and the molecule to be encapsulated in an aqueous solution of surfactant. When the ethanol diffuses in the aqueous phase, tiny individual oil droplets form, and because of the

Figure 2. Anionic polymerization of alkylcyanoacrylate initiated by the OH^- from the dissociation of the water molecule



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contact with water at the oil/water interface, the polymerization of the alkylcyanoacrylate takes place on the droplet surface (Gallardo, 1993). This method is mainly adapted for the encapsulation of oily soluble substances. However, surprisingly, highly water-soluble molecules such as insulin could be entrapped in these nanocapsules with high encapsulation yields (up to 97%) (Aboubakar., 1999).

Water containing nanocapsules were prepared by interfacial polymerization of the alkylcyanoacrylate in water-in-oil microemulsions. In these systems, water swollen micelles of surfactants of small and uniform sizes are dispersed in an organic phase. To prepare nanocapsules, the monomer is added to the oily phase of the already prepared microemulsion.

The anionic polymerization of the alkylcyanoacrylate is initiated at the surface of the water swollen micelles and the polymer formed locally to make the shells of the nanocapsules. In the method first introduced by Gasco and Trotta, the microemulsions were prepared with hexane as the organic phase and Aerosol-OT as the surfactant. Both of these constituents are not compatible for the development of an acceptable drug carrier system. Thus, the method was recently adapted to microemulsions formulated with more biocompatible compounds, but still quite high concentrations of surfactant (up to 14 wt%) are required for their preparation (Watnasirichaikul, 2000). The nanocapsules obtained by this method are dispersed in an organic medium and can mainly be used for oral administration. Indeed, for intravenous administrations, it is necessary to transfer the nanocapsules into an aqueous continuous phase. Such a transfer still remained a problem until recently, since the total elimination of the organic phase and the redispersion of the nanocapsules, in water is a difficult task to achieve avoiding the aggregation of the nanocapsules. Recently, Lambert et al. proposed to perform this operation by ultracentrifugation of the nanocapsule dispersion over a layer of pure water. Using this simple approach, the nanocapsules transferred from the organic phase to the aqueous phase without any aggregation problem during the centrifugation. In addition, this technique allows the elimination of the excess surfactant, which remains in the organic phase. This nanoencapsulation method has special interest for the encapsulation of water soluble molecules such as peptides and nucleic acids including antisense oligonucleotides (Lambert et al., 1998).

Preparation of Nanoparticles Using a Polymer

In this group of methods, the nanoparticles are obtained from a polymer, which was prepared according to a totally independent method. This approach presents the major advantage that the polymers entering the composition of the nanoparticles are well characterized and their intrinsic physicochemical characteristics will not depend on the conditions encountered during the preparation of the nanoparticles as it can be the case with the previously described methods. Most methods starting from polymers have taken advantages of the physicochemical properties of the polymer used in terms of its solubility or its faculty to form a gel under certain conditions. Basically, two approaches are followed. One is based on the spontaneous formation of colloidal particles of the polymer that are then stabilized in a second step of the procedure. The second approach is based on the adaptation of methods initially developed to make microparticles. In this case, the goal is to reduce the size of the particles formed with these methods. A third approach leading to the formation of very specific particles named SupraMolecular BioVectors by their authors (De Miguel, 1995) is described separately. The different ways to produce nanoparticles from a polymer is summarized in Figure 3.

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Methods Based on the Spontaneous Formation of the Nanoparticles

Spontaneous formation of nanoparticles can be achieved by taking advantage of the solubility and gelling properties of a dissolved polymer. Usually, the step allowing polymer colloidal particles to form is reversible, and it is necessary to complete the procedure by a second step required to stabilize the particles.

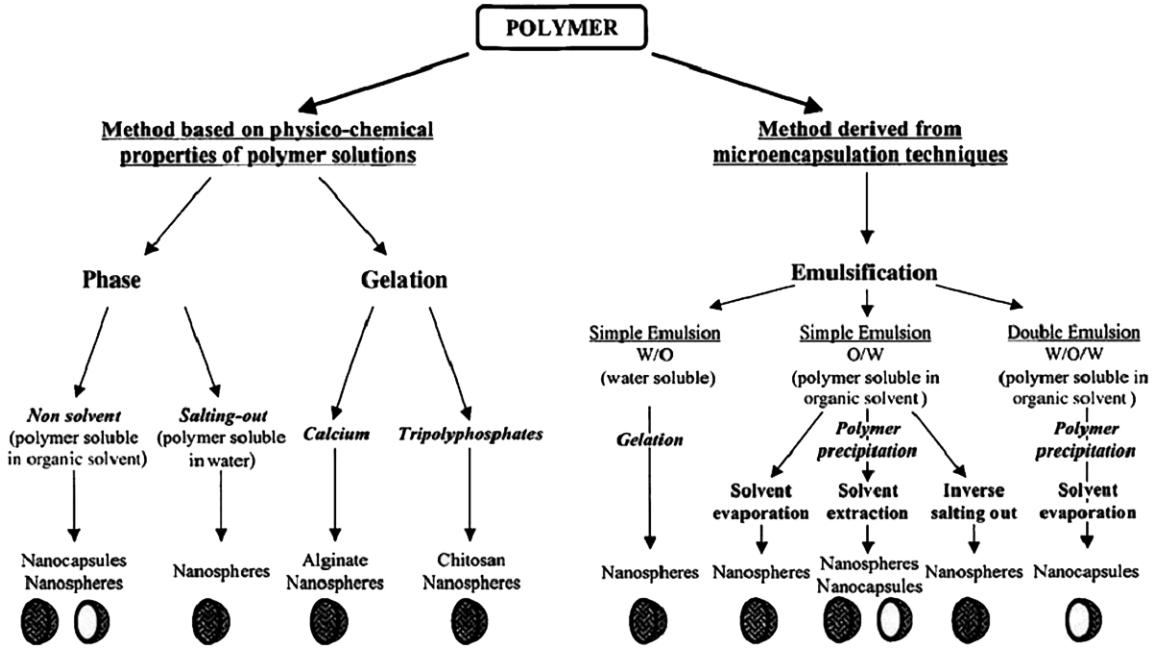
Based on the solubility properties of a polymer, the general principle is to prepare a solution of the polymer and to induce a phase separation by the addition of a non-solvent of the polymer or by a salting-out effect. The occurrence of the phase separation can be followed by turbidimetric measurements or investigated using ternary phase diagrams (Stainmesse, 1990). Phase separations leading to polymer colloid particles are usually obtained with diluted solutions of polymers. In a ternary phase diagram, it corresponds to a small domain.

Using higher polymer concentrations in the solvent, the colloidal particles formed at the limit of the phase separation as followed by turbidimetric measurements in the case of proteins. To facilitate the formation of colloidal dispersion of polymer particles, it is better to induce the phase separation in totally miscible solvent–non-solvent systems. At that stage, the particles form spontaneously and quasi-instantaneously.

Once the proper conditions to obtain the polymer colloidal nanoparticles are identified, the particles must be stabilized. This is usually achieved either by the elimination of the polymer solvent by evaporation or by chemical cross-linking of the polymer as it is the case with proteins (Weber, 2000).

This method, also known as the nanoprecipitation method, can be applied to numerous synthetic polymers. In general, the polymer is dissolved in acetone and the polymer solution is added into water. The acetone is then evaporated to complete the formation of the particles. Surface active agents are usually added to water to ensure the stability of the polymer particles. This easy technique of nanoparticle

Figure 3. Summary of the different methods to prepare nanospheres and nanocapsules from a polymer
W/O: water-in-oil, O/W: oil-in-water, W/O/W: water-in-oil-in-water



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preparation was scaled up for large batch production. It leads to the formation of nanospheres. Nanocapsules can easily be prepared by the same method just by adding a small amount of an organic oil in the polymer solution. When the polymer solution is poured into the water phase, the oil is dispersed as tiny droplets in the solvent–non-solvent mixture and the polymer precipitates on the oil droplet surface. This method leads to the preparation of oil-containing nanocapsules and can be valuably used for the encapsulation of liposoluble drugs. Techniques based on the use of proteins are much more adapted to the encapsulation of hydrosoluble compounds and were recently developed to produce gelatin nanospheres as carrier systems for gene delivery.

Based on the gelation properties displayed by certain polymers, nanoparticles are formed spontaneously by controlling the gelation process. This approach has been developed with alginate and chitosan that form highly water-swollen gels. The gelling agents for these two natural polysaccharides are respectively calcium and tripolyphosphate. Nanoparticles are formed in a certain domain of concentrations of the polysaccharide and of the gelling agent. With the alginate, it has been shown that nanoparticles can be prepared when the respective concentrations of alginate and calcium are comprised in the domain of the pregel stage of the alginate gelling process (alginate 0.6 wt%, calcium chloride 0.9 mM). At this composition, tiny particles of gels form resulting from inter and intramolecular aggregations of alginate molecules caused by the interaction with calcium. These aggregates are stabilized by the formation of a polyelectrolyte complex with polylysine. Alginate and chitosan nanoparticles are interested for nucleic acid and protein delivery as recently reviewed by (Vauthier & Couvreur, 2000).

Methods Derived from Microencapsulation Techniques

Methods derived from microencapsulation techniques require the formation of an emulsion as a first step of the procedure. To produce nanometer-scale-sized particles, the size of the emulsion droplets must be small enough. This can be achieved by the use of special equipments such as high pressure homogenizers and microfluidizers. The energy input produced by these apparatus is important and is mainly due to high turbulence and cavitation forces. It allows an efficient dispersion of the polymer solution in the continuous phase. Once the desired emulsion is prepared, the formation of the nanoparticles can be induced according to two routes including the gelation of the polymer and the precipitation of the polymer either by solvent displacement or by solvent evaporation. Gelation can be induced by increasing the pH, adding calcium, or decreasing the temperature of emulsions containing respectively, chitosan, alginate, and agarose. In the solvent displacement technique, the emulsion is formed with a solvent of the polymer, which is partially soluble in water and with an aqueous phase saturated with the solvent. Once the emulsion is formed with ethylacetate as an example, it is diluted by the further addition of water to displace the solvent from the dispersed phase inducing the polymer precipitation. This principle was also developed on the base of an inverse salting out method. In this procedure, a solution of polymer in acetone is dispersed in an aqueous phase containing a high concentration of salt to keep the acetone nonmiscible with water. Just by diluting the emulsion with a large amount of pure water, the acetone is then extracted from the dispersed phase inducing the polymer precipitation. The total elimination of the acetone can be achieved by evaporation. Finally, precipitation of the polymer solubilized in the dispersed phase can also be induced by the removal of the solvent by evaporation. In this method named emulsification solvent evaporation, the polymer solvent diffuses through the aqueous continuous phase and evaporates at the air/water interface. The emulsion should remain under agitation during the time required for the total evaporation of the solvent. These methods produce nanospheres.

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To make nanocapsules, small amounts of oil can be added in the dispersed phase of the emulsion that will form the nanocapsules by solvent displacement. Nanocapsules can also be prepared with the solvent evaporation technique from a double water-in-oil-in-water emulsion. The removal of the organic solvent of the intermediate phase of this double emulsion by evaporation causes the polymer to precipitate at the surface of the inner aqueous phase. Whereas nanocapsules made by the solvent displacement method is more adapted for the encapsulation of lipophilic compounds, this last method allows the encapsulation of hydrosoluble compounds.

Preparation of SupraMolecular BioVector

SupraMolecular BioVector consists of polysaccharide nanospheres surrounded by a bilayer of phospholipids (De Miguel, 1995). At the origin, these systems were developed to mimic the low density lipoproteins that are natural colloidal structures encountered in the blood circulation and designed for the transport of cholesterol and cholesterol esters in vivo. SupraMolecular Bio-Vector are prepared in several steps including the functionnalization and chemical cross-linking of a polysaccharide (starch or dextran), the purification and the drying of the modified polysaccharide followed by the fragmentation of the resulted powder under high pressure to produce small polysaccharide nanoparticles. Finally, a lipid bilayer is adsorbed on the surface of the nanoparticles. The chemical modification of the polysaccharide forming the core of the Supra Molecular BioVector lead to various possibilities in terms of the type of molecules that can be associated with such a carrier system. Indeed, the polysaccharide core can be either negatively or positively charged or even neutral opening large application potential.

Preparation of Surface Modified Nanoparticles

Once intravenously administered, the body distribution of the nanoparticles is controlled by their surface properties. Indeed, despite their small size, nanoparticles display an enormous specific surface area that makes the interaction with the surrounding medium very important especially for their fate in vivo. Thus, the preparation of surface modified nanoparticles received much attention during the last decade to produce nanoparticles that are able to circulate for a long period of time in the blood stream at first and, more recently, to achieve an effective targeting of the device or to improve their bioadhesivity to mucosae. Nanoparticles that are able to circulate for a long time in the blood stream should not be recognized by macrophages of the mononuclear phagocyte system. To achieve this goal, at least one of the two major known mechanisms involved in the recognition of foreign particles by macrophages should be avoided. These two mechanisms include the particle opsonisation and the complement activation, which consists in protein adsorption and subsequent recognition by macrophages. A barrier to protein adsorption could be achieved by creating an efficient barrier of steric hindrance, therefore, by coating or adsorbing hydrophilic polymers to nanoparticle surface.

Nanospheres coated with poly(ethylene glycol) were first obtained by the simple adsorption of triblock copolymers of poly(ethylene glycol)–poly(propyleneglycol)–poly(ethylene glycol) on the surface of already prepared nanospheres. To improve the stability of the poly(ethylene glycol) coating, nanospheres were prepared by nanoprecipitation or by emulsification solvent evaporation using copolymers of poly(lactic acid)–co-poly (ethylene glycol) or of poly(alkylcyanoacrylate)–co-poly(ethylene glycol). Finally, poly (ethylene glycol) can initiate the polymerization of alkylcyanoacrylate to produce poly(ethylene glycol)-coated poly-(alkylcyanoacrylate) nanoparticles by emulsion polymerization. To

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make nanoparticles able to escape complement activation, Passirani et al., proposed to coat nanospheres with heparin. This compound, which is a polysaccharide, is a physiological inhibitor of complement activation in vivo. Heparin-coated poly(methylmethacrylate) nanoparticles were prepared by emulsion polymerization. In the method, the radical polymerization of methylmethacrylate was initiated by heparin according to an original method involving cerium ions and allowing heparin to covalently attach to poly(methylmethacrylate) (Passirani, 1998; Passirani,1999).

The next step now is the development of targeted nanoparticles toward a specific cell type. This has recently been investigated by Stella et al., who prepared poly(alkylcyanoacrylate) nanoparticles showing residues of folic acid on their surface. These nanoparticles will be used to target cancer cells overexpressing a membrane receptor for the folic acid. The targeting moiety, consisting on the folic acid, was grafted on the surface of poly(aminopoly(ethylene glycol) cyanoco-hexadecylcyanoacrylate) nanoparticles that were obtained by nanoprecipitation (Stella et al., 2000). In another way, chitosan was used as a coating agent for nanoparticles to improve their bioadhesive properties after oral and nasal administration. Indeed, chitosan is known to have bioadhesive properties as well as an interesting absorption enhancing capacity.

3. CHARACTERIZATION OF NANOPARTICULATE DRUG CARRIERS

Nanoparticles can be characterized by all the different physico chemical techniques that apply for polymer colloids. Concerning the development procedure of nanoparticles as drug carriers, the main physico chemical parameters that are investigated are the shape, the size, the surface properties, the density, and the concentration of the particles. The size as well as the size distribution are important parameters to be determined to achieve safe intravenous administration. Surface properties are also important to consider as nanoparticles display considerable specific surface area responsible for the interactions with the surrounding medium. Finally, the density and the concentration are required to deduce the specific surface area of the particles together with the size.

Nanoparticles can be visualized using different microscopy techniques. Transmission electron microscopy is usually applied to nanoparticles after negative staining with phosphotungstate acid or with uranyl acetate after it has been checked that the staining agents do not modify the particles. Recent progress in transmission electron microscopy now allows direct observations of the nanoparticles without the use of any staining agent that may cause artefacts in some cases. In particular, direct observations of the nanoparticles after a sample of the nanoparticle dispersion has been freezing at very low temperature can be carried out by cryotransmission electron microscopy. Scanning electron microscopy is performed on samples coated with a thin layer of gold metal to produce the contrast. These techniques as well as those based on atomic force microscopy give useful images of the nanoparticles showing their shape. Measurements of the size and of the size distribution require determination of the diameters number of individual nanoparticles that may be assisted by the use of valuable image analysis softwares. The internal structure of the nanoparticles can be observed by freeze fracture and cryotransmission electron microscopy.

Generally, mean size and size distribution of nanoparticles are evaluated by quasi-elastic light scattering also named photocorrelation spectroscopy. This method is based on the evaluation of the translation diffusion coefficient, D, characterizing the Brownian motion of the nanoparticles. The nanoparticle hydrodynamic diameter, pH is then deduced from this parameter from the Stokes Einstein law. Other techniques can be used to determine the size and the size distribution of the nanoparticles. The field

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flow fractionation method is based on the separation of particles according to their size in a thin glass channel in which the flow carrying on the nanoparticles is submitted to an external perpendicular force produced either by a crossed flow or a sedimentation. This technique, which can be applied for particles in a wide range of size (10 nm to several hundred *m*m), will gain more attention in the future for size determination and also for nanoparticle surface analysis. Size and size distribution of nanoparticles can also be determined by size exclusion chromatography performed on appropriate gels. This approach, requiring less equipment than the previous methods, presents the main limitation that only particles having a diameter lower than 120nm can be characterized by this method. As mentioned earlier, surface characteristics of the nanoparticles are of primary importance for the interaction of the nanoparticles with the surrounding medium. The main nanoparticle surface characteristics that are considered are the charge, the hydrophilicity, the chemical composition and the capacity to absorb proteins and to induce complement activation.

The charge of the nanoparticle surface is usually evaluated by the measurement of their zeta potential, which gives information about the overall surface charge of the particles and how it is affected by changes in the environment. Zeta potential is affected by the surface composition of the nanoparticles, the presence or the absence of adsorbed compounds, and the composition of the dispersing phase, mainly the ionic strength and the pH. The hydrophilicity of the nanoparticle surface can be evaluated by hydrophobic interaction chromatography. This technique, based on affinity chromatography, allows a very rapid discrimination between hydrophilic and hydrophobic nanoparticles. The nanoparticles are passed through a column containing a hydrophobic interaction chromatography gel. The nanoparticles that are retained by the gel and only eluted after the addition of a surfactant are considered as hydrophobic, whereas the nanoparticles that do not interact with the gel and that are directly eluted from the column are considered as hydrophilic. Apart from the hydrophobic interaction chromatography, the field flow fractionation techniques recently appeared to present interesting potential for the characterization of nanoparticles with different surface characteristics.

X-ray photoelectron spectroscopy (ESCA) can be use to determine the chemical composition of the nanoparticle surface. This technique is a very useful tool for the development of surface modified nanoparticles providing a direct evidence of the presence of the components that are believed to be on the nanoparticle surface.

The capacity of the nanoparticles to adsorb proteins and to activate the complement in vivo after intravenous administration will influence the fate of the carrier and its body distribution. To approach this aspect, in vitro tests have been developed to investigate the profile of the type of serum proteins that adsorbed onto the nanoparticle surface after incubation in serum and to evaluate the capacity of the nanoparticles to induce complement activation. The analysis of the protein adsorbed onto the nanoparticle surface can be performed by 2D-polyacrylamide gel electrophoresis. This technique allows the identification of the proteins that adsorbed onto the nanoparticle surface. To evaluate modifications of the composition of the adsorbed protein with time, a faster method based on capillary electrophoresis can also be used. Finally, the activation of the complement produced by nanoparticles can be evaluated either by a global technique or by a specific method measuring the specific activation of the component C3. In the global technique, nanoparticles are incubated with serum and, after the incubation, the remaining non-activated complement in the serum is evaluated using a red blood cell lysis test. The concentration of nanoparticle in the dispersion can be deduced from gravimetric determination or by turbidimetric measurements based on the application of the Mie's law. Density of the nanoparticles is evaluated either by pycnometry or by isopycnic centrifugation (Quintanar-Guerrero, 1999).

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4. PHARMACEUTICAL APPLICATIONS OF NANOPARTICLES

Nanoparticles were first developed in the mid-seventies by Birrenbach & Speiser. Later on, their application for the design of drug delivery systems was made available by the use of biodegradable polymers that were considered to be highly suitable for human applications. At that time, the research on colloidal carriers was mainly focusing on liposomes, but no one was able to produce stable lipid vesicles suitable for clinical applications. In some cases, nanoparticles have been shown to be more active than liposomes due to their better stability. This is the reason why in the last decades many drugs (e.g., antibiotics, antiviral and antiparasitic drugs, cytostatics, protein and peptides) were associated to nanoparticles (Birrenbach & Speiser, 1976).

Intravenous Administration

Fate of Nanoparticles and Their Content after Intravenous Administration

the main interest of nanoparticles is their ability to achieve tissue targeting and enhance the intracellular penetration of drugs. After intravenous administration, nanoparticules are taken up by the liver, spleen and to a lower extent the bone marrow. Within these tissues, nanoparticles are mainly taken up by cells of the mononuclear phagocyte system (MPS). The uptake occurs through an endocytosis process after which the particles end up in the lysosomal compartment where they are degraded producing low molecular weight soluble compounds that are eliminated from the body by renal excretion. As a result of the MPS site specific targeting, avoidance of some organs was made possible, thus reducing the side effects and toxicity of some active compounds. Due to their strong lysosomal localization, one could imagine that nanoparticles are not suitable to target to the cytoplasm. To avoid their trapping within the lysosomal compartment, several compounds able to destabilize the lysosomal membrane were added to the nanoparticulate systems (e.g., cationic surfactant) allowing some drugs to be delivered to the cytoplasm. Recently, to avoid MPS uptake, several groups have developed a strategy consisting of the linkage to the nanoparticles of poly(ethylene glycol) derivatives. (Peracchia, 1999) This linkage results in a lower uptake of nanoparticles by the MPS and in a longer circulation time. As a consequence, these so-called stealth_ nanoparticles would be able to extravasate across endothelium that becomes permeable due to the presence of solid tumors.

Application to the Treatment of Intracellular Infections

Intracellular infections were found to be a field of interest for drug delivery by means of nanospheres. Indeed, infected cells may constitute a ''reservoir'' for micro organisms, which are protected from antibiotics inside lysosomes. The resistance of intracellular infections to chemotherapy is often related to the low uptake of commonly used antibiotics or to their reduced activity at the acidic pH of lysosomes. To overcome these effects, the use of ampicillin, a β lactam antibiotic, bound to nanospheres was proposed as endocytozable formulation.(66) The effectiveness of polyisohexylcyanoacrylate (PIHCA) nanospheres was tested in the treatment of two experimental intracellular infections.

Firstly, ampicillin-loaded nanospheres were tested in the treatment of experimental Listeria monocytogenes infection in congenitally athymic nude mice, a model involving a chronic infection of both liver and spleen macrophages. After adsorption of ampicillin onto nanospheres, the therapeutic activity of

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ampicillin was found to increase dramatically over that of the free drug. Bacterial counts in the liver were at least 20-fold reduced after linkage of ampicillin to PIHCA nanospheres. In addition, nanoparticulate ampicillin was capable of ensuring liver sterilization after two injections of 0.8 mg of nanospheres bound drug, whereas no such sterilization was ever observed with any of other regimens tested. Reappearance of living bacteria in the liver after the end of the treatment was probably due to a secondary infection derived from other organs such as the spleen, which was not completely sterilized by the treatment. Secondly, nanosphere-bound ampicillin was tested in the treatment of experimental salmonellosis in C57/BL6 mice, a model involving an acute fatal infection. All mice treated with a single injection of nanoparticle-bound ampicillin survived, whereas all control mice and all those treated with unloaded nanospheres died within 10 days post infection. With free ampicillin, an effective-curative effect required three doses of 32mg each. Lower doses (3 _ 0.8mg and 3 _ 16 mg) delayed but did not reduce mortality. Thus, the therapeutic index of ampicillin, calculated on the basis of mice mortality, was increased by 120-fold when the drug was bound to nanospheres.

In order to clarify the mechanism by which nanospheres improved the antimicrobial efficacy of ampicillin, Forestier et al., have compared in vitro the efficacy of ampicillin bound to poly(isobutylcyanoacrylate) (PIBCA) nanospheres with that of free ampicillin in terms of survival of L. monocytogenes in mouse peritoneal macrophages. After 30 h of incubation, nanospheres-bound ampicillin decreased the number of viable bacteria by 99% as compared to the controls whereas with free ampicillin, the number of bacteria was slightly lower than in the controls (Forestier et al., 1992). Nanoparticle-ampicillin thus appeared to bemuchmore effective than free ampicillin for inhibiting intracellular growth of L. monocytogenes. With in vitro Salmonella typhimurium infected macrophages, the situation was a little bit more complicated since the bactericidal effect of ampicillin-bound PIHCA nanospheres was poor although the intracellular capture of ampicillin was dramatically increased and its efflux in the extracellular medium reduced. In another study, confocal microscopy and transmission electron microscopy were used to establish the intracellular traffic of ampicillin bound-PIHCA nanospheres and its relation with the bacteria within the subcellular compartments. The data obtained clearly demonstrated the active uptake by phagocytosis of ampicillin-bound PIHCA nanospheres by murine macrophages and their localization in the same vacuoles as the infecting bacteria, but in a restrictive way. Thus, it was difficult to understand the limited bactericidal effect of ampicillin-bound nanospheres. The most probable explanation is to be found in the resistance mechanism of S. typhimurium involving the inhibition of the phagosome–lysosome fusion, which lets some bacteria in phagosomes free of nanospheres. If this proposed hypothesis (inhibition of phagosome–lysosome fusion) is correct, the dramatic efficiency observed in vivo should rather be due to the specific targeting of the infected tissues (rich in reticuloendothelial cells), than to an efficient intracellular targeting as it could be supposed.

In order to eliminate both dividing and non-dividing bacteria, a fluoroquinolone antibiotic, ciprofloxacin, has been associated with PIBCA and PIHCA nanospheres. In an animal model of persisting Salmonella infection, although an effect on the early phase of the infection was observed, neither free nor nanospherebound ciprofloxacin was able to eradicate truly persisting bacteria. Since they accumulate in the MPS, nanospheres hold promise as drug carriers for the treatment of visceral leishmaniosis. Thus, it has been shown that PIHCA nanospheres can be used as a carrier of primaquine whose activity was increased 21-fold against intracellular Leishmania donovani when associated with nanospheres. A part of the activity was attributed to the fact that phagocytosis of nanospheres led to the induction of a respira-

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tory burst, which was more pronounced in infected than in non-infected macrophages. Dehydroemetine is also one of the drug candidates for this treatment but has some side effects involving the heart, which were reduced after linkage with nanospheres.

Application to the Treatment of Cancer

When given intravenously, anticancer drugs are distributed throughout the body as a function of the physicochemical properties of the molecule. A pharmacologically active concentration is reached in the tumor tissue at the expense of massive contamination of the rest of the body. For cytostatic compounds, this poor specificity raises a toxicological problem, which presents a serious obstacle to effective therapy. The use of colloidal drug carriers could represent a more rational approach to specific cancer therapy. In addition, the possibility of overcoming multidrug resistance might be achieved by using cytostatics-loaded nanospheres. The antitumor efficacy of doxorubicin-loaded nanospheres was first tested using the lymphoid leukemia L-1210 as a tumor model. In this study, one intravenous injection of doxorubicin-loaded PIBCA nanospheres was found to be more effective against L1210 leukemia than when the drug was administered in its free form following the same dosing schedule. Although the increased life span (ILS %) of mice injected with doxorubicin-loaded PIBCA nanospheres was twice as high as the ILS % for free doxorubicin, there were no long-term survivors.

The effectiveness of doxorubicin-loaded PIHCA nanospheres against L1210 leukemia was even more pronounced than that of doxorubicin loaded onto PIBCA nanospheres. The drug toxicity was markedly decreased when it was bound to this sort of nanospheres, so that impressive results were obtained with this formulation at doses for which the therapeutic efficiency of free doxorubicin was completely masked by the overpowering toxicity of the drug. Furthermore, preliminary experiments suggested that one i.v. bolus injection of doxorubicin-loaded nanospheres was more active, in L1210-bearing mice, than perfusion of the free drug for 24 h.

The superiority of doxorubicin targeted with the aid of poly(alkylcyanoacrylate) nanospheres was later confirmed in a murine hepatic metastases model (M5076 reticulosarcoma). Irrespective of the dose and the administration schedule, the reduction in the number of metastases was much greater with doxorubicin-loaded nanospheres than with free doxorubicin, particularly if treatment was given only when the metastases were well established. The improved efficacy of the targeted drug, as clearly confirmed by histological examinations, shows that both the number and the size of the tumor nodules were lower when doxorubicin was administered in its nanoparticulate form. Furthermore, liver biopsies of animals treated with the nanosphere-targeted drug showed a lower cancer cell density inside tumor tissue. Necrosis was often less widespread with the nanosphere-associated drug than in the control group and the group treated with free doxorubicin.

Studies performed on total homogenates of livers from both healthy and metastases-bearing mice showed extensive capture of nanoparticulate doxorubicin by the liver; no difference in hepatic concentrations was noted between healthy and tumor-bearing animals. In order to elucidate the mechanism behind the enhanced efficiency of doxorubicin-loaded nanospheres, doxorubicin measurements were made in both metastatic nodules and neighboring healthy hepatic tissue. This provided quantitative information concerning the drug distribution within these tissues. During the first 6 h after administration, the exposure of the liver to doxorubicin was 18 times greater for nanosphere-associated doxorubicin. However, no special affinity for the tumor tissue was detected and the nanospheres were seen by electron microscopy to be located within Kupffer cells (macrophages). However, at later time-points, the

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amount of drug in the tumor tissue increased in nanosphere-treated animals to 2.5 times the level found in animals given free doxorubicin. Since uptake of nanospheres by neoplastic tissue is unlikely, this increase in the doxorubicin concentration in tumor tissue probably resulted from doxorubicin released from healthy tissue, in particular Kupffer cells. Hepatic tissue could play the role of drug reservoir from which prolonged diffusion of the free drug (from nanospheres entrapped in Ku¨ pffer cell lysosomes) toward the neighboring malignant cells occurs.

This hypothesis raises the question of the long-term effect of an 18-fold increase of doxorubicin concentration in the liver. Although toxicological data have shown that doxorubicin-loaded nanospheres were not significantly or unexpectedly toxic to the liver in terms of survival rate at high doses, body weight loss, and histological appearance, this possibility should be borne in mind, especially since a temporary depletion in the number of Kupffer cells, and hence the ability to clear bacteria, was observed in rats treated with doxorubicin-loaded liposomes. A systematic study using unloaded poly(alkylcyanoacrylate) nanoparticles confirmed a reversible decline in the phagocytic capacity of the liver after repeated dosing, as well as a slightinflammatory response. Nanoparticle-associated doxorubicin also accumulated in bone marrow, leading to a myelosuppressive effect. However, this tropism of carriers might be useful to deliver myelostimulating compounds such as granulocyte colony stimulating factor to reverse the suppressive effects of intense chemotherapy. Nanospheres are also captured by splenic macrophages. In this study, the spleen architecture was shown to play a role in the localization of the nanospheres: in mice, uptake was mainly observed in metallophilic macrophages of the marginal zone whereas in rats, which have sinusoidal spleens similar to that of humans, particles were found in the red pulp macrophages.

On the other hand, alteration of the drug distribution profile by linkage to nanospheres can, in some cases, considerably reduce the toxicity of a drug because of reduced accumulation in organs where the most acute toxic effects are exerted. This concept was indeed illustrated with doxorubicin, which displays severe acute and chronic cardiomyopathy. After intravenous administration to mice, plasma levels of doxorubicin were higher when the drug was adsorbed onto nanospheres and at the same time the cardiac concentration of the drug was dramatically reduced. In accordance with the observed distribution profile, doxorubicin associated with nanospheres was found to be less toxic than free doxorubicin. The ability of tumor cells to develop simultaneous resistance to multiple lipophilic compounds represents a major problem in cancer chemotherapy. Cellular resistance to anthracyclines has been attributed to an active drug efflux from resistant cells linked to the presence of transmembrane P-glycoprotein, which was not detectable in the parental drug-sensitive cellline. Drugs, such as doxorubicin, appear to enter the cell by passive diffusion through the lipid bilayer. Upon entering the cell, these drugs bind to P-glycoprotein, which forms transmembrane channels and uses energy from ATP hydrolysis to pump these compounds out of the cell. To solve this problem, many authors have proposed the use of competitive P-glycoprotein inhibitors, such as the calcium channel blocker verapamil, which are able to bind to P-glycoprotein and to overcome pleiotropic resistance. However, since the adverse effects of verapamil are serious, its clinical use to overcome multidrug resistance is limited.

During the past few years, many studies have been devoted to evaluating the antitumor potential of carrier-drug complexes (Brasseur, et. al., 1980). The effect of nanospheres loaded with doxorubicin, resistance to which is known to be related to the presence of P-glycoprotein, was evaluated. The cytotoxicity of free-doxorubicin, doxorubicin- loaded PIHCA nanospheres (NP-Doxorubicin) (mean diameter 300 nm), and nanospheres without drug (NP), against sensitive (MCF7) and multidrug resistant (Doxorubicin R MCF7) human breast cancer cell lines was compared. MCF7 cells were more sensitive to free-doxorubicin than Doxorubicin R MCF7 cells with a 150-fold difference in the IC50. No significant

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difference was observed in the survival rate of MCF7 treated with free-Doxorubicin or NPdoxorubicin. In contrast, for doxorubicin R MCF7, the IC50 for doxorubicin was 130-fold lower when NPdoxorubicin were used instead of free-doxorubicin. These results indicated that nanospheres provided an effective carrier for introducing a cytotoxic dose of doxorubicin into the pleiotropic resistant human cancer cell line Doxorubicin R MCF7.

Complementary experiments, conducted with other sensitive and resistant cell lines, have confirmed this efficacy of nanospheres. Doxorubicin resistance was circumvented in the majority of the cell lines tested, and some encouraging results were obtained in vivo in a P388 model growing as ascites. Further studies were undertaken to elucidate the mechanism of action of polyalkylcyanoacrylate nanospheres. The incubation time and number of particles per cell were important factors and, when PIBCA nanospheres were used, doxorubicin accumulation within P388/ADR resistant leukemic cells was increased compared with free drug, although no endocytosis of nanospheres occurred. On the other hand, when the less rapidly degradable PIHCA nanospheres were used, reversion was observed in the absence of increased intracellular drug. The degradation products of poly (alkylcyanoacrylate) nanospheres (mainly poly(cyanoacrylic acid)) were also able to increase both accumulation and cytotoxicity of doxorubicin, although they were soluble in the culture medium. Hence, the reversion of resistance seems to be due both to the adsorption of nanospheres on the cell surface and to theformation of a doxorubicin-poly(cyanoacrylic acid) ion pair, which facilitates the transport of the drug across the cell membrane.

In the light of the results obtained with doxorubicinloaded nanospheres in the liver metastases model described earlier, (Chiannilkulchai, 1989) the role of macrophages as a reservoir for doxorubicin was tested in a two-compartment coculture system in vitro with both resistant and sensitive P388 cells. Even after prior uptake by macrophages, doxorubicin-loaded PIBCA nanospheres were able to overcome resistance. However, this reversion was only partial. It was decided to take advantage of the particulate drug carrier offers to associate an anticancer drug and a compound capable of inhibiting the P-glycoprotein. This approach was tested with doxorubicin and cyclosporin A bound to the same nanospheres and was found to be extremely effective in

reversing P388 resistance (Soma, 1999). The association of cyclosporine A with nanospheres would ensure that it reaches the same sites as the anticancer drug at the same time and would also reduce its toxic side effects.

As early as 1986, researcher observed that other colloidal carriers, like nanocapsules, administered by the IV route in rabbits, were taken up rapidly by organs of the mononuclear phagocyte system (Khouri et al., 1986). One application that takes advantage of this uptake concerns nanocapsules of (muramyl tripeptide cholesterol) (MTP-Chol). This immunostimulating agent is able to activate macrophages and induce toxicity toward tumor cells, and would therefore be a useful agent to treat metastatic cancer. The mechanisms by which activated macrophages arrest tumor proliferation include production of nitric oxide and TNF-*a*. It was showed in in vitro models of rat alveolar macrophages and RAW 264.7 mouse monocyte macrophage line that nanocapsules based on poly(D,L-lactide) containing MTP-Chol are more efficient activators than the free drug. This action could be due to an intracellular delivery of the immunomodulator encapsulated in nanocapsules after phagocytosis and to an intermediate transfer of the drug to serum proteins (Seyler, 1999). This system has also demonstrated its efficiency in vivo; in fact (Barratt et al. 1994), reported that antimetastatic effects of nanocapsules contained MTP-Chol in a model of liver metastases. Some antimetastatic activity was also seen after oral administration.

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Nanospheres for Oligonucleotide Delivery

Oligodeoxynucleotides are potentially powerful new drugs because of their selectivity for particular gene products in both sense and antisense strategies. However, using antisense oligonucleotides in therapeutics is a challenge to pharmaceutical technology because of their susceptibility to enzymatic degradation and their poor penetration across biological membranes. Nanoparticulate preparations might be an interesting alternative because of better stability in the presence of biological fluids. In the case of nanospheres made of synthetic polymers (poly(alkylcyanoacrylate), poly (lactic acid)), since oligonucleotides have no affinity for the polymeric matrix, association with nanoparticles has been achieved by ion pairing with a cationic surfactant, cetyltimethylammonium bromide (CTAB) adsorbed onto the nanoparticle surface (Fattal, 1998). Oligonucleotides bound to poly(alkylcyanoacrylate) nanospheres in this way were protected from nucleases in vitro and their intracellular uptake was increased (Chavany, 1994). In addition, nanospheres were able to concentrate intact oligonucleotides in the liver and in the spleen (Nakada, et. al. 1996). Antisense oligonucleotides formulated in this way were able to specifically inhibit mutated Ha-ras-mediated cell proliferation and tumorigenicity in nude mice (Schwab, 1994).

This approach has recently been applied to the association of a phosphodiester antisense oligonucleotide directed against the 30 non-translated region of the PKC*a* gene with nanospheres prepared from PIBCA. These nanospheres were able to inhibit PKC*a* neoexpression in cultured Hep G6 cells. Nanospheres containing oligonucleotides have also been formulated from a naturally occurring polysaccharide, alginate, which forms a gel in the presence of calcium ions. In this case, the oligonucleotides penetrate into the gel matrix by reptation, thus providing a high loading yield and good protection against nuclease (Aynie, 1999).

Subcutaneous/Intramuscular Administration

Subcutaneous administration of nanoparticles was achieved mainly for the delivery of peptides and vaccines. It allows slow release of the entrapped drugs therefore reducing the number of administrations, increasing blood half-life of the active drug, and finally, in some cases, reducing side effects.

PIBCA nanospheres were injected subcutaneously to rats. Autoradiographic pictures obtained after using radiolabelled polymer have shown a progressive staining reduction in the muscular tissue suggesting that nanospheres were slowly biodegraded. In the same study, nanospheres were found to release a peptide (GRF) in a sustained manner. Comparison of the

AUC of free GRF and GRF-loaded nanospheres showed that in addition to the slow release process nanospheres were able to improve the bioavailability of the peptide. This improvement could be attributed to the fact that free administered GRF is very quickly metabolized at the injection site, whereas it is partly protected from massive enzymatic degradation when it is administered associated with nanospheres (Gautier, 1992).

A few examples of the use of nanospheres as adjuvant for antigens/allergens delivery were described in the literature. The main advantage of this approach is to design single shot vaccine. In this case the drug carrier has to remain at the site of administration and deliver either continuously or pulsatively the antigen. The use of slowly degradable polymers (PLA, PMMA) is suitable for this application, since peptide or protein release is more adequate. Poly(methyl methacrylate) were first investigated as adjuvants for injectable vaccines. These nanospheres were claimed to be biodegradable after subcutaneous or intramuscular injection and shown to exhibit very powerful adjuvant properties for a number of antigens.

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However, the adjuvant properties were shown to be better when the antigen was incorporated during the polymerization process than when adsorbed onto nanospheres (Mahmoudi et al., 2010). When comparing the effect of PMMA with other polymers (polystyrene and 2-hydroxyethyl methacrylate/methyl methacrylate copolymer, HEMA: MMA), it was also demonstrated that a decrease in particle size and an increase in the hydrophobicity of nanospheres increased the antibody response after immunization against influenza whole and split virus, bovine serum albumin, and HIV2 split virus (Kreuter, 1978).

Oral Route

There are numerous reports showing that uptake and translocation of nanoparticles and microparticles take place after oral administration to animals (Jani, 1989). Different mechanisms have been proposed to explain the translocation of particulate material across the intestine: i) uptake via Peyer's patches or isolated lymphoid follicles; ii) intracellular uptake; and iii) intracellular/paracellular passage. The uptake of poly(alkylcyanoacrylate) nanocapsules by Peyer's patches has been shown by Damge´ et al., (Damge et al., 1988) When administered in the lumen of an isolated ileal segment of the rat, polylalkylcyanoacrylate nanocapsules were found preferentially over Peyer's patches through which they passed massively and rapidly.Nanocapsules were clearly visible in M-cells and in intercellular spaces around the lymph cells. Intracellular uptake of nanospheres has been proposed by Kreuter, Muller and Munz (Kreuter, 1989; Muller & Munz, 2000) based on electron-microscopic autoradiographic investigations showing radioactivity into epithelial and goblet cells after oral administration of poly(hexylcyanoacrylate) (PHCA) nanospheres labeled with 14C. The translocation of particles by a paracellular pathway has been evidenced in a study done by Aprahamian et al., using PIBCA nanocapsules. Nanocapsules were filled with an iodinized oil (lipiodol) in order to render them detectable using a scanning electron microscope equipped with an energy-dispersive X-ray spectrometer (Aprahamian et al., 1987). When they were administered in an isolated segment of a dog jejunum, they appeared as vesicles associated with intraluminal mucus. Subsequently, they were observed in intravillus capillaries in close contact with red cells or adsorbed to the inner wall of endothelial cells. Among these three mechanisms and according to many studies involving nanoparticles made of other biodegradable and nondegradable polymers, translocation via the uptake in Peyer's patches seems to be the major pathway for a rapid and substantial passage after oral administration of nanoparticles. Although it might exist in certain situations, the passage of particles between the absorptive cells is rather less likely if the barrier of tight junctions has not been disrupted. Although there are abundant reports from various independent workers showing evidence of absorption of particulate systems by the gastrointestinal tract, the oral absorption of nanoparticles remains a controversial issue. However, even if a more clear estimation of the quantity of absorbed particles is needed as well as a better understanding of the factors affecting particles uptake, it must be concluded that translocation of small sized particles like poly(alkylcyanoacrylate) nanoparticles is possible. The question remains if the extent of particle translocation is compatible with a strategy of drug administration with therapeutic perspectives. This will be discussed below.

Oral Delivery of Peptides and Proteins and Vaccines

Poly(isobutylcyanoacrylate) nanocapsules were shown 10 years ago to be able to encapsulate insulin and to increase its activity as assessed by a reduction of glycemia. Several aspects of this phenomenon are surprising: encapsulation of a hydrophilic drug in the oily core of nanocapsules; reduction of glycemia

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was only obtained with diabetic animals; hypoglycaemia appeared two days after a single administration and was maintained for up to 20 days depending on the insulin doses, although the amplitude of the pharmacological effect (minimum level of blood glucose) did not depend on the insulin dose. Damge´ et al. (Damge et al., 1990) and Lowe and Temple (Lowe and Temple, 1994) suggested that nanocapsules could protect insulin from proteolytic degradation in intestinal fluids, based on the protection of encapsulated insulin, observed in the presence of different enzymes in vitro. Later studies showed that insulin did not react with the alkylcyanoacrylate monomer during the formation of nanocapsules and was located within the oily core rather than adsorbed on their surface.

The capacity of insulin nanocapsules to reduce glycemia could be explained by their translocation through the intestinal barrier, as suggested by Damge´ et al., (Damge et al. 1997) for example by paracellular pathway or via M cells in Peyer's patches (Michel, 1991). Recently, the use of Texas Red_-labeled insulin allowed this translocation to be visualized more readily (Aboubakar, 2000). One hour after oral administration, nanocapsules reached the ileum. The presence of fluorescent areas within the mucosa and even in the lamina propria suggested that insulin-loaded nanocapsules could cross the intestinal epithelium. Although this passage is certainly an important factor, it does not explain the duration of the hypoglycemia. This prolonged action could be due to the retention of a part of the colloidal system in the gastrointestinal tract. Interestingly, a prolonged hypoglycemic effect was also observed with insulin entrapped in poly(alkylcyanoacrylate) nanospheres when these were dispersed in an oily phase containing surfactant. This suggests that some components of nanocapsules could act as promoters of absorption. Recently, Damge´ et al. showed that the incorporation of octreotide, a somatostatin analogue, in poly(alkylcyanoacrylate) nanocapsules also improved and prolonged the therapeutic effect of this peptide, after administration by the oral route (Damge et al., 1997).

Even if the main limitation to oral administration of poly(alkylcyanoacrylate) nanoparticles is that their passage through the intestinal barrier is probably restricted and sometimes erratic, they represent an interesting tool for oral delivery of antigens. Indeed, M-cells appear to be the main site for the uptake of poly(alkylcyanoacrylate) nanoparticles after oral administration (Damge et al., 1997).and, furthermore, it is generally accepted that limited doses of antigen are sufficient for a mucous immunization. In fact, oral delivery of antigens may be considered as the most convenient means of producing an IgA antibody response. However, it is importantly limited by enzymatic degradation of antigens in the GI tract and, additionally by their poor absorption (Yildirimera et al., 2011). Thus, it has been postulated that the use of micro- or nanoparticles for the oral delivery of antigens should be efficient if those systems are able to achieve the protection of the antigenic molecule. Poly(alkylcyanoacrylate) nanoparticles have been shown to enhance the secretory immune response after their oral administration in association with ovalbumin (O'Hagan, 1989). This result was not fully reproduced in the case of poly(acrylamide) nanoparticles loaded with the same antigen. It was postulated that in the case of poly(acrylamide) nanospheres, much of the antigen was located at the surface of the polymer and could have been degraded during its passage through the gut. The relatively high surface concentration of ovalbumin adsorbed onto poly(butylcyanoacrylate) nanospheres may have reduced the ability of the proteolytic enzymes in the gut to gain access to and to degrade the antigen, resulting in a greater antigen availability.

Ocular Delivery

The anatomical structure and the protective physiological process of the eye exert a strong defense against ocular drug delivery. This is the reason why conventional ocular dosage forms exhibit extremely low

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bioavailability. Limited absorption of the drug through the lipophilic corneal barrier is mainly because of short precorneal residence time due to the tear turn-over, rapid nasolacrimal drainage of instilled drug from the tear fluid, and non-productive absorption through the conjonctiva. Only a small proportion (1–3%) of the applied drug penetrates the cornea and reaches intraocular tissues. For these reasons, it is necessary to develop efficient and more acceptable ocular therapeutic systems.

Different strategies can be carried out to improve the precorneal residence time and/or penetration ability of the active ingredient. Among them, one approach consists of using colloidal drug delivery systems such as nanoparticles. Initial studies carried out with nanocapsules, as ocular drug carriers, attempted to increase the penetration of lipophilic drugs into the eye by prolonging the precorneal residence time, as observed with other colloidal systems, liposomes and nanospheres. These studies, which concerned antiglaucomatous agents, such as betaxolol, carteolol, and metipranolol encapsulated in nanocapsules, only showed a reduction of the non-corneal absorption (systemic circulation) leading to reduced side-effects as compared with the free drug (Losa, 1993). These systemic side-effects are due to a poor ocular retention of drugs that are directly absorbed into the systemic circulation by conjunctival and nasal blood vessels. In two cases (carteolol and betaxolol), encapsulation in nanocapsules produced an improved pharmacological effect (reduction of intraocular pressure) than produced by free drug and nanospheres (although the penetration of nanocapsules was not tested) and reduced cardiovascular systemic side-effects. Metipranolol showed the same activity alone and associated with nanocapsules but, as in the case of carteolol and betaxolol, its side-effects were reduced. When betaxolol was used, the nature of the polymer making up the nanocapsule wall was found to be important, and poly (*e*-caprolactone) was more efficient than PIBCA or poly(lactic-co-glycolic acid) (Marchal-Heussler, 1993).

Calvo et al., explored the mechanisms of interaction of nanocapsules with ocular tissues to better understand the pharmacological responses obtained with antiglaucomatous agents. By confocal microscopy, they showed that poly(*e*-caprolactone) nanocapsules could specifically penetrate the corneal epithelium by an endocytic process without causing any damage to the cells, in contrast with PIBCA nanoparticles, the uptake of which was associated with cellular lysis (Calvo et al., 2001). These results explained the improved therapeutic effect and the reduction of systemic side-effects as a result of drug loss through the conjunctiva provided by poly(*e*-caprolactone) nanocapsules by increasing corneal epithelium penetration of lipophilic drugs. Calvo et al., also excluded the influence of the oily inner structure in the activity of the nanocapsules, in the light of the absence of differences in penetration between nanospheres and nanocapsules (Park et al. 2015; Afrooz et al., 2015), in contrast with Marchal-Heussler et al., who observed a better therapeutic effect with nanocapsules than with nanospheres (Marchal-Heussler et al. 1992). Moreover, Calvo et al. demonstrated with indomethacin-loaded nanocapsules that the colloidal nature of the carrier was the main factor influencing its ocular bioavailability. The same authors were also interested in the influence of the nature and the charge of the surface of nanocapsules on their physical stability and on their ocular bioavailability. They found that coating the negatively charged surface of poly(*e*-caprolactone) nanocapsules with cationic polymers could prevent their degradation caused by the adsorption of lysozyme, a positively charged enzyme found in tear fluid (Calvo et al. 1997). Moreover, they noticed that a cationic polymer, chitosan, adsorbed on the surface of nanocapsules was able to provide the best corneal drug penetration without any local intolerance as compared to another positively charged polymer. This was achieved by a combination of effects: Penetration of particles into the corneal epithelial cells, mucoadhesion of positively charged particles onto negatively charged membranes, and a specific effect on the tight junctions (Calvo et al. 1997).

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This effect of improvement of ocular absorption was also reported by Calvo et al. 1996 with the immunosuppressive peptide cyclosporin A. The corneal level of the drug was increased fivefold as compared with an oily solution of the drug owing to a highly loaded nanocapsule preparation, also containing poly (*e*-caprolactone). The efficacy of this topical formulation has also been observed on a penetrating keratoplasty rejection model in the rat (Holzwarth et al., 2014) and also proposed an alternative preparation of cyclosporin nanocapsules based on poly(alkylcyanoacrylate) dispersed in poly(acrylic acid) gel able to drastically reduce toxicity of poly(alkylcyanocrylates) on the cornea and to promote absorption of the drug (Harper et al., 2015).

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Chapter 7
Non-Ionic Surfactant Vesicles (Niosomes) as New Drug Delivery Systems

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ABSTRACT

Lipid vesicular systems composed of hydrated amphihiles with or without bilayer inducing agents such as cholesterol. On the basis of used amphiphilic molecule different nomenclature are used as liposomes, ufasomes and niosomes. Nonionic surfactants with mono-, di- or trialkyl chains form niosomes which are lipid vesicles with more chemical stability in comparison with phospholipids of liposomes. Both hydrophobic and hydrophilic chemicals can be encapsulated in niosomes as a new drug delivery system. This drug carrier system could have administered via injection, oral, pulmonary, vaginal, rectal, ophthalmic, nasal or transdermal routes with penetration enhancing potential. This chapter presents a detailed explain about niosome forming components, methods of preparation and routes of administration. Many examples for drug delivery potential of niosomes are also available in this review. Vaccine adjuvant and genetic substances vector capabilities are not given here.

INTRODUCTION

There are versatile lists of amphiphilic compounds for production of lipid vesicles which on the basis of used amphiphiles or structural compounds, different nomenclature has been used such as liposomes (Kaminskas et al. 2012), niosomes (Moazeni et al. 2010; Akbari et al. 2013), ufasomes (Patel et al. 2011), ethosomes (Liu et al. 2012), eliposomes (Lattin et al. 2012) and so on. In lipid vesicular systems, polar headgroups of amphiphilic molecules are oriented into aqueous compartments and their lipophilic parts form bilayer membranes. Niosomes are bilayer vesicles composed mainly of hydrated non-ionic surfactants in addition to, in many cases, cholesterol (CHOL) or its derivatives (Pardakhty and Moazeni 2013). Cosmetic industry was the place for the first account of niosome production (Handjani-Vila et Al. 1979) after which a large number of niosome applications in drug, vaccine and gene delivery have been

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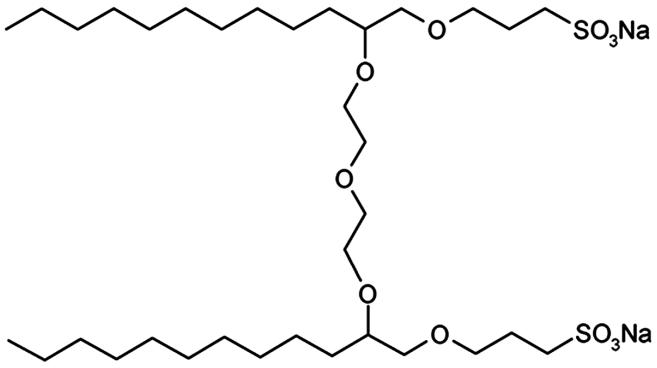
explored. While most niosomes are in the nano or sub-micron (colloidal) size range, not many authors used the "nanoniosome" or "nanovesicle" titles in their published articles due to introduction the new nanotechnology related phrases during the few recent years. Niosomes are capable to encapsulate both hydrophilic and lipophilic substances which the first ones usually are entrapped in vesicular aqueous core or adsorbed on the bilayer surfaces, and lyophobic molecules are encapsulated by their partitioning into the lipophilic domain of the bilayers. Incorporation of charged molecules into non-ionic surfactant vesicles forms charged or hybrid niosomes (Sennato et al. 2008). Vesicles can be also prepared from the mixed cationic and anionic surfactant systems such as cetyltrimethylammonium bromide and sodium lauryl sulfate (CTAB-SLS) (Aiello et al. 2010). These types of vesicles are presented as "cat-anionic" or "catanionic" vesicles. Catanionic vesicles can also be formed from electrostatic interaction between an ionized drug, such as diclofenac (Zhao et al. 2013) or alprenolol (Dew et al. 2012) and a positively/negatively charged surfactant.

Non-ionic surfactants have more chemical stability against oxidation and are less thermo-labile in comparison to phospholipids, the main constituent of liposomes (Fang et al. 2001; Tarekegn et al. 2010) thus less significant care in handling and storage are needed. Furthermore, greater versatility and lower cost make this type of vesicles more attractive in drug, gene and vaccine delivery (Florence 1993). From the pharmaceutical manufacturing stand of view the superiority of niosomes is simple methods required for industrialized and large–scale production of them without the use of pharmaceutically unacceptable solvents (Sahin 2007). In this chapter the most important aspects of niosomes and drug delivery potentials will be reviewed. Application of niosomes as gene or vaccine delivery tools will not discussed in this chapter.

Amphiphiles (Surfactants)

Following the application of some forms of energy such as mechanical or heating, the formation of niosomes is a self-assembly process due to high interfacial tension between aqueous medium and the lipophilic alkyl chain(s) resulted in the association of non-ionic surfactant monomers into vesicles (Uchegbu and Vyas 1998). Concurrently, the hydrophilic headgroups of amphiphilic molecules make water mediated interactions counter the previous formed force, finally result in bilayer formation. The

Figure 1. α,ω-trioxyethylene-bis(sodium 2-dodecyloxy-propylenesulfonate), an anionic gemini surfactant, used for preparation of niosomes
Muzzalupo et al. 2008.



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energy required to form vesicles with amphiphile molecules has three contributors related to the surface energy, the mechanical energy due to overpressure and the chemical potential excess (Manconi et al. 2005).

Formation of niosomes needs at the first step to an amphiphilic molecule, but in many cases the presence a wedge shaped molecule such as CHOL is essential to turn the micellar structure of surfactant aggregates to bilayer arrangement (Pozzi et al. 2009). The lipophilic moiety of amphiphile molecule may be contain one (Varshosaz et al. 2003), two (Okahata et al. 1981)or three (Yoshioka et al. 1994; Jain and Vyas 1995; Jain et al. 2005)alkyl or perfluoroalkyl(Zarif et al. 1994)groups or in some cases a single steroidal group(Echegoyen et al. 1988).

Amino acid-based surfactants (Guedj et al. 1994; Polidori et al. 1994), glycolipids (Polidori et al. 1997) and crown ether compounds (Echegoyen, Hernandez et al. 1988; Darwish and Uchegbu 1997; Muzzalupo et al. 2007; Paolino et al. 2007)were used to form niosomes in aqueous hydration media. More recently gemini surfactants, composed of two amphiphilic molecules connected to each other by a spacer, were also used to form niosomes (Muzzalupo et al. 2008; Paolino et al. 2008) (Figure 1).

The flexibility of compounds capable for vesicle forming is due to different and various polar head groups attached to saturated or unsaturated alkyl chain(s) composed of 12 to 18 carbon atoms (C_{12}-C_{18}). However, here the main non-ionic surfactant classes used for niosome production will be presented which are alkyl ethers, alkyl esters, alkyl amides andsome other amphipathic compounds such as fatty acids.

Alkyl Ethers

Surfactant I (polyglyceryl-3 cethyl ether) has one n-alkyl chain and was the first amphihpile used for niosomes preparation (Handjani-Vila, Ribier et al. 1979). This amphiphile has been used for preparation of niosomes encapsulating adriamycin (Rogerson et al. 1987) and diclofenac sodium (Türker et al. 2005). Özer et al. (Özer et al. 2013) showed diclofenac sodium niosomes formulated by Surfactant I/ CHOL could bear different γ-radiation doses.

Hofland et al. (1992); Hofland et al. (1993); Hofland et al. *1994) reported the formation of a series of vesicles composed of n-alkyl polyoxyethylene ether surfactants(C*n*EO*m*) mainly designed for topical delivery of therapeutic compounds. Van Hal et al. (van Hal et al. 1996)used C*n*EO*m*, in which the number of oxyethylene units (*m*) ranged from 3 to 7 and the number of carbon atoms (*n*) ranged from 12 to 18, in order to obtain a systematic variation in length of alkyl chain and headgroup. They showed without CHOL in C_nEO_3 surfactants the vesicle formation was probable by sonication method but increasing the polyoxyethylene units to C_nEO_7 put an end to vesicle formation. Bouwstra et al (Bouwstra et al. 1997) also reported the vesicle forming ability of $C_{14}EO_5$ and $C_{16}EO_5$ in the absence of CHOL. Pardakhty et al. (2007) prepared vesicles composed of $C_{16}EO_2$ (Brij® 52), $C_{18}EO_2$ (Brij 72), $C_{18}EO_{10}$ (Brij 76), $C_{9=9}EO_2$ (Brij 92) and $C_{9=9}EO_{10}$ (Brij 97) without any added CHOL by film hydration method. In contrast with their results, Suwakul et al. (Suwakul et al. 2006)reported the lack of niosome formation with the mentioned surfactants in the absence of CHOL. This is maybe caused by different used methods for preparation of vesicles and unlike encapsulated drug's nature; Suwakul's group utilized direct sonication of molten lipids, in addition to use of propylthiouracil, a lyophobic drug which interact with bilayer formation ability of the applied surfactants and even made the bilayer less accommodating for CHOL molecules (Suwakul, Ongpipattanakul et al. 2006).While we used film hydarttion method with a water soluble molecule, insulin(Pardakhty, Varshosaz et al. 2007).

Various lipophilic drugs such as cycloslporin-A(Niemiec et al. 1995; Waranuch et al. 1998), erythromycin (Jayaraman et al. 1996), estradiol (Hofland, van der Geest et al. 1994), finasteride (Tabbakhian

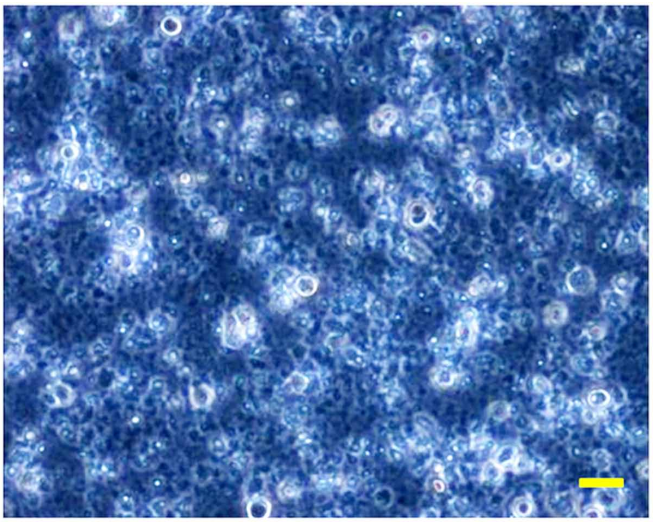
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et al. 2005; Tabbakhian et al. 2006) minoxidil (Balakrishnan et al. 2009), tretinoin (Manconi et al. 2002; Manconi et al. 2003; Manconi et al. 2006), triamcinolone acetonide (Sankar et al. 2013), and some water soluble ones like α-interferon (Niemiec, Ramachandran et al. 1995), cimetidine (Lieb et al. 1994), and recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) (Memisoğlu et al. 1997),were encapsulated in C_nEO_m containing vesicles. All the mentioned vesicular formulations were formulated for topical delivery to stratum corneum, follicular (pilosebaceous units) and the other parts of the dermis. Hofland, van der Geest et al. (1994) studied permeation of estradiol from C_nEO_m niosomes. They showed that direct contact between niosomes and skin was imperative to exert the highest effect on drug transport. This effect even reported when pretreatment of the skin withnon-occlusive C_nEO_m niosomes enhanced the iontophoretic transport of R-apomorphine through human abdominal skin (Li et al. 2001). Hydroxychloroquine niosomes composed of Brij 98/CHOL were also formulated for topical management of oral lichen planus (Bendas et al. 2013).

Alkyl Esters

Among the alkyl esters, the most important and frequently used surfactants are sugar esters. These surfactants contain a sugar group as the polar headgroup of the amphiphilic molecule. The sugar moieties can be sucrose, glucose, sorbitan, methyl glucose, mallitol, or other sugars. The hydroxyl moieties of the sugars are partially esterified with alkyl chains. Kiwada et al. (KIWADA et al. 1985; Kiwada et al. 1985)used alkyl glycosides for preparation of vesicles and Schenk et al. (Schenk et al. 1989)worked on vesicles of sucrose ester surfactants. Sucrose cocoate, a mixture of fatty acid sucrose esters, was used for vesicle formation and encapsulation of sulfadiazine or diclofenac, both for topical delivery in nanoniosome forms (Tavano et al. 2010). Manconi et al. (Manconi et al. 2006)also reported the formation of niosomes composed of decylpolyglucoside with or without CHOL entrapping methylene blue as a hydrophilic model compound.

Figure 2. Dark field microscopy photomicograph of Leishmania major recombinant gp63 (r-GP63) multilamellar vesicles (MLVs) containing Span 60/cholesterol (70:30 molar ratio); magnification 40×40, bar = 5 μm



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Sorbitanmono esters (Span®) can be known as the basic and the most widely applied surfactants used for niosome preparations (Figure 2). Many articles could be found in literature about using sorbitan ester niosomes for entrapping different compounds with various physicochemical and therapeutic properties such as acetazolamide (Guinedi et al. 2005; Aggarwal et al. 2007), acyclovir(Attia et al. 2007), insulin (Khaksa et al. 2000; Ning et al. 2005), methotrexate (Chandraprakash et al. 1990; Chandraprakash et al. 1992), doxorubicin (Uchegbu et al. 1995; Uchegbu et al. 1996); vincristine (Parthasarathi et al. 1994), hydroxycamptothecin (Shi et al. 2006; Hong et al. 2009), vasoactive intestinal peptide (Dufes et al. 2004) and salicylic acid (Junyaprasert et al. 2008).

Generally, by increasing the hydrocarbon chain length from C_{12} to C_{18} and also more rigidity in bilayers by saturation of hydrophobic chain (stearate vs. oleate) in sorbitan esters vesicles, the encapsulation of water soluble molecules such as insulin (Varshosaz, Pardakhty et al. 2003), colchicine (Hao et al. 2002), salicylic acid and p-hydroxyl benzoic acid (Hao and Li 2010) or poorly soluble compounds such as flurbiprofen (Mokhtar et al. 2008)will be increased. Sorbitan esters can form vesicles in the presence or absence of molecules which operate co-operatively to form bilayer membranes; for instance, Span 60 with C_{18} mono-alkyl chain has been used for vesicle formation alone (Varshosaz et al. 2003), with CHOL (Suwakul, Ongpipattanakul et al. 2006) or CHOL/cholesteryl poly-24-oxyethylene ether (Solulan C24) (Uchegbu, Double et al. 1995). Kato et al. prepared temperature-sensitive fusogenic niosomes composed of Span 80 alone, or with CHOL (Kato et al. 2008).

Shi, Fang et al. (2006) used different poly (methoxypolyethyleneglycol cyanoacrylate-co-n-hexadecyl cyanoacrylate) (PEG-PHDCA) acted as surface modification materials, Span 60 and CHOLto encapsulate hydroxycamptothecin in "stealth nanoniosomes". The tumor inhibition rate of PEG5000-PHDCA niosomes (92.5 nm) at a dose of 2 mg/kg was five times that of drug injection at 4 mg/kg. Used PEG was likely to fold on the surface of the niosomes due to its compressibility, which is favorable for niosomes to prevent aggregation and improve its ability to evade macrophages uptake (Shi, Fang et al. 2006). Hong et al. (Hong, Zhu et al. 2009) modified the stealth nanoniosomes by coupling periodate-oxidated transferrin to terminal amino group of PEG to produce the active targeting vesicles with average diameters of 116 nm. Compared with injected free drug/non-stealth and stealth niosomes, transferrin holding PEG-PHDCA niosomes demonstrated the strongest cytotoxicity to three carcinomatous cell lines (KB, K562 and S180cells), the greatest intracellular uptake especially in nuclei, the highest tumor concentration and largest are under the intratumoral hydroxycamptothecin concentration curve, as well as the most powerful anti-tumoractivity with the inhibition rate of 71% against S180 tumor in mice.

Yoshioka, Sternberg et al. (1994) studied the vesicle forming ability of sorbitan monoesters and a sorbitan triester (Span 85). Sorbitan tri-stearate (Span 85) was also utilized for rifampicin niosome preparation (Jain and Vyas 1995). Increasing the amount of CHOL led to decrease in the entrapment efficiency of rifampicin due to rigidity increase of niosomal bilayers following CHOL content increment (Baillie et al. 1985).

Even though the polyoxyethylene sorbitan esters (Tween®) are more soluble and have higher hydrophilic lipophilic balance (HLB) values in comparison to sorbitan esters, this class of surfactants have been used for vesicle preparation in combination with sufficient amount of CHOL (Carafa et al. 1998; Di Marzio et al. 2011; Di Marzio et al. 2012). Carafa et al. (2006) reported the formation of pH sensitive nanoniosomes made from polxoxyethylene sorbitan mono-laurate (Tween 20), CHOL and its derivative, cholesteryl hemisuccinate, as pH-sensitive molecule. More recently a novel class of compounds based on Tween 20 surfactant bearing different pH-sensitive head groups (i.e., glycine, N-methyl-glycine, N,N-dimethyl-glycine) were used successfully for delivery of Na calcein, a carboxylic fluorescent dye,

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to human hepatoblastoma (HepG2) cell line (Masotti et al. 2010). Di Marzio et al. (Di Marzio, Marianecci et al. 2011) studies suggest that the Tween 21/CHOL nanovesicle is also a pH-sensitive system. The pH-sensitivity and more bilayer fluidity of Tween 21 vesicles could be related to the lower content of counter ions and associated waters of hydration near the head groups, bearing only 4 PEG units, in comparison with Tween 20, bearing 20 PEG units.

Nano size niosomes composed of polyoxyethylene sorbitan monostearate (Tween 61) and CHOL were used for encapsulation of azelaic acid and its derivatives (Panyosak et al. 2009), diclofenac diethylammonium (Manosroi et al. 2008), glucose (Manosroi et al. 2008), and rice bran extract (Manosroi et al. 2012). Manosroi et al. (2010) reported the entrapment enhancement of three peptides including bacitracin, insulin and bovine serum albuminin neutral, anionic and cationic Tween 61 niosomes.

Utilizing of combination of sorbitan esters and their polyethoxylated derivatives has led to stable and efficient vesicles encapsulating of ciprofloxacin (Moazeni, Gilani et al. 2010)with low cytotoxicity against alveolar human carcinoma cell line (A549) suggesting a good aerosol drug delivery system. Combinations of different Spans and Tweens with CHOL were also successfully utilized to encapsulate ciprofloxacin in nanoniosomes for enhance intracellular delivery of this flouroquinolone (Akbari, Abedi et al. 2013; Akbari et al. 2015); the high physical stability of the prepared niosomes shown as little size change during the storage may be was due to the steric stabilization of vesicles following the incorporation of polyoxyethylene bearing surfactants, Tweens, in the formulations. Desai and Finlay (Desai and Finlay 2002) demonstrated the potential of encapsulating all-trans-retinoic acid in niosomes composed of Span 20 or Span 60 with Tween 80 and delivering it as an inhaled aerosol by nebulization. Such a combination, even though without CHOL and in the presence of Span 80/Tween 80/PEG 6000/water has been experimented for vesicle forming ability by transformation of lamellar liquid crystal structure to vesicular ones enable for encapsulation both hydrophilic (ribavirin) and hydrophobic (ibuprofen) substances (Liu and Guo 2007). Devaraj et al. (2002) prepared different niosomal formulations in which a combination of polysorbates (Tweens), polyglyceryl-3-diisostearate and different fatty alcohols used for vesicle formation and encapsulation of anti-inflammatory drug, ketorolac. Combination of Span 20 or Tween 20/ polyglycero-6-oleate was also used for stable nano size niosomes enable to encapsulate three model peptide or proteins (Cortesi et al. 2007). Span and Tween niosomes were also used for encapsulation of poorly water soluble antifungal drug, itraconazole (Alomrani et al. 2015). Rat abdominal skin permeation studies showed Span niosomes had enhancement penetration of itraconazole compared to its solution; however, it was less in magnitude than that of Tweens.

Alkyl Amides

Zarif, Gulik-Krzywicki et al. (1994) studied the supramolecular assemblies or vesicles produced by applying alkyl galactosides and glucosides incorporating amino acid spacers. They also reported the formation of perfluoralkyl glycolipid-based vesicles (Zarif 2002). Uchegbu (Uchegbu 1998)reported the formation of nanoniosomes composed of palmitoyl muramic acid/CHOL/Solulan C24 which were loaded with doxorubicin by the use of ammonium sulfate gradients. The biodistribution of prepared 200 nm vesicles in male Balb/c mice after i.v. administration were found to avoid liver uptake but not splenic uptake when compared to drug loaded Span 60 niosomes. N-palmitoylglucosamine (NPG) niosomes were also prepared by sonication of NPG/ Span 60/CHOL/ Solulan C24 which had a z-average mean diameter of 164 nm (Dufes et al. 2000).

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Other Amphiphilic Compounds

Many other compounds with surfactant-like behavior have been used for vesicle formation; for example, long chain fatty acids such as oleic acid can be used for vesicle formation which usually named as unsaturated fatty acid vesicles or 'ufasomes' (Gebicki and Hicks 1973). Intranasal lyophilized gels containing ufasomes were prepared from oleic acid to circumvent the low and erratic absorption of orally administrated cinnarizin (Salama and Aburahma 2015).

Covalently bound alkyl amino acids with efficient amphiphilic properties have been used for vesicle formation (Neumann et al. 1987).

Gopinath et al. (2004) utilized one ascorbic acid derivative, ascorbyl palmitate, for vesicle formation and *in vitro* transdermal delivery of azidothymidine by these vesicles which named as 'aspasomes'. Conacher et al. (2001) has also developed drug delivery vehicles called 'bilosomes', which are vesicles prepared from bile salts such as deoxycholate for oral immunization with peptide and protein antigens.

BILAYER INDUCING AGENTS

Cholesterol

The most common additive found in niosomal systems is cholesterol (CHOL) which is known to abolish the gel to liquid phase transition of liposomal and niosomal systems, resulting in less leakiness of the vesicles and improved niosomes stability (Junyaprasert, Teeranachaideekul et al. 2008). Surface pressure measurements on monolayers of nonionic surfactant/CHOL mixtures demonstrated a condensing effect of CHOL as evidenced by the decrease in the effective area per molecule as the CHOL content of the monolayer increased. This effect might have attributed to the accommodation of CHOL in the molecular cavities formed by surfactant monomers assembled into vesicles and is responsible for the observed decreased permeability of CHOL-containing membranes compared to CHOL free membranes (Devaraj et al. 2002). CHOL is used to complete the hydrophobic moiety of high HLB single alkyl chain non-ionic surfactants for vesicle formation (Masotti, Vicennati et al. 2010). In general, it has been found that a molar ratio of 1:1 between CHOL and non-ionic surfactants is an optimal ratio for the formulation of physically stable niosomal vesicles (Nasseri 2005). Some reports denote the formation of monohydrate or anhydrous CHOL crystals among the surfactant/CHOL bilayers (Caracciolo et al. 2008). The minimum amount of CHOL required to form vesicles without evoking surfactant aggregates or other irregular structures depended on the type of surfactant and it's HLB (van Hal, Bouwstra et al. 1996; Uchegbu and Vyas 1998).

Other Bilayer Inducing Agents

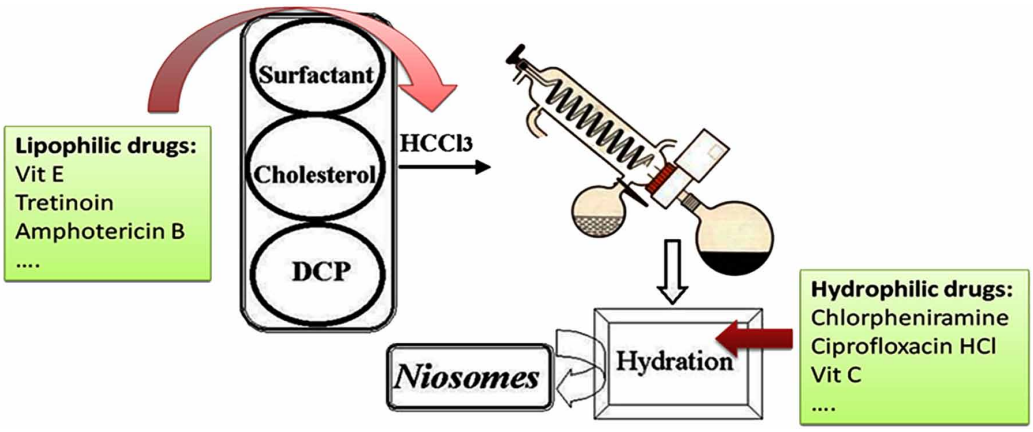
Non-ionic surfactant vesicles have been formed with different lipid compositions without CHOL. In Table 1, a complete list of these compounds was presented. The resultant vesicles showed different physicochemical behaviors; for example, polyhedral niosomes were formed from systems containing $C_{16}G_2$ (hexadecyl diglycerol ether) and Solulan C24 alone, while cholesterol-containing systems formed spherical vesicles (Arunothayanun et al. 2000).

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Table 1. Different compounds utilized for niosome preparation in the absence of cholesterol

Bilayer Additives	Used Surfactant	Encapsulated Compound	Reference(s)
Soybean phosphatidylcholine	Span 80	------	(Kato, Walde et al. 2008)
PEG 400	Span 80	Ribavirin or ibuprofen	(Hua and Liu 2007)
PEG 6000	Tween 80/Span 80	Hemoglobin	(Liu et al. 2007)
PEG 6000	Tween 80/Span 80	Ribavirin or ibuprofen	(Liu and Guo 2007)
Poly-24-oxyethylene cholesteryl	Hexadecyl diglycerol	5(6)-carboxyfluorescein	(Arunothayanun, Bernard et al. 2000)
ether (Solulan C24)	ether (C16G2)		
Fatty alcohols	Tween 20, 40, 60, 80	Ketorolac tromethamine	(Devaraj, Parakh et al. 2002)
(C12, C14, C16, C18, and C16+18)			
Absolute ethanol	Span 20, 40, 60, 80	Flurbiprofen (proniosome)	(Mokhtar, Sammour et al. 2008)

Figure 3. Thin film hydration (hand shaking) for preparation of MLV niosomes



Methods of Niosome Preparation

Generally, there are two classes of niosome or liposome preparation methods; the first set includes dissolving the whole lipids in organic solvent(s) for molecular level mixing of the bilayer constituents, then removing the organic solvent and hydration of formed lipid thin films or surfaces by an aqueous medium. The second series of vesicle formulation comprises the direct mixing of lipids and hydration medium, usually in high elevated temperature, which have omitting the hazardous effect of trace residuals of organic solvents on entrapped substance or biologically applied environments.

Detailed preparation methods which use organic solvents are presented here:

1. **Thin Film Hydration (Hand Shaking):** In this method all lipids are dissolved in an organic solvent, typically chloroform. The solvent is evaporated under reduced pressure and the hydration phase containing water soluble components is added at a temperature higher than phase transition temperature of used surfactants (Figure 3). This is the most common method of niosome preparation and for instance was reported for encapsulating of nimesulidea (Shahiwala and Misra 2002).

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2. **Reverse-Phase Evaporation:** Vesicle forming ingredients are dissolved in chloroform/diethyl ether and water in oil emulsion is formed by sonication of two phase's mixture. The organic solvent(s) is evaporated in a rotary evaporator and the resultant viscous niosomal suspension is diluted by adding aqueous phase. This technique was reported for preparation of acetazolamide niosomes (Guinedi, Mortada et al. 2005).
3. **Ether Injection:** Lipids are dissolved in diethyl ether and slowly injected into the preheated aqueous phase (about 60°C) by a needle. Under this condition, the organic solvent is evaporated and niosomal suspension is formed as used for entrapment of some newly synthesized beta-blockers (Piplani, Kumar et al. 2015).
4. **Dehydration Rehydration Method:** The prepared vesicles are freeze dried (lyophilized) and then rehydrated by appropriate aqueous dehydration rehydration (DRV) medium (Tafreshi and Mirdamadi 2015). We used this method for encapsulation of N, N-diethyl-meta-toluamide, as an insect repellant, in niosomes (Amanatfard et al. 2015).
5. **Solvent Evaporation from Double Emulsion Droplets:** By using a microfluidic flow focusing device to form the double emulsion (monodisperse water–oil–water emulsion droplets) following by organic solvents evaporation from the oil shells, giant vesicles are created (Foster et al. 2010).
6. Comprehensive lipid vesicle preparation techniques without need to use an organic solvent are described below:
7. **Heating and Sonication of Lipids:** A mixture of vesicle forming lipids is melted in a glass test tube. The pre-warmed aqueous phase containing the drug is then added to the molten lipid. The mixture is immediately sonicated at elevated temperature in an ultrasonic bath followed by vortexing. This method was used for encapsulation of propylthiouracil (Suwakul, Ongpipattanakul et al. 2006).
8. **Homogenization of Lipids:** Initially, a combination of lipids and absolute ethanol is heated in tightly sealed glass vials in water bath (55–60°C). To each of the formed transparent solutions, the specified amount of drug containing hot distilled water (55–60°C) is added while warming in the water bath till a clear or translucent proniosomal solution is obtained. By addition an appropriate buffer, niosomes are formed and processed with a high-pressure homogenizer. Niosomes of tenofovir, an anti-HIV drug, were formulated by this technique (Zidan et al. 2011).
9. **Lamellar Liquid Crystal (LLC) Transformation:** This method is based on the microstructure and transformation of surfactant/polymer mixtures such as Tween 80, poly(ethylene glycol) (PEG) 6000, Span 80 and water which are vortex mixed to prepare the LLC at specific mass ratio (Liu and Guo 2007). PEG aqueous solution is then added to the LLC and the samples are mixed by sonicating to prepare niosomes.
10. **Heating (Mozafari) Method:** This method involves the hydration of the lipid vesicle components in an aqueous medium followed by the heating of these components, in the presence of glycerol (3% v/v), up to *c.*120°C (Pardakhty and Moazeni 2013).
11. **Application of Super Critical CO_2 ($scCO_2$):** Lipids and aqueous phase containing drug are added into the view cell and the temperature is raised to 50°C. CO_2 gas is introduced into the view cell and pressure and temperature in the view cell are maintained at 200 bar and 50±1°C, respectively. The pressure was released after 30 min and the niosomal dispersion is obtained (Manosroi, Chutoprapat et al. 2008). Rapid expansion of supercritical solution (RESS)-based process coupled with a gas ejector again uses $scCO_2$ in elevated temperature to change the dissolved surfactant into multilamellar or unilamellar niosomes (Wagner and Rizvi 2015).

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12. **Inert Gas Bubble:** Introduction of inert gas (N_2) bubbles into a coarse dispersion of non-hydrated lipids produces stable lipid vesicular dispersions with a mean particle size in the range between 0.2 and 0.5 μm (Talsma et al. 1994).
13. **Microfluidic Hydrodynamic Focusing:** Surfactant mixture dissolved in a water miscible solvent such as isopropyl alcohol and aqueous phase are vigorously mixed in microchannels under high hydrodynamic pressure (Lo et al. 2010). The resultant nanoscaled niosomes had narrower size distributions in comparison with traditional methods.
14. **Electroformation of Vesicles:** This procedure is based on swelling of membrane-forming lipid deposit on an electrode assisted by applied alternating electric fields to generate vesicles in aqueous solutions of the amphiphilic molecules (Okumura and Iwata 2011). The resulting giant vesicles of 30–50 μm, some as large as 100 μm, were formed with the standard setup.

Niosomes as New Drug Delivery Systems

Niosomes are currently used as versatile drug delivery systems with many pharmaceutical applications. Various routes for niosome administration are available which in this section some examples for therapeutic medicines incorporated in such vesicular systems are listed. In transdermal route of drug administration, many vesicles have been derived from proniosomal formulations which were more stable and appropriate dosage forms for topical application.

Nasal Route

Nasal mucosa offers advantages to deliver drugs to brain via olfactory route thus provides rapid onset of drug action and hence faster therapeutic effect (Ravouru et al. 2013; Illum 2015). Niosomes of sumatriptan succinate were prepared and evaluated for nasal absorption using an ex vivo animal model, showing 78.1% of the drug absorption (Gayathri Devi and Udupa 2000). Folic acid, a water soluble vitamin, showing difficulty in crossing blood-brain barrier (BBB), was successfully encapsulated in niosomes and administered through nasal cavity of rats (Ravouru, Kondreddy et al. 2013).

Ophthalmic Route

There are some problems of conventional ocular therapy, such as short residence time, loss of drug through nasolacrimal drainage, impermeability of corneal epithelium and frequent instillation (Guinedi, Mortada et al. 2005). To overcome these limitations, colloidal drug delivery systems, such as niosomes, have been evaluated.

Aggarwal et al. (Aggarwal et al. 2004)prepared acetazolamide-loaded Span 60/ CHOL niosomes and evaluated their intraocular pressure (IOP) lowering potential in adult male rabbits. Results of the study indicated that it was possible to develop a safe (as indicated by corneal toxicity studies) and physiologically active topical niosomal formulation of acetazolamide relative in efficiency to the newer local carbonic anhydrase inhibitor, dorzolamide. Selected acetazolamide niosomal formulations composed of Span 60/ CHOL were tested for their intraocular pressure lowering activity on normotensive albino rabbits and the results were compared with that of plain niosomes and acetazolamide solution (1%) prepared using polyethylene glycol 400 (7%, v/v) and propylene glycol (53%, v/v) (Guinedi, Mortada et

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al. 2005). Multilamellar acetazolamide niosomes formulated with Span 60/CHOL in a 7:4 molar ratio were found to be the most effective and showed prolonged decrease in IOP.

Niosomes prepared by reverse phase evaporation (REV) method loaded with acetazolamide were coated with 0.05% Carbopol934P in order to increase the retention time of the drug in the eye of New Zealand albino rabbits (Aggarwal, Pal et al. 2007). Microdialysis method for determination of drug levels in aqueous humor showed a significant broadening of peak time and concentration extent for the bioadhesive coated niosomal formulation. Similar results were reported for brimonidine tartrate, an α2-adrenergic agonist indicated in open angle glaucoma, when entrapped in Span 60/CHOL niosomes (Prabhu et al. 2010).

The potential ocular irritancy and/or damaging effects of niosomal formulations containing gentamicin sulfate were evaluated by observing them for any redness, inflammation, or increased tear production, upon application to the eyes of albino rabbits (Abdelbary and El-gendy 2008); no sign of irritation for all tested niosomal formulations.

Conjunctival and corneal tolerability assessment of ocular naltrexone niosomes on the hen's egg test-chorioallantoic membrane (HET-CAM) and bovine corneal opacity and permeability (BCOP) models showed good ocular tolerability and minimal ocular tissue irritation (Abdelkader et al. 2010; Abdelkader et al. 2011; Abdelkader et al. 2012).

Atenolol (Abu Hashim et al. 2014), cyclopentolate (Perini et al. 1996), ganciclovir (Mutha et al. 2013), ofloxacin (Gupta et al. 2010), pilocarpine (Sheena et al. 1997), prednisolone (Gaafar et al. 2014), some newly synthesized beta blockers (Piplani, Kumar et al. 2015), tacrolimus (Li et al. 2014) andtimolol (Kaur et al. 2010) were also successfully entrapped in niosomes and evaluated for ophthalmic drug delivery.

Oral Route

The delivery of peptide and protein therapeutic agents to the systemic circulation through oral administration is hindered by numerous barriers, including proteolytic enzymes, sharp pH gradients and low epithelial permeability (Lee and Yamamoto 1989). Niosomes could be used to overcome these barriers and improve protein absorption following oral delivery. Yoshida et al. (Yoshida et al. 1992) investigated oral delivery of 9-desglycinamide-8-arginine vasopressin entrapped in niosomes in an *in vitro* intestinal loop model and reported that stability of peptide increased significantly. Animals treated with oral niosome-encapsulated insulin (100 IU/Kg) showed decreased levels of blood glucose and elevated serum insulin, which in the case of Brij 92 vesicles, hypoglycemic effect was significant ($P < 0.05$) (Pardakhty et al. 2011). More recently, Span 60/CHOL/N-trimethyl chitosan (TMC) niosomal nanostructure has been used to enhance insulin permeability through Caco-2 cell monolayer 4-fold, compared with insulin alone (Moghassemi et al. 2015).

Niosomes also could be used for improvement of botanical extract oral absorption and distribution; the in vivo distribution study of *Ginkgo biloba* extract nano-vesicles composed of Tween 80/Span 80/CHOL showed that the flavonoid glycoside content in heart, lung, kidney, brain, and blood of rats treated with niosomal carrier system was greater than those treated with the oral *Ginkgo biloba* extract tablet (Jin et al. 2013).

Di Marzio et al. (Di Marzio et al. 2013)prepared polysorbate 20 nano-niosomes for oral delivery of unstable or poorly soluble compounds by film hydration associated with sonication in order to reduce the size down to sub-micron range. These vesicles were stable in different pH and in simulated gastrointestinal media with high mucoadhesion properties.

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Niosomes could be used to enhance the aqueous solubility of poorly soluble compounds, thus improving their oral bioavailability. *In vivo* study on rats revealed five-time increment in bioavailability of ganciclovir after oral administration of Span60/CHOL (3:2 molar ratio) as compared with tablet (Akhter et al. 2012). Sezgin-Bayindir et al. (2015) formulated candesartan cilexetil-loaded niosomes and mixed niosomes with promising water solubility and bile solution stability promotion of entrapped compound. Developed niosomal griseofulvin improved the oral bioavailability and maintained therapeutic level of drug for a longer period of time as compared to free griseofulvin in albino rats (Jadon et al. 2009). Acyclovir (Attia, El-Gizawy et al. 2007), carvedilol (Arzani et al. 2015), celecoxib (Nasr 2010), gliclazide (Tamizharasi et al. 2009), paclitaxel (Bayindir and Yuksel 2010), piroxicam (Abdallah et al. 2011) and tenofovir disoproxil fumarate (Kamboj et al. 2014)are some drugs which their oral bioavailabilities were enhanced after encapsulation in niosomal formulations. Cationic niosomes were also used for improving oral bioavailability and lung targeting efficiency afteroral or intravenous administration of azithromycin in rats, respectively (Non-Lecithoid 2014).

Parenteral Route

Many researchers used parenteral route of administration such as intramuscular injection of luteinizing hormone releasing hormone (LHRH) vesicles (Arunothayanun et al. 1999) and subcutaneous injection of norfloxacin niosomes (Abdelaziz et al. 2015) for evaluation of prepared drug delivery systems. However, anticancer therapy and brain targeting are two main goals which could be used for effective niosomal drug delivery and here would be focused on.

Anticancer chemotherapy by using vesicular systems have many benefits such as reduced organ toxicity (O'brien et al. 2004), enhanced antineoplastic efficacy (ElBayoumi and Torchilin 2009), prolonged circulation of vesicular carriers (Cosco et al. 2009)and less mortality in patients (Hamilton et al. 2002). Gude et al. (2002) prepared cisplatin-loaded niosomes and investigated them for antineoplastic activity in experimental metastatic model of B16F10 melanoma. Theophylline and its combination effect with free cisplatin and niosomal cisplatin were also carried out in the same model. Treatment with niosomal cisplatin (1 mg/kg) and combination of the same with theophylline (15 mg/kg) showed significant reduction in the number of lung nodules as compared to untreated control as well as with free cisplatin (1 mg/kg).

Carboplatin was incorporated into niosomes and the therapeutic efficacy of prepared vesicles against S-180 lung neoplasm model was evaluated (Lu et al. 2003). Antitumor effect was increased and differed significantly ($p<.05$) compared with the original drug of the same dose. Cisplatin-loaded niosomes (CP-NMs) were prepared under optimized conditions with Span 40 and cholesterol and their anticancer efficacy was tested in rabbits bearing VX2 sarcoma (Yang et al. 2013). The rabbits locally injected with the CP-NMs gave significantly superior results of inhibition of tumor growth, much lower mortality and improved results of body weight change and inhibition of tumor metastasis to inguinal lymph nodes and liver compared to those treated in the same way with the drug solution.

The blood–brain barrier (BBB) is the most important barrier impeding drug transport into the brain via the blood circulation (Chen and Liu 2012). Many attempts have been done to overcome this barrier for effective drug delivery into brain such as administration of lipid vesicles; both conventional niosomes or niosomal surface decorated with a targeting agent, such as N-palmitoylglucosamine (NPG), permit an enhanced drug delivery to brain parenchyma (Paolino et al. 2011). Niosomal formulation functionalized with NPG loaded with doxorubicin was intravenously injected in rats for reducing drug accumulation in heart and increasing brain concentration of drug (Bragagni et al. 2012). I.v. injection of vasoactive

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intestinal peptide (VIP)-loaded glucose-bearing niosomes to mice confirmed the presence of intact VIP in brain after administration of VIP-loaded niosomes, but not after administration of VIP solution (Dufes, Gaillard et al. 2004).

A niosomal formulation, functionalized with NPG, was also developed as potential brain targeted delivery system for an endogenous neuropeptide, dynorphin-B (Bragagni et al. 2014); i.v. administration of dynorphin-B optimized niosomal formulation to mice (100 mg/kg) showed a pronounced antinociceptive effect, significantly higher ($P< 0.05$) than that given by i.v. administration of the simple solution of the peptide.

Recently, Span-Tween 60/CHOL niosomes of ascorbic acid and α-tocopherol were i.v. administered in male rats with cerebral ischemia-reperfusion induced by middle cerebral artery occlusion for 30 min (Varshosaz, Taymouri et al. 2014); i*n vivo* results showed that the used niosomes had more neuroprotective effects against cerebral ischemic injuries than free ascorbic acid.

Pulmonary Route

Pulmonary administration of drug-loaded vesicles may represent an interesting approach for both the targeting of drugs to lungs and the improvement of the passage of drugs through the mucus barrier and hence of their intracellular uptake (Carafa, Di Marzio et al. 2006). One of most targeted lung diseases for colloidal drug delivery is tuberculosis, due to emersion of Mycobacterium multi-, single- and extensive-drug resistance infections in humans (Garg et al. 2015). Jain and Vyas (Jain and Vyas 1995) injected micron-sized rifampicin-loaded niosomes through the caudal vein into albino rats. More than 60% of the drug was localized in the lungs after 3 h. Rifampicin niosomes attained substantially higher drug concentrations in thoracic lymph nodes when administered through the intraperitoneal (i.p.) route suggesting compartmentalization of the drug took place in the lymphous tissue when administered through this route of administration (Jain et al. 2006).

However, for pulmonary route of administration, promising results were obtained after biological evaluation of selected pyrazinamide niosomal formulations on guinea pigs infected with *Mycobacterium tuberculosis* (El-Ridy et al. 2011). Encapsulation of ethambutol hydrochloride in niosomal formulations for the treatment of tuberculosis also showed higher efficacy and safety compared with the free drug (El-Ridy et al. 2015). The organ distribution pattern of rifampicin (Mullaicharam and Murthy 2004) and gentamicin sulfate (Mullaicharam and Murthy 2006)from niosomes was also studied in albino rats; after intratracheal administration of niosomal-entrapped drugs, a 145-time and 27-time increase in drug accumulation capacity of rifampicin and gentamicin niosomes to free drug solution in lungs was observed, respectively. Mehta and Jindal (Mehta and Jindal 2013) used Tyloxapol, a biocompatible surfactant, for preparation of nano-sized niosomes with high encapsulation efficiencies of antituberculosis drugs (rifampicin, isoniazid and pyrazinamide).

Topical Route

The ability of a particle to diffuse through the *stratum corneum* (for particles of the same charge) depends mainly on its size and viscoelastic properties (Babu et al. 2009). Niosomal bilayers could fuse in *stratum corneum*, the main barrier impeding drug transport through human skin (Hofland, van der Geest et al. 1994), resulting to penetration enhancing effect of lipid vesicular systems both for water soluble or insoluble compounds. Alvi et al. (Alvi et al. 2011)reported that vesiculization of 5-FU not

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only improved the topical delivery, but also enhanced the cytotoxic effect of 5-FU in actinic keratosis and non-melanoma skin carcinoma. There are many clinical applications of topical niosomes and proniosomes for treatment of acne, cutaneous inflammation, cutaneous leishmaniasis, hair loss and alopecia, local anesthesia, psoriasis, skin and nail fungal infections, vitiligo and whitening effect (Rahimpour and Hamishehkar 2012; Hamishehkar et al. 2013; Rahimpour et al. 2015). Proniosomes are liquid crystalline-compact niosomal hybrid that can be hydrated to form niosomal dispersion instantly before use (Rahimpour, Kouhsoltani et al. 2015).

Asadi et al. (Asadi et al. 2012) prepared and evaluated topical anti-leishmanial effect of paromomycin niosomes in balb/C mice model with promising results. Non-steroidal anti-inflammatory drugs such as acelofenac (Nasr et al. 2008; Solanki et al. 2010; Srinivas et al. 2010), diclofenac(Raja et al. 1994; Manosroi et al. 2008; Özer, Turker et al. 2013), flurbiprofen (Reddy and Udupa 1993; Mokhtar, Sammour et al. 2008),indomethacin (Noori 2012), ketorolac (Alsarra et al. 2005), meloxicam (El-Menshawe and Hussein 2013) andpiroxicam (Solanki et al. 2007; Chandra and Sharma 2008; AHMED et al. 2013)have been formulated as niosomal or proniosomal formulation for enhancement of drug transport through skin after transdermal route of administration.

Minoxidil is a lipophilic drugs approved by FDA for topical use in androgenic alopecia. For increasing skin latency and penetration, many reports are available for niosomal encapsulation and application of minoxidil (Mura et al. 2007; Balakrishnan, Shanmugam et al. 2009; Mura et al. 2009; Mura et al. 2011; Mali et al. 2013). Recently, by a randomized and controlled double-blind clinical trial, we endorsed the superiority and better efficacy of minoxidil niosomes in comparison to conventional formulation (Shamsi Meymandi et al. 2014). Manosroi et al. reported in vivo hair growth promotion activity of gel containing niosomes loaded with the *Oryza sativa* bran fraction (Manosroi et al. 2012). The *in vitro* permeation, skin retention and hair follicular transport of finasteride were reported by Tabbakhian et al. (Tabbakhian, Daneshamouz et al. 2005; Tabbakhian, Tavakoli et al. 2006).

A combination of iontophoresis and vesicular entrapment have been used for transdermal transport enhancement of, finasteride (Prasanthi and K Lakshmi 2013), isosorbide dinitrate (Jain et al. 2011) and triamcinolone acetonide (Sankar, Babu et al. 2013).

Vaginal Route

Ning et al. (Ning, Guo et al. 2005) prepared insulin-loaded Span 40 and 60 niosomes and investigated the hypoglycemic effects and insulin concentrations after vaginal administration of vesicles to rats in comparison to subcutaneous administration of insulin solution. The same research group developed alternative formulations (liposomes/niosomes) for the vaginal administration of clotrimazole to provide sustained and controlled release of appropriate drug for local vaginal therapy, were evaluated as delivery vehicles (Ning et al. 2005). The prepared liposomes/niosomes were incorporated into 2% carbopol gel, and the systems were evaluated for drug stability in phosphate-buffered saline (pH 7.4) and simulated vaginal fluid at 37 ± 1°C.

Zidan and Habib prepared tenofovir niosomal gel to overcome the low permeability of this antiretroviral drug through mucosal membranes. Vesicular size, shape and surface charge, drug entrapment efficiency, *in vitro* release, and skin permeation were used to assess the gels. In addition, their biological performance on *Lactobacillus crispatus* viability and mucoadhesion to porcine vaginal tissue was also assessed with promising results.

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CONCLUSION

Niosomes are widely used by research scientists for improving drug pharmacokinetic, targeting, and penetration through biological membranes. Intracellular delivery of therapeutic compounds, enhancing the stability and solubility of vast range of chemical compounds and reducing the toxic effect of some active therapeutic agents are also utilized by niosomal drug delivery. This lipid vesicular system are also widely used in cosmetic industry and can be used for vaccine and gene delivery.

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Chapter 8
Cancer Drug Delivery: Pharmacogenetics, Biomarkers, and Targeted Therapies

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ABSTRACT

Advancements in cancer drug delivery have led to the development of personalized oncology care through molecularly-driven targeted therapies. Understanding molecular and cellular mechanisms which drive tumor progression and resistance is critical in managing new treatment strategies which have shifted from empiric to biomarker-directed therapy selection. Biomarker-directed therapies have improved clinical outcomes in multiple malignancies as monotherapy and in combination with other treatment modalities, however the changing scope of treatment options presents new opportunities and challenges for research. Furthermore, pharmacogenetics may provide a rationale method of personalizing anticancer drug dosing and supportive care management for oncology patients. This chapter reviews biomarker classifications and pharmacogenetics in anticancer therapy and supportive care. Examples of biomarker-directed therapies and clinical assays, in addition to future directions of molecular profiling in oncology therapy management are discussed.

INTRODUCTION

Novel methods of individualizing cancer drug delivery and selection are critical to improve patient outcomes given the large heterogeneity in drug response that exists across the cancer patient population. Until recently, the majority of genomic cancer research has been in discovery and validation; however, as our knowledge of tumor molecular profiling improves, the implementation of genomic cancer medicine in the clinic becomes increasingly tangible, paralleled with the development of dozens of targeted cancer therapies (Tran et al., 2012). Our current understanding of cancer at the molecular level has resulted in a

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shift from characterizing tumors solely based on their anatomical location and histology to consideration of their molecular profile, opening an array of possibilities for a targeted approach to cancer therapy (Macconaill, Van Hummelen, Meyerson, & Hahn, 2011).

Pharmacogenetic biomarkers found within the tumor and the host offer valuable information for personalizing anticancer drug delivery. As the number of clinical assays available to test for pharmacogenetic biomarkers increases, it is imperative for clinicians to understand the therapeutic implications of mutations occurring within these molecular pathways to aid in drug selection and delivery. This chapter aims to summarize clinically relevant pharmacogenetic biomarkers, which may be used to personalize cancer therapy selection and dosing, in addition to a review of pharmacogenetics in supportive care management related to the treatment of cancer-related symptoms. Tables 1 and 2 summarize clinically relevant somatic and germ-line pharmacogenetic biomarkers and drug targets, and their respective clinical assays available in practice.

BACKGROUND: BIOMARKERS OVERVIEW

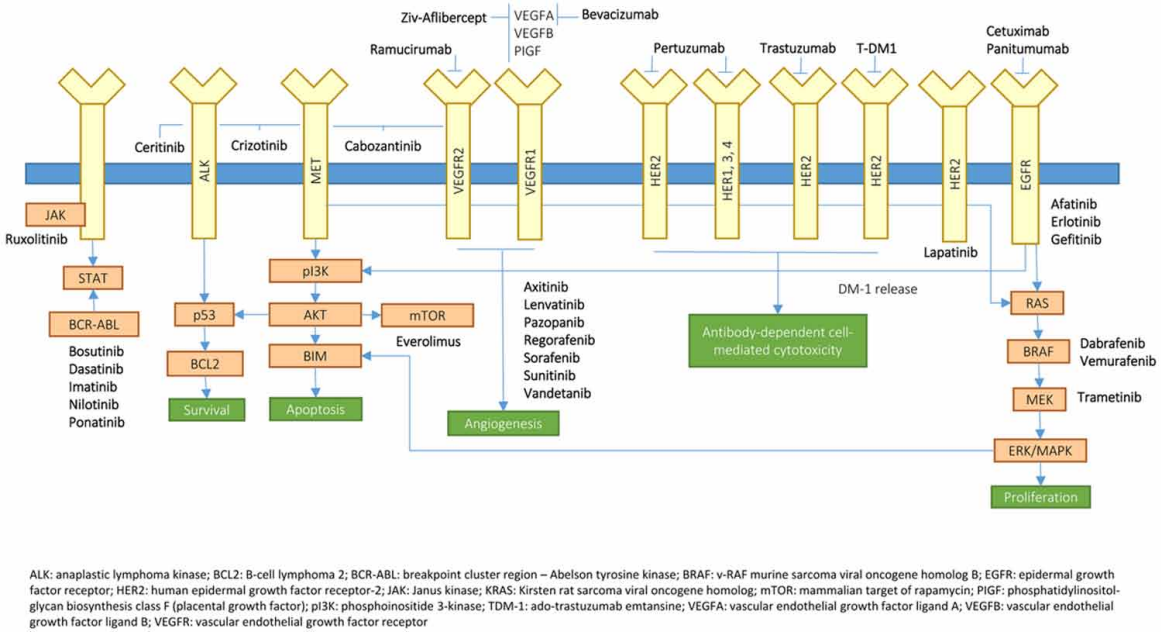
DNA analysis for pharmacogenetic purposes can be performed with either somatic or germ-line DNA. The major difference between somatic and germ-line DNA is the origin of the mutated cell (Patel, Mandock, & McLeod, 2014). While germ-line DNA is found in germ cells (sperm or egg) and therefore inherited and transmitted to the offspring, somatic DNA is found within the tumor after conception and subsequently not passed on to offspring, i.e. acquired after birth. Germ-line DNA is readily obtained by blood samples, and variations, if present, will occur homogenously throughout any randomly drawn blood sample within the same individual. Somatic DNA must be obtained by tumor biopsy and is therefore subject to sample selection. Common practice is to obtain one biopsy from the primary tumor for molecular analysis, as it was assumed that tumors were homogenous and the section sampled accurately represented the complete tumor composition. However, there is evidence to suggest that intra-tumor heterogeneity exists within cancer, where molecular analysis of one biopsy site may differ from another biopsy site (Gerlinger et al., 2012). Additionally, somatic mutations may change or evolve during cancer progression, resulting in a significant challenge in applying routine cancer pharmacogenetics to the clinic. While germ-line DNA is particularly useful for determining the pharmacokinetic behavior of a drug by understanding variations in drug metabolizing enzymes and/or transporters, somatic DNA is particularly useful in determining the pharmacodynamic effect of a drug and ultimately tumor response (Deenen, Cats, Beijnen, & Schellens, 2011).

Cancer biomarkers can be broadly categorized into two classifications: prognostic and predictive. A prognostic biomarker is mainly associated with disease outcome in the absence of treatment (i.e. Oncotype Dx, Mammaprint), while a predictive biomarker is valuable in assessing drug response (i.e. *ALK* [anaplastic lymphoma kinase], *BCR-ABL*, *EGFR* [epidermal growth factor receptor] (Mandrekar & Sargent, 2009; Patel, 2014).

Alternatively, some biomarkers may be classified as both prognostic and predictive (i.e. human epidermal growth factor receptor-2 [*HER2*], KRAS [Kirsten rat sarcoma viral oncogene homolog], and *BRAF* [v-RAF murine sarcoma viral oncogene homolog B]). Figure 1 highlights several strategic growth pathways, which cancer cells can activate through somatic mutations, and their respective targeted therapies. Pharmacodynamic biomarkers, a subset of predictive biomarkers, are useful in measuring the treatment effects of a drug on the tumor or on the host and can be used to guide dose selection; examples

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Figure 1. Summary of somatic biomarkers and targeted therapies



ALK: anaplastic lymphoma kinase; BCL2: B-cell lymphoma 2; BCR-ABL: breakpoint cluster region – Abelson tyrosine kinase; BRAF: v-RAF murine sarcoma viral oncogene homolog B; EGFR: epidermal growth factor receptor; HER2: human epidermal growth factor receptor-2; JAK: Janus kinase; KRAS: Kirsten rat sarcoma viral oncogene homolog; mTOR: mammalian target of rapamycin; PIGF: phosphatidylinositol-glycan biosynthesis class F (placental growth factor); pI3K: phosphoinositide 3-kinase; TDM-1: ado-trastuzumab emtansine; VEGFA: vascular endothelial growth factor ligand A; VEGFB: vascular endothelial growth factor ligand B; VEGFR: vascular endothelial growth factor receptor

include dihydropyrimidine dehydrogenase (*DPYD*), thiopurine-S-methyltransferase (*TPMT*) and uridine diphosphate glucuronosyl transferase 1A1 (*UGT1A1*) amongst others (Patel, 2014; Sawyers, 2008).

[This figure depicts examples of key signaling pathways and downstream effects of somatic biomarker molecular targets. One example pathway is the inhibition of vascular endothelial growth factor (VEGF) which can disrupt tumor angiogenesis through multiple mechanisms. Bevacizumab inhibits VEGF ligand A, ziv-aflibercept acts as a decoy receptor for VEGF ligands A and B and placental growth factor (PIGF), and ramucirumab inhibits the interaction between VEGF receptor 2 and its ligands. Although small molecule tyrosine kinase inhibitors such as axitinib, lenvatinib, pazopanib, regorafenib, sorafenib, sunitinib, and vandetanib all target VEGF, these agents also have additional targets that differentiate indications and potential adverse effects. Other signaling pathways include inhibition of anaplastic lymphoma kinase (ALK), HER2, and EGFR which can lead to changes in tumor survival, apoptosis, antibody-dependent cell-mediated cytotoxicity, and proliferation.]

SOMATIC PHARMACOGENETIC BIOMARKERS

ALK

Anaplastic lymphoma kinase (ALK) is a member of the insulin receptor tyrosine kinase family (RTK), first found to be associated with the (2;5)(p23;q35) chromosome translocation in Ki-1 lymphoma or anaplastic large cell lymphoma. The fusion of the echinoderm microtubule-associated protein like-4 (*EML4*) gene with the *ALK* gene was identified in 2007 from an inversion in chromosome 2 that juxta-

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poses the 5' end of the *EML4* gene with the 3' end of the *ALK* gene. This protein allows activations of the RAS-MEK-ERK, Janus kinase 3 (JAK3)-STAT3, and PI3K-AKT pathways (Iragavarapu et al., 2015).

The *ALK* translocation is detected using fluorescence in situ hybridization (FISH) as the gold standard assay in the United States. Other assays include, immunohistochemistry (IHC) and reverse transcription polymerase reaction of cDNA (RT-PCR). The overall incidence of *ALK* translocations reported in the NSCLC population ranges from 2 to 7% and occurs independently of *EGFR* or *KRAS* mutations. Similar to *EGFR* mutations, *ALK* rearrangements in NSCLC are associated with clinical and histopathologic features. Patients with *ALK* gene rearrangements are relatively younger, male, and never or light smokers (<10 pack years) (Iragavarapu et al., 2015).

Crizotinib is a small molecular tyrosine kinase inhibitor (TKI) that competitively binds to and inhibits *ALK* and is approved by the Food and Drug Administration (FDA) for the treatment of locally advanced or metastatic *ALK*–positive NSCLC. In a phase III trial with treatment-naïve patients, crizotinib showed a statistically significant improvement in progression-free survival (PFS) (10.9 vs. 7 months), response rate (74 vs. 45%), and quality of life compared to standard chemotherapy with pemetrexed plus either cisplatin or carboplatin (Solomon et al., 2014). In a phase III trial with patients previously treated with one prior platinum-based chemotherapy regimen, crizotinib improved PFS (7.7 vs. 3 months, $p<0.05$) and objective response rate (ORR) (65% vs. 20%; $p <0.05$) compared to single-agent pemetrexed or docetaxel (Shaw et al., 2013). Crizotinib is generally continued until there is evidence of disease progression or unacceptable toxicity. The most common toxicities include visual disturbances, nausea, diarrhea, edema, and fatigue.

Although the majority of patients with *ALK*-positive NSCLC derive substantial benefit from crizotinib, the majority of patients will develop resistance to crizotinib during the first year. Mechanisms of resistance to crizotinib may be due to the development of new, secondary *ALK* mutations, amplification of the *ALK* fusion gene, or activation of alternating pathways that bypass the *ALK* pathway. Secondary *ALK* mutations include *L1196M*, which results from the substitution of leucine by methionine at position 1196 of the *ALK* kinase domain and alters the ATP-binding site of *ALK*, and *C1156Y*, which results in the substitution of a cysteine by tyrosine at position 1156. *KIT* gene amplification has been identified as a potential bypass-signaling pathway (Iragavarapu et al., 2015).

More recently, ceritinib, a second generation *ALK*-inhibitor that is approximately 20 times more potent than crizotinib received accelerated FDA approval for the treatment of patients with metastatic *ALK*-positive NSCLC who are resistant or unable to tolerate crizotinib. The efficacy of ceritinib was established in a multicenter, single-arm, open-label clinical trial where patients with metastatic *ALK*-positive NSCLC who progressed while receiving or were intolerant to crizotinib had an improvement in ORR of 58% and 56% in crizotinib naïve and resistant cases, respectively (Shaw et al., 2014). Both agents have similar adverse effects and are metabolized by the cytochrome P450 enzyme system.

As evident by crizotinib and ceritinib, the drug development paradigm for highly targeted therapies is changing, allowing earlier, accelerated approval of exceedingly effective therapies, years before phase III randomized studies are completed. Alectinib is a second generation *ALK* inhibitor that was approved in Japan for the treatment of patients with recurrent or relapsed *ALK*-positive NSCLC, and is now FDA approved following Breakthrough Therapy Designation by the United States for patients who progressed on crizotinib. Clinical trials of new *ALK* inhibitors that can potentially overcome resistance mechanisms in addition to combination targeted therapy with EGFR, TRK, FAK, and ROS1 are ongoing (Iragavarapu et al., 2015; Seto et al., 2013).

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BCR-ABL

The *BCR-ABL* chromosomal abnormality is formed by a translocation between chromosomes 9 and 22, resulting in a fusion between the breakpoint cluster region (*BCR*) and the *c-ABL* oncogene (the Philadelphia chromosome, t(9;22) (q34.1;q11.21)) (Heisterkamp, Stam, Groffen, de Klein, & Grosveld, 1985). The chimeric *BCR-ABL* tyrosine kinase (TK) is constitutively active, triggering signal transduction pathways associated with cell survival. The translocation occurs in >95% of chronic myeloid leukemia (CML) cases, 25% of adult acute lymphocytic leukemia (ALL) cases, and 3% of pediatric ALL cases (de Klein et al., 1982; Fielding, 2011). The discovery of the TKIs, imatinib, nilotinib, dasatinib and most recently, bosutinib and ponatinib, have resulted in mean cytogenetic response rates > 90% (Jabbour et al., 2011). Although these TKIs have slightly different mechanisms, they primarily exert their activity by binding to the *ABL* domain of the TK and preventing the binding of ATP, thereby preventing phosphorylation and subsequent activation of various downstream substrates. Imatinib, nilotinib, and ponatinib all bind to the inactive conformation of the *ABL* kinase, preventing the conformational switch to the active form. Dasatinib inhibits both the active and inactive conformations and bosutinib inhibits both *SRC* and *ABL*. Nilotinib has a higher affinity for the *ABL* kinase domain than imatinib, resulting in greater potency and selectivity (Bixby & Talpaz, 2009). Both nilotinib and dasatinib are >100-fold more potent than imatinib in vitro.

Roughly 10% of patients will initially present with resistance to TKIs, and close to 20% will develop resistance within five years (Mauro, 2006). Primary resistance is observed when a patient fails to achieve a desired response to treatment (up to 25% of patients in the chronic phase of CML) and secondary resistance occurs when patients with an initial response ultimately relapse (up to 80, 50, and 15% at around two years for patients in blast crisis, accelerated phase or chronic phase of CML, respectively). The principal mechanisms of resistance to TKIs are point mutations in the kinase domain of the *BCR-ABL* protein, resulting in the inability of the TKI to bind to the *BCR-ABL* protein. The T315I mutation of the *ABL* gene represents the highest magnitude of resistance, as current TKIs require threonine at position 315 in order to bind to their targets (Gorre et al., 2001).

A newer compound, ponatinib (a pan-*BCR-ABL* inhibitor), is the only TKI shown to be effective in overcoming resistance mediated by the T315I mutation. Although ponatinib received accelerated approval by the FDA for the treatment of CML in adults with resistance or intolerance to prior tyrosine kinase inhibitor therapy, its indication has been narrowed due to the risk for life-threatening vascular complications, thrombosis, and hepatotoxicity (Bixby & Talpaz, 2009; T. Zhou et al., 2011).

BRAF

BRAF mutations have been identified in a wide range of cancers including 40 to 60% of malignant melanomas, 45% of papillary thyroid cancer, 10% of colorectal cancers, and 3-5% of lung cancers and gynecologic cancers. Mutations result in a substitution of glutamic acid at amino acid 600 (*V600E*) in 80 to 90% of cases or a substitution of lysine for valine (*V600K*) in 10 to 20% of cases, causing downstream signaling through the mitogen-activated protein kinase (MAPK) pathway (Chapman et al., 2011). Inhibition of the MAPK pathway in patients whose tumors contain a *BRAF V600* mutation using either a *BRAF* inhibitor and/or a *MEK* inhibitor is a targeted therapeutic approach to the treatment of advanced

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melanoma. As such, all patients with advanced melanoma should have their tumors assayed for the presence of a *BRAF* mutation. *BRAF* mutations are more frequent in patients with early age at tumor onset, truncal primary, and lack of chronic skin damage. Patients with a *BRAF V600* mutation and bulky disease, visceral metastases, elevated serum LDH, or a poor performance status are more likely to have rapidly progressive disease and a shorter overall survival (Chapman et al., 2011).

BRAF inhibitors have been shown to have a shorter time to response for the treatment of advanced melanoma compared to immunotherapy such as ipilimumab, however, the duration of response is short and almost all patients eventually have disease progression. There are currently two *BRAF* inhibitors that are FDA approved for the treatment of advanced melanoma, vemurafenib and dabrafenib. In a phase III trial comparing vemurafenib to dacarbazine in previously untreated, *BRAF* V600E mutation-positive patients, vemurafenib improved overall survival (OS) compared to dacarbazine (13.6 vs. 9.7 months, HR 0.70, 95% CI 0.57-0.87) (McArthur et al., 2014). Dabrafenib increased median PFS compared to dacarbazine (median 5.1 vs. 2.7 months, HR 0.33, 95% CI 0.20-0.54, $p<0.0001$) (Hauschild et al., 2012). Similar to vemurafenib, the most frequent grade 2 or greater toxicities were dermatologic. Dabrafenib must be taken on an empty stomach and has active metabolites, less squamous cell carcinoma, rash, photosensitivity, and increased pyrexia, hyperglycemia, and hypophosphatemia compared to vemurafenib (Trinh, Davis, Anderson, & Kim, 2014).

Resistance quickly develops in roughly 20-25% of *BRAF* mutant, metastatic melanoma patients treated with vemurafenib, with a mean time to diagnosis of first lesion at approximately 10 weeks. A number of mechanisms have been elucidated, including the paradoxical activation of the MAPK pathway through *RAS* mutations, elevation of pERK, and additional acquired mutations (*NRASQ61, MEK1*) (Su et al., 2012).

A potent inhibitor of *MEK1* and *MEK2*, trametinib, has been studied as monotherapy and in combination with dabrafenib to overcome resistance due to downstream effects in the MAPK pathway. Blocking a paradoxical activation of the MAPK pathway with *MEK* inhibition may also reduce the incidence of skin toxicity. The combination of dabrafenib and trametinib resulted in prolonged PFS (median 11.0 vs. 8.8 months, HR 0.67, 95% CI 0.53-0.84), higher ORR (69 vs. 53%) and prolonged OS (median 25.1 vs. 18.7 months, HR 0.71, 95% CI 0.55-0.92) compared with dabrafenib alone. The combination is also associated with less toxicity, but increased pyrexia and chills (Flaherty et al., 2012). OS was significantly increased in the combination when compared against vemurafenib in previously untreated patients with *BRAF V600* mutation-positive melanoma (one-year survival rate 72 vs. 65%, HR for death 0.69, 95% CI 0.53-0.89) (Robert, Karaszewska, et al., 2015).

Patients with *BRAF*-mutated colorectal tumors tend to have significantly shorter PFS and OS compared to wild-type patients, and also have the potential to impair the effects of EGFR-inhibitor therapy in *KRAS* wild-type patients (Di Nicolantonio et al., 2008). The benefit observed with vemurafenib in melanoma patients was not evident in colorectal cancer (CRC) patients harboring the *BRAF* mutation, indicating the significant influence of tumor origin and microenvironment on treatment response. Early phase studies suggest EGFR inhibition in addition to vemurafenib may overcome the resistance mechanisms demonstrated in CRC (Prahallad et al., 2012). The data for BRAF inhibition in lung cancer is scarce, although case reports have demonstrated clinical activity with vemurafenib (Peters, Michielin, & Zimmermann, 2013). Interim results of a phase II study of dabrafenib in *BRAF* V600E-positive NSCLC patients who failed at least one line of chemotherapy showed early antitumor activity with an ORR of 54% (Planchard et al., 2013).

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C-KIT

The CD117 antigen is almost universally expressed by gastrointestinal tumors (GISTs) and is part of the KIT transmembrane receptor tyrosine kinase (RTK) that is the product of the KIT proto-oncogene, *c-KIT* (Rubin, Fletcher, & Fletcher, 2000). The *c-KIT* proto-oncogene encodes a receptor TK, which binds to stem cell factor ligand, allowing for the development of melanocytes, erythrocytes, germ cells, and mast cells, ultimately resulting in dimerization, autophosporylation, and signal transduction (Yasuda et al., 2007). More than 80% of GISTs express a mutation in the *c-KIT* gene that leads to oncogenic signaling in the cell; 75% of the *c-KIT* mutations affect exon 11, while other mutations affect exon 9, 13, or 17. However, uncontrolled *c-KIT* activation may be present in the absence of *c-KIT* mutations, which occurs in 4 to 5% of cases. *C-KIT*-negative GISTs may be attributed to transcriptional silencing mechanisms or different activating mutations (e.g. platelet-derived growth factor receptor alpha [*PDGFRA*]) (Emile et al., 2004). Adult patients with confirmed GIST that are *c-KIT* or *PDGFRA* mutation-negative usually have tumors localized the stomach, whereas GISTs expressing *c-KIT* mutations are often larger in size and more frequently invade adjacent tissues (Heinrich et al., 2003).

Imatinib inhibits several targets including *BCR-ABL, c-KIT,* EPHA2, PDGFR, and *SRC*. By binding to the ATP-binding pocket required for phosphorylation and activation of *c-KIT* and PDGFR, imatinib has induced dramatic clinical benefit for the treatment of GIST (Balachandran et al., 2011). The partial response rate for GISTs harboring exon 11 *c-KIT* mutations is approximately 80-85% when administering imatinib, whereas patients with tumors containing an exon 9 *c-KIT* mutation, or no detectable mutation, have partial response rates closer to 45-50% (Heinrich et al., 2003). Studies show a rapid response as early as 24 hours after the first dose of imatinib. Long-term follow-up studies show an estimated 10-year survival rate of 22% (Demetri et al., 2014).

Although imatinib is highly effective in GIST, patients may acquire resistance through secondary mutations in *c-KIT* with an average median time to progression of about 2 to 3 years. Use of more potent or multi-targeted inhibitors that target other receptors like PDGFR such as sunitinib can help overcome resistance (Demetri et al., 2006; Reichardt et al., 2012). Regorafenib was most recently approved in 2013 for patients who are no longer responsive to imatinib and sunitinib (Demetri et al., 2013).

EGFR

Epidermal growth factor receptor (EGFR, also called dHER1 or erbB-1) tyrosine kinases are cell surface receptors that dimerize to activate and control intracellular signaling via the PI3K-AKT and RAS-MEK-ERK pathways (Yarden & Shilo, 2007). *EGFR* mutations are present in about 15% of NSCLC adenocarcinoma in the United States and occur more frequently in Asians and non-smoking females (Shepherd et al., 2005; Thatcher et al., 2005). Deletions in exon 19 and a missense mutation at exon 21, resulting in an arginine to leucine substitution (*L858R*), account for 90% of all *EGFR* mutations and are associated with a higher response rate to EGFR TKIs (gefitinib, erlotinib, afatinib) compared to traditional chemotherapy (Lynch et al., 2004).

Currently, screening for *EGFR* mutations is used to select stage IV NSCLC patients who may receive erlotinib in the first-line setting. In 2013, the FDA approved a companion diagnostic test for erlotinib and authorized expanded approval for first-line use in patients with metastatic NSCLC that test positive for the *EGFR* activating mutation. Data from 13 phase III trials comparing EGFR TKIs and platinum-based chemotherapy show prolonged PFS in patients with *EGFR*-positive advanced NSCLC, however,

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no significant increase in OS was noted (Lee et al., 2013). Erlotinib improved PFS (13.1 vs. 4.6 months, HR 0.16; 95% CI 0.10-0.26) and ORR (83 vs. 36%) compared with standard gemcitabine plus carboplatin in patients receiving initial therapy for advanced NSCLC with *EGFR* mutations (C. Zhou et al., 2011). Compared to platinum-based doublet therapy, erlotinib also increased PFS (median 9.7 vs. 5.2 months, HR 0.37, 95% CI 0.25-0.54) with no significant difference in OS (Rosell et al., 2012).

Afatinib was FDA approved in 2013 for first-line therapy of metastatic NSCLC in *EGFR* mutation-positive patients. It covalently binds to the kinase domains of EGFR (ErbB1), HER2 (ErbB2), and HER4 (ErbB4) and irreversibly inhibits tyrosine kinase autophosphorylation, resulting in downregulation of ErbB signaling. This irreversible binding allows for enhanced activity in resistant tumors that have progressed after initial *EGFR* inhibitor therapy (Miller et al., 2012). Data from two phase III trials did not show a statistically significant increase in OS when comparing afatinib to chemotherapy for NSCLC (median 25.8 vs. 24.5 months, HR 0.91, 95% CI 0.75-1.11). Mutation analysis showed a significant benefit in OS and PFS in patients with exon 19 deletion and a significant benefit in PFS but not OS for patients with *L858R* mutations (Yang et al., 2015).

Patients initially responding to EGFR TKIs can subsequently develop disease progression due to acquired resistance. Two primary mechanisms of resistance to EGFR inhibitors include a secondary point mutation in *EGFR* (*T790M*) that blocks the capacity for erlotinib to inhibit the receptor, and the amplification of *MET*, which activates similar downstream signaling pathways. *MET* amplification is thought to account for resistance in 5 to 20% of patients who progressed after treatment with erlotinib or gefitinib (Bean et al., 2007). Multiple agents have also been developed for patients that have acquired a resistance mutation with *T790M* and have limited benefit from EGFR tyrosine kinase inhibition, including AZD9291 (61% response rate in *T790M* mutation-positive patients) and rociletinib (59% response rate in *T790M* mutation-positive patients) (Sequist et al., 2015; Yang et al., 2015). Combination therapy may be beneficial in patients whose tumors contain an *EGFR* driver mutation (Herbst et al., 2011).

HER2

HER2 is a transmembrane glycoprotein EGFR that belongs to a family of four receptors EGFR/HER1, HER2, HER3, HER4) responsible for cell growth, survival and differentiation through signal transduction activation of the PI3K/Akt and RAS/RAF/MEK/MAPK pathways (Arteaga et al., 2012).

HER2 status represents both a prognostic and predictive biomarker. Amplification of HER2 occurs in about 20% of patients with early stage breast cancer. Prior to the introduction of HER2-directed therapies, HER2 overexpression was associated with high recurrence rates and increased mortality. A high level of HER2 overexpression by either 3+ immunohistochemical staining (IHC) for the HER2 protein or evidence of *HER2* gene amplification by fluorescence in situ hybridization (FISH >2.0), is a strong predictive factor for sensitivity to HER2-targeted agents (Arteaga et al., 2012).

Trastuzumab binds to the extracellular domain of the HER2 receptor and is thought to work by several potential mechanisms including antibody-dependent-cell-mediated cytotoxicity (ADCC), inhibition of cleavage of the extracellular domain of the receptor, inhibition of ligand-dependent dimerization, and inhibition of downstream signaling pathways (Arteaga et al., 2012). Trastuzumab is recommended in the adjuvant setting as monotherapy after completion of chemotherapy, in combination with paclitaxel or docetaxel or given concurrently with carboplatin and docetaxel. A meta-analysis including eight randomized clinical trials showed that the combination of trastuzumab with adjuvant chemotherapy produced a significant benefit in disease-free survival (DFS) (HR 0.60, 95% CI 0.50-0.71), OS (0.66,

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95% CI 0.57-0.77), locoregional recurrence (OR = 0.53), and distant recurrence (OR = 0.62), as compared to chemotherapy alone (Moja et al., 2012). Trastuzumab in the neoadjuvant setting also improves pathological complete response rates and event-free survival in patients with locally advanced or early stage breast cancer. In addition, it is approved in metastatic *HER2*-positive disease in combination with paclitaxel, docetaxel or as monotherapy.

Mechanisms of resistance to trastuzumab include loss of or increased HER2 expression, increased ligand (TGF-α) expression, steric hindrance of the antibody interaction, inhibition of binding by extracellular domain fragments cleaved from the HER2 receptor, and alternate signaling pathways. Incomplete HER family blockade could allow another HER receptor to compensate when one receptor is blocked. *PIK3CA* and *PTEN* expression may affect the sensitivity of tumor cells to trastuzumab and lapatinib. Variability of human breast cancer cell lines with regard to growth factor receptors, co-amplification of other genes, and acquired drug resistance phenotypes may be limitations to correlating resistance and HER2 overexpression (Arteaga et al., 2012).

Lapatinib is a small molecule TKI that reversibly inhibits intracellular HER2 and EGFR (HER1) by suppressing tyrosine autophosphorylation and downstream pathways. Studies have demonstrated that lapatinib may have additive or synergistic activity if combined with trastuzumab with better central nervous system penetration. Use of lapatinib has led to significant improvements in time to progression in metastatic HER2 positive breast cancer in combination with capecitabine compared to capecitabine alone (HR=0.49; P<0.001) (Geyer et al., 2006). Resistance to lapatinib may include survival pathways leading to upregulation of transcription factors leading to increased estrogen receptor signaling and an estrogen receptor-independent mechanism of acquired autoresistance (Arteaga et al., 2012).

Pertuzumab is a monoclonal antibody that binds the extracellular dimerization domain of HER2 and prevents it from binding to itself or other members of the EGFR family. It is administered in combination with trastuzumab for more complete HER2 inhibition. The combination of trastuzumab, pertuzumab, and a taxane showed an improvement in median PFS compared with placebo (19 vs. 12 months; HR 0.62, 95% CI 0.51-0.75), improvement in overall response rate (80 vs. 69%), and median OS (56.5 vs. 40.8 months, HR 0.68, 95% CI 0.56-0.84) (Baselga et al., 2012).

Most recently, ado-trastuzumab emtansine (TDM-1), an antibody-drug conjugate composed of trastuzumab, a thioether link, and an extremely potent antimicrotubule agent, DM1, was approved for the treatment of metastatic breast cancer. Compared with trastuzumab plus docetaxel, ado-trastuzumab emtansine showed a longer median PFS (14 vs. 9 months, HR 0.58, 95% CI 0.36-0.97) and higher ORR of 64 vs. 58% (Hurvitz et al., 2013).

KRAS

KRAS belongs to a group of small GTP-binding proteins, known as the RAS superfamily or RAS-like GTPases. More than 150 *RAS*-like genes have been identified in mammalian genomes. Activating mutations in the *KRAS* gene impair the ability of the KRAS protein to switch between active and inactive states, leading to cell transformation and increased resistance to chemotherapy and biological therapies targeting EGFR. *KRAS* gene point mutations have been detected in pancreatic carcinomas (>80%), colon carcinomas (40–50%), and lung carcinomas (30–50%), but are also present in biliary tract malignancies, myeloid leukemias, endometrial, cervical, bladder, liver, and breast cancers. The Qiagen Therascreen *KRAS* RCQ PCR kit is FDA-approved to detect seven different mutations of the *KRAS* gene within exon 2, however testing for all *RAS* mutations may be preferred (Jancik, Drabek, Radzioch, & Hajduch, 2010).

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The National Comprehensive Cancer Network (NCCN) recommends testing for *KRAS* and *NRAS* exons 2, 3, and 4 in patients being considered for an anti-EGFR agent.

The *KRAS* genes associated with colon cancer appear most often in codons 12 (28%) and 13 (8%) of exon 1 and less frequently in codon 61. Mutations in codon 12 are more frequent in primary metastatic carcinoma, suggesting a more aggressive phenotype. A mutation in codon 13 has been shown to be associated with reduced survival rates. Expression of *KRAS*, mainly on exon 2 (codons 12 and 13) has been associated with upregulated carcinoembryonic antigen (CEA) expression, disturbance of epithelial cell polarization, and increased adhesiveness of colon cancer cells (Jancik, Drabek, Radzioch, & Hajduch, 2010). *KRAS* mutation status is the most important predictor of resistance to EGFR-inhibitors used in colon cancer, cetuximab or panitumumab; both the median PFS of cetuximab-treated patients and OS found to be superior in a *KRAS* wild-type group than in a *KRAS* mutant group (31 vs. 10 weeks, and 16 vs. 7 months, respectively) (Lievre et al., 2008).

Cetuximab is approved for use in combination with FOLFIRI (fluorouracil, leucovorin, irinotecan) for first-line treatment of patients with *KRAS* wild-type CRC. Patients with wild-type *KRAS* treated with cetuximab had a median OS of 23.5 vs. 20 months and PFS of 9.9 vs. 8 months compared to those patients who did not receive cetuximab (Van Cutsem et al., 2009). Cetuximab is also approved for squamous head and neck cancer.

In 2006, a humanized monoclonal anti-EGFR1 IgG2 antibody, panitumumab, was approved for the treatment of patients with metastatic CRC who progressed after chemotherapy. Patients with metastatic CRC who were previously untreated and had *KRAS* mutations had a predicted response to panitumumab when added to FOLFOX (fluorouracil, leucovorin, oxaliplatin) compared to FOLFOX alone. The mutations were associated with inferior PFS and OS (Douillard et al., 2013). In a phase III trial, panitumumab monotherapy was compared to best supportive care and *KRAS* mutations were detected using polymerase chain reaction on DNA from tumor. PFS of patients with wild-type compared to mutant *KRAS* gene tumors was 12 vs. 7 weeks, showing that panitumumab monotherapy efficacy in metastatic CRC is confined to patients with wild-type *KRAS* tumors and *KRAS* status should be considered in patients who may be candidates for therapy. (Amado et al., 2008). Mutations in the *KRAS* gene in codons 12 and 13 were detected in 21% of NSCLC. *RAS* gene (exon 1: codons 12, 13, or 61) with or without increases in *EGFR* copy numbers did not derive benefit from erlotinib in NSCLC and had about a 96% chance of disease progression (Massarelli et al., 2007).

PD-1

Programmed death 1 protein (PD-1) is an immune checkpoint receptor that is expressed by activated T cells and binds to its ligands, PD-L1 and PD-L2, to provide a negative signal for T cell activation and proliferation. Anti-PD-1 monoclonal antibodies such as nivolumab and pembrolizumab disrupt the interaction between PD-1 and its ligands to provide an immune antitumor response. In contrast to CTLA-4-mediated inhibition, which is a central event, PD-1-mediated inhibition can occur peripherally in the tumors, providing a potential mechanism for adaptive immune resistance. Checkpoint inhibitor pathways have a mechanism of action that is not limited to one tumor or tissue type. Tumor expression of PD-L1 in early trials appears to predict a higher response rate, longer PFS and OS compared to tumors that do not express PD-L1; however, sensitivity of assays and appropriate cutoff values for expression are heavily debated (Pardoll, 2012).

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Pembrolizumab was studied in patients with advanced melanoma in three dose cohorts and showed an overall ORR of 33%, one-year OS of 66%, and median PFS 4.4 months (Duad, Ribas, Robert, & al., 2015). Pembrolizumab regimens (10 mg/kg every 2 or 3 weeks) also significantly prolonged PFS when compared to ipilimumab (47.3% and 46.6% for pembrolizumab, respectively, and 26.5% for ipilimumab) with an OS HR of 0.63-0.69 at one year (Robert, Schachter, et al., 2015).

Nivolumab received accelerated FDA-approval in December 2014 based on tumor response and durability of response in patients with previously treated metastatic melanoma. In a phase III trial comparing nivolumab to investigators choice chemotherapy (dacarbazine or carboplatin and paclitaxel) in patients with unresectable or metastatic melanoma who progressed on ipilimumab and a *BRAF*-inhibitor (if *BRAF* mutation-positive), nivolumab improved ORR compared with chemotherapy (32% vs. 10%, 95% CI 3.5-23.1) with a longer duration of response (median not reached vs. 3.5 months with chemotherapy) (Weber et al., 2015). In previously untreated patients with wild-type *BRAF* mutations, OS was significantly increased in patients treated with nivolumab compared to dacarbazine (one-year OS rate 73% vs. 42%, HR 0.42, 99.8% CI 0.25-0.73) (Robert, Long, et al., 2015). The combination of nivolumab and ipilimumab in patients with wild-type *BRAF* mutations showed an improved ORR of 61% vs. 11% ($P<0.001$) compared to ipilimumab monotherapy, however a higher rate of grade 3 to 4 drug-related adverse effects occurred with combination therapy (54% vs. 24%) compared to monotherapy (Postow et al., 2015).

Based on current published clinical trials, both pembrolizumab and nivolumab seem to be more effective than ipilimumab in patients regardless of *BRAF* mutation status, with a more tolerable safety profile, and a possible biomarker for response (anti-PD-L1), however additional studies are ongoing to determine their place in therapy.

The FDA indication for nivolumab was expanded to include the treatment of metastatic, squamous NSCLC in March 2015 based on a single-arm trial in patients who progressed after receiving platinum-doublet therapy with an ORR of 14.5% (95% CI 8.7-22.2) (Rizvi et al., 2015). There have also been positive preliminary results in the second-line non-squamous NSCLC setting after failure of platinum-based doublet chemotherapy and TKI with a 27% risk reduction in death with nivolumab compared to docetaxel (Spigel et al., 2015). PD-1 inhibition continues to be a target of interest for new cancer drug development for both hematologic and oncologic tumor types, in addition to identifying novel combinations of chemotherapy and/or targeted therapy with immunotherapy.

VEGF

Vascular endothelial growth factor (VEGF) promotes angiogenesis through endothelial cell proliferation and migration. The biological effects of VEGF are mediated by two receptor tyrosine kinases, VEGFR-1 and VEGFR-2 (Ferrara, Gerber, & LeCouter, 2003). Genetic variability in the promoter regions of VEGF affects the activity and expression of the VEGF protein, which may impact the benefit of targeted VEGF inhibitor therapy (Schneider, Radovich, & Miller, 2009). No biomarkers have been validated to date to identify patients who are more likely to benefit from VEGF inhibitor therapy, however many agents have been recently approved in multiple malignancies (Gerger, LaBonte, & Lenz, 2011).

The VEGF family contains VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PLGF). VEGF-A was the first pharmacologically targeted VEGF family member and acts selectively on vascular endothelial cells. VEGF-A expression is regulated by hypoxia, whereby the promotion of new blood vessel formation occurs to respond to reduced tissue oxygenation. The pro-angiogenic

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role of VEGF is inhibited by anti-VEGF and anti-VEGF-receptor antibodies, soluble VEGF "decoy" receptors, and small molecule TKIs (Ferrara, Gerber, & LeCouter, 2003).

Bevacizumab is a humanized monoclonal antibody directed against VEGF that targets VEGF-A. A pooled analysis of trials comparing chemotherapy with and without bevacizumab in the first-line setting of metastatic CRC showed a 19% reduction in risk of death (HR 0.81, 95% CI 0.70-0.93), however OS improvement was modest (median 19.8 vs. 17.6 months) (Hurwitz et al., 2013). Bevacizumab is FDA approved for the treatment of metastatic CRC, both as first-line in combination with fluorouracil-based chemotherapy and as second-line after progression on a first-line bevacizumab-containing regimen (Bennouna et al., 2013). Bevacizumab is also FDA approved in cervical cancer, glioblastoma, NSCLC, ovarian, and renal cell cancer.

Soluble "decoy" receptors consisting of native VEGFR sequences can bind to VEGF and prevent its interaction with VEGFRs on tumor or endothelial cells. Aflibercept is a recombinant fusion protein that contains the VEGF-binding domains of VEGFR1 and VEGFR2 fused to the Fc domain of human IgG1 and acts as a decoy receptor for VEGF, inactivating receptor binding to VEGF-A and VEGF-B (Holash et al., 2002). Aflibercept has a higher affinity for VEGF-A than bevacizumab and is approved for use in combination with FOLFIRI for the treatment of metastatic CRC that is resistant to or has progressed on an oxaliplatin-containing regimen. Patients who progressed during or within six months of receiving oxaliplatin-containing chemotherapy with or without bevacizumab had longer OS and PFS if treated with FOLFIRI plus aflibercept compared to FOLFIRI plus placebo (13.5 vs. 12.1 months and 6.9 vs. 4.7 months, respectively), regardless of bevacizumab exposure (Van Cutsem et al., 2012).

More recently, an antibody that targets the cell surface receptor for VEGF (VEGFR) has been approved to block VEGF signaling. Ramucirumab is a recombinant IgG1 monoclonal antibody that prevents the interaction of VEGF receptor 2 (VEGFR2) and its ligands. Two trials have shown survival benefit for treatment of second-line advanced or metastastic gastric or gastroesophageal junction adenocarcinoma for ramucirumab as monotherapy or in combination with paclitaxel. In one trial, patients with previously treated advanced or metastatic gastric or gastroesophageal junction adenocarcinoma and treated with either ramucirumab had an improved median PFS (2.1 vs 1.3 months) and OS (5.2 vs. 3.8 months, HR 0.78, 95% CI 0.60-0.998) compared to placebo (Fuchs et al., 2014). In another trial, weekly paclitaxel plus ramucirumab in patients who had disease progression after first-line platinum fluropyrimidine-based combination therapy for metastatic gastric or gastroesophageal junction adenocarcinoma had an OS benefit of 9.6 vs. 7.4 months (HR 0.807, 95% CI 0.768-0.962), PFS (4.4 vs. 2.9 months), and ORR (28 vs. 16%) compared to placebo (Wilke et al., 2014). Based on these results, the FDA approved ramucirumab both as a single agent and in combination with paclitaxel for advanced or metastatic gastric of gastroesophageal junction adenocarcinoma in the second-line setting. This indication was also expanded to include treatment of second-line NSCLC (median OS 10.5 vs. 9.1 months in patients treated with docetaxel plus ramucirumab compared to docetaxel plus placebo after progression on platinum doublet therapy for NSCLC) and second-line treatment of metastatic colorectal cancer (median OS 13.3 vs. 11.7 months and PFS 5.7 vs 4.5 months in patients receiving FOLFIRI plus ramucirumab compared to FOLFIRI plus placebo) (Garon et al., 2014; Tabernero et al., 2015).

Oral, small molecule VEGF inhibitors are available, but may have targets in addition to VEGF. The broader specificity is due to the structural similarity of the catalytic ATP-binding site region. This broad activity may enhance the action of VEGF inhibitors by allowing inhibition of multiple signaling pathways, however there could be additional toxicity based on other targets. These agents typically have shorter half-lives compared to monoclonal antibodies. Some examples of multi-targeted VEGF

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inhibitors and their targets include: axitinib (VEGFR1-3), lenvatinib (VEGFR, RET, FGFR), pazopanib (VEGFR1-3, PDGFR, FGFR1 and 3, c-KIT), sorafenib (VEGFR2, FLT3, PDGFR, FGFR-1), sunitinib (c-KIT, VEGFR1-3, PDGFR-α, PDGFR-β, FLT3, CSF-1R, RET), regorafenib (VEGFR1-3, RET, c-KIT, PDGFR, FGFR), and vandetanib (VEGFR, RET, EGFR). These agents have approvals for the treatment of multiple malignancies such as renal cell carcinoma and thyroid carcinoma (Zhang, Tan, Ding, Xin, & Jiang, 2012; Zhong & Bowen, 2011).

Although VEGF inhibitors have been shown to prolong survival in multiple malignancies, the benefit often does not last longer than a few months, potentially due to tumor resistance or adaptation. Tumor production of alternative angiogenic factors, such as fibroblast growth factor (FGF) and human growth factor (HGF), may contribute to resistance to VEGF inhibition (Ebos, Lee, & Kerbel, 2009).

GERM-LINE PHARMACOGENETIC BIOMARKERS

CYP2D6 and Tamoxifen

Tamoxifen is a selective estrogen receptor modulator and is widely used to treat hormone receptor positive breast cancer. Tamoxifen is a prodrug that requires bioactivation to the more active metabolite, endoxifen, via the hepatic enzyme cytochrome P450 2D6 (CYP2D6). The gene coding for this enzyme, *CYP2D6*, is highly polymorphic, where the**1* allele is the wild-type allele resulting in normal enzyme activity (i.e. extensive metabolizers [EM]), while **3*, **4*, and **5* result in null enzyme activity (i.e. poor metabolizers [PM]), and the **9*, **10*, and **17* alleles result in reduced enzyme activity (i.e. intermediate metabolizers [IM]). Patients with multiple copies of the functional allele are considered ultrarapid metabolizers (UM). The most common variant allele, **4*, is present in roughly 18% of Caucasians, moderately present in African Americans (7-9%), and rarely present in Asians (<1%) (Jin et al., 2005). The intermediate **10* allele is present in 40% of patients of Japanese origin, while the **17* allele is present in about 25% of patients of African origin (Bradford, 2002; Jin et al., 2005).

A retrospective cohort study of over 1300 patients demonstrated higher recurrence rates for PMs and IMs compared to EMs (29% and 20.9% vs. 14.9%, respectively) (Schroth et al., 2009). Subsequently, a prospective, genotype-guided study demonstrated a significant increase in endoxifen exposure in PMs/IMs as the dose was increased from 20 mg to 40 mg daily. While a significant difference in endoxifen levels was observed between EMs and IMs at the standard dose, this difference was diminished after doubling the dose in IMs, whereas concentrations in PMs were still significantly lower compared to IMs/EMs even at the 40 mg dose (Irvin et al., 2011). The influence of endoxifen exposure on response was not addressed in this study, limiting the clinical translation of these findings on breast cancer recurrence. However, studies have demonstrated that patients with the lowest endoxifen concentrations have the highest risk of relapse, suggesting that a therapeutic threshold may be needed for optimal benefit (Madlensky et al., 2011; Saladores et al., 2014). This data further implies that PMs and IMs may be unable to achieve this therapeutic threshold at standard tamoxifen doses.

Controversy exists on the clinical utility of *CYP2D6* testing as two of the largest studies investigating the *CYP2D6*-tamoxifen relationship and breast cancer recurrence demonstrated no association between genotype and recurrence rates (Rae et al., 2012; Regan et al., 2012); however, flaws in the genomic study design, such as use of tumor DNA as opposed to germ-line DNA, have been noted by pharmacogenetic experts (Nakamura et al., 2012). Subsequently, Rae et al. identified concordance rates >95% between

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CYP2D6 genotypes obtained from tumor-derived and germ-line DNA from 122 breast cancer patients (Rae et al., 2013). Alternatively, Goetz et al. genotyped nearly 1200 breast tumor samples and demonstrated loss of heterozygosity (LOH) in over a third of samples, while also documenting that non-malignant tissue did not exhibit LOH (Goetz et al., 2015). Investigators also reported that while there was approximately 20% discordance between tumor and buccal cell genotypes, there was 100% concordance between non-malignant and buccal cell genotypes (Goetz et al., 2015), suggesting that breast tumor tissue should not be used to genotype for *CYP2D6* and may result in false genotypes. Lastly, the development of oral endoxifen may bypass the pharmacogenetic issues presented (Ahmad, Ali, Ahmad, Sheikh, & Ahmad, 2010). Advocates of *CYP2D6* genetic testing indicate that until prospective, adjuvant trial data is available, the current evidence is sufficient to accept the *CYP2D6*-tamoxifen pharmacogenetic relationship in postmenopausal women (Brauch et al., 2013); however, the appropriate dose adjustment and ultimate impact on breast cancer recurrence appear to be unclear.

DPYD and 5-Fluorouracil

Dihydropyrimidine dehydrogenase (DPD), the rate-limiting enzyme involved in the metabolism of 5-fluorouracil (5-FU) to 5,6-dihydrofluorouracil, has been shown to exhibit polymorphic potential (Toffoli & Cecchin, 2007). Given that 80% of 5-FU is metabolized by DPD, a loss of enzyme activity has the potential to extend the half-life of 5-FU by up to 100-fold, enhancing the prolonged cytotoxic effect of the agent, including side effects such as severe neutropenia and diarrhea. However, the frequency of null enzyme activity is very low at <1%, while about 5-7% have intermediate enzyme activity (Saif, Ezzeldin, Vance, Sellers, & Diasio, 2007). More than 30 variants of *DPYD*, the gene coding for DPD, have been identified and result in functional changes in enzyme activity (Van Kuilenburg, Meinsma, Zoetekouw, & Van Gennip, 2002). The polymorphic variant most commonly associated with DPD deficiency is a splice-site mutation, *IV14+1G>A* (also referred to as *DPYD*2A*), located on the exon 14-flanking region and causing skipping of 165 base pairs (Toffoli & Cecchin, 2007).

In a multicenter trial, approximately 700 patients with various cancers and treated with 5-FU were genotyped for *DPYD*. Investigators observed a positive predictive value of 0.46 for overall toxicity in patients carrying the *DPYD*2A* variant; however, of interest was a gene/sex-interaction resulting in an OR for overall toxicity of 41.8 ($P<0.0001$) for male patients but only 1.33 ($P>0.05$) for female patients (Schwab et al., 2008). A smaller study demonstrated that 60% of 60 patients who had experienced severe 5-FU-related toxicity had decreased DPD enzyme activity, independently of genotype (Van Kuilenburg, Meinsma, Zoetekouw, & Van Gennip, 2002). In a prospective genotype-guided clinical trial, investigators administered an initial 5-FU dose reduction of $\geq$ 50% with subsequent dose titration based on clinical tolerance in CRC patients carrying the *DPYD*2A* allele. Safety was compared with 45 historical *DPYD*2A* controls having administered 5-FU at the full dose. Of 1,700 patients screened, only 17 were heterozygous for the variant allele. Results demonstrated that the risk of grade $\geq$ 3 toxicity was reduced from 68% in historical controls to 15% and drug-induced death was reduced from 10 to 0% in patients receiving genotype-guided dosing. Furthermore, 5-FU exposure in *DPYD*2A* carriers at reduced doses was comparable to fully dosed non-carriers, supporting the role of genotype-guided therapy to decrease drug toxicity (M.J. Deenen et al., 2011). Most recently, a systematic review of candidate genes associated with capecitabine (the oral prodrug of 5-FU) toxicity in 927 CRC patients demonstrated that global toxicity was associated with the *DPYD* alleles *2846T>A* and **2A* (combined OR, 5.51; $P = 0.0013$) (Rosmarin et al., 2014).

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Based on the available literature, it is evident that patients with *DPYD* variants (or DPD deficiency) are at an increased risk of 5-FU induced toxicity; however, measuring catalytic DPD activity in peripheral blood mononuclear cells is challenging. Standardized cut-offs for deficient DPD activity are not readily available and different laboratories may use different thresholds for activity (Yen & McLeod, 2007). Furthermore, there are implications on cost and resources as the minor allele frequency is very low resulting in a high number of patients needed to screen to prove a beneficial impact on reducing toxicity. Additionally, experimental protocols for genotyping are often laborious, intensive and limit the number of patients who can be tested (Yen & McLeod, 2007), ultimately limiting the utility of genetic testing in clinical practice. Nonetheless, the Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines provide recommendations for dosing based on DPD enzyme activity: patients with normal enzyme activity require no dose adjustment; patients with decreased enzyme activity require at least a 50% dose reduction, followed by titration of dose based on toxicity or exposure, and patients with complete enzyme deficiency should receive an alternative drug (Caudle et al., 2013).

TPMT and 6-Mercaptopurine

Thiopurine-S-methyltransferase (TPMT) is a cytosolic enzyme ubiquitously expressed in the human body and catalyzes the S-methylation of thiopurines, including 6-mercaptopurine (6-MP), into inactive compounds. 6-MP is one of the backbone agents used to treat ALL in both children and adults. It inhibits the formation of purine ribonucleotides, ultimately leading to cell death (McLeod, Lin, Scott, Pui, & Evans, 1994). Severe hematological toxicity, including myelosuppression, is the most common adverse effect associated with 6-MP (McLeod, Krynetski, Relling, & Evans, 2000). The *TPMT* locus is subject to genetic polymorphisms with roughly 6-11% of the population being heterozygous and 0.2%-0.6% being homozygous for the variant allele. *TPMT *2* and **3* alleles account for approximately 95% of the defective TPMT activity (Stanulla et al., 2005). The risk of myelosuppression is significantly elevated in patients who harbor *TPMT* genetic variants, given elevated circulating thioguanine nucleotide levels (Stanulla et al., 2005).

A study of 180 children with ALL treated with a 6-MP-based regimen demonstrated that wild-type, heterozygous, and homozygous-deficient patients were able to tolerate the full dose of 6-MP for 84%, 65%, and 7%, of the total weeks of therapy, respectively. Additionally, a statistically significant inverse relationship between thioguanine nucleotide concentrations and *TPMT* was observed, with an average concentration (pmol/8 $\times$ 10^8 erythrocytes) of 417, 963, and 3,565 in *TPMT* homozygous wild type, heterozygous, and homozygous deficient patients, respectively (Relling et al., 1999). In a prospective study, investigators determined that the OR for patients with at least one variant allele and receiving a 30-70% dose reduction based on genotype was 1.30 (equivalent to the risk seen in wild type patients receiving full dose 6-MP), whereas the OR of toxicity for patients with at least one variant allele and receiving standard 6-MP dosages (no dose adjustment for genotype) was 4.0 (Stocco et al., 2009). In a comprehensive phenotype-genotype study of 1214 healthy volunteers, investigators identified a clearly defined trimodal distribution of TPMT activity with 0.6% deficient, 9.9% intermediate and 89.5% normal to high. Four novel variants were identified resulting in 17 individuals with intermediate TPMT activity discordant to genotype. Taking these variants into account, the overall concordance rate between genotype and phenotype was 98.4% (Schaeffeler et al., 2004).

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Given the available data, *TPMT* genetic testing is routinely utilized to identify ALL patients with defective enzyme activity (Relling et al., 2011). CPIC guidelines recommend an initial 10-fold dose reduction of 6-MP in patients with two inactive alleles and reduced doses (30-70% of the full dose) in heterozygous patients (Relling et al., 2013).

TYMS and 5-Fluorouracil

Thymidylate synthase (TS), a critical enzyme involved in DNA synthesis, serves as the primary target of 5-FU. *TYMS*, the gene coding for TS, contains 28-base pair tandem repeat units found in the 5' untranslated region (UTR), acting as an enhancer to the *TYMS* promoter (Toffoli & Cecchin, 2007). As 5-FU is involved with the inhibition of TS, low *TYMS* expression induced by *TYMS 2R* (two repeats) or the 6-base pair (bp) deletion in the *TYMS* 3'-UTR would subsequently increase the cytotoxic effects of 5-FU, whereas high expression induced by *TYMS 3R* (three repeats) or 6-bp insertion in the 3'-UTR would decrease the efficacy of 5-FU [73].Approximately 25% of European patients are homozygous for *TYMS 3R/3R*, 20% are homozygous for *2R/2R*, and 55% are heterozygous for *2R/3R*.

In a study of 65 patients with rectal cancer treated with 5-FU-based preoperative chemoradiotherapy, patients harboring the *TYMS 3R/3R* genotype achieved a 22% down-staging rate, whereas *2R/2R* patients achieved a 60% down-staging rate (Villafranca et al., 2001). In a genotype-guided clinical trial of 135 patients with rectal cancer, investigators administered 5-FU chemoradiation plus irinotecan to patients with the *TYMS 3R/3R* or *3R/4R* genotypes, i.e. the poor-risk group (27.4%), and administered standard 5-FU chemoradiation alone to those with *2R/2R, 2R/3R*, and *2R/4R*, i.e. the good-risk group (72.6%). Down-staging and complete tumor response rates were 64.4% and 20% for good risk and 64.5% and 42% for poor-risk patients, respectively, suggesting that the addition of irinotecan may help overcome 5-FU resistance. However, the addition of irinotecan in good risk patients was not addressed (Tan et al., 2011). Lastly, in a large analysis of 927 colorectal cancer patients treated with capecitabine or another 5-FU-based regimen, investigators identified that global toxicity (grades 0/1/2 vs. grades 3/4/5) was associated with the common polymorphisms *TYMS 2R/3R* and *3'UTR 6bp ins-del* (combined OR, 1.31; $P = 9.4 \times 10^{-6}$) (Rosmarin et al., 2014). A large number of studies support the association between *TYMS* and 5-FU response/toxicity (Patel, Fuchs, Owzar, Chen, & McLeod, 2013); however, a paucity of prospective, interventional clinical trials exists, owing to the current lack of clinical utility. Despite availability of clinical assays, large prospective, interventional trials are warranted to confirm *TYMS* as a predictive biomarker for 5-FU therapy.

UGT1A1 and Irinotecan

Uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) conjugates SN-38, the active component of irinotecan, a topoisomerase I inhibitor, to a glucuronide inactive metabolite, SN-38G. More than 60 polymorphisms in *UGT1A1* have been identified, most of which result in TA (thymine-adenine) repeats in the promoter region of the gene. The wild type allele is referred to as *UGT1A1*1*, while the most common and studied variant, *UGT1A1*28*, results in up to a 70% reduction in enzyme activity (other less frequent variants include **6*, **60*, and **93*). The frequency of the **28* allele is 39% in patients of European, 16% of Asian, and 43% of African origins. Roughly 10–20% of Caucasians and African Americans are homozygous for **28*, while less than 5% of Asians are homozygous for **28* (Perera, Innocenti, & Ratain, 2008). A meta-analysis demonstrated a significant increase in grade 3/4 neutropenia for **28/*28*

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patients at medium (150-250 mg/m^2) and high doses (>250 mg/m^2) (OR=3.22, P=0.008 and OR=27.8, P=0.005, respectively) (Hoskins, Goldberg, Qu, Ibrahim, & McLeod, 2007). The current U.S. FDA approved package insert includes the *UGT1A1 *28/*28* genotype as a risk factor for severe neutropenia.

Given the genetic basis for irinotecan intolerance, investigators performed a prospective, genotype-guided phase I study in metastatic colorectal cancer patients receiving FOLFIRI. Patients were stratified by *UGT1A1* genotype and received dose escalations to the maximum tolerated dose (MTD). Results demonstrated that the MTDs of irinotecan were 370 and 310 mg/m^2 in **1/*1* and **1/*28*, respectively, suggesting higher tolerable doses in wild-type and heterozygous patients compared to the standard 180 mg/m^2 dose currently administered to all patients as part of the routine FOLFIRI regimen (Toffoli et al., 2010). A subsequent and similar genotype-guided dose escalation study determined the MTD to be 390, 340, and 150 mg/m^2 in **1/*1, *1/*28,* and **28/*28*, respectively (Marcuello et al., 2011). Although not powered to detect differences in clinical outcome, the first study identified a significantly higher response rate in patients treated at doses ≥ MTD vs. those treated at doses < MTD (65% vs. 25%; P<0.05) (Toffoli et al., 2010). Similarly, the second study demonstrated significantly higher response rates in patients treated at ≥ 260 mg/m^2 (median dose) compared to those receiving < 260 mg/m^2 (67% vs. 24%; P<0.05), in addition to a significant difference in time to disease progression (16 vs. 7 months, respectively; P<0.05) (Marcuello et al., 2011). A similar trial of patients receiving irinotecan monotherapy demonstrated fixed MTDs of 850 mg, 700 mg, and 400 mg in **1/*1*, **1/*28* and **28/*28* patients. Interestingly, at the MTD for each genotype, dosing by genotype resulted in similar SN-38 areas under the curve (AUCs; $r^2 = 0.0003$; P=0.97), while the irinotecan AUC was correlated with the actual dose ($r^2 = 0.39$; P<0.001) (Innocenti et al., 2014).

While it is evident that *UGT1A1 *28/*28* patients are at an increased risk of toxicity and dose reduction to ≤150 mg/m^2 in these patients is appropriate, the optimal irinotecan dose in **1/*1* and **1/*28* patients is unknown and is currently under investigation in a phase II genotype-guided study of irinotecan dosing in metastatic CRC patients receiving FOLFIRI plus bevacizumab (Clinicaltrials.gov: NCT02138617).

SUPPORTIVE CARE PHARMACOGENETIC BIOMARKERS

Antidepressants

Antidepressant drugs are widely prescribed to cancer patients to treat depression and anxiety disorders, however, only one-third of these patients exhibit a beneficial therapeutic response (Rush et al., 2006). Potential reasons for lack of drug response include clinical heterogeneity, diagnostic uncertainty, environmental and social factors, and genetic factors.

The heterogenous response to antidepressant drugs, particularly tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs), may be in part due to differences in cytochrome P450 activity, by which the majority of antidepressants are metabolized by. The main enzymes involved in antidepressant drug metabolism are CYP2D6, CYP2C9, CYP2C19, CYP3A4 and CYP1A2, with CYP2D6 involved in the metabolism of approximately half of the commonly prescribed psychotropic drugs (Staddon, Arranz, Mancama, Mata, & Kerwin, 2002). Studies with nortriptyline showed that *CYP2D6* poor metabolizers (PMs) reached therapeutic plasma levels with initial starting doses, whereas escalated doses would result in a higher risk of toxicity. Alternatively, patients with functional (extensive metabolizers [EMs]) or ultra-rapid metabolizer (UM) phenotypes required higher doses to reach thera-

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peutic plasma levels (Bertilsson, Dahl, Dalen, & Al-Shurbaji, 2002). Another study also identified an association between *CYP2D6* genotype and plasma level concentrations of venlafaxine, however, did not find any such association for desvenlafaxine. When subjects received venlafaxine 75 mg, the area under the concentration-time curve (AUC) and maximum concentration (Cmax) of O-desmethylvenlafaxine (the primary active metabolite) were 445% and 434% higher, respectively, in *CYP2D6* EMs compared with PMs ($P \leq 0.001$) (Nichols, Focht, Jiang, Preskorn, & Kane, 2011).

Variations in the *CYP2C19* gene have also been shown to be associated with antidepressant response, including citalopram, imipramine and clomipramine. Additionally, tertiary amines (e.g. amitriptyline) are mainly metabolized by CYP2C19 to desmethyl metabolites (e.g., nortriptyline). Therefore, variations in CYP2C19 metabolism may modulate antidepressant activity and side effects through the pharmacological actions of the tertiary amines. Serotonin reuptake inhibition has been shown to be more pronounced in *CYP2C19* PMs due to the decreased conversion to nortriptyline (Hicks et al., 2013). In addition, *CYP2C19* alleles have been shown to affect escitalopram metabolism (Jin et al., 2010; Noehr-Jensen et al., 2009) including a positive association in the European consortium project Genome-Based Therapeutic Drugs for Depression (GENDEP) (Huezo-Diaz et al., 2012). Investigators identified that those homozygous for the *CYP2C19*17* allele (UM phenotype) had a significantly lower mean escitalopram concentrations (approximately 2-fold, $P = 0.0001$) and a higher mean metabolic ratio ($P = 0.0003$) than EMs, while those homozygous for alleles conferring the PM phenotype had a higher mean escitalopram concentration than EMs (1.55-fold, $P = 0.008$). Furthermore, the side-effect profile of amitriptyline appears to be dependent on a combination of alleles coding for *CYP2D6* and *CYP2C19* (Huezo-Diaz et al., 2012).

A larger analysis combined samples from four studies and found an association between *CYP2D6* alleles and treatment response to venlafaxine (Lobello et al., 2010). Specifically, investigators identified that, compared with PMs, EMs had significantly greater mean changes from baseline on 4 of 5 depression rating scales (all 4 comparisons, $P \leq .020$) and a greater percentage experiencing response or remission ($P \leq .015$) (Lobello et al., 2010). However, other studies have found no such association for either *CYP2D6* or *CYP2C19* alleles (Grasmader et al., 2004; Serretti et al., 2009). Particularly, an analysis in the large STAR*D sample set did not find an association with either *CYP2D6* or *CYP2C19* and response to citalopram (Fava et al., 2003). As a result, the clinical translation and use of these genes in practice has been limited and larger prospective studies are needed to fully elucidate these effects. Nonetheless, CPIC guidelines provide dosing recommendations for amitriptyline, desipramine, doxepin, imipramine, nortriptyline and trimipramine (see https://www.pharmgkb.org/page/cpic). Other genetic determinants of response to antidepressant therapies include variations in p-glycoprotein, monoamine metabolic enzymes, 5-HT genes, dopamine genes, and norepinephrine genes; however, these have not been validated and further research is needed to elucidate the role of these genes in response to therapy (Narasimhan & Lohoff, 2012).

Antiemetics

Chemotherapy-induced nausea and vomiting (CINV) is one of the most debilitating adverse drug effects experienced by patients treated with cytotoxic chemotherapy (Blower, 2002; Rojas & Slusher, 2012). Ineffective CINV control occurs in $\geq 25\%$ of all cancer patients despite antiemetic administration (Feyer & Jordan, 2011). Three classes of medications are considered standard to treat CINV: 5-hydroxytryptamine (5HT; serotonin) type 3 (5HT3) receptor antagonists (5HT3-RAs), neurokinin 1 receptor antagonists, and corticosteroids (Basch et al., 2011; Roila et al., 2010). Variability in emetic control with 5HT3-RAs

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has resulted in the examination of the genetic variance in 5HT3 receptor subunits, genetic differences in cytochrome P450 metabolism of the various 5HT3-RAs, and polymorphisms associated with drug transporters (Blower, 2002; Fasching et al., 2008; Tremblay et al., 2003; Yaakob, Malone, Exintaris, & Irving, 2011).

The five subunits that make up the 5HT3 receptor pentamer have been identified as 5HT3A, 5HT3B, 5HT3C, 5HT3D, and 5HT3E (Bruss et al., 2000; Krzywkowski, Jensen, Connolly, & Brauner-Osborne, 2007; Yaakob, Malone, Exintaris, & Irving, 2011). The subunits and the genes coding for them have become the target of studies of genetic variation to explain suboptimal control with 5HT3-RAs (de Wit, Aapro, & Blower, 2005; Gandara et al., 1998; Machu, 2011; Ward, Kotasek, & McKinnon, 2008; Yaakob, Malone, Exintaris, & Irving, 2011). A study demonstrated that patients who were homozygous for the polymorphism, -100_-120delAAG promoter region deletion in the start codon of 5HT3B, had a much higher frequency of vomiting than other patients (Tremblay et al., 2003). Multivariate analysis further identified that poor CINV control associated with the deletion was not due to the level of chemotherapeutic emetic risk or co-administered medications (Tremblay et al., 2003). Another study assessed 5HT3C receptor gene polymorphisms and CINV incidence post ondansetron and dexamethasone administration in 110 breast cancer patients treated with anthracyclines. Results identified a SNP in the gene *K163N*, which was associated with increased incidence of poorly controlled CINV in 50% of *CC* homozygous patients (P=0.009) as compared with 22% of *AA* or *AC* patients (Fasching et al., 2008). The clinical implications of the genetic variability within the individual subunits of the 5HT3 receptor remain to be seen. Using genetic variations in 5HT3 receptors to predict efficacy from 5HT3-RAs is limited owing to the need for validation across larger and more diverse populations. The 3C, 3D, and 3E subunits have failed to produce any statistically significant variations that could impact clinical decision making.

CYP2D6 is notable as a major metabolic pathway for several of the 5HT3-RAs. The 5HT3-RAs ondansetron, palonosetron, tropisetron, and dolasetron rely on CYP2D6 metabolism in differing degrees, whereas granisetron does not rely on CYP2D6 metabolism. Therefore, the identification of a patient's *CYP2D6* phenotype may help guide clinician choices in antiemetic therapy. The weakest control of CINV with 5HT3-RAs is seen in the UM population who rapidly metabolize these medications resulting in loss of efficacy (de Wit, Aapro, & Blower, 2005; Ingelman-Sundberg, 2004; Tzvetkov et al., 2012). A study reported that UM patients had a significantly higher mean number of vomiting episodes compared to IMs, PMs, and EMs receiving cyclophosphamide, cisplatin, or carboplatin (2.3 ± 2.5 vs 0.2 ± 1.0 ($P<0.001$) episodes of vomiting, respectively). In addition, UM patients have experienced severe nausea despite use of ondansetron or tropisetron (Kaiser et al., 2002). Other antiemetic options remain for suspected UM patients with poor CINV control. Granisetron is the only 5HT3-RA that does not involve CYP2D6 in its metabolism; thus, conferring a reasonable option in a suspected UM patient (Bloomer et al., 1994), in addition to other antiemetics such as dexamethasone, lorazepam, prochlorperazine, etc.

Lastly, the ATP-binding cassette subfamily B member 1 (ABCB1) transporter (also known as p-glycoprotein, a multi-drug-resistant protein) functions as an efflux pump for a variety of substances, including ondansetron (Babaoglu et al., 2005; Ishikawa, Hirano, Onishi, Sakurai, & Tarui, 2004). Given that ABCB1 is thought to be an active component of the blood brain barrier, the potential of genetic variations to influence antiemetic activity is of importance. The *ABCB1 3435C>T* variant has been correlated with significantly reduced expression of ABCB1, resulting in increasing concentrations of medications that require ABCB1 for drug efflux (Babaoglu et al., 2005; Ishikawa, Hirano, Onishi, Sakurai, & Tarui, 2004). A study demonstrated that 93%, 48% and 56% of patients who were treated with granisetron and were homozygous for the *TT*, *CT* and *CC* genotypes, for the *3435C>T* variant ex-

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perienced a significantly higher rate of CINV control (P<0.05), respectively. The presence of the *3435 C>T* variant did not significantly improve CINV control in the ondansetron group, and had no effect in the tropisetron group (Babaoglu et al., 2005). Lastly, a study of 215 children with acute myeloid leukemia (AML) treated with high-dose cytarabine and pre- and post-chemotherapy ondansetron identified a significant association between *ABCB1* polymorphisms and antiemetic efficacy in the acute phase, but not in the delayed phase (He et al., 2014). Specifically, the *CG* haplotype (*C3435T* and *G2677T*) was associated with a higher risk of grade 3/4 nausea and vomiting (P=0.003 and P= 0.026, respectively). After adjustment for patient-specific clinical factors in a multivariate analysis, the *CG* haplotype was identified as a predictive marker of grade 3/4 CINV (P = 0.003 and P = 0.039, respectively). In addition, a significant association between the *3435CC* genotype and grade 3/4 vomiting in AML patients was observed (P = 0.016) (He et al., 2014).

Although polymorphisms exist that may explain variability in 5HT3-RA efficacy, the only current clinically actionable gene may be *CYP2D6*, given the test is readily available in practice and may be used to guide 5HT3-RA regimen choices. Further prospective studies are needed to replicate remaining polymorphisms, such as those in the 5HT3 receptor and drug transporters (i.e. ABCB1).

Opioids

CYP2D6 is the primary metabolic enzyme responsible for the activation of weaker opioids (codeine, tramadol, oxycodone and hydrocodone) into stronger opioids (morphine, o-desmethyltramadol, oxymorphone and hydromorphone, respectively). As mentioned previously, the **3*, **4*, and **5* alleles account for approximately 95% of the poor metabolizer (PM) phenotype, while multiple copies of a functional allele result in the ultra-rapid metabolizer (UM) phenotype (most often occurring with **1* or **2* duplications) (Bernard, Neville, Nguyen, & Flockhart, 2006).

Codeine and tramadol rely almost exclusively on CYP2D6 for activation to their active metabolites, morphine and O-desmethyltramadol, respectively. The clinical analgesic effect of codeine is mainly attributed to its conversion to morphine, which has a 200 times higher affinity and 50 times higher intrinsic activity at the mu-opioid receptor than codeine itself (Mignat, Wille, & Ziegler, 1995). Case reports of codeine fatalities in *CYP2D6* UMs (Ciszkowski, Madadi, Phillips, Lauwers, & Koren, 2009; Gasche et al., 2004; Koren, Cairns, Chitayat, Gaedigk, & Leeder, 2006; Madadi et al., 2011; Madadi et al., 2010; Madadi & Koren, 2008) resulted in a black box warning being issued in 2013, stating that codeine use in certain children with the UM phenotype may result in life-threatening adverse events or death. As a result, several U.S. institutions require preemptive *CYP2D6* genotyping prior to codeine administration. Similarly, a case report of tramadol-induced respiratory depression was reported in a UM patient who also had renal impairment (Stamer, Stuber, Muders, & Musshoff, 2008). Alternatively, it has been demonstrated that the analgesic activity of tramadol in PM patients is significantly reduced (Musshoff, Madea, Stuber, & Stamer, 2006; Stamer et al., 2003; Stamer et al., 2007; Stamer, Stuber, Muders, & Musshoff, 2008). In a prospective study of nearly 300 patients recovering from abdominal surgery, the percentage of non-responders was significantly higher in the PM group (46.7%) compared with the EM group (21.6%; p=0.005) (Stamer et al., 2003).

Oxycodone and hydrocodone are metabolized by CYP2D6 to more potent metabolites, oxymorphone and hydromorphone, respectively, and by CYP3A4 to inactive metabolites, noroxycodone and norhydrocodone (Stauble et al., 2014). In one study, *CYP2D6* PMs required significantly larger cumulative oxycodone doses for equianalgesic effect compared to EMs (25 mg vs. 16 mg; P=0.005) (Stamer et al.,

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2013). Similarly, a study showed that PMs achieved 8-fold lower hydromorphone concentrations vs. UMs, which significantly correlated with pain relief (Stauble et al., 2014). Another study demonstrated that *CYP2D6* UMs experienced 40% higher pain tolerance thresholds compared to EMs, while PMs had a 20-30-fold lower pain tolerance compared to EM/IMs (Samer et al., 2010). Furthermore, drug-gene and drug-drug interactions may overlap, resulting in additive effects on drug disposition and ultimately therapeutic response and/or toxicity.

Catechol-O-methyltransferase (COMT) is responsible for the inactivation of catecholamines (dopamine, adrenaline and norepinephrine), which are involved in pain modulation. As a result, genetic variability in *COMT* can contribute to pain sensitivity. It has been shown that a common variant allele (*1947G>A*) (allele frequency 48%) results in a three- to four-fold reduction in COMT enzyme activity (Lotta et al., 1995). A study of 207 Caucasian cancer patients demonstrated that patients with the *GG* genotype required significantly more morphine (155 mg/24 hours) compared to *AG* (117 mg/24 hours) and *AA* genotypes (95 mg/ 24 hours) ($P<0.05$) (Rakvag et al., 2005).

Lastly, the mu-opioid receptor, the primary site of action of opioids, is encoded by the gene opioid receptor-like 1 (*OPRM1*). The *OPRM1 118A>G* polymorphism (allele frequency 8-17%) results in less effective opioid analgesia. One study demonstrated that cancer patients with the *GG* genotype required higher morphine doses for pain relief compared to *AA* patients (Klepstad et al., 2004). Another study showed that patients undergoing total knee arthroplasty with the *GG* genotype consumed approximately 60% more morphine than patients who were heterozygous or homozygous wild-type (Chou et al., 2006).

CPIC guidelines (Crews et al., 2014) provide pharmacogenetic-based dosing recommendations for codeine, while the Dutch Pharmacogenetics Working Group (DPWG) (Samer, Lorenzini, Rollason, Daali, & Desmeules, 2013) provides guidelines for codeine, oxycodone and tramadol (amongst other classes of drugs metabolized by CYP2D6, such as antidepressants). Understanding genetic variations in *CYP2D6*, *COMT* and *OPRM1* may allow for better analgesia selection and dosing.

Voriconazole

Voriconazole is a triazole antifungal agent commonly used to treat and prevent invasive fungal infections (IFIs) in patients undergoing allogeneic hematopoietic stem cell transplant (HSCT) and other hematological conditions. The half-life of voriconazole is estimated to be approximately 6 to 9 hours; however, this may be highly variable due to the drug's non-linear saturable pharmacokinetics, which results in high inter-patient variability in exposure. Voriconazole steady state concentrations are estimated to reach at 5-7 days, with an optimal therapeutic concentration of 1.0-5.5 mg/L. Overall, only 50% of patients achieve the pre-defined therapeutic range by 5-7 days (Hicheri, Cook, & Cordonnier, 2012; Troke, Hockey, & Hope, 2011), while as many as 50% of patients treated with prophylactic doses of 200 mg twice daily have sub-therapeutic levels (<1.0 mg/L) (Trifilio et al., 2007).

Therapeutic drug monitoring (TDM) of voriconazole is useful due to unpredictable pharmacokinetics, the drug's narrow therapeutic index, and serum concentrations that are associated with both efficacy and toxicity (Troke, Hockey, & Hope, 2011). Furthermore, drug interactions and genetic polymorphisms in *CYP2C19,* the gene coding for the drug metabolizing enzyme of voriconazole which inactivates the parent compound, have an effect on serum concentrations (Mikus, Scholz, & Weiss, 2011). Sub-therapeutic voriconazole trough concentrations (<1.0 mg/L) have been associated with therapeutic failure (Hamada, Seto, Yago, & Kuroyama, 2012). In a prospective treatment trial, patients randomized to receive TDM-guided dosing had a significantly greater percentage of complete or partial response

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compared to the non-TDM group (81% vs. 57%, respectively; P=0.04) (Park et al., 2012). However, another study demonstrated a significant association between IFI-related mortality and initial trough concentrations ≤0.35 mg/L at day 5 (OR 11.0; P=0.004), even when TDM was subsequently utilized to target a therapeutic concentration, suggesting TDM alone may have limitations such as low initial trough concentrations and better methods of preemptive dose individualization are warranted (Miyakis, van Hal, Ray, & Marriott, 2010).

The greatest risk of voriconazole failure due to inadequate drug concentrations is in *CYP2C19 *1/*17* and **17/*17* UMs who rapidly inactivate voriconazole (Hamada, Seto, Yago, & Kuroyama, 2012; Hassan et al., 2011; Hicks et al., 2014; Owusu Obeng, Egelund, Alsultan, Peloquin, & Johnson, 2014; Pieper et al., 2012; Wang et al., 2014). *CYP2C19 *1/*1* (extensive metabolizer; EM) patients are expected to metabolize voriconazole normally, whereas patients homozygous for **2* and/or **3* are considered PMs and may achieve higher voriconazole concentrations (although the correlation between high concentrations and toxicity is inconsistent, and toxicity is often reversible). The frequency of UMs, EMs, IMs and PMs in the European population is approximately 31%, 42%, 19% and 3%, respectively (Owusu Obeng, Egelund, Alsultan, Peloquin, & Johnson, 2014). A study demonstrated that nearly 20% of 747 routine TDM samples had voriconazole concentrations ≤0.2 mg/L, and 80% of subjects with these sub-therapeutic concentrations harbored at least one *CYP2C19*17* allele (Hassan et al., 2011). Therefore, the greatest concern for voriconazole is sub-therapeutic concentrations in UM patients. A population pharmacokinetic model was used to simulate various dosage regimens in order to optimize therapeutic dosing according to *CYP2C19* status and found that voriconazole dosed 200 mg by mouth twice daily in PM patients was comparable to 300 mg by mouth twice daily in non-PM patients (Wang et al., 2014). Although there is a paucity of data on the direct influence of *CYP2C19* genotype-guided voriconazole dosing on clinical outcomes, substantial evidence exists to support the relationship between both genotype-exposure and exposure-response (Owusu Obeng, Egelund, Alsultan, Peloquin, & Johnson, 2014). Studies examining the clinical utility of prospective, *CYP2C19*-guided voriconazole dosing are currently underway.

FUTURE RESEARCH DIRECTIONS

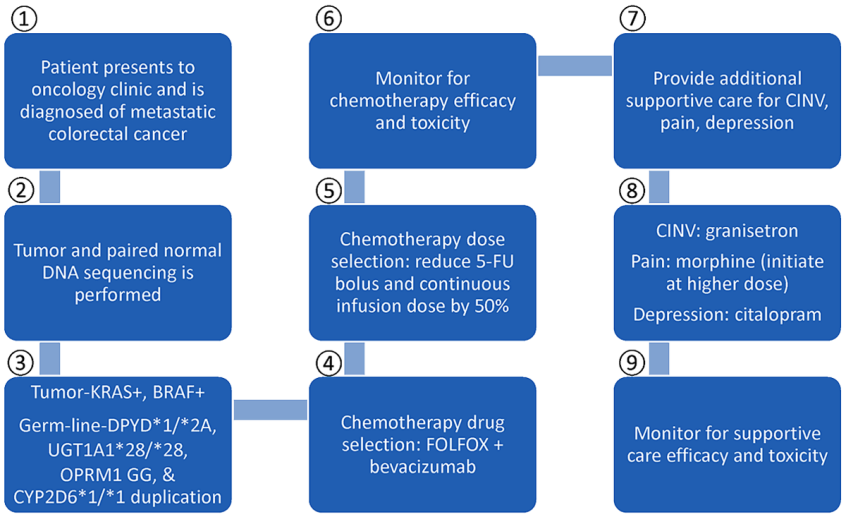
More than 100 drugs approved by the U.S. FDA include pharmacogenetic biomarkers in the drug label, the majority of which are related to cancer therapies. With declining costs and faster turnaround times for next generation sequencing, enormous amounts of molecular data will be generated for each patient. The results from thousands of patients being profiled from around the world will provide critical information on drug-gene associations and clinical outcomes. Future research on clinical implementation of these molecular results will be pivotal in translating molecular profiling from bench to bedside. However, for pharmacogenetic tests to be adopted in clinical practice, it must provide reliable, actionable and predictive information that the clinician would not have otherwise known. Figure 2 summarizes an example of personalized cancer medicine and how a clinician can translate pharmacogenetic test results into actionable prescribing decisions.

[This diagram summarizes how pharmacogenomic information, including both somatic and germline, can be used to personalize anticancer and supportive care pharmacotherapy.

1. The patient presents to clinic, a biopsy is obtained, and the pathologist reviews the results with the multidisciplinary team.

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Figure 2. Example of personalized cancer medicine: translating pharmacogenomic results into actionable prescribing decisions



DPYD-dihydropyrimidine dehydrogenase; UGT1A1-uridinediphosphate glucuronosyl transferase 1A1; OPRM1-mu-opioid receptor gene; CYP2D6-cytochrome P450 2D6; 5-FU-5-fluorouracil; FOLFOX- 5-FU, oxaliplatin, leucovorin; CINV-chemotherapy-induced nausea and vomiting;

2. The patient has confirmed metastatic CRC and both tumor and paired normal DNA are sent for DNA sequencing.
3. Next generation sequencing identifies a KRAS and BRAF mutation in the tumor, in addition to a DPYD, UGT1A1, OPRM1 and CYP2D6 mutation in the germ-line DNA.
4. Because the patients' tumor has a KRAS mutation, EGFR inhibitor therapy is ruled out, and because the patient has a UGT1A1 mutation, they would be at an increased risk for irinotecan-induced toxicity, therefore, FOLFIRI is ruled out. The recommendation is to initiate first-line therapy with FOLFOX plus bevacizumab.
5. Because the patient has a DPYD mutation, the 5-FU bolus and continuous infusion dose are reduced by 50% to avoid excessive toxicity.
6. The patient is closely monitored for drug efficacy and toxicity.]
7. The patient experiences CINV, pain and depression, therefore, supportive care is needed.
8. Given the CYP2D6 duplication, the optimal choice of a serotonin receptor antagonist would be granisetron for CINV, morphine (or another opioid not metabolized by CYP2D6) for pain (consider higher dose due to OPRM1 GG genotype, which confers increased pain sensitivity), and citalopram (or another antidepressant not metabolized by CYP2D6).
9. Finally, monitor closely for supportive care efficacy and toxicity.

Reliance on prospective, randomized controlled clinical trials (RCTs) as the sole method of justifying implementation may not be realistic. While RCTs are generally considered the gold standard, the delay in conducting and interpreting results may potentially deprive patients of life-saving or extending therapies. Initiatives such as the American Society of Oncology's (ASCO) CancerLinq (http://cancerlinq.org/), NCI Match (http://www.cancer.gov/about-cancer/treatment/clinical-trials/nci-supported/nci-match),

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ASCO's TAPUR study (http://www.asco.org/practice-research/targeted-agent-and-profiling-utilization-registry-study) and other biomarker-driven basket trials will provide critical information to better assign targeted therapies to molecular aberrations and ultimately allow evaluation of clinical outcomes for eventual translation into clinical practice. Furthermore, germ-line DNA from patients enrolled into cancer clinical trials and the drug development process should be exploited to retrospectively discover and validate germ-line pharmacogenetic associations, with subsequent pilot studies to investigate the clinical implementation of promising variants.

CONCLUSION

A concerted effort should be made by researchers and clinicians to implement pharmacogenetic and biomarker-driven therapies in clinical practice. The examples outlined in this chapter provide evidence for the use of both somatic and germ-line DNA to personalize anticancer therapy and supportive care management. As more driver mutations are being discovered and the pharmaceutical industry continues to develop more and more targeted therapies, clinicians must be aware of the molecular mechanisms which perpetuate tumor growth, and an understanding of how molecularly targeted therapies can be effective in slowing or stopping cancer progression. National and institutional treatment pathways should make a focused effort to incorporate molecular profiling and biomarker driven therapies where appropriate, which will allow for the promise of personalized drug delivery in the oncology patient population.

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ENDNOTE

[1] Estimated frequency in the European/Caucasian population for most frequently reported variants.

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APPENDIX

Table 1. Summary of FDA-approved targeted therapies, biomarkers and clinical assay availability.

Biomarker	Therapy	FDA-Approved Indication(s)	Clinical Assay(s) Available
ALK	ceritinib	NSCLC	Vysis ALK Break Apart FISH probe kit
	crizotinib	NSCLC	
BCR-ABL	bosutinib	CML	BCR-ABL Mutation Analysis test
	dasatinib	ALL, CML	
	imatinib	ALL, CML, GIST	
	nilotinib	CML	
	ponatinib	ALL, CML	
BRAF	dabrafenib	Melanoma	Cobas 4800 V600E THxID BRAF test
	vemurafenib	Melanoma	
BTK	ibrutinib	MCL, CLL, WM	No Clinical Assay Available
CDK 4/6	palbociclib	Breast	No Clinical Assay Available
c-KIT	imatinib	ALL, CML, GIST	c-KIT pharmDX
	regorafenib	CRC, GIST	
	sunitinib	RCC, GIST, pNET	
CTLA-4	ipilimumab	Melanoma	No Clinical Assay Available
EGFR	afatinib	NSCLC	EGFR pharmDx Cobas EGFR Mutation Assay
	erlotinib	NSCLC	
ER/PR	anastrazole	Breast	ER/PR pharmDx TargetPrint
	exemestane	Breast	
	letrozole	Breast	
	tamoxifen	Breast	
HER2	ado-trastuzumab emtansine	Breast	HercepTest[1], Pathway Insite PathVysion SPOT-Light HER2 CISH
	lapatinib	Breast	
	pertuzumab	Breast	
	trastuzumab	Breast, Gastric	
JAK	ruxolitinib	Myelofibrosis, PCV	JAK2 V617F Mutation Detection Assay HTScan JAK2 Kinase Assay Kit
KRAS	cetuximab	CRC, NSCLC, HNC	Cobas KRAS Mutation Test Therascreen KRAS RGQ PCR Kit DxS KRAS Mutation Test Kit, Genzyme's KRAS Mutation Analysis
	panitumumab	CRC	
MEK	trametinib	Melanoma	No Clinical Assay Available
mTOR	everolimus	Breast, pNET, RCC	No Clinical Assay Available
	temsirolimus	RCC	

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Table 1. Continued

Biomarker	Therapy	FDA-Approved Indication(s)	Clinical Assay(s) Available
PARP	olaparib	BRCA-mutated ovarian	No Clinical Assay Available
PD-1	pembrolizumab	Melanoma	No Clinical Assay Available
	nivolumab	Melanoma, NSCLC	
SMO	vismodegib	BCC	No Clinical Assay Available
VEGF	aflibercept	CRC	No Clinical Assay Available
	axitinib	RCC	
	bevacizumab	Cervical, CRC, GBM, NSCLC, Ovarian, RCC	
	cabozantinib	MTC	
	lenvatinib	Thyroid	
	pazopanib	RCC, STS	
	ramucirumab	Gastric	
	regorafenib	CRC, GIST	
	sorafenib	RCC, HCC, DTC	
	sunitinib	RCC, GIST, pNET	
	vandetanib	MTC	

BBT: basal cell carcinoma; CLL: chronic lymphocytic leukemia; CML: chronic myeloid leukemia; CRC: colorectal; DTC: differentiated thyroid cancer; GBM: glioblastoma; GIST: gastrointestinal stromal tumor; HCC: hepatocellular carcinoma; HNC: head and neck cancer; MCL: mantle cell lymphoma; MTC: medullary thyroid carcinoma; NSCLC: non-small cell lung cancer; PCV: polycythemia vera; pNET: pancreatic neuroendocrine tumor; RCC: renal cell carcinoma; STS: soft-tissue sarcoma; WM: Waldenstrom's macroglubulinemia

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Table 2. Overview of select germ-line biomarkers used for personalized cancer medicine

Biomarker	Therapy	Tumor	Clinical Assay(s) Available	Frequency of Variant/ Biomarker[1]
CYP450 2D6	tamoxifen amitriptyline nortriptyline clomipramine desipramine fluoxetine paroxetine venlafaxine imipramine codeine oxycodone hydrocodone tramadol ondansetron	Breast (tamoxifen) Any (for antidepressants and opioids)	AmpliChip Cytochrome P450 Genotyping test, xTAG CYP2D6, P450-Glo	*4 allele – 18% *10 allele – 6% Gene duplication (ultrarapid metabolizer) – 5%
DPYD	5-fluorouracil capecitabine	CRC, gastric, breast	Theraguide 5-FU DPD Enzyme Assay	*2A variant – 2%
TPMT	6-mercaptopurine	ALL	TPMT Enzyme diagnostic test, Prometheus TPMT genetic test	*2 allele – 0.4% *3A allele – 5% *3C allele – 0.5%
TYMS	5-fluorouracil	CRC	Theraguide 5-FU	2R – 46% 3R – 54%
UGT1A1	irinotecan nilotinib	Leukemia, CRC	Invader UGT1A1 Molecular Assay, INFINITI UGT1A1 Assay	*28 – 38%
CYP2C19	amitriptyline citalopram clomipramine imipramine voriconazole	Any	CYP2C19 Testing Spartan Rx Verigene CYP2C19 Test INFINITI CYP2C19 Assay xTAG CYP2C19	*2 allele – 15% *3 allele – <1% *17 allele – 25%

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Chapter 9
CNS Targeted Nanoparticle Drug Delivery:
CNS Drug Delivery

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ABSTRACT

The idea of formulating brain permeable nanoparticles stems from the need to treat various neurological disorders like Parkinson's disease, Alzheimer's disease, schizophrenia, depression and brain tumors. Neuropeptides, antibiotics, anticancer drugs and many CNS active drugs cannot cross blood brain barrier (BBB). Studies have revealed that when these drugs are loaded on to nanoparticles they not only cross BBB, but also exhibit decreased side effects. The drug can be dissolved, dispersed, encapsulated inside the nanoparticle or attached on to surface of nanoparticles. In 1995, dalargin was the first drug to be delivered across blood brain barrier (BBB) using polysorbate 80 coated nanoparticles. The size of nanoparticles is usually between 10-1000nm. For crossing BBB it should be less than 300 nm.

INTRODUCTION

In the past few years, several high profile global pharmaceutical companies have shutdown major research activities within the neuroscience area. This is because the development of new psychotherapeutic drugs has become a high risk activity with little success. CNS drug development takes considerable amount of time. Out of 5000 potential candidate molecules, only 250 reach pre-clinical evaluation. Out of 250 reaching the pre-clinical stage, 10 reach clinical development. Out of these 10 molecules entering clinical development, only one gets approved by the regulatory authorities and get to the market. The time for development of one drug molecule takes 12-15 years. The cost of developing a new drug is typically US$10-15 billion. Moreover, CNS drugs usually fail at later stage of clinical trials, after significant investment has been made (Wegner & Rujescu, 2013). The new drug molecule should possess desired pharmacological activity, selectivity at receptor site to avoid undesirable side effects, optimal bioavail-

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ability, ability to penetrate the blood-brain barrier. The BBB and the efflux transporters are the main cause of failure of most of the CNS drugs under clinical trial.

Ehrlich and his colleagues very the first to describe the existence of blood-brain barrier (BBB) in 1885. They showed that intravenous injection of acidic dye tryptan blue was not able to color brain and spinal cord. This gave an indication of presence of some barrier which separated the CNS from rest of the body (Ehrlich, 1885). The existence of a barrier at the level of cerebral vessels was first postulated by Bield and Kraus (1898) and Lewandowsky (1900) based on the observation that the intravenous injection of cholic acids or sodium ferrocyanide had no pharmacological effects on the CNS, whereas neurological symptoms occurred after intraventricular application of the same substances. After the development of electron microscope, it was found that cerebral endothelial cells were morphologically different from other endothelial cells in the body. The tight junctions between the endothelial cells forms the cellular basis of BBB. The brain capillary endothelial cells are surrounded by astrocytes, microglial cells, pericytes. BBB protect brain from general circulation and toxic substances. Astrocytes are periendothelial structures which play an important role in brain homeostasis. They are involved in maintenance of potassium ion levels, inactivation of neurotransmitters, regulation and production of growth factors and cytokines (Ehrlich,1885). Damage to the BBB or alterations in transport systems may facilitate pathogenesis of many CNS diseases, including HIV-associated CNS dysfunction. HIV-1 infection can result in neuropathologic changes in about one half of infected individuals and also can result in damage to the BBB. HIV-1 and the HIV-1 viral proteins, Tat and gp120, cause alterations in the integrity and function of the BBB through both paracellular and transcellular mechanisms (McRae, 2016).

Mechanisms of Transport Across BBB

The transport of solute molecules across the BBB membrane takes place by four different mechanisms:

1. **Paracellular Diffusion:** This is a non-saturable and non-competitive process occurring between cells. The presence of tight junctions in brain endothelial cells greatly restricts paracellular diffusion. Only small water-soluble molecules can diffuse through the BBB.
2. **Transcellular Diffusion:** It is also non-saturable and non-competitive. And takes place across cells. Molecules which are highly lipophilic and have molecular weight less than 450 Da undergo transcellular diffusion.
3. **Carrier-Mediated Transport:** These carriers are membrane bound and facilitate active transport of specific nutrients across the BBB. Glucose transporters GLUT1 and GLUT3 are of great importance as glucose is main energy source of brain. Monocarboxylate transporters are another important carrier systems for lactate and pyruvate. Similarly specialized carriers exist for amino acids and vitamins. Drugs or prodrugs can cross BBB through this transport mechanism by mimicking nutrients (Ohtsuki & Terasaki, 2007).
4. **Receptor-Mediated Endocytosis:** It facilitates the transport of large endogenous proteins and hormones across the BBB. It is mediated by receptors present on the luminal side of the barrier. Specific receptors have been identified for insulin, insulin-like growth factors, angiotensin II, folates and transferrin. Lipids can also be internalized in the brain in the form of low-density lipoproteins (LDL), which are recognized and endocytosed by the endothelial cells, due to the expression of apolipoproteins on the surface of the LDL (Yoshikawa & Pardridge, 2001).

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The massive brain microvasculature has a total surface area of 20 m^2 and a total length of 640 km. The brain capillaries present no fenestrae, few pinocytosis vesicles, and particular tight junctions, also known as zonula occludens, controlling the movement of molecules through the paracellular pathway. This comprises an almost impermeable barrier for drugs administered through the peripheral circulation (Kreuter, 2013). Only small molecules can diffuse through it. BBB prevents transport of many important drugs from blood to brain including anticancer drugs, antibiotics, drugs for treatment of Alzheimer's and Parkinson's disease. It has been a real challenge in developing drugs for CNS disorders (Jain, 2009). A number of methods have been developed to overcome this barrier. Chemical modifications of the drugs, such as increasing their lipophilicity, turn them more prone to be internalized in the brain. Osmotic opening of the tight junctions, direct surgical administration of drugs into the brain, use of prodrugs, use of carrier systems like antibodies or liposomes: Use of molecular tools to bind the drugs such as small immunoglobulins, liposomes or nanoparticles that will act as Trojan horses favouring the drug delivery in brain (Pardridge, 2015).

POLYMERIC NANOPARTICLES

In 1995, nanoparticles were first time used to deliver drugs across the BBB following intravenous administration. The underlying mechanism was found to be receptor-mediated endocytosis followed by transcytosis. Their was no evidence of tight junction opening of brain endothelial cells. Nanoparticles coated with surfactants like polysorbate-80 or poloxamers 188 take advantage of physiological receptor-mediated transport across the BBB. Once nanoparticles enter the blood, they get covered by a corona of biomolecules. These biomolecules are responsible for interaction of nanoparticles with the cellular processes. The physical adsorption of apolipoprotein A-I and /or E mediate interaction with LDL receptors on the BBB. The covalent attachment of apolipoprotein A-I and /or E or of transferring, insulin or antibodies against the respective receptors also enables a similar nanoparticle-mediated drug transport across the BBB (Ohtsuki & Terasaki, 2007).

Apolipoprotein E was chemically linked to human serum albumin nanoparticle (HAS-NP). Loperamide, which does not cross the blood-brain barrier but exerts antinociceptive effects after direct injection into the brain, was used as model drug. This preparation induced antinociceptive effects in the tail-flick test in ICR mice after i.v. injection. In contrast, nanoparticles linked to apolipoprotein E variants that do not recognize lipoprotein receptors failed to induce these effects. These results indicate that apolipoprotein E attached to the surface of nanoparticles facilitates transport of drugs across the blood brain barrier, probably after interaction with lipoprotein receptors on the brain capillary endothelial cell membranes (Michaelis *et al.*, 2006). HAS-NP with different vectors, transferrin, and apoE have been used in two independent series of experiments. Similar results were achieved for the delivery of loperamide to the CNS in both cases (Ulbrich *et al.*, 2009). Transferrin (Tf) is a single glycoprotein synthesized in the liver, and is the main iron-containing protein in blood plasma. Upon combination with Fe^{2+}, its molecular structure changes and it acquires binding affinity for the transferrin receptor (TfR) (Widera et al., 2003; Yang et al., 2009). TfR is widely expressed in capillary endothelial cells of the BBB, and targeted administration *via* the Tf-TfR pathway is a promising method of drug delivery (Xing *et al.*, 2016).

Polymeric nanoparticles are solid colloidal systems of 1-1000nm size, composed of biocompatible copolymers that have low solubility in water (Craparo *et al.*, 2011; Kumar *et al.*, 2010). Synthetic polymers like poly(alkyl cyanoacrylates); polyesters such as polylactides (PLAs); poly (D,L-lactide-co-

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glycolate) (PLGA) have been used for preparation of nanoparticles. Nanoparticles made of PLA and PLGA are synthesized by emulsion-diffusion and precipitation, whereas poly(alkyl cyanoacrylates) nanoparticles are synthesized by emulsion polymerization and nanoprecipitation method (Craparo *et al.,*2011). Nanoparticles are able to transport substances adsorbed onto their surface or covalently bound or incorporated into their polymer matrix, or encapsulated in them. It is difficult to reveal localization of the incorporated substance, therefore the terms "nanospheres" and "nanocapsules" are not commonly used, instead the term "nanoparticles" is generally used (Denora *et al.*, 2009). Nanoparticles present in the blood are quickly cleared because of opsonization by RES cells. Decreasing the size of nanoparticles increases duration of their circulation in the blood (Pinto *et al.*, 2006). However, adsorption of surfactants on their surface has been shown to be more effective for prolongation of their circulation time in blood (Kreuter, 2001). Modification of the surface of the nanoparticles by adsorption onto their surface or covalent linking of hydrophilic polymers, such as PEG, polysorbate 80, or polysaccharides, leads to an increase in the circulation of nanoparticles in the blood, whereas incorporation of molecules that recognize cellular determinants helps in penetration of nanoparticles through the BBB (Kreuter *et al.*, 1995).

PLGA is a block co-polymer made of polylactic acid (PLA) and polyglycolic acid (PGA) that has been approved by the FDA and European Medicine Agency. PLGA has excellent biocompatibility, in the human body it is hydrolyzed into lactic and glycolic acids, which are naturally-occurring metabolites. PLGA nanoparticles can be easily modified to accommodate a range of functional or targeting options. However, PLGA NPs without modifications also have a number of intrinsic drawbacks. Their negative surface charge inhibits makes cellular uptake. They have a relatively short blood circulation time and they do not pass through the BBB without further modifications (Cai *et al.*,2016).

Polybutylcyanoacrylate nanoparticles were used to facilitate transport of drugs through the BBB (Kreuter *et al.*, 1995). Kreuter and Alyautdin, 2000 performed in vivo experiments that demonstrated the possibility for brain delivery of peptide dalargin using polybutylcyanoacrylate nanoparticles coated with polysorbate 80. It was shown in subsequent experiments with H^3-dalargin that the absence of the polymeric coating of nanoparticles significantly reduced the delivery of the peptide to the brain (Schroeder *et al.*, 2000). The polar hydrophilic drugs tubocurarine (Alyautdin *et al.*, 1998) and neostigmine, P-gp substrates loperamide (Alyautdin *et al.*, 1997) and doxorubicin (Steiniger *et al.*, 2004) and the nerve growth factor (NGF) a protein have also been delivered to the brain through the BBB using this transport system (Kurakhmaeva *et al.*, 2009). These data support the fact that adsorption of polysorbates onto the surface of nanoparticles leads to changes in the characteristics of nanoparticle distribution. Analysis showed that penetration of nanoparticles is provided by stimulation of receptor-mediated endocytosis; primarily, by interaction of nanoparticles with LDL receptors on brain endothelial cells (Wohlfart *et al.*, 2012). The reason for such interaction may be due to adsorption of endogenic LDL from the plasma by polysorbate 80. The role of apoE has been demonstrated by Michaelis *et al.*, 2006. Two types of albumin nanoparticles one coated with polysorbate 80 and other with covalently bound apoE on their surface exhibited approximately equal ability to transport drugs through BBB. It has been found that polysorbate absorbed on the surface of polymeric nanoparticles has three effects: it allows for an increase in the duration of nanoparticle circulation in the blood by decreasing the chance of being captured by RES cells; it indirectly stimulates receptor-dependent endocytosis; and it directly influences Tight junctions of the BBB (Kreuter, 2004; Kreuter *et al.*, 2003; Kreuter *et al.*, 2002).

Rivastigmine, a reversible cholinesterase inhibitor used for treatment of Alzheimer's disease. It was shown that, compared to the free drug, rivastigmine-loaded poly(n-butylcyanoacrylate) (PBCA) nanoparticles (Riv-NP), particularly after being coated with polysorbate 80 (Riv-NP + PS80), accumulated to

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a greater extent in the brain. It was postulated by Wilson *et al.*, 2008 that this could translate into a better efficacy of rivastigmine. It was found that, compared to Riv-NP, Riv-NP + PS80 accumulates in a significantly lesser amount in the liver, which could potentially limit organ-specific toxicity and drug–drug interactions. Delivery of antineoplastic agents across the BBB has been shown to be improved by utilization of a nanoparticles. Gemcitabine, has proven to have beneficial effect on solid tumors such as non-small-cell lung cancer, pancreatic cancer, and breast cancer. Following incorporation into PBCA and coating with polysorbate 80 (Gem-PBCA + PS80),compared to gemcitabine alone, was found to have significantly improved antiproliferative activity on C6 glioma cells in an *in vitro* assay. The survival time was significantly increased in rats treated with Gem-PBCA + PS80 as compared to those treated with free gemcitabine (Wang *et al.*, 2009). Polymeric nanoparticles coated with polysorbate 80 have been used successfully to transport tacrine (Wilson *et al.*, 2008) and antitumor agent doxorubicin to the brain (Gulyaev *et al.*, 1999). It has been shown both *in vitro* and *in vivo* that polymeric nanoparticles penetrated the BBB, accumulated in the brain parenchyma resulting in strong antitumor effects (Kreuter and Gelperina, 2008). The polymers used as matrices for nanoparticles includes albumin, PLA, PLGA, chitosan, solid lipids, and polyethyleneimines. The main advantages of these polymers are low toxicity, biodegradability, presence of functional groups on the surface, processing stability, and the possibility to influence the excretion rate of drug molecules (Olivier, 2005). The development of novel and smart polymers have led to increase in number of transport vectors that can ferry drug molecules across BBB. Similar to the use of apoE as a vector for receptor-mediated transport, transferrin (OX26) or antibodies to transferrin receptor, insulin and antibodies to insulin, and TAT proteins have been used as targeting agents. Transferrin provides iron transport in the organism; therefore, there is a relatively high concentration of this protein in the plasma, which decreases its transport potential. Antibodies situated on the surface of nanoparticles interact with transferrin receptors in ECs, making it possible to deliver nanoparticle-receptor complex across BBB through transcytosis (Pardridge, 2006). Transferrin has been used to transport albumin nanoparticles containing loperamide through the BBB. The possibility of drug transport to the brain using antibodies to human insulin receptor was first demonstrated by Pardridge in monkeys in 1995. Ulbrich et al demonstrated, in 2011, the possibility for transporting the opioid agonist loperamide through the BBB in experiments using albumin nanoparticles with insulin attached to their surface. Antibodies to the insulin receptor inhibited transport of loperamide to the brain (Ulbrich *et al.*, 2011). TAT peptide is one of the most frequently used vectors for drug transport to the brain. This peptide, via a domain composed of nine to 16 amino acids, may penetrate biological membranes using mechanisms that are independent of transporter- or receptor-mediated transport (Torchilin, 2008) PLA nanoparticles, containing ritonavir and coated with TAT protein, increased the concentration of the drug in the brain 800 times (Rao *et al.*, 2008). Besides this, recent evidence suggests the usage of another cell penetrating peptide (CPP), syncytin-B (SynB), as a vector for drug transport across the BBB (Tian *et al.*, 2012). This group of investigators showed very promising data with nanoparticles that were conjugated to SynB peptide using PEG and gelatin–siloxane. Another protein, gH625, which is a membrane-perturbing domain in the glycoprotein gH of Herpes simplex virus type I, was conjugated onto polystyrene nanoparticles for transport across the BBB. This study was performed in vitro and can be applied to the design of one or more systems of drug delivery to the brain (Guarnieri *et al.*, 2013). Interesting data regarding CPP penetration, which delivered antibodies to the prion protein into mouse brain, were obtained (Skrlj *et al.*, 2013). Placing serotonin on the surface of a nanoparticle led to recognition by the serotonin receptor on the surface of cells, thereby stimulating endocytosis; in the same way, DNA was delivered to the cell (Gopal *et al.*, 2011). Both liposomes (Mora *et al.*, 2002) and

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polymeric nanoparticles (Nowacek *et al.,* 2009) were successfully used for cell-mediated drug transport. This approach of drug delivery to the brain has several advantages. Targeting of drug to disease sites, resulted in prolonged drug half-lives and time-controlled drug release (Gendelman *et al.*, 2008). The concept of polymeric nanoparticles seems to be conventional, but surface conjugation of these carriers with peptides have improved their targeting capabilities as can be easily see in Table 1. It can be clearly seen that these versatile carriers can be developed as diagnostics for Alzheimer's disease.

Novel Non-Viral Vector for Brain Targeting

The application of viral vectors in drug delivery is restricted by issues such as toxicity, and their is utmost need for safe and effective treatment. Liposomes are a new non-viral vector for the delivery of drugs to

Table 1. Brain permeable theranostic polymeric nanoparticles

Drug/Bioactive Molecule	Polymer Used/ Stabilizer	Surfactant Coating	Particle Size	Results	References
Dalargin	Polybutylcyanoacrylate	Polysorbate-80	260 nm	Significant & Prolonged Anesthesia	Kreuter and Alyautdin, 2000
Tubocurarine (Positively charged)	Polybutylcyanoacrylate/ dextran 70 KDa	Polysorbate-80	230 nm	Significant increase in brain concentration	Alyautdi *et al.,* 1998
Loperamide	PLGA	Glycosylated heptapeptide	155 nm	100 times increase in brain concentration	Vergoni *et al.*, 2009
Dopamine	PLGA	Polysorbate-80	215 nm	Significant and sustained increase in antinociceptive effects	Pahuja *et al.*, 2015
Paclitaxel	PLGA	PEG	114 nm	Improved penetration into brain	Nance *et al.*, 2012
(125)I-clioquinol	Polybutylcyanoacrylate	--------	200 nm	Promising delivery vehicle For SPECT/PET amyloid imaging (Alzheimer's disease)	Kulkarni *et al.*, 2010
Sulpiride	PEG-PLA	Thiolated cationized bovine serum ablbumin conjugation	329nm	Improved concentration in brain and dose reduction	Parikh *et al.,* 2010
Rifampicin, Isoniazid, Pyrazinamide,Ethambutol combination	PLGA	------	186-290 nm	Significant and prolonged drug levels in brain	Pandey & Khuller, 2006
Efavirenz, Lopinavir, Ritonavir antiretroviral combination	PLGA	-------	138.3±55.4 nm	Significant and suatained levels in brain for 28 days.	Destache *et al.*, 2010
Model drug Coumarin-6	PEG-PLA	Penetratin conjugation	100 nm	Enhanced brain uptake	Xia *et al.,*2012

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the brain, such as doxorubicin, which can be entrapped in the aqueous phase of the liposome (Schnyder et al., 2005; Xu, 2011; Gao et al., 2013). Brain-derived neurotrophic factor (BDNF) is widely expressed in the central nervous system, especially in the hippocampus and cortex (Bertrand, 2010; Qin, 2011). BDNF and its receptor TrkB play an important role in regulating various biological properties, including neuronal survival, differentiation, growth and plasticity (Ploughman et al., 2009; Tuinstra, 2012; Zhang, 2012). In the developing brain, endogenous BDNF levels are very low; therefore, in cases of brain injury (such as ischemia, degeneration and inflammation) during development, administration of exogenous BDNF is a promising treatment approach. However, as a macromolecular protein, exogenous BDNF cannot directly traverse the blood-brain barrier (BBB) or other biomembranes to exert its therapeutic function (Vijender, 2015). Recently it was investigated whether BDNF could be transported across the blood-brain barrier by tail-vein injection of liposomes conjugated to transferrin (Tf) and polyethylene glycol (PEG), and carrying BDNF modified with cytomegalovirus promoter (pCMV) or glial fibrillary acidic protein promoter (pGFAP) (Tf-pCMV-BDNF-PEG and Tf-pGFAP-BDNF-PEG, respectively). Both liposomes were able to traverse the blood-brain barrier, and BDNF was mainly expressed in the cerebral cortex. BDNF expression in the cerebral cortex was higher in the Tf-pGFAP-BDNF-PEG group than in the Tf-pCMV-BDNF-PEG group. This study demonstrates the successful construction of a non-virus targeted liposome, Tf-pGFAP-BDNF-PEG, which crosses the blood-brain barrier and is distributed in the cerebral cortex (Xing *et al.*, 2016).

Cell-Mediated Drug Transport

Immunocytes including mononuclear phagocytes (dendritic cells, monocytes and macrophages), neutrophils, and lymphocytes) are highly mobile; they have the ability to migrate across impermeable barriers and release their drug cargo at sites of infection or tissue injury. Thus immune cells can be exploited as trojan horses for drug delivery. Using cells as delivery vehicles enables targeted drug transport, and prolonged circulation times, along with reductions in cell and tissue toxicities. For successful cell-mediated drug delivery, high drug loading into cell-carriers, efficient preservation of entrapped therapeutic agents against disintegration and clearance in the host cells, drug triggered release at the site of action, efficient homing of cell-carriers to a disease site, safety of cell-based drug formulations for the whole organism are some of the prerequisite which needs to be achieved. The design of nanocarriers for cell-mediated drug delivery differ from those used for conventional drug delivery systems. Two different approaches are presently utilized in cell-mediated drug delivery: genetically modified cell-carriers producing therapeutically active molecules; and drug loaded cell-carriers used as "Trojan horses" to deliver the drug to the disease site. This disease combating strategy that can be applied to a spectrum of human infectious, cancerous, and degenerative disorders (Batrakova *et al.,* 2011).

To target circulating mononuclear phagocytic cells, negatively-charged nano-sized liposomes were formulated encapsulating serotonin, a BBB impermeable neurological drug. In vitro and in vivo studies confirmed the ability of monocytes to carry nano-sized liposomes loaded with serotonin across the BBB (Afergan *et al.*, 2008). There was also evidence of in vitro delivery of catalase to the brain using a polymeric nanocarrier. Catalase was immobilized on a polyethyleneimine-poly(ethylene glycol) copolymer, and such "nanozymes" were phagocytosed by bone-marrow-derived macrophages (Batrakova *et al.*, 2008). The polymer protected the enzyme from being activated inside the immune cell, while the macrophage delivered the antioxidant enzyme to the brain to reduce oxidative stress and increase survival of dopaminergic neurons in patients with Parkinson's disease.

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Efflux Transporters in Brain

Efflux transporters contribute significantly in maintaining brain homeostasis by eliminating toxic endogenous and exogenous substances. The efflux transporters of the ATP-binding cassette (ABC) P-glycoprotein (Pgp) can actively transport large quantities of lipophilic drugs out of the brain capillary endothelial cells. Some of the other ABC efflux transporters are multidrug resistance protein (MRP) and breast cancer resistance protein (BCRP). ABC efflux transporters are beneficial in reducing or preventing adverse effects of drugs and neurotoxic substances. But they also limit distribution of drugs that are valuable in treating CNS disorders. Moreover, neurological disorders such as epilepsy may be associated with overexpression of ABC efflux transporters, resulting in resistance to therapeutic medication. Hence, modulation of ABC efflux transporters forms a novel strategy to enhance the penetration of drugs into the brain (Löscher and Potschka, 2005).

Another very popular ATP-dependent drug transport protein P-glycoprotein (P-gp) is present on the luminal side of BCEC. It is a 170-kDa membrane-associated glycoprotein that actively transports structurally diverse molecules. It has been established, both in vitro and in vivo, that BBB. P-gp can prevent accumulation of drug molecules in the brain, by expelling them from the cells. Inhibition of the P-gp function can be obtained through down-regulation of its expression at transcriptional or translational levels, or by altering membrane targeting after synthesis of the P-gp protein, or through chemical inhibition of transporter functions. The first two approaches have been less developed, whereas chemical inhibition of the transporter functions has been extensively studied for improving permeability across BBB (Tsuji and Tamai,1997; Schinkel, 1999). An alternative approach is use of nanoparticles to carry the drug into the endothelial cells by endocytosis, thereby bypassing the P-gp drug efflux mechanism (Miller *et al.*, 2008).

The activity of P-gp, is increased in pathological conditions like epilepsy, stroke (Spudich *et al.*, 2006; Yang and Rosenberg,2011*)*. This reduces drug efficacy. P-gp actively transport out phenytoin, levetiracetam, lamotrigine, and Phenobarbital ((Denora *et al.,* 2009*)*. In the case of HIV infection, transactivating transcriptor (TAT) proteins from virion stimulate P-gp activity (Hayashi *et al.*, 2005). Same is true for antitumor drugs like paclitaxel, vincristine and ivermectin. In transgenic mice lacking P-gp, the concentration of vincristine and ivermectin in the brain increased 80–100-fold than that in normal mice. Paclitaxel is effective on tumor cells in vitro, but does not show same activity in vivo because of P-gp efflux. The concentration of paclitaxel can be significantly increased by simultaneous administration of paclitaxel and an inhibitor of P-gp, PSC833 (Denora *et al.,* 2009*)*. The first-generation inhibitors of P-gp, verapamil, diltiazem and cyclosporine, are used to increase brain delivery of viral protease inhibitors, paclitaxel and itraconazole, docetaxel, digoxin, and imatinib. But, they had limitations, like low affinity to P-gp, toxic effects due to high doses of these inhibitors, and simultaneous inhibition of liver microsomal enzymes (CYP3A). A second-generation P-gp inhibitor, an analog of cyclosporine valspodar 10, has great affinity for the transporter, with reduced toxicity and decreased effects on liver microsomal enzymes. A third generation of P-gp inhibitors, including elacridar 11, zosuquidar 12, and tariquidar 13, are devoid of effect on liver microsomal enzymes (CYP3A). Modulation of BCRP/ABCG2 activity also has significant clinical importance due to the possibility of mutual overlapping with P-gp (Laquintana *et al.,*2009*).* Elacridar 11 and the proton pump inhibitor pantoprazole are known inhibitors of BCRP (Litman *et al.*, 2000). Inhibition of P-gp is desirable for delivery of therapeutic molecules but

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would at the same time open the BBB to potentially toxic substances. This risk can be acceptable for the treatment of patients with cancer, but should be carefully weighed with long-term therapy of chronic CNS diseases (Alyautdin *et al.*, 2014).

ROLE OF NANOTECHNOLOGY IN TREATMENT OF BRAIN TUMORS

Glioblastomas belong to the most aggressive human cancers with short survival times. It is currently being treated with radiation therapy and surgery. One of the most important problems regarding the treatment of Glioblastomas is the presence of blood brain barrier (BBB) which inhibits the efficient drug delivery into central nervous system (Huse *et al.*, 2013). Nanotechnology can deliver therapeutic drugs into CNS by crossing the BBB. There are different types of nanoparticles (Nps) which can be manipulated for clinical applications as a treatment for CNS-related disorders (Pourgholi *et al.,*2016). Steiniger *et al.*, 2004 have shown that doxorubicin bound to polysorbate-coated nanoparticles crossed the intact blood-brain barrier, thus reaching therapeutic concentrations in the brain. Additionally, binding to nanoparticles may reduces systemic toxicity of doxorubicin. The standard therapy for GBM is surgery followed by radiation and chemotherapy with temozolomide (TMZ). Treatment of GBMs remains a challenge, largely due to the fast degradation of TMZ, inability to deliver an effective dose of TMZ to tumors, and lack of target specificity which may cause systemic toxicity (Zhang *et al.*, 2012). Recently Fang *et al.*, 2015 presented a simple method to synthesize a nanoparticle-based carrier that can protect TMZ from rapid degradation in physiological solutions and can specifically deliver them to GBM cells through the mediation of a tumor targeting peptide chlorotoxin (CTX). This nanoparticle, namely NP-TMZ-CTX, had a hydrodynamic size of less than 100 nm, exhibited sustained stability in cell culture media for up to two weeks, and could accommodate stable drug loading. The NP contains a biotin-functionalized chitosan core that is covalently conjugated with TMZ that were linked to GBM-specific, surface-bound CTX ligands with fast, stable, and stoichiometric biotin-neutravidin interactions (Petros and Desimone, 2010). NP-TMZ-CTX showed higher uptake in GBM cells and was more effective in cell killing than non-targeted NPs and free drug. It was further demonstrated that NP-TMZ-CTX can cross the BBB and deliver TMZ into avascular region of brain. NP-TMZ-CTX offers hope to deliver effective doses of TMZ specifically to GBM cells while minimizing dose-limiting toxicity on other healthy tissues. This NP-based delivery system can be broadly applied to various applications as it can be tailored to incorporate various therapeutics that are otherwise unstable or insoluble in physiological conditions (Fang *et al.*, 2015).

CONCLUSION AND FUTURE PROSPECTS

Biologics such as antibodies because of their large size are unable to cross BBB. They can be made to cross BBB by re-engineering them as an IgG fusion protein. The IgG is a monoclonal antibody against endogenous BBB receptor i.e transferring receptor. So the biologic is ferried into the brain by receptor mediated endocytosis. Many of ligand targeted nanomedicines for brain targeting have progressed into clinical trials. The fusion of classical pharmacology with nanotechnology has opened up many different

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approaches for new generation of neuroactive drugs to access BBB. An example is targeted drug delivery to brain of BDNF using liposome as non-viral vector. Green synthesized nanoparticles can also be used in discovery of biomarkers and improvement of diagnosis. This forms the basis for new drug development for neurological disorders. It further explores new methods for drug delivery across blood-brain barrier. Nanotechnology based implants are being developed to facilitate regeneration of the nervous system. It is being speculated that femtolasers, nanorobots and other nanodevices will bring advancement in neurosurgery. Finally, the progress of BBB permeability of drugs and biologics are highly dependent on highly precise *in vitro* BBB models which have good prediction power. These models are extremely useful for investigating intracellular sorting mechanism of receptors. This requires microscopy at subcellular resolution. This will also help in studying vesicular compartments involved in transcytosis and finding new transcytosis receptors. All the molecules designed for BBB penetration cannot be tested in animals due to ethical considerations. Many models have been designed to mimic BBB *in vitro*. But none can replace *in vivo* investigations completely. Hence, great care should be exercised in the analysis and interpretation of the results obtained with cultured cells and extrapolating it to the native BBB *in vivo*.

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Chapter 10
Engineered Gellan Polysaccharides in the Design of Controlled Drug Delivery Systems

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ABSTRACT

Natural polysaccharides are getting increasing attention in the development of pharmaceutical dosage forms due to their encouraging reports on nontoxicity and biodegradability. Natural gums can also be engineered to have better materials for drug delivery system design. Gellan gum originates from microbial fermentation and has been declared as safe by US FDA for human consumption. It possesses gelling ability in presence of multivalent earth metal cations and thus enabled the design of mutiparticulate drug delivery systems in completely aqueous environment avoiding the use of organic solvents. Due to faster drug release profiles of divalent cation-induced gellan gum particles, nowadays chemically modified forms of gellan polysaccharide are currently being investigated for the controlled release of drugs. This chapter discusses the factors contributing to the varying gelling characteristics of gellan gum and the recent developments in its chemical modification towards the fabrication of novel controlled drug delivery devices.

INTRODUCTION

Polymers are compounds with high molecular masses formed by monomers. The word poly means ‘many’ and meros mean ‘units or parts’ in Greek. They consist of different functional groups. Because of their unique properties, polymers are extensively used in pharmaceuticals. The new technology in polymer-based drug release system offer possibilities in the administration of drugs. Pharmaceutically, these polymers are used as a binder in tablets, flow controlling agents in liquids, suspension and emulsions, as film coating agents to mask unpleasant taste of drug, to enhance drug stability, and to modify

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the release characteristics of the drug. The rate of drug release from a matrix product depends on initial drug concentration and relaxation of the polymer chains, which overall displays a sustained release pattern (Uhrich, Cannizzaro, & Langer, 1999).

Natural polysaccharides and their derivatives represent a group of polymers widely used in pharmaceutical dosage forms. Various kinds of natural gums are used in the food industry and are regarded as safe for human consumption. These polysaccharides are obtained usually as plant exudates containing various sugars other than glucose and having significant quantities of oxidized groups in adjunct to their normal polyhydroxy format. In many cases, water-soluble polysaccharides are components of land and marine plants and their seeds. These material results from normal metabolic processes, and many times, they represent the reserve carbohydrate in that system (Bhardwaj, Kanwar, Lal, & Gupta, 2000). Natural gums are often preferred to synthetic materials due to their nontoxic nature, low cost, and free availability. Furthermore, natural gums have been modified to overcome certain drawbacks, like uncontrolled rate of hydration, thickening, drop in viscosity on storage, microbial contamination, and the like (Durso,1980).

Polysaccharides are a diverse class of polymeric materials of natural (animal, plant, microbial) origin formed via glycosidic linkages of monosaccharides (Shukla & Tiwari, 2012). Depending upon the nature of the monosaccharide unit, polysaccharides can have a linear or branched architecture. In addition to structural diversity, polysaccharides have a number of reactive functional groups, including hydroxyl, amino, and carboxylic acid groups, indicating the possibility for chemical modification (Liu, Jiao, Wang, Zhou, & Zhang, 2008). Moreover, polysaccharide molecular weight can vary between hundreds and thousands of Daltons, further increasing diversity (Saravanakumar, Jo, & Park, 2012). Also owing to their native presence within the body, most polysaccharides are subject to enzymatic degradation. Through enzyme catalysis, polysaccharides can be broken down to their monomer or oligomer building blocks and recycled for use as storage, structural support, or even cell signaling applications (Jain, Gupta, & Jain, 2007). For example, glycosidases are common, constituting 1%–3% of the human genome (Rempel & Withers, 2008), and can readily catalyze the hydrolysis of many different glycosidic linkages. In contrast to glycosidase, other enzymes (for example, hyaluronidase is specific for the polysaccharide hyaluronic acid) are more polysaccharide specific. Of note, some polysaccharides are particularly susceptible to degradation by lysosomal enzymes, including esterases, and proteases, following endocytosis pathway (Mehvar, 2003). Thus, enzymatic degradation provides a mechanism of release for therapeutics associated with polysaccharide-based carrier systems.

Mucoadhesion refers to the interaction of a material with a mucosal layer, such as in the gastrointestinal (GI) tract, nasal pathway, or airway. Many polysaccharides possess mucoadhesive property and thus, numerous investigators have explored their use for oral drug delivery. For neutral or negatively charged polysaccharides, hydrogen bonding provides the basis for mucoadhesion (Reddy, Mohan, Satla, & Gaikwad, 2011).

Any pharmaceutical formulation usually contains two types of ingredients one is the active ingredient and other components are excipients. The excipients help in the manufacturing of dosage form and also improve physicochemical parameters of the dosage form. They can influence drug release characteristics and preferably should be compatible, non-toxic, stable, economical etc. They are broadly classified as natural polymers and synthetic polymers. Due to their wide range of applications, the selection of polymer constitutes a vital step in designing any dosage form. Nowadays, due to many problems associated with drug release and side effects, manufacturers are inclined towards using natural polymers (Kulkarni, Butte, & Rathod, 2012).

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They are amenable to chemical modifications, potentially biodegradable, and also biocompatible with few exceptions (Satturwar, Fulzele, & Dorle, 2003). The specific application of biopolymers in pharmaceutical formulations include their use in the manufacture of solid monolithic matrix systems, implants, films, beads, microparticles, nanoparticles, inhalable and injectable systems as well as viscous liquid formulations (Pandey & Khuller, 2004; Chamarthy & Pinal, 2008; Alonso-Sande, Teijeiro, Remuñán-López & Alonso, 2009). The polymeric materials have fulfilled different roles such as binders, matrix formers or drug release modifiers, film coating formers, thickeners or viscosity enhancers, stabilizers, disintegrants, solubilizers, emulsifiers, suspending agents, gelling agents and bioadhesives in these dosage forms (Guo, Skinner, Harcum, & Barnum, 1998).

Controlled drug delivery is the use of formulation components and devices to release a therapeutic agent at a predictable rate *in vivo* when administered by an injected or non-injected route. Controlled drug delivery occurs when a polymer, whether natural or synthetic, is judiciously combined with a drug or other active agent in such a way that the active agent is released from the material in a predesigned manner. The release of the active agent may be constant over a long period, it may be cyclic over a long period, or it may be triggered by the environment or other external stimulus. In any case, the purpose is to achieve more effective therapies while eliminating the potential for both under and overdosing. The role of many of the original controlled-release systems was to achieve a delivery profile that would yield a high blood level of the drug over a long period of time. With traditional tablets or injections, the drug level in the blood follows a profile in which the level rises after each administration of the drug and then decreases until the next administration. The key point with traditional drug administration is that the blood level of the agent should remain between a maximum value, which may represent a toxic level, and a minimum value, below which the drug is no longer effective. In controlled drug delivery systems, designed for long-term administration, remaining constant, between the desired maximum and minimum, for an extended period of time (Torchilin, 2001). Depending on the formulation and the application, this time may be anywhere from 24 hours (Procardia XL) to 1 month (Lupron Depot) to 5 years (Norplant) (Shaik, Korsapati, & Panati, 2012).

For decades microbial exopolysaccharides have been invaluable ingredients in the food industry, as well as having many attractive pharmaceutical and chemical applications. The biopolymer gellan is a more recent addition to the family of microbial polysaccharides that is gaining much importance due to its novel property of forming thermo-reversible gels when heated and cooled. It has applications in diverse fields in the food, pharmaceutical and many other industries (Giavasis, Harvey, & McNeil, 2000).

Gellan gum is an anionic, high molecular weight (5,00,000Da), deacetylated exocellular polysaccharide gum produced as a fermentation product by a pure culture of *Pseudomonas elodea* (Jansson, Lindberg, & Sandford, 1983), with a tetrasaccharide repeating unit of one α-L-rhamnose, one β-D-glucuronic acid and two β-D-glucose residues. The native polysaccharide is partially esterified with L-glycerate and acetate (Kuo, Mort, & Dell, 1986) but the commercial product Gelrite® has been completely de-esterified by alkali treatment (Morris, 1995; Kang, Veeder, Mirrasoul, Kaneko, & Cottrell, 1982). It usually contains small amount of nitrogen-containing compounds resulting from fermentation procedures. The glucuronic acid is neutralized by the presence of potassium, calcium, and magnesium ions. The relative concentrations of these ions will control the physical properties of the gum material such as gel strength, melting point and setting point. The production organism is an aerobic, gram-negative bacterium, which has been very well characterized and demonstrated to be non-pathogenic. This organism was found during an extensive screening programme seeking naturally occurring hydrocolloids with useful properties. Gellan gum has the characteristic property of temperature-dependent and cation-induced gelation. This

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gelation involves the formation of double helical junction zones followed by a gellan gum aggregation of the double helical segments to form a three-dimensional network by complexation with cations and hydrogen bonding with water.

The acetyl groups in native gellan gum are removed by alkaline treatment to produce deacetylated gellan gum. Acyl substituents affect the rheology, and gel characteristics (Kang & Veeder, 1982). There are two types of deacetylated gellan gum differentiated on the basis of degree of deacetylation: high acyl gellan gum (partially deacetylated) and low acyl gellan gum (highly deacetylated) (Sanderson & Clark, 1983a). The high acyl form produces soft very elastic and non-brittle gels, while the low acyl form produces firm, non-elastic and brittle gels. In 1991, toxicological evaluation of gellan gum was done at the thirty-seventh meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in Geneva. Results from a limited study on tolerance to gellan gum in humans indicated that oral doses of up to 200 mg per kg of body mass administered over a 23-day period did not elicit any adverse reactions. Toxicological studies show that gellan gum is relatively nontoxic. An acute oral toxicity test on rat found that the LD_{50} of gellan is higher than 5000 mg/kg, and an eye irritation test indicated gellan to be safe for eye contact. Such evidence led to gellan gum being given FDA approval for use in foods on November 25, 1992 (Pszczola, 1993).

This chapter discusses the gelling properties of gellan gum, background information on gellan gum-based drug delivery systems, their merits, and demerits, factors controlling gelling behavior of gellan gum, and the latest developments in the area of controlled drug delivery system design using modified gellan polysaccharides.

BACKGROUND

In the design of pharmaceutical dosage forms, naturally occurring polysaccharides are currently being preferred over their synthetic counterparts because they are nontoxic, biodegradable, freely available, and less expensive (Bhardwaj et al., 2000). Over the last few years, a great deal of attention has been paid to the development of polysaccharide-based hydrogel beads through ionotropic gelation technique. They have been found useful as potential carriers for controlled drug delivery. One of the advantages of this technique is that the drug encapsulation in the beads can be accomplished in an eco-friendly aqueous environment (Patil, Kamalapur, Marapur, & Kadam, 2010). In addition, multiunit systems avoid the vagaries of gastric emptying and different transit rates through the gastrointestinal tract, thereby, releasing drugs more uniformly and preventing exposure to a high drug concentration, when compared to single unit dosage form on chronic dosing (Follonier & Doelkar, 1992). The anionic biopolymer sodium alginate (Das & Senapati, 2008; Kikuchi et al., 1999; Yegin et al., 2007) has been investigated in detail for its unique nature of forming hydrogel beads with divalent calcium ions. However, calcium alginate beads erode/disintegrate rapidly in simulated intestinal fluids leading to quick release of theloaded drugs (Acarturk & Takka, 1999). Further, the gel strength of alginate beads was improved by the formation of polyelectrolyte complex with positively charged amines such as poly-l-lysine (Ueng et al., 2000), polyethyleneimine (Halder, Maiti, & Sa, 2005), and chitosan (Anal & Stevens, 2005) for the controlled delivery of drugs. But the results were not so encouraging. Chitosan is at the origin a neutral polysaccharide whose amine functional group can be protonated in acidic pH conditions and become one of the little polysaccharides which are positively charged. The ability of chitosan to form hydrogel beads in presence of negatively charged sodium tripolyphosphate has also been investigated for controlled drug

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delivery (Aydin & Akbu˘ga, 1996; Srinatha, Pandit, & Singh, 2008). However, higher porosity of sodium tripolyphosphate/chitosan beads led to rapid release of drug in acidic dissolution medium (Gupta & Ravi, 2000). Even, the pectin beads have some drawbacks due to their rapid *in vitro* drug release (Bodmeier, Oh, & Pramar, 1989). Thus, it was evident that none of the polymeric bead systems seems suitable as an oral controlled release system. Due to its ability to form hydrogel beads in presence of divalent cations, particularly Ca^{+2} ions, gellan gum has been investigated for the controlled release of various kinds of drugs. Gellan gum gels are stable in low pH solution but swell in weakly basic solutions and calcium gellan allowed releasing the drug rapidly in phosphate buffer (El Fattah, Grant, Gabr, & Meshali, 1998).

In a study by Reddy and Tammishetti (2002), the efficiency of divalent and trivalent metal ions to form durable carboxymethyl guar gum beads was compared. They reported that due to higher valency, the cross-linking rate for trivalent aluminum (Al^{+3}) ions with carboxymethyl guar gum was faster than divalent calcium (Ca^{+2}) ions. The optimum salt concentration that provided maximum bovine serum albumin retention in the beads was found to be much lower for Al^{+3} ions. Furthermore, the beads cross-linked with Al^{+3} ions released the protein over a longer duration in simulated intestinal fluid, than those cross-linked with Ca^{+2} ions. Thus, the faster cross-linking rates with Al^{+3} ions could be very useful to load higher percentage of drug into the beads; otherwise the loaded drug would escape into cross-linking medium with time. Considering relatively fast drug release rate of Ca^{+2}/gellan beads in phosphate buffer solution, a novel glipizide-loaded bead system was developed by Maiti et al. (2011) through ionotropic gelation of gellan with trivalent Al^{+3} ions A maximum of 97.67% drug entrapment efficiency was achieved. The beads released only 10% drug in acidic medium in 2h; however it was found 38-47% for Al^{+3}/gellan beads in weakly alkaline medium. Al^{+3}/gellan beads could be useful carriers for the controlled oral delivery of glipizide.

Gellan gum has the potential to form gels in presence of mono- or divalent cations. Because of the presence of free carboxylate groups in GG, it becomes anionic in nature and thus, would possess the characteristic property of undergoing ionic gelation in the presence of mono- and divalent cations. However, the affinity for divalent cations is much stronger than the monovalent cations (Sanderson & Clark, 1983b). The mechanism of gelation involves the formation of double helical junction zones followed by aggregation of double helical segments to form a three-dimensional network by complexation with cations and hydrogen bonding with water (Grasdalen & Smidsrød, 1987).

This property has enabled possible applications of GG in the field of drug delivery. GG alone or its modified form has been studied extensively as a vehicle for ophthalmic delivery of drugs (Sanzgiri et al., 1993; Rozier, Mazuel, Grove, & Plazonnet, 1997; Carlfors, Edsman, Peterson, & Jornving, 1998) and other sustained release formulations viz. tablet binder (Ike-Nor, Ofoefule, & Chukwu, 2006), disintegrants (Antony & Sanghavi, 1997; Shah, & Jani, 2010), implantable films (Li, Kamath, & Dwivedi, 2001), and especially oral hydrogel beads (Santucci et al., 1996; Kedzierewicz, Lombry, Rios, Hoffman, & Maincent, 1999; Singh & Kim, 2005; Agnihotri, Jawalkar, & Aminabhavi, 2006; Babu, Sathigari, Kumar, & Pandit, 2010).

In view of the characteristic property of cation-induced gelation, it has been widely used in the formulation of *in situ* gelling ophthalmic preparations (Sanzgiri et al., 1993; Rozier et al., 1997) as well as *in situ* gelling oral sustained release formulations (Kubo, Miyazaki, & Attwood, 2003; Miyazaki, Kawasaki, Kubo, Endo, & Attwood, 2001; Miyazaki, Aoyama, Kawasaki, Kubo, & Attwood, 1999). Quigley and Deasy (1992) developed the sustained release beads containing GG for controlled release of sulfamethizole prepared by the hot extrusion method into the chilled ethyl acetate. The GG beads containing salbutamol sulfate was also studied for sustained release applications (El Fattah et al., 1998). Recently,

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Kedzierewicz et al. (1999) have developed the GG beads for the controlled release of propranolol hydrochloride and found that the drug release was rapid. However, the ionotropic gelation with GG offers new opportunities in the field of bioencapsulation. Chan et al. (2002) recently reported that a combination of calcium and zinc ions could produce more sustained release of drugs from the alginate microspheres compared to calcium ions alone. Agnihotri et al. (2006) prepared and evaluated gellan gum beads. Gellan gum beads containing cephalexin were prepared by extruding the dispersion of cephalexin and gellan gum into a solution containing a mixture of calcium and zinc ions (counterions). Beads were prepared by changing experimental variables such as pH of the counterion solution and amount of cephalexin loading in order to optimize process variables on the final % drug entrapment efficiency, release rates, size, and morphology of the beads. Beads were spherical in shape, with the average bead size ranging from 925 to 1183 μm as measured by the laser light scattering technique. Cephalexin entrapment of up to 69.24% was achieved. *In vitro* release studies were performed in 0.1N HCl or pH7.4 phosphate buffer and the release of cephalexin was achieved up to 6h.

Alhaique et al. (1996) prepared gellan capsules by Ca^{+2} induced gelation of this polysaccharide around a core containing starch, calcium chloride and a model drug. Results obtained indicated that gellan is suitable for the formulation of sustained release capsules and that solvent uptake by the dried capsules is most likely the main factor capable of affecting the rate of delivery from the tested preparations.

Kedzierewicz et al. (1999) prepared the beads according to the technique of ionotropic gelation. Gellan gum beads of propranolol hydrochloride, a hydrophilic model drug, were prepared by solubilising the drug in a dispersion of gellan gum and then dropping the dispersion into calcium chloride solution. The droplets formed gelled beads instantaneously by ionotropic gelation. The drying of the gel beads was carried out by either freeze drying or oven-drying at 37°C for 24h. Very high entrapment efficiencies were obtained (92%) after modifying the pH of both the gellan gum dispersion and the calcium chloride solution. The drug release from gellan gum beads (2.09-3.35 mm) was studied. The beads could be stored for 3 weeks in a wet or dried state without modification of the drug release. Oven-dried beads released the drug somewhat more slowly than the wet or freeze-dried beads. The drug release from oven-dried beads was slightly affected by the pH of the dissolution medium. Gellan gum could be a useful carrier for the encapsulation of fragile drugs and provides new opportunities in the field of bioencapsulation.

In order to evaluate the influence of the dissolution medium on drug release, the results obtained in buffer at pH6.8 were compared with results from a medium at pH2.0. The results showed that the wet beads released the drug (maximum 80%) in 15 min in both dissolution media. In contrast, drug release from oven-dried beads was slightly affected by the pH. This small increase in the delivery rate observed after 7min in the acidic environment cannot be related to the different solubilities of the drug at pH 2.0 and 6.8, because the maximum amount of drug released from the wet beads was the same in the two dissolution media. It could be explained by modifications of the gel structure due to the competition between H^+ and Ca^{+2} ions which could affect the swelling rate of the beads and consequently the penetration of the solvent into the beads. Indeed, reduced ionization of carboxyl group results in decreased polymer extension in aqueous medium and a modification of gel structure, which affects the overall release mechanism, may occur (Deasy & Quigley, 1991). This hypothesis was supported by the non-swelling of the beads in medium at pH2.0. Although the drug was released more quickly in medium at pH2.0, the total amounts released were the same in the two media and never greater than either 50% for the beads prepared with the gum dispersion at normal pH or 80% when the gum dispersion was alkalinized to pH12.

Low-acyl gellan is cross-linked more effectively with divalent cations than the monovalent cations (Morris, Gothard, Hember, Manning, & Robinson, 1996). Divalent metal ions bind to pair of carboxylic

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group on nearby helices, whereas monovalent metal ions bind individual helices (Tang, Tung, & Zeng, 1996). The strongest gellan gels were obtained when the [cation]/[carboxylate] ratio corresponds to 0.5 for divalent and 10-30 for monovalent. At optimum concentration of respective cations, gellan form stronger gel with Ca^{+2} than Mg^{+2}. Low-acyl gellan forms gel at temperatures between 30 and 50°C. The high-acyl gellan has poor aggregation properties; it gels at around 70°C. Low-acyl gellan forms hard and brittle gel, whereas high-acyl gellan forms soft and elastic gel. Below pH3.6, gellan forms acid gel, which has lower modulus and hardness value.

The high-acyl gellan has poor cation-mediated aggregation. The acetyl groups present on the surface of the duplex block helix-helix aggregation, and the steric effect of bulky glyceryl group repels the carboxylic groups, making it incapable of accommodating site-bounded cations (Mortika, Fukuba, Kumeno, Nakahma, & Nishinari, 1991).

Patil et al. (2006) prepared gellan beads by ionotropic gelation method which involves very mild processing conditions and can be accomplished in an aqueous, organic solvent-free environment. The drug-loaded beads were spherical with size range of 0.85-1.8 mm. The drug entrapment efficiency of various batches was in the range of 72.52-92.74%. Calcium-gellan beads containing diclofenac sodium showed pH-dependent swelling and drug release properties. Swelling and drug release were significantly higher in pH7.4 phosphate buffer than 0.1N HCl. The swelling ratio for beads was up to 22 and 3 for phosphate buffer and 0.1N HCl, respectively. Cumulative diclofenac sodium release from calcium-gellan beads was 12-35% in 0.1N HCl within 2h, whereas complete drug release was observed within 3-4h in pH7.4 phosphate buffer. Studies indicated that, in addition to drug: polymer ratio, pH of cross-linking solution and speed of agitation also influence the formation and properties of calcium-gellan beads. The pH of manufacturing liquid is more important for drugs having pH-dependent solubility and gums having pH-dependent gelling. The effect of speed of agitation is more influential for the polysaccharides showing slow rigidization during cross-linking process. Release of diclofenac sodium from calcium-gellan beads in phosphate buffer was not only dependent on drug solubility but also governed by formation of drug precipitate, which occurred during processing.

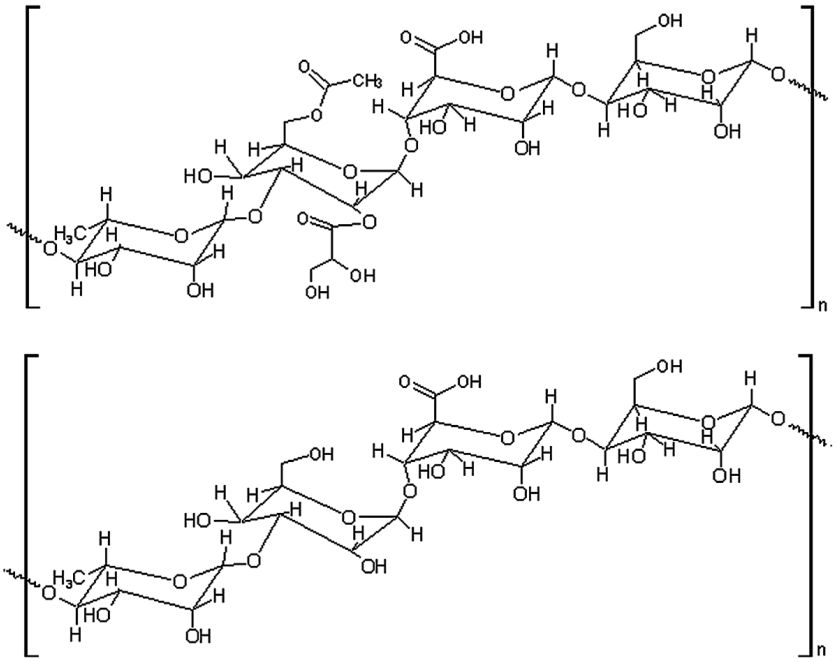
The native form of gellan gum contains two acyl substituents, namely acetate and glycerate, both of which are located on the same glucose residue and, on average, there is one glycerate and a half acetate group per every tetrasaccharide repeating unit (Figure 1).

The varying substitutions lead to a difference in the gelling potential and, therefore, microencapsulation efficiency of its native and deacetylated forms (Chandrasekaran, Radha, & Thailambal, 1992). The gelling mechanism of gellan gum is inducible by cations as well as temperature dependent. In aqueous solution, the gelation of gellan gum is usually accompanied by a two-step process, which involves formation of double helices from random coil chains ('coil-to-helix transition') and an aggregation of pairs of double helices. Furthermore, the coil-helix transition is greatly affected by the electrostatic interaction with co-existing cations in the solution (Kanesaka, Watanabe, & Matsukawa, 2004), whereas the aggregation behaviour appears to be affected by the pH of solution (Horinaka, Kani, Hori, & Maeda, 2004). In the case of monovalent cations, the gelation is mainly contributed by the screening of the electrostatic repulsion between the ionized carboxylate groups (COO-) on the gellan chains (Nakajima, Ikehara, & Nishi, 1996).

On the other hand, in the case of divalent cations, the gelation and aggregation of gellan gum occurs via a chemical bonding between divalent cations and two COO- groups belonging to glucuronic acid molecules in the gellan chains, in addition to the screening effect. Although a great deal of effort has been made in understanding the gelation mechanism of gellan gum in the presence of various monovalent

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Figure 1. Chemical structure of native gellan gum (top) and deacetylated gellan gum (bottom) (O'Neill, Silvendran, & Morris, 1983)



and divalent cations; only a few studies have explored the microencapsulation efficiency of gellan gum in the presence of divalent cations other than Ca^{+2} (Tang, Tung, & Zeng, 1997).

Singh & Kim (2005) evaluated the effects of calcium (Atomic number=20), magnesium (12), copper (29), zinc (30) and barium (56) ions on the encapsulation efficiency of deacylated gellan gum using azathioprine as a model immunosuppressive drug. The information from this study is not only relevant from a microencapsulation perspective, but also from a therapeutic point of view, as both azathioprine and certain transition metals (e.g. copper) have been suggested to have anti-rheumatic properties (Fini, Feroci, & Fazio, 2001).

Spherical beads containing azathioprine were prepared from deacetylated gellan gum by ionotropic gelation method. One molar solution of various divalent chlorides ($MgCl_2$, $BaCl_2$, $CaCl_2$, $CuCl_2$ and $ZnCl_2$) and two additional concentrations of $CaCl_2$ (2.5M and 5.0M) were used as ionotropic media. Drug solubility was also determined in these ionotropic media. Solubility in various divalent chloride solutions (1.0M) suggests that azathioprine forms complex with Ca^{+2}, Zn^{+2}, and Cu^{+2}, while there might be a formation of poorly water-soluble chelates with Mg^{+2} and Ba^{+2} as solubility in these media were less than in deionized water. The encapsulation efficiency of gellan gum was much higher in the presence of transition elements (Cu^{+2} and Zn^{+2}) when compared to alkaline earth metal ions (Ca^{+2}, Mg^{+2} and Ba^{+2}). Higher concentrations of Ca^{+2} decreased the encapsulation efficiency of gellan gum in a nearly proportional manner. The correlation between encapsulation efficiency and pH of the ionotropic media was negative, although the solubility of azathioprine seems to be independent of the pH of the ionotropic medium. Overall, the results suggested that drug encapsulation efficiency of deacetylated gellan gum was largely affected by the concentration and nature of various divalent cations (e.g. atomic number, valency or electropositivity, co-ordination property, etc.) and pH of the ionotropic medium (Singh & Kim, 2005).

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Gellan beads have the following advantages: i) nontoxic when taken orally ii) since the dried gellan beads have the property of reswelling, they can act as controlled release system iii) since their property of reswelling is susceptible to environmental pH, acid sensitive drugs incorporated into the beads would be protected from gastric juice (Coviello, Matricardi, Marianecci, & Alhaique, 2007).

However, porosity gives gellan beads not only fast release of incorporated drugs but also a very low efficiency of incorporation of low molecular weight drugs except for sparingly soluble drugs. Therefore, it appears that gellan beads can be used for a controlled release of sparingly water soluble drugs or macromolecular drugs. Multiparticulate delivery systems spread out more uniformly in the gastrointestinal tract and reduce the local irritation with respect to single unit dosage forms, such as tablets.

Babu et al. (2010) explored the possible applicability of gellan macro beads as an oral controlled release system of a sparingly soluble drug, amoxicillin. They investigated the effect of drug loading, stirring time, polymer concentration, electrolyte ($CaCl_2$) concentration, curing time etc. influencing the preparation of the gellan gum macro beads and the drug release from gellan gum beads. Optimal preparation conditions allowed 91% drug incorporation efficiency (gellan gum: 2%w/v; amoxicillin: 2%w/v; $CaCl_2$ concentration: 0.2M; and curing time: 5min). The gellan beads are stable in low acidic pH but swell and disintegrate in intestinal pH. The swelling and disintegration of calcium gellan beads are dependent on the pH of the dissolution medium and solubility of the drug entrapped in the gellan beads. The release rate of amoxicillin from gellan beads was in the following order: 0.1N HCl> phosphate buffer>distilled water. Amoxicillin was released from gellan beads similar to ibuprofen as in earlier studies although alginate beads are not swelled and disintegrated in gastric fluids (Lee & Min, 1996).

The percent of amoxicillin released in 0.1N HCl was higher due to higher solubility when compared to lower solubility in phosphate buffer. The shape of the gel beads did not change in appearance even on completion of drug release studies. This suggests that the gel structure was maintained throughout the release process. The rapid drug release from gellan matrices in acid medium can also be explained by a change in the matrix properties on contact with acid. The calcium ions in gellan beads were totally discharged in an acid environment and the carboxyl groups were shifted to an un-ionized form. This might alter the matrix properties, including the release characteristics of the incorporated drug. The swelling of gellan matrices in phosphate buffer (pH6.8) resulted from the exchange of hydrogen ions with sodium ions of the dissolution medium (Kim & Lee, 1992). The rapid swelling and aqueous solubility of gellan and amoxicillin, respectively, might be the cause of the increased drug release in phosphate buffer than in distilled water.

When gellan beads are placed in the dissolution media, three processes occur. First, the gellan beads imbibe water, rehydrate and perhaps swell. Second, as water penetrates the gel network, amoxicillin saturates the water in the gel. Third, amoxicillin diffuses through the water filled gelled network. On both a molecular and macroscopic scale the gellan beads are porous. The cross linked regions create a three-dimensional pore structure which will be filled with the medium. If calcium forms more extensive cross linking (at increasing calcium concentration), the molecular porosity may be reduced and both the extent of rehydration and the rate of drug release are reduced. During drug release, the gellan beads become macroscopically porous because the dissolved drug particles would leave water filled cavities in the gel. A significant number of the drug particles originally suspended in the gellan beads would be in physical contact with each other and the individual cavities left after dissolution would become continuous water filled tortuous channels. The formation of the channels depends on the extent of drug loading in the gellan beads. The percolation limit, the drug load above which the channels become continuous, has been estimated to be 30-40% (Fernandez-Hervas, Vela, Holgado, Cerro, & Rabasco, 1995).

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Release study of the gellan beads in all three media showed an initial burst concentration of amoxicillin. This may be due to crystals of the drug on the bead surface or may be due to an unequal distribution of drug within the gellan beads which results in slightly higher concentration of drug at the surface of the gellan beads. These may arise as follows. When gelation occurs, water is expelled from the gellan beads because the gel contracts during cross linking. Furthermore, as the gellan beads are dried, water migrates to the bead surface. These water fluxes may carry amoxicillin molecules and alter the drug distribution within the gellan bead, which will be reflected as an increased concentration of drug particles on or just beneath the surface of the dry gellan beads. Since water uptake by the gellan beads during release and dissolution of amoxicillin occurs radially inwards, the initial rate of release is high. The rate decreases with time because of the greater diffusional path length and possibly because of the lower concentration of amoxicillin towards the bead centre. The radial pattern of drug dissolution in the gellan is visible as a translucent gel layer at the edge surrounding a solid drug core.

Change in curing time did not significantly affect the release rate constant, but drug concentration, polymer concentration and electrolyte concentration significantly affected the release rate of amoxicillin from the beads. The gellan macro beads may be suitable for gastro retentive controlled delivery of amoxicillin. The encapsulation efficiency of amoxicillin beads increased as the polymer and drug concentration were increased. The entrapment efficiency was determined as a function of different initial amoxicillin (0.5 - 2.0 g) loading. Around 90% of amoxicillin was incorporated when the initial drug loading was at 2% (w/v); the entrapment efficiency increased with increasing drug concentration. Although the entrapment efficiency values seem low, it has to be kept in mind that the encapsulation process is carried out in an aqueous environment, where the drug is sparingly soluble. The entrapment efficiency of amoxicillin also increased as a function of the polymer concentration (1-3%, w/v). Approximately 84% of amoxicillin was incorporated at the polymer concentrations 3.0 percent (w/v). Since the amount of the drug extracted depends on the duration for which the beads remain in the cross linking solution, the duration of the cross linking process has to be strictly determined. To assure the reproducibility of drug loading, the curing time was maintained constant (5 min) in the preparation. When the percent of drug (0.5 to 2%) and polymer (1 to 3%) were increased the beads were still round but their diameter increased with the percent of drug and polymer. This effect can be explained by an increase in the viscosity of the gellan solution which therefore flowed more slowly out of the syringe. Consequently, the drops and then the beads had a larger diameter. The entrapment efficiency of amoxicillin into gellan beads was studied as a function of curing time. Excessively long curing time might result in low entrapment efficiency of soluble drugs in gellan beads. Due the overall aqueous environment, the curing time may be one parameter strongly influencing the entrapment efficiency. Therefore, in order to decrease the leakage of the incorporated amoxicillin in to the calcium chloride solution during bead preparation, the curing time was reduced from 15 min to 5 min. It was observed that curing for 5 min gave good entrapment efficiency (84.4%) and proper gelling of the polymer. Increasing the curing time from 5 to 15 min resulted in low entrapment efficiency and consequently decreased size of beads. The drug loading decreased as the concentration of cation was increased (0.1-0.4M). As gelation proceeds, water is expelled due to cross links formed by the cations. Therefore a more cross linked structure may show a larger water loss. Dissolved portion of amoxicillin in the beads could be lost from the gellan beads during curing by simple diffusion and the expulsion of water will cause convective loss of drug molecules (Ostberg, Lund, & Graffner, 1994). The mean diameter of gellan beads decreased with increased concentration of calcium, due to more extended gel bead shrinkage, caused by syneresis, during curing in a similar manner to calcium alginate (Oestberg & Graffner, 1992).

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Release of amoxicillin from gellan beads cured for 15 min was somewhat slower than that from beads that had been cured for 5 and 10 min. However, the pattern of release showed very little variation between gellan bead samples cured for longer than 5 min. Therefore, it appears that the optimum curing time of the gellan beads could be taken as 5 min, as there is no statistically significant difference in the release rates of bead with curing times between 5 and 15 min ($p > 0.05$). The release from gellan beads made at 0.4M calcium chloride solution was lower than gellan beads made at 0.2 and 0.1M calcium chloride solutions. The release rate of amoxicillin in phosphate buffer was increased with drug loading. The increase in drug release from beads containing high concentrations may be due to the decrease in the polymer/drug ratio. As polymer concentration increased, the release rate decreased due to hindrance of swelling and disintegration of gellan beads in intestinal fluids]. Increased gellan density per unit volume may also result in decrease in the mesh size within the gel, affecting amoxicillin release. This result is consistent with that of Kikuchi et al. (1997) for alginate beads.

The potential role of gellan in controlled drug release and adsorption in stomach has also been examined. Wataru et al. (2003) studied sustained delivery of paracetamol by gellan and sodium alginate formulations, and reported that the bioavailability of paracetamol from the gels formed *in situ* in the stomach of rabbits following oral administration of the liquid formulations prepared from gellan gum and sodium alginate was similar to that of a commercially available suspension containing an identical dose of paracetamol. Use of gellan gum for controlled bioavailability of ophthalmic formulations has also been proposed. Sanzgiri et al. (1993) have shown that gellan-based ophthalmic solutions have longer residence time in tear fluid than saline solution. Sidda et al. (2007) formulated *in situ* gellan gum-based gels with ciprofloxacin hydrochloride as a drug. The gel formulation containing both ciprofloxacin and gellan gum showed a prolonged drug release pattern. Gellan gum was evaluated as a binding agent in lactose-based tablets containing metronidazole or paracetamol. The binding properties of the gum were compared with acacia and gelatin. Granules were prepared by the conventional wet granulation method. The results indicated that though the hardness of tablets containing gellan gum was lower than that containing gelatin or acacia, gellan gum can be employed in the formulation of normal release of metronidazole and paracetamol with moderate hardness, low friability and good disintegration and dissolution properties (Ike-Nor et al., 2006).

Due to its characteristic property of temperature-dependent and cation-induced gelation, gellan has been used in the formulation of eye drops which gelify on interaction with the sodium ions naturally present in the eye fluids. Gelrite® solution, a novel ophthalmic vehicle, gels in the presence of mono or divalent cations. In the conjunctival sac 'ion-activation' of the sol/gel transition is accomplished by the lacrimal fluid. A 0.6% Gelrite® vehicle has been compared to an equiviscous solution of hydroxyethylcellulose (HEC) using timolol maleate as a drug probe. *In vitro* release rates of timolol from HEC and Gelrite® gel were similar. *In vivo*, the formation of the gel in the presence of tear fluid cations prolonged precorneal residence time and increased ocular bioavailability of timolol in the cornea, aqueous humor and iris and ciliary body of albino rabbits (Rozier, Mazuel, Grove, & Plazonnet, 1989).

A methylprednisolone (MP) ester of gellan (gellan-MP) was synthesized. Sustained release dosage forms evaluated were gellan-MP films, gellan films with physically incorporated MP and eye drops of MP suspended in a 0.6% w/w/ gellan dispersion in water. The control dosage form was a suspension of MP in normal saline. *In vitro* release of MP from the test dosage forms was determined in a pH 7.4 phosphate buffer at 32 °C using a rotating bottle apparatus. MP concentrations in the tear fluid of New Zealand white rabbits were measured after ocular application of the dosage forms. Compared with the MP suspension control, the gellan-MP films yielded an approximately 4-fold higher area under tear

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fluid concentration vs time curve, AUC_{0-8h}, but exhibited a tendency to slip out of the eye due to a high degree of swelling. The *in vivo* release from films containing physically incorporated MP showed higher variability and provided mean AUC_{0-8h} values approximately equal to the control values. The gellan eye drops containing MP yielded 2.6-fold higher AUC_{0-8h} values than the control and also provided ease of administration. Gellan solutions might thus provide a versatile vehicle for ocular sustained release of drugs (Sanzgiri et al., 1993).

Recently, gellan based intra-gastric *in situ* gelling system for controlled delivery of amoxicillin has been reported (Rajinikanth, Balasubramaniam, & Mishra, 2007). The aim of this study was to develop a new intra-gastric floating *in situ* gelling system for controlled delivery of amoxicillin for the treatment of peptic ulcer disease caused by *Helicobacter pylori* (*H. pylori*). Gellan based amoxicillin floating *in situ* gelling systems (AFIG) were prepared by dissolving varying concentrations of gellan gum in deionized water containing sodium citrate, to which varying concentrations of drug and calcium carbonate, as gas-forming agent, was added and dissolved by stirring. The formulation variables like concentration of gellan gum and calcium carbonate significantly affected the *in vitro* drug release from the prepared AFIG. The *in vivo H. pylori* clearance efficacy of prepared AFIG in reference to amoxicillin suspension following repeated oral administration to *H. pylori* infected Mongolian gerbils was examined by polymerase chain reaction (PCR) technique and by a microbial culture method. AFIG showed a significant anti-*H. pylori* effect in the *in vivo* gerbil model. It was noted that the required amount of amoxicillin for eradication of *H. pylori* was 10 times less in AFIG than from the corresponding amoxicillin suspension. The results further substantiated that the prepared AFIG has feasibility of forming rigid gels in the gastric environment and eradicated *H. pylori* from the gastrointestinal tract more effectively than amoxicillin suspension because of the prolonged gastrointestinal residence time of the formulation.

Another latest report was associated with the use of a blend of two natural polymers for ionic crosslinking and obtaining hydrogel beads (Prezotti, Cury, & Evangelista, 2014). Gellan gum/pectin mucoadhesive beads containing ketoprofen were prepared by ionotropic gelation using Al^{+3} as crosslinker. Beads having diameter of 728-924μm exhibited a drug entrapment efficiency of 52.22–88.78%. The erosion of beads was greater in acid media while swelling was more pronounced in pH7.4. Drug release was dependent on pH and was 20-34% in pH1.2 acidic medium, while in pH7.4 buffer solution; the drug release was sustained up to 6h.

Thorough literature scrutiny suggested that deacylated gellan gum can be used for the design of sustained release ophthalmic preparation and oral hydrogel beads due to its Calcium ion induced gelling ability. However, the prior art suggested that calcium gellan beads alone could not control the drug release for a longer duration which is desirable from better therapeutic point of view. Some remedial actions were taken by some workers with the variation in type and concentration of gelling ions and other formulation/processing variable. However, these changes were still insufficient for gellan beads to be suitable for the controlled delivery of drugs. Lot of workers put their effort to make effective controlled drug delivery systems for a variety of drugs, each having their own advantages or disadvantages. Therefore, it would wise decision if some chemical modifications can be done on the native gellan gum and the drug delivery systems be designed.

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MODIFIED GELLAN POLYSACCHARIDE IN DRUG DELIVERY

Recalling the drawbacks of metal ion induced gelled beads in the inability to control drug release rate in simulated gastrointestinal fluids, this section has been devoted to highlight the recent advancements in the field of drug delivery using chemically modified gellan polymer. This section has been described under different subhead according to different type of chemical modifications that have been done to overcome accompanying problems of calcium gellan beads.

Grafted Gellan

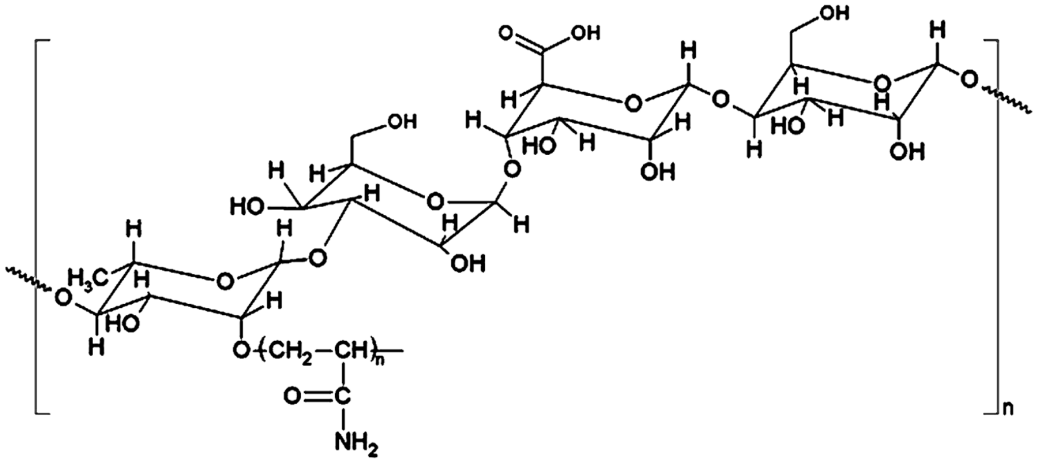
Recently, the polyacrylamide-grafted copolymer of natural polysaccharides has entered the arena of prolonged drug delivery. Graft copolymer is described as a macromolecular chain, where the main polymer backbone has branches of another polymeric chain emanating from different points along its length (Zohuriaan-Mehr, 2005). It can easily be converted into its ionic form through hydrolysis of the side chain amide groups resulting in pH-sensitive or smart copolymer (Kaur & Chatterji, 1990; Qiu & Park, 2001). However, its degree of pH sensitivity depends on the number of ionisable functional groups (Soppimath & Aminabhavi, 2002). This fascinating technique may be considered as an approach to achieve novel polysaccharide-based materials with combined properties of natural and synthetic polymers. Furthermore, the evolution of ionotropic gelation technique has enabled their preparation in multi-particulate form in an aqueous, eco-friendly environment without the need for expensive, toxic organic solvents. As the pH of the GI tract varies from acidic (stomach) to weakly alkaline (intestine), the smart hydrogel particles can dictate swelling, and consequently release the drug from their network (Satish, Satish, & Shivakumar, 2006).

Maiti and his coworkers (2012) synthesized gellan gum (GG) graft copolymer and developed hydrogel beads for controlled delivery of salbutamol sulfate. Acrylamide was grafted onto GG polymer by free radical polymerization following the method reported earlier (Pourjavadi, Sadeghi, & Hosseinzadeh, 2004). Initially, 100 ml of 1% (w/v) GG solution was prepared in a 500-ml three-necked round bottom flask by flushing a slow stream of nitrogen gas and gently heating to 80°C for 4h. Then, 0.12 mol of acrylamide and 0.002 mol of ammonium peroxodisulphate were added to the reaction flask and nitrogen was purged for 1h to allow the grafting reaction. The resulting graft copolymer was then filtered, re-precipitated with methanol for removing the un-reacted monomers, and dried at 50°C. The copolymer was hydrolyzed in 0.9M NaOH solution at 75°C for 1h and purified in methanol. The hydrolyzed copolymer beads were prepared by ionotropic gelation with aluminium ions. The smart hydrogel reticulates (1.43-1.56 mm) seemed spherical and showed a maximum of 65% drug retention. The drug release rate was inversely proportional to $AlCl_3$ strength in simulated intestinal milieu (pH7.4), but approximated a proportional relationship with drug load. The hydrogel particles liberated only 10–17% content in simulated gastric milieu (pH1.2) in 2h. The release data correlated well with the pH-dependent swelling of hydrogel and indicated the anomalous drug diffusion mechanism. They concluded that smart hydrogel had the potential to prolong drug release mimicking the variable pH of the gastrointestinal tract.

The graft copolymer of acrylamide and gellan gum was prepared by microwave-assisted (at 480 W), free radical induced polymerization method by Vijan et al. (2012). The probable chemical structure was given in Figure 2. Tablets were prepared by wet granulation method and an anti-diabetic drug metformin hydrochloride was incorporated in grafted gum along with excipients (PVP-K 30). *In vitro* studies showed extended drug release up to 8h. They suggested that microwave assisted cerric (IV) induced

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Figure 2. Possible structure of poly (acrylamide) grafted gellan copolymer



graft co-polymerization is an easy, efficient, less time consuming and reproducible (due to the supply of fixed energy form microwave) method for the development of graft co-polymer which can be used as an rate controlling polymer for the development of sustained release dosage from.

Covalently Crosslinked Gellan

Chemical crosslinking of polysaccharide is highly versatile method with good mechanical stability. During crosslinking counterions diffused into the polymeric and crosslinking agent reacts with polysaccharides forming either intermolecular or intramolecular linkages. The gellan gum-based drug delivery devices were reported by Maiti et al. (2011).

Due to relatively faster drug release from Ca^{+2}/gellan beads in phosphate buffer solution, a combination of ionotropic gelation with trivalent Al^{+3} ions and covalent cross-linking with glutaraldehyde (GA) was adopted for designing a novel hydrogel bead system. Glipizide (30%, w/w) was dispersed uniformly into 1.25% (w/v) gellan gum aqueous solution and extruded dropwise into slightly agitated 5% (w/v) aluminum chloride ($AlCl_3$) solution containing 0.08% (w/v) Tween 80. Following an incubation period of 10 min, the beads were isolated and air-dried. The beads were then treated with glutaraldehyde (GA) solution (pH2.0) at 50°C for 2h. The cross-linked beads were removed and washed with distilled water repeatedly to remove non-reacted GA. A maximum of 97.67% drug entrapment efficiency was achieved; however GA-treatment reduced the same by 11.89%. All the beads released only 10% drug in acidic medium in 2h; however it was only 15% for GA-treated beads in weakly alkaline medium. The anomalous drug transport mechanism shifted to super case II transport for GA-treated beads, where the polymer relaxation phenomenon was dominant. They stated that GA-treated beads could be useful carriers for the controlled oral delivery of glipizide.

Later on, Kulkarni et al. (2013) developed simvastatin-loaded gellan gum-carrageenan composite polyspheres by ionotropic gelation/covalent crosslinking method. The polyspheres had rough and dense surface. The drug entrapment efficiency of the particles prepared by ionic crosslinking was higher than those prepared by dual crosslinking. The *in vitro* drug release study indicated that the ionically cross-

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linked polyspheres discharged the drug quickly whereas, dual crosslinked polyspheres extended the drug release for longer period. The polyspheres effectively reduced the elevated total serum cholesterol and triglycerides in Wistar rats.

Complex Coacervation

During the last ten to twelve years the focus of a number of pharmaceutical scientists has been on the use of polyelectrolyte complex (without the need of toxic crosslinkers) for the sustained/modified delivery of drugs. Use of cationic chitosan in the formation of polyelectrolyte formation with anionic polymers is not new. Polyelectrolyte complexes of chitosan with pectin, xanthan, alginate, gelatin, hyaluronic acid etc have been extensively reported in the literature. However, very few articles have been found reporting the formation of gellan-chitosan polyelectrolyte complex (PEC). Dixit et al. (2011) explored their potential for oral sustained/controlled/ stomach specific delivery of water soluble and insoluble drugs. They prepared beads by extruding aqueous solution of gellan gum (with or without drugs) into chitosan solution (pH3.5). Prepared beads exhibited poor encapsulation and burst release for metronidazole, while very high encapsulation and extended release was observed for metronidazole benzoate in simulated gastric fluid (pH1.2). From metronidazole base containing formulations, almost all the drug was released within 2h. Overall, gellan-chitosan beads showed poor retardant capacity of drug release for metronidazole whereas good retardant capacity was observed for metronidazole benzoate.

Floating emulsion PEC beads (gellan gum-gelucires-chitosan) were prepared by combining the complex coacervation technique with emulsion gelation (Dixit, Verma, Mishra, Verma, & Pandit, 2012). Here, an emulsion of gellan with gelucire 39/01 and 50/13 containing $CaCO_3$ (with or without drug) was extruded dropwise into chitosan solution (0.6% w/v) and were isolated after 15min and then dried. Floating gellan-chitosan PEC beads were prepared in the similar manner but without gelucires. The beads showed excellent buoyancy along with significantly improved encapsulation efficiency (92.78%) and sustained release of metronidazole for 8h when compared to floating gellan-chitosan PEC beads prepared in the similar manner but without gelucires. Experimental data showed that floating emulsion PEC beads may form a potential stomach site specific drug delivery system for the delivery of highly water soluble drugs with an absorption window in the upper gastrointestinal tract.

Oxidized Gellan

Polysaccharide-based hydrogels have been proven promising in tissue engineering applications due to their good biocompatibility, controllable properties and abundance, and are therefore in great demand as therapeutic cell vehicles. For tissue engineering purposes, however, gellan-based hydrogels need to be modified in order to meet the requirement of encapsulating living cells while maintaining their injectability, because the gelling point of this temperature-dependent gel is too high (above 42°C). In the presence of cations (e.g., Na^+, K^+, and Ca^{+2}), aqueous solutions of gellan undergo thermo-reversible gelation when cooled to below $T_{gelation}$, forming a gel that is stable below $T_{melting}$. Therefore, the gellan solution is theoretically available to be injected into a defect site and form a gel *in situ* by cooling treatment. But in physiological cationic conditions, the $T_{gelation}$ of commercially available and unmodified gellan is too high (>42°C), and must be decreased to suspend the mammalian cells at physiological temperature (37.5°C) for injectable purposes. Gellan (Phytagel) was mixed with deionized water (5g gum powder in 500 ml) and heated to 70°C for 3–5h to obtain a homogeneous aqueous solution. After the gellan solution was

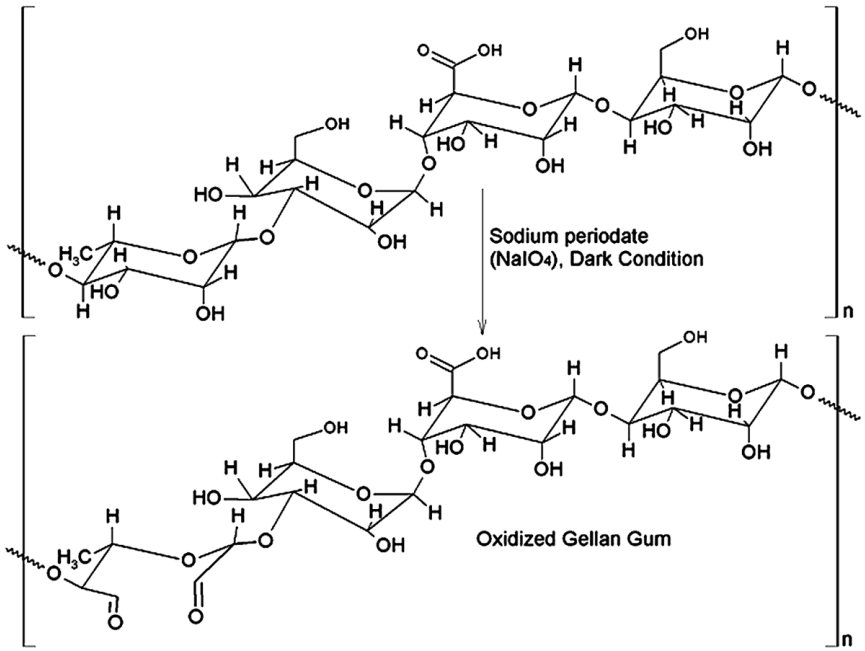
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cooled to 40°C, sodium periodate (0.25 M $NaIO_4$) was then added at different dosages (3-7.2ml) with gentle stirring and reacted for 1–120h to achieve various oxidization degrees. An equimolar ethylene glycol was added at predefined reaction time points to stop the oxidation reaction. The resulting solution was purified by Sephadex G25 SEC. Finally the product was lyophilized. The chemical structure of oxidized gellan gum was predicted in Figure 3. Chondrocytes were then seeded into the modified gellan gels. Their cytocompatibility and the capability to promote *in vitro* tissue regeneration were evaluated. Notably, chondrocytic constructs based on modified gellan gel were kinetically monitored for 150 days which showed superiority for long-term cartilaginous development in terms of many aspects such as cell proliferation and specific matrix formation. They showed that modified gellan was able to retain the chondrocytes viability, enhance the extracellular matrix (ECM) secretion and maintain normal phenotype. A novel injectable hydrogel was successfully developed basing on gellan gum, the molecular size of which had been chemically reduced and optimized to alter the working gelling point to the cell-favorable range and simultaneously maintain the injectable processability for transplantation. The chondrocytic cell-laden gellan gel was kinetically tested for nearly half a year. The results indicated that such modified gellan gels have superior performance in long-term culture *in vitro* in comparison with other popularly employed gels such as agarose. This new hydrogel-based scaffolding system was capable of providing a promising platform for cartilaginous regeneration (Gong et al., 2009).

Interpenetrating Polymer Network (IPN)

A considerable interest has been given to the development of IPN hydrogels for the controlled release of drugs. An IPN is a composite of two polymers, which is obtained when at least one polymer network is synthesized or cross-linked independently in the immediate presence of the other. Such systems are

Figure 3. Periodate oxidation to cleave the adjacent dihydroxyl groups in the gellan polysaccharide chain



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known to increase the phase stability and, thus, enhance the mechanical properties of the final product. Agnihotri and Aminabhavi (2005) developed IPN of gellan gum for oral controlled drug delivery of carvedilol, an antihypertensive drug. Gellan gum and poly (vinyl alcohol) IPN microspheres containing carvedilol were prepared by the emulsion cross-linking method. In this method, poly (vinyl alcohol) was dissolved in hot water at 80°C, and after cooling, gellan gum was added (total polymer concentration was 4%, w/v). Carvedilol was dissolved in 2ml absolute alcohol and slowly added to the above polymer solution under stirring. This produced a fine precipitation of carvedilol, which was stirred further to get a uniform suspension. This solid-in-water suspension was emulsified into light liquid paraffin in the presence of 0.5% Span 80 at 300 rpm for 5 min. Then, glutaraldehyde (GA) and 1 ml of 5N HCl was added slowly, and the reaction was continued for 2h. Hardened microspheres were separated by filtration and washed with *n*-hexane followed by acetone. The microspheres were dried at 50°C for 24h. Carvedilol, Scanning electron microscopy confirmed the spherical nature and smooth surface morphology of the microspheres. Mean particle size of the microspheres ranged between 230 and 346 mm. Carvedilol was successfully encapsulated up to 87% in the polymeric matrices. The drug release continued up to 12h in simulated intestinal fluid. It was found that the drug release rate relied upon the amount of GA used for cross-linking, i.e., drug release was slower for those formulations in which a lower amount of GA was used compared to those in which a higher amount was used. This was due to higher cross-linking density of the matrix which hindered the easy transport of drug molecules through the matrix. It was seen that carvedilol release from the microspheres was faster in the case of simulated gastric fluid compared to simulated intestinal fluid. This was attributed to higher saturation solubility of carvedilol in gastric fluid compared to intestinal fluid.

Thermoresponsive gellan gum-poly(*N*-isopropylacrylamide) semi-interpenetrating polymer networks was prepared by Mundargi et al. (2010) and evaluated the controlled release property of atenolol, an antihypertensive drug from this network. Scanning electron microscopy indicated spherical nature and smooth surfaces of the microspheres with some debris attached on their surfaces. Mean particle size was between 34 and 76 μm. *In vitro* drug release performed at 25°C and 37°C indicated temperature-dependency of drug release, which was extended up to 12h. The reports on IPN systems were encouraging and thus, had a splendid outlook in the design of gellan-based controlled drug delivery devices.

Amphiphilic Copolymer

Like low molecular weight surfactant molecules, amphiphilic copolymer can also form micellar structures with higher physical stability and can also solubilize poorly soluble drugs in the micellar core and therefore can act as controlled drug delivery carriers. Maiti et al. (2014a) prepared gellan copolymer by conjugating hydrophobic cetyl group to the backbone of native gellan polymer. Native gellan polymer (3%, w/v) was dispersed in DMF and stirred for 1.5h. To this, sodium hydride (1.67%, w/v) was added at 10°C. After 20min, cetyl chloride dispersion in DMF (6.67%, w/v) was added to the resulting polysaccharide mixture and stirred continuously for additional half an hour. Then, the reaction product was transferred into 50ml of water and adjusted to pH7.0 with controlled addition of 0.01N glacial acetic acid. Cetyl gellan copolymer thus formed was separated by filtration, washed with ethanol for purification and oven-dried at 40°C. The reaction scheme can be briefly described as follows:

$$\text{Cetyl alcohol} + SOCl_2 = \text{Cetyl chloride} + SO_2 + HCl$$

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$$\text{Polysaccharide} - OH + NaH = \text{Polysaccharide} - ONa + H_2$$

$$\text{Polysaccharide} - ONa + \text{Cetyl} - Cl = \text{Polysaccharide} - O - \text{Cetyl}(C_{16}) + NaCl$$

Gellan alkoxide was formed when gellan gum reacted with sodium hydride. In order to remove excess hydride, the final product was dispersed into water. In contact with water, NaH converted into sodium hydroxide (NaOH), and released traces of hydrogen gases. Finally, etherification reaction between gellan alkoxide and cetyl chloride yielded the desired amphiphilic copolymer. Considering stability aspects, the copolymer micelles seemed suitable for practical purposes due to its low CAC value (1.02mg/l). Higher CAC value was reported for dextran-*g*-poly (ethyleneglycol)-C_{16} polymer (6.5-12.5mg/l) (Francis, Cristea, Yang, & Winnik, 2005) and stearic acid-*g*-chitosan copolymer (8.5 mg/l) (Li et al., 2008).

The micelles were prepared by dispersing the amphiphilic copolymer in distilled water. The drug was loaded into micellar core by solvent evaporation method. Both drug-free and drug-loaded micelles looked spherical in water. It was argued that driving force behind self-association of amphiphilic molecules was related to reduction of free energy of the system upon removal of hydrophobic fragments from the aqueous surroundings and subsequent formation of micellar core. The z-average diameter of the drug-loaded micelles ranged between 1287nm and 1599nm. However, cyclosporine-loaded dextran-*g*-poly (ethylene glycol)-C_{16} copolymer micelles showed relatively smaller diameter (10-22nm) (Francis et al., 2005). The mean size of drug-loaded stearic acid-*g*-chitosan copolymer micelles was 173.7nm (Li et al., 2008). Self-assembled poly (ε-caprolactone)-*g*-dextran nanoparticles had an average diameter of 80-128nm (Bajgai, Aryal, Lee, Park, & Kim, 2008). Probably, gellan polysaccharide and cetyl chains induced larger steric hindrances over a larger volume for the polymer of higher molecular weight (Baldwin & Chien, 1988) and thus led to the formation of higher particle size. Hydrophobic simvastatin solubilized into core of the copolymer micelles to an extent greater than its intrinsic water solubility (2.54μg/ml). Increasing copolymer: drug weight ratio above 1:1 actually led to reduction of water solubility of the drug. The micellar solubility of drug was highest at the lowest copolymer: drug weight ratio (1:1). Compared to its inherent solubility, the micellar solubility was increased by ~355 fold. At the end of 8h, a maximum 60% drug was found to release in phosphate buffer solution. The micellar carriers could control hyperglycemia for a prolonged period.

Further, the work of Maiti et al. (2014b) demonstrated the drug solubilization power of a novel amphiphilic gellan co-polysaccharide. Anti-rhinitis micellar solution of budesonide was prepared. Herein, a long alkyl chain (C18) was successfully grafted onto gellan polysaccharide by etherification reaction. The dispersion of co-polysaccharide in water led to formation of spherical, nanomicellar structures. Depending upon the co-polysaccharide: drug weight ratio (1:1, 1:2 and 1:3), a maximum drug loading (>95%) was noted at the lowest level. The nanomicelles were in the range of 371–750 nm and showed negative zeta potential (48.3 to 67.2 mV) values indicating their stability in aqueous system. They exhibited a longer dissolution profile in simulated nasal fluid (pH 5.5). The co-polysaccharide micellar system showed potential in controlling intranasal delivery of budesonide for the symptomatic relief of anti-rhinitis (Maiti, Chakravorty, & Chowdhury, 2014).

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FUTURE DIRECTIONS

The polysaccharide can be modified in different ways in order to get functional biomaterials for controlled drug delivery. The development of pure modified polymers, free of residual toxic chemicals used in the synthetic process is a challenge to the pharmaceutical scientist before designing novel drug delivery systems. The biopolymers resemble natural living tissue more than any other class of synthetic biomaterials due to their high water content; furthermore, the high water content of the materials contributes to their biocompatibility. In this regard, the gellan gum can be carefully modified as a suitable material for tissue engineering applications. Though natural polymers are biodegradable, their modified forms may not show biodegradability and biocompatibility. The tailored materials must be evaluated for these properties. Some polysaccharides may possess innate bioactivity and hence, its derivative should be evaluated for any possible bioactivity and toxicity profiles. The most exciting opportunities in polymer drug delivery lie in the arena of responsive delivery systems to deliver a drug precisely to a targeted site. The "intelligent" or "smart" polymers that respond to external stimuli may play important role in drug delivery since they may dictate not only where a drug is delivered, but also when and with which interval it is released thus, new synthetic methods have been used to prepare homo-and co-polymeric hydrogels for a wide range of drug, peptide, and protein delivery applications.

Novel graft, block and comb -like copolymers can be synthesized for several interesting applications of such systems in the treatment of diabetes, osteoporosis, cancer or thrombosis. Moreover, several types of linkages can be made depending on the desired speed of cross-linking and biodegradability of the resulting conjugates. New promising methods of delivery of chemotherapeutic agents using hydrogels can also be developed. Now, it is not hard to say that a new generation of drug delivery systems can be developed by scissoring the gellan gum structures. However, the modification techniques can also be applied to other polysaccharide also and we can get better material for drug delivery. Besides, the *in vivo* evaluation of modified material-based delivery systems will be one of the future directions, in order to fully understand their biological fate and *in vivo* drug delivery efficacy. Successful development of the novel formulations using modified polysaccharides will obviously require assimilation of a great deal of emerging information about the chemical nature and physical structure of these new materials and regulatory considerations. Depositing billions of drug molecules in the blood or gut and allowing the hapless molecules to locate their target, by uncontrolled diffusion, is surely a therapeutic strategy of yesterday and not of tomorrow. Guiding sufficient numbers of molecules in sufficient time directly to their targets is the future. Biopolymers may help this endeavor and will continue to enable this effort in the foreseeable future.

CONCLUSION

Natural polysaccharides have received increasing interest year after year due to their nontoxic and biodegradability. In this chapter, it was clearly seen that more and more new functional gellan gum materials have been synthesized in order to control the drug release in simulated biological fluids. Their highly porous structure can easily be tuned by controlling the cross-linking density in gel matrix and by synthesizing novel materials.

The selection of polymer plays an important role in drug manufacturing. But, while selecting polymers care has to be taken regarding its toxicity, drug compatibility and degradation pattern. Chemical

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modification of polymers enables the development of new drug delivery systems which improves the therapy and treatment. The large variety of applications as well as the steadily increasing number of research workers engaged in studies of Gellan gum due to their unique properties, have made significant contributions to many types of formulations and suggest that the potential of Gellan gum as novel and versatile will be even more significant in future.

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KEY TERMS AND DEFINITIONS

Amphiphilic: Chemical compound possessing both hydrophilic (water-loving) and lipophilic (fat-loving) properties.

Complex Coacervation: Refers to the formation of a dense macroion-rich phase when solutions of two oppositely charged polymers are mixed.

Controlled Release: Release of drugs in biological fluids in a predictable manner over an extended period.

Engineered Polymer: The materials obtained after scissoring the native polymer by chemical reaction.

Graft Copolymer: Macromolecular chain with one or more species of block connected to the main chain as side chain.

Hydrogels: Natural or synthetic polymer network that imbibes water and swell but do not dissolve in aqueous biological fluids.

Ionotropic Gelation: A process where polymeric chain is cross-linked with multivalent cations. These may include, calcium, zinc, iron and aluminium.

Natural Polymer: Refers to high molecular weight substances of natural origin (plant, algae, microbial, marine) whose structure is composed of multiple repeating units of sugar moieties.

Polysaccharide: Polymeric carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic linkages.

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Chapter 11
Improving Pharmaceutical Care through the Use of Intelligent Pharmacoinformatics

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ABSTRACT

The expansion of drug-related problems urged healthcare organizations to adopt Pharmacoinformatics to signal, analyze and report Adverse Drug Reactions (ADRs). Data for this study have been compiled from local and international sources such as WHO. The study resulted into the development of an intelligent multi-agent decision support system including a process model, a multi-agent architecture and an integrated data processing model with clear description of agent functionalities. The model reflects three main modules: a data capture and update module, diagnosis module and a pharmaceutical care and drug monitoring module. The study also reflected on the practical and managerial environment of the model and the basic considerations to be taken into account. The study also provided some important recommendations.

INTRODUCTION

The use of information systems for the improvement of healthcare is gaining paramount importance. They increase information accessibility to healthcare providers (Makus, 2001) and increased convenience for patients (Fitzgerald, Piris & Serrano, 2008; Glaser & Foley, 2008). Their deployment by pharmaceutical care assists in the assessment and management of therapeutic outcomes in patients as well as in detecting, signaling, evaluating, and solving potential and actual drug-related problems (including adverse drug events or drug interactions). Within the context of a wider hospital management system, the use of information systems to assist in pharmacy-related decision making is known as "pharmacoinformatics". In addition to databases, such systems make use of different technological settings (including informatics, the internet, and interactive technologies) to assist in improving pharmaceutical care, patient safety and enhancing hospital management processes. They aim at improving the capacity of clinical practitioners

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to efficiently acquire and develop new treatment strategies. The use of such systems by practitioners tends to focus on three main areas: facilitating information exchange, detecting and managing adverse drug events and enabling the supply chain management process.

However, despite the potential outstanding benefits to be gained by hospitals as a result of the deployment of powerful pharmacoinformatics applications, the realization of such applications is still limited. Currently, pharmacoinformatics applications are used as sub-modules of the corporate information system. Their roles tend to be limited to stock control, monitoring drug availability and issuance at outpatient and ward pharmacies. Even for stock control, there seems to be no emphasis on the use of electronic ordering and procurement processes for which no standard operating procedures exist. The analysis of drug therapies and management of prescription inconsistencies are not supported by the current pharmacoinformatics applications in use. Moreover, there is a considerable lack of emphasis on developing and deploying information systems for signaling and detecting adverse drug events manifested in patients and recorded by healthcare professional. While the role of Pharmacovigilance centers in analyzing and managing adverse drug reactions is fundamental, there is a considerable lack in using information systems to establish relationships between such centers and hospitals through which adverse events can be reported and tracked. As a result, hospitals tend to miss the technical support provided by national Pharmacovigilance centers.

Therefore, the aim of this project is to develop a reference multi-agent pharmacoinformatics model to assist in detecting adverse drug events and drug interactions, as well as improving hospital management. The model will be based on the use of multi-agent technologies which has proven to be suitable for tackling large, real-world problems (Gasmelseid, 2006).

1. PROJECT'S OBJECTIVES

This project highlights the different Pharmacoinformatics channels that have been used (by both healthcare professionals and patients) in hospital management and the provision of pharmaceutical care which involves identifying, solving, and preventing potential or actual drug-related problems (DRPs) with regards to a patient's drug therapy.

Despite the fact that there are a number of different approaches to the development of Pharmacoinformatics applications used for decision support, there is a general agreement that decision support systems are developed most successfully by an interactive, prototyping approach. The suitability of this approach for this project stems from the fact that complete information requirements of stakeholders are difficult to identify in advance and may change significantly over time. To achieve the aims of this project emphasis will be made on:

1. The articulation of the issues which can potentially affect hospital management processes and the quality of pharmaceutical care in public hospitals in pursuit of providing insights into how Pharmacoinformatics can potentially affect the future of healthcare.
2. The development of a process mode (including all decision models and processing functions relevant to the organization as a base for the transformation of data into information) and
3. The development of a data processing model (establishing relationships between the system's structure and dynamics.

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2. PROJECT'S SIGNIFICANCE

The significance of the study originates from the following considerations:

1. This study deals with the context of healthcare with emphasis on the improvement of pharmaceutical care processes as basic elements in the delivery of improved healthcare services.
2. The growing importance of the deployment of advanced information systems in response to the fundamental changes exhibited in the healthcare system and its subsystems. The growing incidence of medical errors and adverse drug events, the complications of communications among medical professionals in many hospitals (mainly physicians and pharmacists), and the escalation of drug procurement costs, are signs that motivate the emphasis on pharmacoinformatics.
3. The emergence of new business models in response to technological advancements, increasing use of medical informatics and communication systems and technologies.

3. METHODOLOGY

The methodology for this study is generally a descriptive analytical survey with both inductive and deductive methods applied. Due to the nature of the study a number of other methods and empirically driven theories capable of dealing with the topic in question, both quantitatively and qualitatively, could also be used. The use of several methods and instruments of research is to allow some flexibility in order to capture the specific relations between the main variables and the factors affecting them. To account for the diversity of information across the different managerial landscapes and to ensure the validity of the instruments of research, Anthony's taxonomy of managerial levels is adopted. The project also made use of the concepts of "information modeling" and agent oriented software engineering methodologies and other "process-centered" and "resource-oriented" approaches for the articulation of variables and the development of the entire model and its corresponding modules.

The majority of the project's data is collected from electronic sources of many healthcare organizations and regulatory agencies such as the National Pharmacovigilance Center at the Food and Drugs Authority (FDA) at the Ministry of health. The study also used a variety of tools and methods of analysis of data provided by international organizations such as WHO. The tools include data matrix, tables, diagrams, models and output of computer programs. To achieve the aims and objectives of this study, data has been compiled from the use of questionnaires and interviews with key personnel in different healthcare organizations across the kingdom. In order to reduce the variance of estimators and gain sampling precision, the technique of optimum allocation has been used.Despite the fact that a wide number of approaches can be used for the development of agent-oriented information systems, the multi agent distributed reference framework proposed in this study follows TROPOS agent oriented software engineering methodology. To improve the face validity of the methods of research as well as the outcomes of the study, close consultation and review for the whole project is done by different experts in many international universities.

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4. REPORT STRUCTURE

This report falls in nine main parts. Part one through part five provide a basic introduction about the main thread of the project and the context of problems to be investigated, project objectives, significance of the project, methodological framework and report structure. Part six is devoted for the development of a conceptual framework guided by a synthesis of related work. While part seven shows the description of the proposed pharmacoinformatics reference model, part eight and nine provide some discussion and recommendations.

5. SOFTWARE AGENTS

Software agents' technology is witnessing an increasing deployment in different sectors such as healthcare, e-business, environmental management, entertainment, telecommunications, e-banking and e-learning, among others. There is a general consensus that software agents interact autonomously and share resources to achieve objectives assigned to them by their users and/or other agents, they also operate individually to achieve personal objectives. Resource sharing and objective realization usually take place within the domain of a specific organizational structure. Because there is no agreement about what constitutes "agency", the conceptualization of agent-based applications and modeling tend to be done by focusing on the qualities possessed by software agents such as autonomy, learning, intelligence, proactivity, reactivity and social ability, among others. Moreover, such qualities-based characterization of agents has been used to develop a typology for agent types as mobile, interface, information, broker, recommendation agents, among others. Software agents' technology has been widely used to perform different tasks and automate different processes in e-commerce, environment, e-banking, airlines, communication and industry.

Software agents are software entities that perform some tasks on behalf of their users, other agents or programs with some degree of autonomy using the appropriate information and communication platforms. Their roles include task delegation, users training, event monitoring, information search, matchmaking and filtering (Gasmelseid, 2009a). They possess important properties such as autonomy, social ability, reactivity and pro-activeness, learning, mobility, benevolence, rationality, independence, cooperation, reasoning, intelligence and adaptivity (Lisa et al, 2001; Persson et al, 2001; Bonarini & Trianni, 2001; Hu & Weliman, 2001). According to Wooldridge and Jennings (1995), autonomy, which represents the main quality, reflects the capacity of agents *to* operate without the direct intervention of humans or others, and have some kind of control over their actions and internal state. Social ability denotes the need that agents should be able to interact with other agents (and possibly humans) via some kind of agent-communication language. Reactivity reflects the ability of agents to perceive their environment and respond in a timely fashion to changes that occur in it. This environment may be the physical world, a user via a graphical user interface, a collection of other agents, the Internet, or perhaps all of these combined. Pro-activeness denotes the ability of agents to act in response to their environment and exhibit goal-directed behaviour by taking the initiative.

Different ways have been used to classify software agents in accordance with their agency attributes and functions. According to Ponnurangam (2005), software agents can be broadly classified into two categories: user agents (direct task-oriented) and service agents (implicit task assistance). Alternatively, agents may be classified according to the tasks they perform, their control architecture, the range and

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effectiveness of their actions, the degree of sophistication of their internal states, or the environment in which agents find themselves (Anumba & Newnham, 2000).

Especially for complex systems, software agents tend to be deployed in the form of a multi-agent organization in which multiple agents interact and exchange information in a decentralized and "social" manner to solve larger and complex problems. Applications such as electronic commerce, traffic control, health care provisioning, portfolio management and telecommunications, prove to be suitable for complex, distributed problems involving a multiplicity of interconnected processes whose solutions demand the allocation of fusion of information and expertise from demographically distributed sources (Sycara 1998). A multi-agent system can be defined as a collection of possibly heterogeneous, computational entities, having their own problem-solving capabilities, and which are able to interact in order to reach an overall goal (Oliveira, et al, 1999). According to Ferber (1999), a multi-agent system is composed of an environment (a space), a set of situated objects (agents) with the possibility of being associated with a position in the environment). It also includes an assembly of agents representing the active entities, an assembly of relations that link objects together. The assembly of operations it includes allows agents to perceive, produce, transform, and manipulate objects in the set of objects and operators with the task of representing the application of these operations and the reaction of the world.

According to Anumba et al (2003), there are several common ways of structuring the agent organizations:

1. **Organizational Structuring:** In which all agents share the same explicit end goal. Due to the fact that only one agent possess a global view of the full task, it is very difficult for peer agents with different goals to resolve their difficulties.
2. **Contracting:** Where a manager agent breaks the entire problem into component problems and then announces each task for contracting agents to make their bids. The manager then reviews the bids and awards the contract. Since contract nets are best used when the problem can be broken down via a well-defined hierarchical nature into a set of tasks, planning has to be done centrally.
3. **Multi-Agent Planning:** Which involves a central arbiter who reviews all potential plans of individual agents and resolve conflict when they exist.
4. **Peer-to-Peer Negotiation:** Where agents communicate directly with each other in the pursuit of achieving both individual and collective goals.

Multi-agent concepts incorporate technological, organizational and decision making issues that support the realization of the objectives of collaborative work in a wider socio-organizational context. In such a context, the objectives of the collaborating parties and their tasks are represented in terms of agents. Such representation goes hand-in-hand with the measures that govern the organizational structure, division of labor and hierarchy of responsibilities at different decision making and organizational levels. Therefore, the agent organization reflects the organization of task assignments together with their related hierarchy of command and goal congruence mechanisms. The diversity of organizational settings, therefore is reflected in the type and functionalities and privileges of the agents interacting within the entire multi-agent organization. The potential and applicability of multi-agent paradigm to support collaborative work is driven by the following considerations. Especially in complex situations, multi-agent configurations facilitate collaboration and manage tasks through the shift of some collaboration tasks to agents and allowing some space for wider interactions and the production of user-created content. Throughout the collaboration process, the collaborating parties can mainstream their tasks in a functional

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way by using functional and task-oriented agents such as the information and interface agents without breaking organizational chains of command. They also contribute to the negotiation of tasks and the use of cooperative distributed applications.

Different software engineering methodologies have been used for the design and development of multi-agent systems such as Gaia, TROPOS, MESSAGE and Multi-agent Systems Engineering methodology (MaSE), among others (Gasmelseid, 2013). While some of the available AOSE methodologies represent an extension to and/or application of existing "conventional" software engineering methodologies (especially object oriented) to agent oriented applications, others have focused on defining a number of models that use the basic guidelines of agent theory as a guide for analyzing and designing agent oriented applications and systems (Iglesias et al, 1998; Wooldridge, et al, 2000; Odell et al, 2000; Kendall, 1999; Lind, 1999). Gaia methodology deals with both the societal (macro) and agent (micro) level aspects of information system design. The main concepts in Gaia are divided into two categories: "abstract" and "concrete". At the high level of conceptualization, "abstract" concepts involve the definition of roles including protocols, permissions, activities and responsibilities. On the other hand, "concrete" concepts put more emphasis on the articulation of agent types, services and acquaintances. The outcomes of the analysis phase are used as inputs for design phase which focuses on creating an agent-based model.

TROPOS methodology pays more attention to understanding the organizational context of the multi-agent information system by focusing on the very early phase of software development in which the system as well as it's context are studied as a larger social-technical system (Bresciani, et al, 2002*;* Perini, et al, 2001; Giunchiglia et al, 2001; Giunchiglia et al, 2002). It includes five phases of software development: early requirements analysis, late requirements analysis, architectural design, detailed design and implementation. Being an organizationally-oriented methodology, TROPOS tries to improve the ability of designers to justify the entire multi-agent system as well as its "traceability" dimensions by enabling artifacts produced during later phases to be clearly referred back to artifacts or requirements produced earlier. MESSAGE methodology is more oriented towards knowledge representation. Most of the knowledge level entity concepts of the MESSAGE methodology fall into three main categories: "Concrete-Entity", "Activity", and "Mental-State-Entity" (Caire, et al, 2001). The main types of Concrete-Entity are: agents, organizations, roles, and resources. The main types of Activity are: tasks, interaction and interaction protocols. The basic type of Mental-State-Entity is the goal. A goal (whether intrinsic or transient) associates an agent with a situation in terms of a rule-based utility function. An analysis model in MESSAGE is contingent upon a number of views describing organization, goal-task, agent-role, interaction, and domain. The Prometheus methodology consists of three phases: system specification, architectural design and detailed design (Padgham & Winiko, 2002a; 2002b). The system specification phase, involves two activities: determining the system's internal and external environment (in terms of percepts denoting incoming information from the environment and actions by which an agent affects its environment), and determining the goals and functionality of the system. The architectural design phase involves three activities: defining agent types, designing the overall system structure, and defining the interactions between agents. The internals of each agent and how it will accomplish its tasks within the overall system are addressed in the detailed design phase which focuses on defining capabilities, internal events, plans and detailed data structure for each agent type identified in the previous step. Multi-agent Systems Engineering methodology (MaSE) is similar to Gaia with respect to generality and the application domain supported, but in addition MaSE goes further to support for automatic code creation through the MaSE tool (DeLoach, 1999; Wood & DeLoach, 2000). It is divided into seven phases: goal

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capture, application of use cases, goal refinement, agent classes' creation, conversations construction, agent class assembly and system design.

Some of these methodologies have been criticized for their limited deployment due to the lack of maturity and their failure to capture the autonomous and proactive behavior of agents, as well as the richness of the interactions (Zambonelli, 2001). Current agent-oriented methodologies focus mainly on multi-agent systems analysis and design, but without providing straightforward connections to the implementation of such systems (Wooldridge, et al 2000; DeLoach, et al 2001). Some of the existing software development techniques are characterized with a fundamental mismatch between the concepts used by object-oriented developers and the agent-oriented view (Wooldridge, 1997). In particular, extant approaches fail to adequately capture an agent's flexible, autonomous problem-solving behavior, the richness of an agent's interactions, and the complexity of an agent system's organizational structures. Most of these methods feature a technology-driven, model-oriented and sequential approach for the development of multi-agent applications that may not be always adequate for the development of agent-oriented systems. Most of them assume (in advance) the suitability of multi-agent technology for the entire problem domain. While the model orientations of methodologies are obvious, the process of model coupling and integration process does not explicitly reflect the links between models (Lind, 1999). Besides as the main issues to be addressed by agent oriented software engineering methodologies (such as autonomy, reactivity, proactive-ness and social ability), the concern for mobility have been growing over time (White, 1997). In spite of the growing diffusion of mobile agent technology, little research has been done to settle "design" directions to be followed in order to determine when mobile agents are convenient to be used or not. However, the current agent oriented software engineering methodologies used for developing multi agent systems do not provide methods to determine in which cases mobile agents should be used. Many of the existing methodologies intentionally do not support intelligent agents; rather, they aim for generality and treat agents as black boxes.

Except for the case of TROPOS and to some extent Gaia, AOSE methodologies tend to focus on system design (basically definition of agents, capabilities and communication) without providing a thorough understanding of the "actual problems" facing decision makers, the causes and consequences of these problems and whether the use of multi agent systems can contribute to the solution space envisioned. Because these methodology are characterized by the limited communication between the "decision maker" and the "designer" and the fact that agent technology has already been decided in advance to be the appropriate paradigm for solving problems, the chance of developing systems on the basis of "imagined" potentials of designers is highly probable.

The use of MESSAGE methodology results into a large amount of data and demands additional processing resources (time, cost and skills) in a way that complicates the process of system design because it is necessary to undertake resource-consuming investigations of the interactions among different "views" and associating them with corporate objectives. Such a consideration may result into complexities with regards to information update and system maintenance. Because of the complex view-coupling process any mistake that exists at later stages of the system development process demands intensive rework that delays design. Although the methodology does not provide in depth emphasis on "problem analysis" and the early phases of decision making, the process of coupling problem dynamics with agent capabilities will be cumbersome. While the process of system analysis and design require extensive user involvement to enrich and enable the articulation of system linkages and agent functionalities, they validity of such methodology will be seriously threatened if such involvement is not maintained appropriately.

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Prometheus methodology does not provide emphasis on the early phases of decision making (decision intelligence and problem-objective orchestration) therefore, the resulting system specifications are not directly related to the basic "information requirements" of the users who "delegate" some of their authorities to "agents. As it is the same limitation in conventional system development and engineering methodologies, the entire system dynamic will be based on the designer's perception of "what will be his information requirements if he were the decision maker for whom the entire agent module or organization provides support" rendering the entire agent-oriented applications to be "operationally" out right failures although "technically sound" and reduce their applicability with regards to the provision of appropriate decision support. Although Prometheus is regarded as supporting the development of intelligent agents in accordance with goals, beliefs, plans, and events (Padgham & Winikoff, 2002), many other methodologies treat agents as "simple software processes that interact with each other to meet an overall system goal" (DeLoach, 2001). Like other methodologies, Prometheus is envisioning implementation through the emphasis on the "automation" of the stages of the methodology rather than the "implementation" of the target system.

Although Gaia is viewed as being neutral with respect to both the target domain and the agent architecture, it does not present a procedure to guide roles and protocols identification when building agent-based systems when it is regarded as an organizational design process. While the analysis models used in Gaia methodology are based on well-defined concepts, they only represent a subset of the concepts required for agent oriented analysis with the design models remain not clearly explained. Although it has been proven as a good approach for developing closed domain agent-systems, the above mentioned restrictions delimit its applicability to open and unpredictable domain of internet applications. The methodology has been extended by adding some organizational concepts such as organizational rules, structures and patterns (Zambonelli et al, 2001).

Because the MaSE methodology is similar to Gaia with respect to generality and application domains supported, it inherits the same limitations. The "process" of using the MaSE methodology tends to be oriented towards the "tool itself" by focusing on the use of diagrams rather than focusing on the orientation of the whole process directed towards the investigation and solution of decision-making problems. On the other hand, MaSE methodology has been characterized by a long system development process that complicates the process of integration in addition to the fact that it does not support the initial phases of decision making.

Multi-agent systems have been widely used in the healthcare sector because they possess a higher capacity to address complex and distributed problems involving a multiplicity of interconnected processes, users, information sources, and locations. The development of agent organizations in the healthcare sector tend to be structured in accordance to organizational structures characterized in functional terms in which superior agents are supported by subordinate ones (e.g., information, interface, task, etc). Superior agents have the capacity to act as "task mangers" to ensure the effectiveness of the entire agent organization in the achievement of corporate objectives based on the functionalities of their corresponding subordinate agents responsible for task performance. While in some cases pharmaceutical care applications are addressed within the context of a wider hospital information system (as a sub-module), there has been a tendency to address pharmacy-related issues in separate applications, such as the use of multi-agent systems for monitoring and reporting of adverse drug events, electronic prescriptions, managing drug therapies and mainstreaming pharmaceutical procurement activities, among others.

The use of software agents in healthcare has been widely cited in e-health, electronic medical records as well as functional applications supporting medical and paramedical business units (e.g., pediatrics,

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internal medicine, geriatrics, pharmacy, nursing, intensive care and emergency, obstetrics, etc.) at the level of hospitals. The use of multi-agent systems in healthcare allows for the realistic modeling of complex, heterogeneous and distributed medical and healthcare systems and environments. They are used to improve the performance of a computerized system in terms of interoperability, scalability and configurability (David, David, & Antonio, 2010), information integration (Yifeng & Bhattacharyya, 2010; Kostkova, et al 2003; Bodenreider, 2004), task automation (such as scheduling of meetings, negotiation and learning (Bireshwar & Mishra, 2010; Hokyin, Minhong, & Huaiqing, 2011).

Godo et al (2003) used the multi-agent systems to monitor the prescription of restricted use antibiotics within the context of an electronic Institution (i.e., hospital) incorporating agents, roles, dialogic framework, scenes, and performative structure. The architecture included six types of (functional) agents: patient (a.k.a guardian angel), physician secretary, laboratory manger, pharmacy expert, and nurse agents. Different scenes were used to address communication among the agents such as: Patients Room, Physicians Room, Laboratory, Pharmacy, Dialog scene, Waiting Room and halls. Based on the patient information acquired from the electronic medical record, the entire agent structure focuses on three main functions: antibiogram authorization, antibiogram results and modification of the entire electronic medical record. However, despite its advantages, the architecture has a limited scope to provide comprehensive pharmacoinformatics assistance.

The use of multi-agent systems for the analysis and signaling of adverse drug events originates from the need for collaboration and exchange of complementary skills from different experts (e.g., epidemiologists, biostatisticians, pharmacists and physicians) for the analysis and interpretation of reports, collecting additional relevant information, and drawing reliable conclusions (Yanqing et al, 2005). Yanqing et al, (2007); (2010) used intelligent agents with a fuzzy recognition-primed decision model to develop a distributed adverse drug reaction detection system. They proposed a multi-agent framework for early detection of adverse drug reactions (ADRs) by utilizing electronic patient data distributed across many different sources and locations. In this framework, intelligent agents assist a team of experts based on a well-known human decision-making model called Recognition-Primed Decision (RPD). They generalized the RPD model to a fuzzy RPD model and utilize fuzzy logic technology to not only represent, interpret, and compute imprecise and subjective cues that were commonly encountered in ADRs but also to retrieve prior experiences reported by patients, physician and hospitals by evaluating the extent of matching between the current situation and a past experience.

Multi-agent systems have also been widely used for the management of supply chains using different technologies (Pathak et al. 2000; Fu & Piplani, 2000; Frey et al., 2003; Davidsson & Wernstedt, 2004; Walsh & Wellman, 2000; Lu & Wang 2008). Govindu and Chinnam (2007) proposed a generic process-cantered methodological multi-agent supply chain management framework to simplify multi-agent systems (MAS) development for supply chain applications. Shirazi and Soroor (2007) presented architecture for strategic information systems. Turcu et al. (2008) discussed the use of multi-agent systems and radio frequency identification (RFID) technologies in healthcare to track pharmaceuticals from the manufacturer, distributor, and pharmacy to the point of administering medications to the patient. Trappey et al. (2009) presented a multi-agents system collaborative production system to support the collaborative and autonomous mold manufacturing outsourcing processes. Seyed et al (2010) focused on merging remote sensing data and population surveys in large, empirical multi-agent models. Gottfried et al, (2011) combine a multi-agent framework with ontology-driven solutions to support and automate the procurement process. Gaurav Jetly et al (2009) developed a multi agent system to simulate the supply chain of the pharmaceutical industry.

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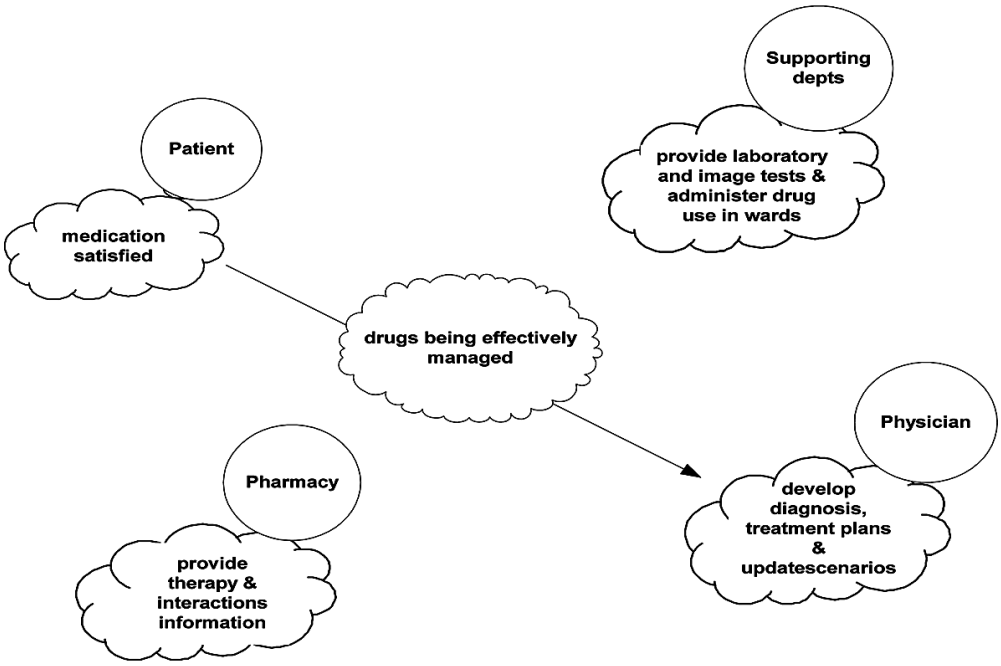
6. MODEL DESCRIPTION

Process Modeling

The use of multi-agent systems is guided by a wide range of agent oriented software engineering methodologies. Using TROPOS, Figure 1 shows the context of interaction among the stakeholders involved (directly or indirectly) in the process of pharmaceutical decision making. Patients, physicians, pharmacists and other supporting departments constitute the stakeholders whose interactions are reflected in the functionality of the multi-agent organization and its model.

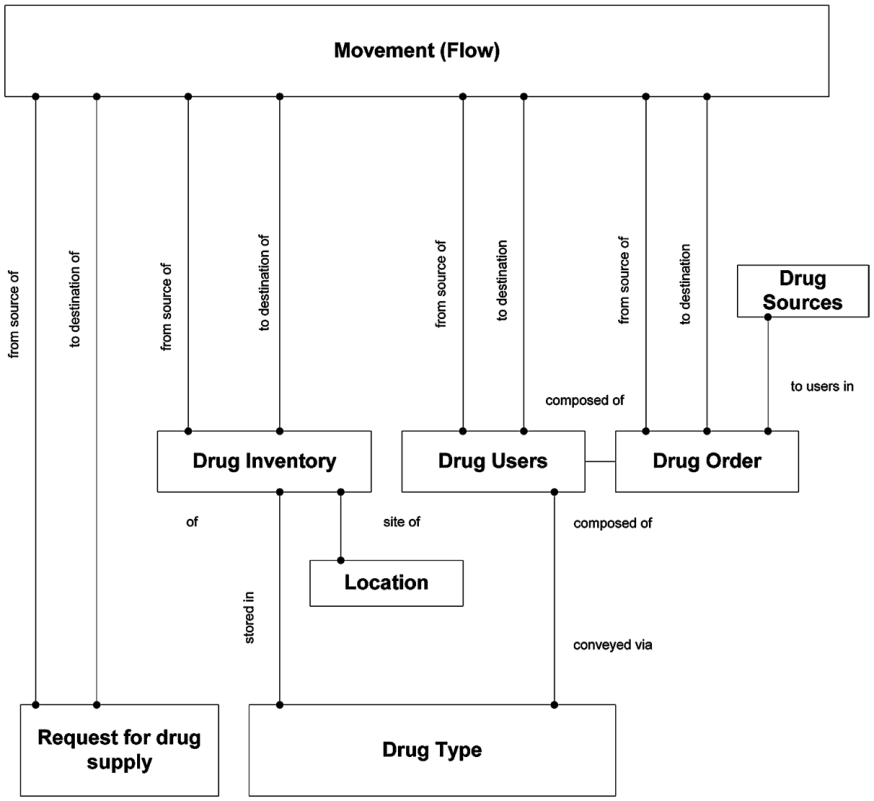
Following a process-cantered approach, the flow of analysis depicted in Figure 2 reflects the set of flows that take place to facilitate communication between patients, physicians, pharmacists and other departments. The flow also depicts the movement of requests and orders in order to ensure drug availability for treatment. As shown in Figure 2, the central entity in the relational conceptualization is the drug order initiated and itemized by the physician (in the form of a prescription), hospital, outpatient or ward pharmacies. It represents an authorization to provide a specified type and quantity of a specified drug from a specific drug source to a specific user. Drug inventories exist at different locations such as hospital, outpatient and ward pharmacies and are capable of satisfying all drug orders using different drug types. The entity representing transactions that moves drugs in, out and through the entire pharmacy network is a movement or a flow. When drugs (flow) are provided against a drug order (prescription), a flow (movement) is recorded by the drug provider from a particular location (e.g., outpatient pharmacy). Should there be an excess flow (drugs) provided by mistake to a patient, it should be monitored and addressed by the "providing" location or other entities in the hospital during the medication course. This

Figure 1. Stakeholders representation and involvement



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Figure 2. Flow analysis in pharmaceutical care systems



may entail another kind of movement transaction to record it. When "stock control" is carried out (with adjustments increasing or reducing the stock balances at different hospitals), an increase can be recorded as a movement to the "inventory" with no "from source of" being indicated and a decrease recorded as a movement from the "inventory" with no "to destination of' being identified.

Multi Agent Architecture

The proposed multi-agent architecture follows the superior-subordinate structure that incorporates superior and subordinate functional agents. It includes interface agents (providing access to databases and knowledge-based systems), information agents (enabling communication and information exchange among health care practitioners), and functional agents (human agent assistants such as physician and pharmacist). In their functional capacity, software agents can assist in the procedure of revising a therapy (getting clinical information, deciding alternative therapies, etc.),

As shown in Figure 3, the proposed multi-agent reference pharmacoinformatics model includes two types of agents:

1. Superior agents representing health organizations (such as hospitals, Pharmacovigilance centres and Food and Drug Authorities (FDAs).. Such agents act on behalf of their organizations as well as their supporting subordinate agents assisting in the implementation of hospital-wide functions

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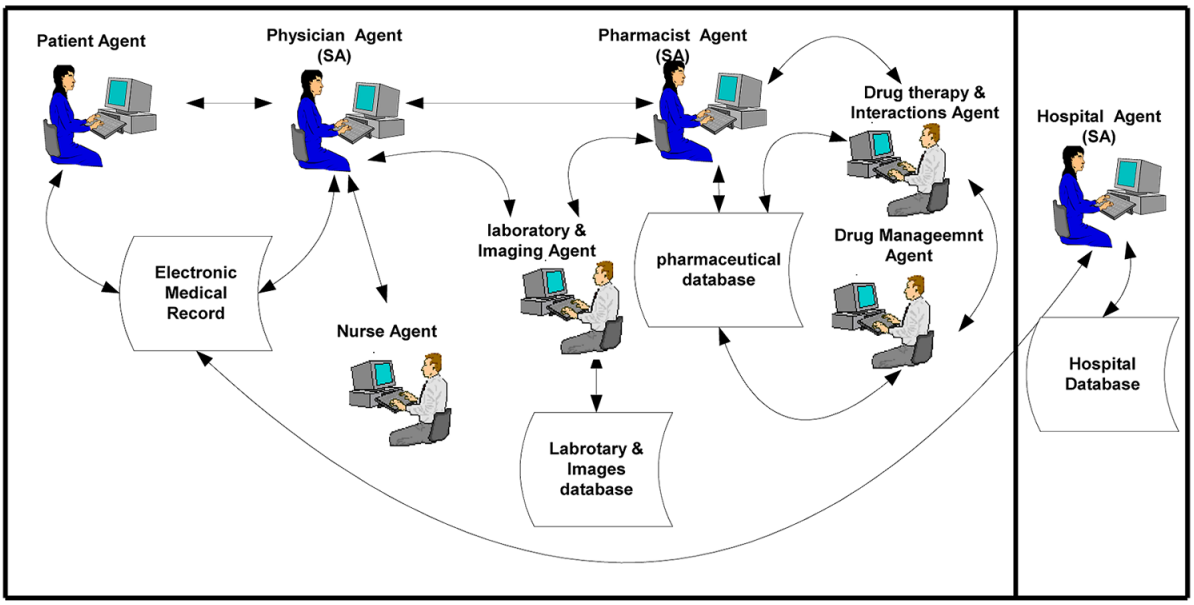
organized in business units such as surgery, paediatrics, etc. The communication process is essential for the assistance in the management of the entire health organization (such as a hospital) or coordination of health organization efforts such as Pharmacovigilance centres and FDAs. In addition to the facilitation and management of internal and external communication, superior agents are responsible for ensuring that organization-wide databases, such as electronic medical records (EMRs), are managed and appropriately accessed.

2. Subordinate agents providing support to their corresponding superior agents as "functional", "information" or "interface" agents. Agents that support departments, physicians, pharmacists and nurses, among others, are examples.

The functionality description of each agent is provided below.

1. **The Hospital Agent (HA):** This is the superior agent responsible for the corporate management of the entire hospital. It works with other supporting subordinate agents who act in the capacity of superior agents for their corresponding subordinate agents. In its capacity as a main superior agent, the hospital agent directs all other agents towards the realization of corporate objectives and represents a link between the agents interacting inside the hospital and other agents or entities in the external environment. To do so, it should be able to access all functional databases and investigate the role of agents and their operating procedures in facilitating joint functionality.
2. **The Patient's Agent (PA):** This is the patient's main agent responsible for exchanging information between the patient and other agents or personnel in the entire healthcare organization, including physicians, medical records, the pharmacy and laboratory. In addition, it allows an interface exchange between the patient, the pharmacist and the physician in case of problems with regards to drug management, after-discharge complexities, and reactions and alerts in relation to appointments, drug usages and confirmations. It also helps in reporting and signaling adverse drug reactions.

Figure 3. Multi-agent architecture



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3. **The Physician Agent (PhA):** This is the agent that supports physicians with regards to their processes, functions, schedules and communication. It allows an interface between the physician, patients, pharmacists, nurses and other laboratory experts dealing with microbiological analyses, pharmaceutical screening and drugs ordering, modification and administration. Exchanging alerts among physicians and other experts, such as pharmacists and patients, are also addressed. The agent also interacts with the patient's agent with regards to appointments, diagnosis, reporting of adverse drug reactions (especially post-discharge) and the confirmation and modification of drugs.
4. **The Laboratory and Images Agent (L&IA):** This is the agent that supports data acquisition, processing and communication to support laboratory and imaging professionals in the hospital. It takes care of the laboratory data (laboratory test requests and results), as well as imaging requests and results. The L&IA updates of the entire laboratory and imaging database and improves the availability and accessibility of information in a comprehensive hospital information system. It interacts with other agents such as the PA, PhA and PrA with regards to the requests and results of laboratory test requests.
5. **The Pharmacy Agent (PrA):** This is the main agent in the pharmaceutical setting because of its functional expertise. In addition to its role in enabling the implementation of pharmaceutical functions, it plays a significant role in facilitating information acquisition and communication, analysis, reporting and recommendation of different alternative medication scenarios using technical expertise and patient's data. It liaises between the hospital agent and its subordinate agents with regards to drug therapies, pharmaceutical and medical analyses, as well as signaling, analyzing and reporting of adverse drug reactions. Since it is responsible for the management of pharmaceutical care at the level of the entire health organization, the pharmacy agent gets support from the following subordinate task agents:
    a. **Drug Therapy and Interactions Agent (DT&I):** The drug therapy and interactions agent works as a subordinate agent for the pharmacy agent. It uses information from the patient's EMR, medical and laboratory test results, and prescription records to assist in managing drug therapies. In addition to reporting drug therapies and confirming them, this agent has the responsibility to recommend changes in medications and drugs based on a data-mining algorithm. It is responsible for screening drug therapies and signalling adverse drug reactions occurring for hospitalized and discharged patients throughout the medication process, in collaboration with the PA and PhA. Upon verification, this agent informs the pharmacy agent to take necessary actions.
    b. **Drug Management Agent (DMA):** The drug management agent acts as a subordinate agent for the pharmacy agent and is responsible for drug management at the level of the hospital, outpatient and ward pharmacies, where it manages drug availability, procurement, ordering and removal of near-expiry drugs. Pharmacy data recorded by the DMA at the level of the hospital pharmacy include name of drug, drug's serial number, drug code, drug components, drug close substitute, dose, quantity available in pharmacy, producing company (with updateable contact address), drug importing company (with updateable contact address), drug's date of production, expiry date, drug food interactions, drug-drug interactions, pharmacist on duty, etc.

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6. **The Nurse Agent:** This is the subordinate agent responsible for collecting medication instructions from the physician agent and managing drugs by monitoring drug availability in wards in accordance with the medical order forms forwarded to it. This agent uses a standard operating procedure to monitor and request routine drugs and ad-hoc requests in collaboration with the DMA.

Multi-Agent Pharmacoinformatics Data Processing Model

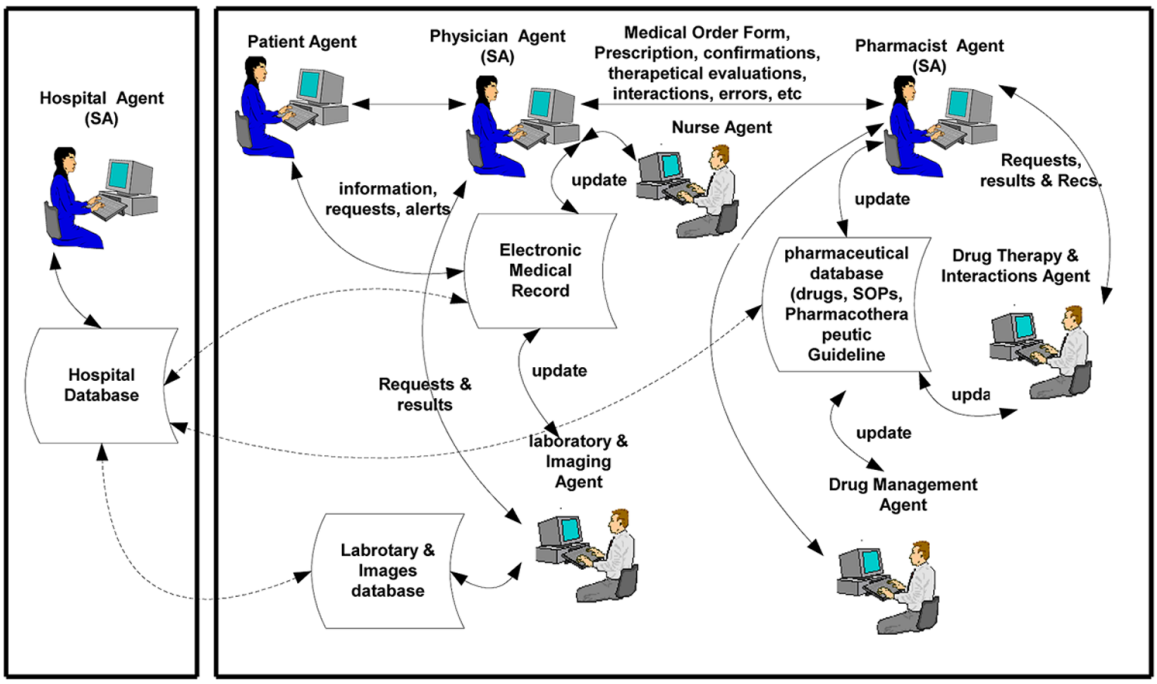
The reference model is implemented through three main modules:

1. Data capture and update (implemented through the functionalities of the agents representing the patient, physician, laboratory and images, and nurses),
2. Diagnosis (implemented through the agents representing physicians and pharmacists), and
3. Drug monitoring (therapies, interactions analyses and requests for supply implemented through the agents representing physicians and pharmacists). The interactions shaping the functionality of these modules are shown in Figure 4. In addition to using different colors to differentiate among superior and subordinate agents, superior agents are labeled with (SA).

Data Capture and Update Module

The process starts with the use of the patient agent to make appointments at the hospital and provide basic information to establish the electronic medical record, which consists of standard set of data about the patients, their diseases and diagnoses, and therapies. For purposes of patient identification, this module

Figure 4. Data processing model



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captures the name, age, addresses of the patient and his/her closest relative(s), the department, room number, room phone, equipments used for the patient in the room (if any), medical records and reports, medical images and the treating physician. Diagnosis data include the patient's medical history, current disease diagnosis and its causes. Current and previous therapies include the current drug(s) used by the patient, dosages, frequencies, types of administration, and start and dates of treatment. Some of these data elements are generated after the consultation by the physician agent as a part of EMR updates. Any change on the patient's electronic medical record done by other staff (such as nurses and image specialists) and their corresponding agents takes the form of data input (e.g. imaging requests or medication instructions) in response to requests made by the physician agent. Updates originating from the pharmacist and his/her corresponding agents concerning any comments about drugs and drug therapies that delay its issuance are a result of the physician agent's approval and authorization. The delay in drug issuance then leads to an automatic update of the electronic medical record that will not require the approval of the physician anymore.

Diagnosis Module

Upon the completion of diagnosis, a prescription is made by the physician and communicated to the patient with a copy to the pharmacy. The issuance of the prescription automatically updates the electronic medical record and informs both the patient agent and the pharmacy agent.

Pharmaceutical Care Analysis and Drug Monitoring Module

This is the module managed through the pharmacy agent and its subordinate agents. It is the core module of the entire multi-agent pharmacoinformatics module as it includes the analysis of drug therapies, detection and signaling of drug interactions and adverse drug reactions, and maintaining drug availability at the different levels of the pharmacy (hospital, outpatient and ward). Upon the receipt of a copy of the prescription by the pharmacy agent, the following supporting agents are activated: the drug allergy and interactions agent (to examine drug therapies) and the drug management agent (to examine drug availability). This process is complex and requires the integration between the pharmaceutical database, electronic medical record and other agents such as the physician agent and the laboratory and images agent for further examination and confirmation.

The pharmaceutical database constitutes information about the:

1. Standard operating procedures that govern the examination of medical documents and requests coming to the pharmacy department, such as medical order forms and requests for drug supplies placed by different units in the hospital. These procedures also govern the interaction between the pharmacy department and other units in the form of document exchanges and requests placed to other internal units (e.g. laboratory) and external organizations (e.g. suppliers, ministry of health and Food and Drug Authority (FDA)). While such procedures are generally related to the functionality of the pharmacy department, they remain an essential part of the hospital standard operating procedures.
2. Hospital Therapeutic Guide, which includes information about all routine drugs and medicines used in the hospital, as well as guidelines for acquiring and managing ad-hoc drugs. It also includes

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detailed recording of drug attributes such as correctness of drug types, dosage, route of administration, frequency, length of use etc.

3. Allergy and microbiological information necessary for making associations between prescribed drugs and diseases, for example, isolated pathogen micro-organisms, and their corresponding sensitivities.
4. Drug groupings created in accordance with diseases and the data individual patients. Each group includes all drugs related to a particular disease (in accordance to drug pharmaceutical attributes and laboratory analyses) for patients with certain allergies and symptoms.

Upon the receipt of the order by the PrA, it examines its "procedural context" and its compliance with the standard operating procedures. Orders in compliance with such procedures are communicated for the corresponding subordinate agents. Non-complying prescriptions are returned by the PhA for further analysis. The DTIA access all prescriptions approved by the PrA. It consults the pharmacy database, HTG, and the patient's electronic medical record to screen order attributes such as appropriateness of drug types, dosages, routes of administration, frequencies and lengths of use; and reports any inconsistencies to the pharmacy agent. These inconsistencies are then communicated to the PhA for further examination. Once the drugs are considered suitable, the DTIA recommends the issuance of these drugs. The DMA examines the availability of drugs by screening and updating the HTG, and drug inventory of routine and ad-hoc drugs. Based on the laboratory and/or diagnostic analysis results posted in the electronic medical record, the PrA can now provide some recommendations to the PhA, such as dosage adjustments, changes in prescribed drugs, and use of medicines. Upon the confirmation of the PhA, the PrA approves the order and interacts with the PhA to update the electronic medical record and the PA for drug administration.

In case of suspected adverse dug events, the DTIA examines the results of general lab analysis of the patient by accessing his/her electronic medical record and informs the PrA to warn the PhA in case of orders prescribed without general lab analysis. The process is also applicable for the detection and signaling of adverse drug reactions reported to the PrA through the PhA in accordance with a signal originating from either the PA or other medical staff involved in medical activities in wards. The (DAIA) examines the event and gives an initial assessment whether it is Type (A) or (B) or (C). In case of type (A), the recommendation posted to the PrA focuses on improving dosage characteristics, since a large proportion of adverse drug reactions are dosage-based and related to the pharmacokinetic properties of drugs. In case of Type (B) detected adverse drug reactions, the DAIA recommends the PrA to consult with both the PhA, and L&IA for further shared discussions because Type (B) events are generally related to the patient's reactions and tend to be allergic, idiosyncratic, immunological, or non-immunological (Meyboom et al, 2000). The same consultation process is required to address Type (C) events since they tend to be serious and may result in significant implications on public health. They originate from the impacts of drugs used for improving the quality of life of patients with serious chronic diseases. The PrA also informs the HA about all reported adverse drug reactions and interactions so as to communicate them to the National Pharmacovigilance Centre.

Drug Management Module

This is the module managed by the Drug Management Agent (DMA) in collaboration with the Nurse Agent (NA), which gets information from the physician agent. The basic aim of this module is to ensure

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the availability of suitable drugs included in the HTG. The nurse agent is concerned with monitoring drugs for patients in wards and ordering them on regular and ad-hoc bases. In the case of regular drugs (i.e. drugs included in the hospital's drug list and HTG, the nurse agent (NA) examines the ward repository for the drugs included in the prescription made by the physician and prepares it for the patient's treatment (if available in stock). If not, it posts a request to the Drug Management Agent (DMA) through the pharmacy agent to screen for drug availability and prepare the medicine for delivery to the requesting ward or post a request (through the hospital agent) for the FDA (in case of public hospitals) or suppliers (in case of private ones) and deliver it to the ward upon arrival. In case of ad-hoc requests, the NA (in consultation with the physician agent) sends the prescription to the pharmacy agent for examination and ordering. In case of detected inconsistencies, if any, the physician agent is informed with recommendations. Upon the receipt of confirmation from the physician agent, the pharmacy agent follows the standard operating procedures to acquire the drug and deliver it to the requesting wards. The DMA monitors drug availability and controls stock at the level of ward repositories and outpatient pharmacies. Such systems provide information about drug orders for in-patients, availability statuses of the drugs through alerts such as 'No quantity', 'Near expiry', 'Re-order', 'restrictions' and requests for authorization and Verification.

7. DISCUSSION

The area of focus of the project is a new research domain where over years, the question of pharmacoinformatics is being misinterpreted and misunderstood by dealing at the process of pharmaceutical care as a function in the entire hospital management process. The study has cleared some of the methodological doubts and gaps with regards to the crystallization and conceptualization of the term by relating it to process of decision making rather than the use of computers in drug production.

The study focused on the development of a multi agent reference pharmacoinformatics model in pursuit of improving hospital management through improved The use of intelligent agents for the improvement of pharmaceutical care processes at the hospital level is gaining importance due to several reasons. The outstanding capabilities shown by intelligent agents in addressing complex problems provide significant assistance to the healthcare in general and pharmacy information systems in particular. Being used as stand-alone applications or within the context of a wider hospital information systems, the use of multi agent pharmacy-related information systems are gaining paramount importance due to their growing roles in improving the acquisition and exchange of drug-related information, examining drug therapies and interactions and integrating information.

The practical managerial context of the model is shown in its potential benefits in improving communication between physicians and other professionals (such as nurses and laboratory) on the one hand and between the physician and the pharmacist, a relation that tend to be complicated in real life. The outcome of such improved communication should be reflected in the quality of medical services and pharmaceutical care as it creates a conducive environment for negotiation, accountability and coordination. The prescription of (suitable) drugs will guided by a pharmaceutical cross check that investigates and alters the compliance with procedures (standard operating procedures) and requirements (such as microbiological analysis) in a way that minimizes medical errors and the occurrence of adverse drug reactions. This will eventually improves signaling and analyzing adverse drug and exchanging information with national Pharmacovigilance centers and attracting technical support.

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However, many issues need to be addressed. The process of agent interactions, consultation and information exchange calls for a clear role description especially for agent-assigned tasks and user-based intervention in accordance with the degree of autonomy allowed for the entire software agent. Semantics, ontologies and knowledge representation are also important considerations that need to be addresses in the light of the problem domain and solution spaces envisioned. Moreover, the preparedness of the medical staff to use intelligent tools and interact in an agent-based organization, willingness to provide inputs and comply with continuous improvement and security requirements are also important issues.

8. RECOMMENDATIONS

In the light of the work done and its outcomes, the following recommendations can be made to help healthcare organizations (hospitals, FDAs, Pharmacovigilance Centres and ministry of health) improve their efficiency in providing high quality pharmaceutical care services:

1. Restructuring hospital-wide processes to reflect the central role of pharmaceutical functions and incorporating clear standard operating procedures to ensure the incorporation of suitable tasks and activities.
2. Adopting an integrated approach for the crystallization of pharmacoinformatics very early in the medication process to ensure its involvement in the analysis of drug therapies, cross-checking prescriptions, alerting physicians and other medical professionals about the non-existence of drug prescription pre-requisites such as general lab analysis and recommending change of drug regimens and the use of free-use drugs wherever applies.
3. Making pharmaceutical care processes more patient oriented to optimize the utility matrix of the stakeholders in the entire healthcare system. As a result, the pharmacoinformatics can be of high importance in improving the knowledge of patients about drugs, the dysfunctional consequences of adverse drug events and their capacity and willingness to report such events.
4. Developing clear hospital-wide information system strategies and information security policies to facilitate effective communication among medical professionals, interactions with patients and, most importantly, interactions with the National Pharmacovigilance Centre of the Federal Food and Drug Authority at the Ministry of Health.

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KEY TERMS AND DEFINITIONS

Adverse Drug Reactions: Complications that occur as a result of drug-related or patient-related factors.

Autonomy: The capacity of agents *to* operate without the direct intervention of humans or others, and have some kind of control over their actions and internal state.

Electronic Medical Record (EMR): The record created by healthcare providers to include all patient-related and medication-specific information that can be used for medical examination, prescription and investigation. Usually it is referenced using the patient's name or number as a key filed.

Healthcare Business Units: All departments and sections that are specialized in the provision of healthcare service at the level of service provision such as specialized departments in hospitals. They constitute the base of the management of pharmaceutical services and the identification of agent-based functionalities.

Information Systems: The collection of physical resources (hardware, software, telecommunication and database) and personnel (such as analysts, designers, developers and programmers) involved in the acquisition, storage, processing, dissemination and provision of information to enable decision makers achieve organizational objectives effectively and efficiently.

Multi-Agent System: A collection of, possibly heterogeneous, computational entities, having their own problem-solving capabilities and which are able to interact in order to reach an overall goal.

Pharmaceutical Care: The set of activities, processes and procedures carried out at different levels of the health care chain in pursuit of improving the quality of pharmaceutical interventions and decisions made.

Pharmacoinformatics: The use of computer based applications for the provision of information and implementation of tasks to assist medical policy makers and professionals in healthcare organizations to control and monitor drug therapies, prescription, use, signaling and analyzing of adverse drug events and stock control.

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Reactivity: The ability of agents to perceive their environment and respond in a timely fashion to changes that occur in it.

Software Agents: Computational entities that interact and cooperate together and use resources collectively in order to achieve their own objectives or the objectives of their owners or other agents.

Superior Agents: High rank software agents operating within the context of the entire software organization. Their basic aim is to ensure that all subordinate agents contribute to the realization of the corporate objective to be met by the multi-agent organization. They exercise control and direction upon their subordinate agents and communicate on behalf of them.

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Chapter 12
Personalized Medicine in the Era of Genomics

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ABSTRACT

Personalized medicine is a model that aims at customizing healthcare and tailoring medicine according to an individual`s genetic makeup. It classifies individuals that differ in their susceptibility to a particular disease or response to a particular treatment into subpopulations based on individual's unique genetic and clinical information along with environmental factors. The completion of Human Genome Project and the advent of high-throughput genome analysis tools has helped in building and strengthening this model. There lies a huge potential in the implementation of personalized medicine to significantly improve the clinical outcomes; however, its implementation into clinical practice remains slow and is a matter of concern. This chapter aims at acquainting readers with the underlying concepts and components of personalized medicine supplemented with some disease-based case studies, discussing challenges and recent advancements in the implementation of the model of personalized medicine.

INTRODUCTION

Personalized medicine is one of the most exciting topics revolutionizing healthcare industry today. It is a concept that has the potential to transform medical interventions by providing effective, tailored therapies based on the genetic profile of an individual by utilizing vast information contained in our genetic code. It simply means studying the person in the framework of disease rather than just studying the disease itself. The kind of genetic makeup a person has, the kind of protein produced in them and bio-signatures or biomarkers present in their genome are considered while tailoring treatment for an

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individual. Offering right drug to the right disease at the appropriate time with the optimal dosage is what is required to improve clinical outcomes and this is what personalized medicine stands for (The Case for Personalized Medicine, 2014)

Traditionally healthcare industry followed a reactive approach to treatment, in which the treatment only begins after signs and symptoms appear. The same prescription is given to different people suffering from the same disease. The physicians only considered family history, social-economic and environmental factors. As a result, every year large number of people die due to severe effects of the medicine and even larger gets hospitalized from adverse effects of medication (ADR-Adverse Drug Reaction). According to a report, patient's response rate to medicines can be as low as 20% depending upon the drug (range= 20-75% response rate). The pharmaceutical industry is still developing medicine based on the observation and the mechanism of the disease. Thus, just treating the disease and not the person.

The modern medical treatment focusses on patient`s disease to investigate exactly the state and the pathophysiology of disease, in order to "select" the most appropriate medication. "One drug size fits all" paradigm has lost its validity and it is also clear now that the drug response varies from patient to patient. Genetics play an important role in individual`s response to a drug and in deciding optimal drug dose. So we have a model that takes into consideration all the aspects affecting a person`s state of health from genetics to lifestyle, termed precision or personalized medicine. The following quote can easily summarize the concept behind personalized medicine *It's far more important to know what person, the disease has than what disease the person has* (Hippocrates of Cos (c. 460 BC – c. 370 BC)).

The development of personalized medicine as an approach to medicine, and as a new dimension in medicine, took off when the human genome was sequenced in 2001. A tectonic shift has started since then which is though subtle and perhaps imperceptible for ordinary individuals, but it is bound to influence the entire backdrop of how we look at our physical and emotional well-being. Modern techniques and the completion of Human Genome project have made it possible to understand the genetic part of the diseases and helped in making significant advancements in the drug development process. Personalized medicine is the new paradigm shift in the field of drugs or medicine which formulates treatment or medicine following genetic testing, proteome profiling, and metabolomic analysis to identify the impact of genetic variations on drug response. However, personalized medicine also requires doctors to collect the patient`s personal information including the genotype, so as to understand the nature of the particular patient. Based on which the doctor will decide the most suitable medical treatment for that particular patient.

In particular, each person has different ability of drug metabolism owing to the activity of their metabolic enzymes. For instance, if a person has very weak enzymatic activity, the drug will not get completely metabolized and its concentration in the blood increases. Contrary to this, if a person has very strong enzymatic activity towards a particular drug, the drug gets metabolized very quickly and its concentration in the blood would remain low. In simpler words, when several patients are prescribed the same dosage of a drug, the actual effect of the drug would vary depending on its enzymatic activity and thus its genotype. Based on this observation, a new process of drug development requires studying the effectiveness of the drug against the genotype, including the adverse effects.

The whole genome is sequenced using next generation sequencing methods and the obtained genomic reads are then filtered, aligned and variants identified. Variants and Mutations are then annotated using publically available bioinformatics resources. Personalized treatment could then be suggested based on the biomarkers present and other molecular aspects.

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It could be possible to assess patient`s susceptibility for diseases such as high blood pressure, stroke, diabetes, cancer etc. On the basis of the collected information, a new type of medical treatment, called "preventive therapy" to prevent these risks is developed. This implies that even though a person is identified as susceptible to a particular disease based on the genomic examination, changes to prevent the disease by performing the preventive therapy which is designed on the basis of personal information, still prevails. The preventive therapy is expected to gain more importance in the near future.

One of the earliest and most important examples of precision medicine is related to breast cancer. In about one-third of the breast cancer patients, HER2 protein is overexpressed which is non-responsive to standard therapy. FDA has approved Trastuzumab for patients with such condition and research in further years showed that it reduced recurrence by about 52% in combination with chemotherapy (The Case for Personalized Medicine,2009). Another example of personalized medicine is known in melanoma. It is shown that a mutation exists in gene BRAF encoding for protein B-Raf, which is responsible for sending signals inside cells for directing its growth in the majority of melanoma patients. Vemurafenib, an inhibitor of B-Raf and the companion BRAF V600E mutation test were approved in 2011 for the treatment of these melanomas. Vemurafenib is only beneficial for patients with a positive test for BRAF V600E mutation (Ascierto et al., 2011).

A non-invasive genetic diagnostic test is available as an alternative to endomyocardial biopsy for heart transplant recipients to help manage the care of patients post-transplant. This test may be helpful for longer-term patient management as it will aid in predicting the risk of rejection and prescribing more precise immunosuppressive drug regimes.

The prevailing medical treatment is exercised majorly in large centralized hospitals where the focus is on "patients of the average population". However, when it comes to personalized medicine, the current medical system may fall short in expectations. If a patient particularly requires the personalized or the preventive therapy, a modern system would be required to fulfill the requirements. In future, a new system is believed to be a reality with the combination of a "family doctor" and a large centralized hospital. In this system, the role of the family doctor would be to convince the patient and establishing mutual trust as well as understanding the patient`s characteristics. On the other hand, the centralized hospital equipped with an advanced and the latest technologies and instruments would be dealing with the patients whom family doctor is unable to handle.

Personalized Medicine or Precision Medicine is a model that customizes and tailors medication according to an individual's genetic information and determines its predisposition to a particular disease or condition. With the help of personalized treatment model, the healthcare industry will be seen shifting its focus from illness to wellness, and from curing disease to maintaining health. The realization of this model is not without challenges such as lack of infrastructure to support the requirements of this model, the lacking recommendations for the implementation and follow-up of personalized therapy, ethical, legal and social issues, economic issues. But on the whole, it seems evident from the outcomes of the traditional trial and error medicinal approach, that personalized medicine was not a promise made hastily, but a realizable dream. Although the investment in precision medicine is not trivial, it is providing the foundation and systematic methods for prospective progress.

This chapter explains the very concept and the key components of the personalized therapy, dwelling deep into behind the scene factors affecting the implementations of the model of personalized medicine. The chapter also focuses on the advancements in other branches of science such as nanotechnology, metabolomics, molecular biology, immunoinformatics and bioinformatics which has significantly added more value to this model.

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PHARMACOGENOMICS AND PHARMACOPROTEOMICS: KEY COMPONENTS OF PERSONALIZED THERAPY

Pharmacogenomics (PGx)uses 'omics' approaches that have led to a revolution in unraveling the underlying cause and mechanism of disease susceptibility, thus providing enormous potential for novel therapeutic strategies.

In general, pharmacogenomics can be defined by splitting the word into two words:

1. **Pharmacology**: The science of drug development and discovery, and
2. **Genomics**: The study of genes and their functional aspects.

Pharmacogenomics – the study of how genetic differences among individuals or alterations in gene expression that are linked to pharmacological function and therapeutic response. It is also sometimes referred to as science differentiating how individual's genetic makeup influences a person's reaction to drugs. The therapeutic response in many disease processes is gene-specific and multifactorial. Therefore, tailoring medication based on patient genomes maximizes efficacy and compliance while avoiding side-effects and drug-drug interactions. Basically, genomics knowledge would help optimize drug therapy for patients that would deliver the best results based on the patient's genotype. Thus, separating responders from non-responders and predicting the efficacy and toxicity of the drug.

PGx involves better pharmaceutical outcomes for patients and possibly finding the right mix of therapy thereby reducing the need for polypharmacy. "Pharmacogenomics" should be implemented in drug development Pipeline. It is introduced in Phase IIA/IIB of clinical trials with the aim of accelerating and facilitating the development of new molecules while reducing the associated risks and costs (Adams, 2008). Thus, aids in understanding how genetic variation among individuals contributes to differences in reactions to the drugs.

PGx play the following role in clinical trials**:**

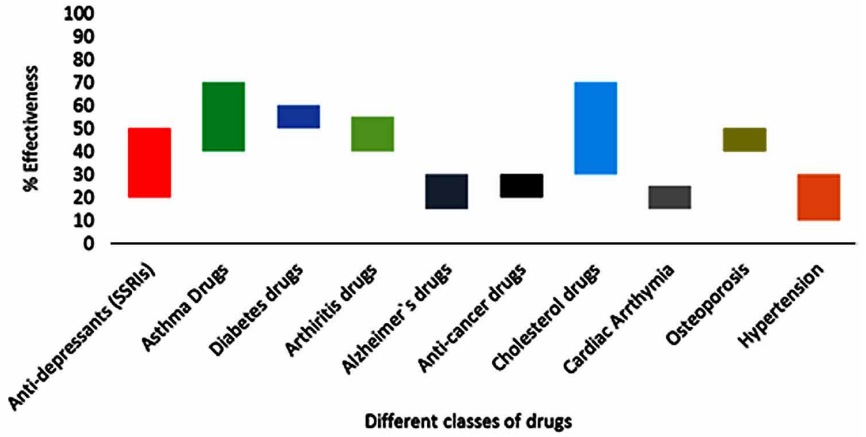
- Identification of variations in a large number of genes that affect drug action.
- Stratification of Patients in Clinical Trials according to genotype.
- Prediction of optimal doses of the drug in different patient populations.
- Reduction in Drug Development time by predicting the populations having good efficacy.
- Prediction of adverse reactions or therapeutic failures based on the Genotype of the patient.
- Prediction of Drug-Drug interactions

In PGx, genomic information is used to study individual responses to drugs (Adams, 2008). The two major determinants for studying drug action in individuals are:

1. How much of a drug is needed to reach its target in the body – *Pharmacokinetics.*
2. How well the target cells such as heart tissue, liver respond to the Drug- *Pharmacodynamics.*

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Figure 1. Advancement in PGxhas allowed for more tailored treatment for a wide range of health problems, including cardiovascular diseases, cancer, HIV/AIDS etc.



Factors Affecting Response to Medication

Genetic variations are not the only determinant of variations in response to the medications but age, sex, diet, intake of supplement drugs can each influence individual`s response to a medication (Personalized Medicine, Coriell Personalised Medicine Collaborative).

Non-Genetic Risks

- Interactions between drugs and lifestyle factors.
- A person's Gender, Age, and Weight.
- Drug-Drug Interactions like taking clopidogrelas a combination therapy with proton pump inhibitors like Prilosec (commonly used to treat heartburn) reduces the clopidogrel effectiveness in preventing blood clots.
- Interact of existing Medical conditions with Medications. For example, a person that has hypertension or high blood pressure cannot take many anti-inflammatory drugs, such as aspirin and steroids, as they could cause an unsafe rise in blood pressure.
- Environmental risk factors such as sun exposure, air quality, and job-related hazards involving exposure to chemicals, radiation, and carcinogens.

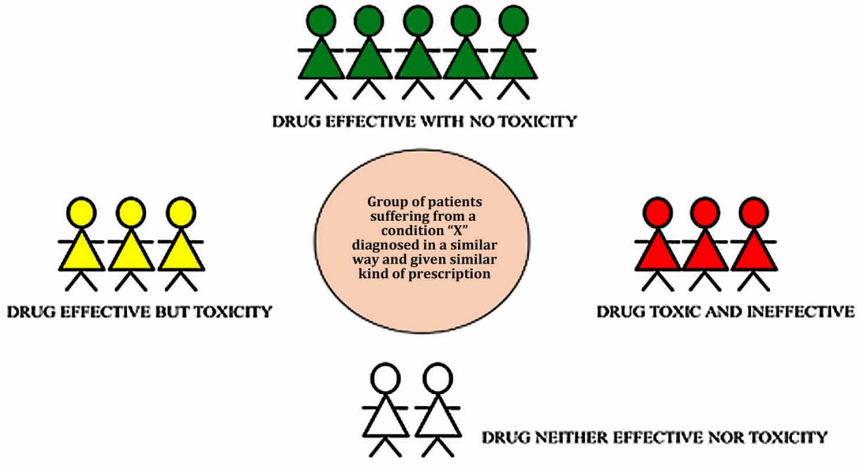
Genetic Risks

Family History for a particular disease may increase the risk of developing a disease.

As pharmacogenetic testing become widely recognized and easily available, precision medicine will make use of the obtained genetic information to evaluate the multifaceted representation of variability in drug response and manifestation of side effects. In conclusion, while adopting pharmacogenomic testing for the design of personalized prescriptions still has hurdles to cross for integration into clinical practice, but the knowledge of drug response at the molecular level, risk factors, and disease susceptibility can increase the susceptibility towards achieving the aim of Precision Medicine.

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Figure 2. Different set of population respond differently to same treatment owing to their genetic differences



Potent Applications

The application of PGx has focused on the studying genetic variations in the population to explore linkages between genes, diseases, and environmental factors, and developed new therapies and diagnostic tests. One such application is mentioned below.

Anti-cancer therapy was the foremost area of medicine to use PGx. Nowadays it is common to treat certain cancers by testing the tumor tissue for its genetic "signature" (Squassina et al., 2010; Xie & Frueh, 2005). The information obtained thus can be helpful for doctors to prescribe the effective therapy based on the tumor's specific genetic makeup. A successful example of this approach was observed in leukemia treatment, breast cancer treatment, and colon cancer treatment. The most promising applications of PGx has also been implemented in defining the risk for adverse effects, dosages and response to medications in Cardiovascular Diseases, Psychiatric Diseases, Infectious Diseases.

Pharmacogenetics (PG)

Most of the articles use the term Pharmacogenetics (PG) and Pharmacogenomics interchangeably though they only differ in the initial approach of science. Pharmacogenetics begins with the unexpected drug response to a given therapy and investigates the genetic cause behind it whereas PGx begins with identifying genetic differences with a population to explain the observed response to certain drugs or to define the susceptibility to a disease.

Potential benefits of PG include discovery and development of drugs for targeted therapies and with minimized adverse effects, more accurately determining drug dosages and development of amalgamated DNA vaccines that could activate the immune system to have the benefits of multiple vaccines with reduced infection risks. It is currently being used for evaluating drug responses in a number of diseases such as cardiological disorders, respiratory syndrome and neurological conditions, investigating fast and slow metabolizers in respect to commonly consumed drugs. It has certain limitations as well. Multiple genes could be responsible for determining the drug response to a drug, thus making drug development process tedious. Identifying genetic variations in each of the individuals that may influence drug me-

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tabolism is very difficult and time-consuming task (Pharmacogenetics/Pharmacogenomics, Fact sheet 25 NSW Government Health).

PG is the study of specific SNPs in specific genes with known functions that could be linked to drug response. Pharmacogenetic testing involves examining SNPs identified in advance, that are linked to Neurotransmitters, receptors or growth factors. Pharmacogenetic testing is an alternative to "Hit-&-Miss" approach of prescribing any drug. The knowledge of patient drug metabolizing gene variants (poor metabolizers, rapid metabolizers, and ultra-rapid metabolizers) can help in finding the appropriate therapy and optimal dosage of many of the commonly prescribed drugs, including Antidepressants, Beta blockers, Warfarin (Scott, 2012).

Determining Drug-Drug Interactions

The wider use of Pharmacogenetic testing is an opportunity for prescribing safety and efficacy. It is a very important and useful tool in predicting which drug will be effective in various patients. For instance, the drug "Plavix" is the best-selling prescription drug that blocks Platelet reception. It is being given to patients who had received a stent in the coronary artery to prevent clotting but Genome Wide Association Studies (GWAS) have identified the gene CYP2C19 in those who are unable to metabolize Plavix (Pharmacogenetics, Wikipedia)

Applying Pharmacogenetics into Clinical Practice

When physicians are equipped well evaluating a patient's genetic make-up will help them diagnose more accurately and prescribe the right drug. The variation extends among people beyond our phenotype (Pharmacogenomics, The University of Utah).

Case Study: An 18-year old person "X" who has been diagnosed with Leukemia type of Cancer involving the bone marrow and blood cells. His doctor wants to start his Chemotherapy as soon as possible.

"Purinethol" is a common chemotherapy drug. It incorporates itself into cancer cells and killing them. While most patients were benefitted from the drug, doctors knew that it canal so cause severe and fatal side effects in certain patients. Previously doctors were unable to understand that why these patients responded so negatively to the drug. After a long time, they discovered that TPMT (Thiopurine Methyltransferase) enzyme is responsible for metabolizing and inactivating the drug, Purinethol. The people also vary in their TPMT enzyme activity level. Because Purinethol is a toxic substance, it is important that it should only remain in the body for a limited amount of time, affecting mainly cancer cells and not the healthy cells. Therefore, before prescribing the drug, it is critical for doctors to know if a patient has enough TPMT enzymes to effectively inactivate Purinethol. If patients are unable to break down Purinethol they will suffer toxic side effects. About 89% of the population has the ability to fully breakdown the toxic anti-cancer drug. This population carries the variation in TPMT responsible for maximum enzymatic activity and will be resistant to severe ill-effects from Purinethol. 10.66% of the population has a TPMT variation with partial activity, leading to the less efficient breakdown of Purinethol. Thus, they will suffer severe side-effects if given a full dose of the drug. However, they will benefit from a much lower dose. 0.33% of the population has TPMT with insufficient enzymatic activity. As a result, Purinethol breakdown will not occur to the greater extent and the toxic substance will persist in the body. So they will suffer fatal side-effects if given any Purinethol.

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Therefore, knowing which genetic variant of TPMT the person "X" has will influence his treatment tremendously. To determine the dosage of Purinethol that "X" will need, his doctor takes a DNA sample from his blood and sends it to a lab. After running a diagnostic test based on SNP Profiling, it was determined that person "X" has 'partial TPMT activity' variation. With this knowledge, he can confidently start "X" on a reduced dosage of Purinethol.

This case study illustrates just one-way doctors can apply PG into clinical practice to deliver with the correct dosage of a chemotherapeutic drug.

Pharmacoproteomics (PP)

Pharmacoproteomics is another key component of personalized medicine along with PG and PGx that uses proteomic technologies to discover and develop novel drugs. Genotyping just provides variations at the DNA level whereas pharmacoproteomics is the functional representation of those variations. Designing therapeutic strategies on the basis of differential protein expression would be more beneficial than just based on genetic polymorphism. Protein chips like microarray chips will be used more frequently in the near future in clinical diagnostics, particularly in Point of Care diagnostics. Proteomics will help in the development of molecular diagnostics, Characterization of complex disorders thereby defining a targeted therapy for a particular biomarker, Drug discovery and development, designing precision medicine based on proteomic profile rather than genetic polymorphism.

PP will be a useful adjunct to PGx and PG in developing the model of personalized medicine by connecting genotype with the phenotype and classifying the patients into responders and non-responders. Several important applications of Pharmacogenomics are already in clinical practice and some of them have the FDA approval (for example, cetuximab/panitumumab and KRAS; vemurafenib and BRAF; warfarin and CYP2C9/VKORC1; abacavir andHLA-B*5701; carbamazepin and HLA-B*1502; thiopurines and TPMT) (Meyer, Zanger, & Schwab, 2013)

METABOLOMIC ANALYSIS FOR PERSONALIZED MEDICINE

Despite technological advancements and enormous financial inputs, PGx has the success that has limitations too like in predicting drug response with absolute certainty using biomarkers. This can be attributed to the fact that PGx takes into account only genotypes and not the phenotypes, which is the result of interactions between genotype and environmental factors such as diet, gut microbiome, nutrition, age, sex, dietary supplements, and lifestyle. Therefore, it becomes critical to determine individual`s phenotype in deciding the correct drug and optimal dosage and thus predicting the response to a given therapy.

The metabolomic/metabolic profiling takes into account the influence of individual`s physiological state, microbiome diversity and functional composition, genetic polymorphism and other environmental factors. Thus, could be useful in predicting the possible outcomes following a therapeutic intervention. A new approach called Pharmacometabolomics is emerging that combines the effect of metabolic profiling and metabolite assessment tools for linking the inherent variations in the metabolite and drug efficacy. Some reviews have typically described the potential of metabolomics, especially pharmacometabolomics in clinical trials and thus personalized medicine (Nicholson, Wilson & Lindon, 2011; Nicholson, Everett & Lindon, 2012).

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One advantage that pharmaco-metabolomics enjoys over the other 'omics' technologies is that the metabolic profile represents the phenotype of the organism and reflects the overall biological impact which no other technology represents. It involves metabolite profiling of the biofluids or fecal extracts in the pre-dose and post-dose individuals to envisage his/her responses for a therapeutic intervention and in the process identifies surrogate markers for subsequent therapies. Furthermore, it is also capable of providing useful drug pharmacokinetic and drug metabolite information for an individual, which can provide a mechanistic understanding of variations in responses.

Pharmacometabolomics along with other 'omics' technologies can be helpful in determining response phenotype, evaluating toxicity and predicting individual response to targeted drug therapy (Kaddurah-Daouk et al., 2010). For example, metabolic profiling was employed to investigate the metabolic pathways of ritonavir which is a protease inhibitor used with HIV chemotherapy (Li, Lu & Ma, 2011). Four bioactivation pathways associated with CYP3A were found to be related to ritonavir-induced toxicity. Additional data obtained from pharmacokinetics for a drug could provide relevant information to determine individual responses for the drug. Recently, to profile pre and post-dose urinary metabolites scientists applied metabolomics approach in individuals given a single toxic dose of APAP-s (acetaminophen sulfate). It was discovered that human subjects with high pre-dose levels of p-cresol (metabolites related to human gut microbiome) had lower concentrations of APAP-S, which is due to the competition of the binding site to the sulfotransferase enzyme. The individuals with lower pre-dose urinary concentrations of p-cresol might be more susceptible to APAP-induced toxicity since less APAP will be conjugated with sulfate to produce APAP-S. The findings indicated that each individual, colonized by a unique assortment of trillions of microbes, could respond to a drug differently, either beneficially or adversely. This study demonstrates that evaluation of metabolic phenotype by metabolic profiling could play an important role in drug toxicity and metabolism, as well as in personalized healthcare system (Clayton et al., 2006).

BIOMARKERS

The sole aim of precision medicine is to utilize the growing understanding of biology so that patients receive the right drug for their disease, at an accurate dose and in adequate time. But personalizing medication includes the use of different biomarkers driven by a decision-making process in which a diagnostic test is essential.

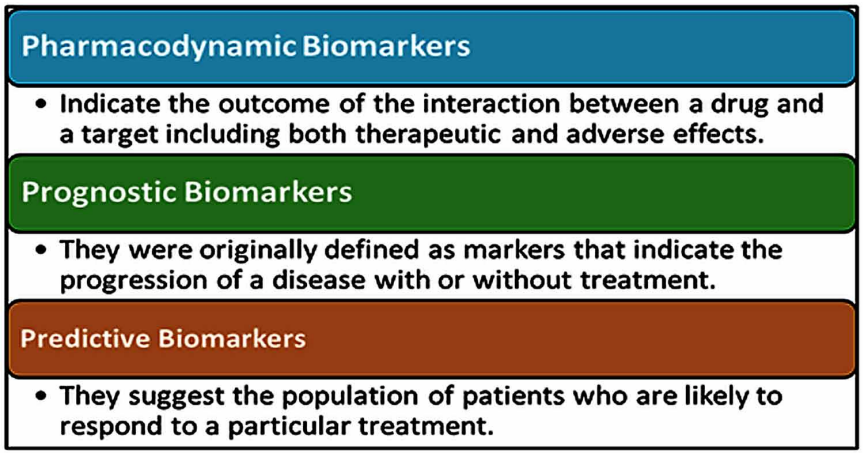
Biomarkers are organic substances that are known to indicate certain biological processes. A biomarker is a term used to indicate a measurable character that can indicate a biological state. Biomarkers vary from one branch of science to others. In the field of medicine, biomarkers such as DNA (copy number variations, methylation states, and mutations), RNA (mRNA, microRNA), Protein (phosphorylation sites, post-translational modifications), polysaccharides, metabolic products, single nucleotide polymorphism and gene variants and cells serve to diagnose diseases (Escamilla, 2013). Tests that screen for biomarkers can help medical practitioners to learn more about the patient's condition, for example, if he or she is afflicted with a certain type of cancer or is he/she at greater risk of suffering a heart attack. In medical diagnostics, biomarkers also serve to detect gene mutations that can cause diseases.

Ideal biomarkers for use in diagnostics and prognostics, and for drug development and targeting, should be measurable, safe, modifiable upon treatment, consistent across different populations, highly specific and sensitive (Issaq, Waybright & Veenstra, 2011).

Clinical Biomarkers can also be categorized as shown in Figure 3.

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Figure 3. Biomarker classification
(Drucker & Krapfenbauer, 2013; Buyse, Sargent, Grothey, Matheson, & de Gramont, 2007)



DNA Sequence-Based (SNPs) Biomarkers

Identifying, cataloging and studying small genetic variations among humans will lead to more efficient drug therapies. Reliable SNPs could serve as biomarkers to predict the response of various drugs.

SNP is defined as the single base change occurring in the DNA sequence such that it is present in at least 1% of the population. SNPs can be of two types:

- **Synonymous SNPs**: The single base change occurring at the DNA level will not reflect in the protein sequence as one amino acid can be coded by different codon triplets.
- **Non-Synonymous SNPs**: The single base change occurring at the DNA level will reflect in the protein sequence, thus leading to altered protein. These SNPs are of much importance in personalized medicine approach.

Finding and Identifying SNPs

It is generally done by following two major approaches:

1. **Genomic Approach:** Large scale projects are started to sequence the whole genome of a large number of people and compared their genome to identify the differences in the sequences. It requires a lot of financial, scientific and computational input to generate as well as store and analyze data. Scientists identify SNPs mainly by two methods:
   - Sequencing complete DNA of a large number of people and then a comparison of results.
   - Primer extension Method.
2. **Functional Approach:** Scientists select a particular disease, then select the genes associated with it, examine them in different sets of people i.e. patients who respond to a drug and the patients who don`t respond to the drug.

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SNPs are categorized into two main categories when studying them in relationship to their effects on drug:

- **Linked SNPs**: They resides on intergenic or intron region but do not affect the function of the protein encoded by the gene. They have some relationship to drug response.
- **Causative SNPs**: These are those SNPs that affects the function of the protein and corresponds to a disease or abnormality or influence the person`s drug response. These are further of two types:
  - **Non-Coding SNPs:** They are present in the regulatory regions of the gene and they affect the gene expression.
  - **Coding SNPs:** They are present in the coding regions or within a gene, change the amino acid sequence in the protein and alters the protein function. [36]

Haplotypes (or Haplotyping)

Characterizing the combination of SNPs in a fragment of DNA. Most of the time a single SNP does not reveal everything about a person. Haplotypes influence the drug response and can be a more accurate predictor of phenotypes than SNPs.

Pharmacogenetics studies on candidate gene have been performed based on haplotype approach. Positive results have been obtained. This approach has also been applied to clinical trials making it the first genetically personalized medicine approach.

Companion Diagnostic (CDx)

Measurement of different markers (RNA, DNA, and/or proteins) needs different diagnostic assays; therefore, different qualification and validation strategies are needed. Pharmaceutical companies are increasingly looking to develop a drug and diagnostic test simultaneously, in a process referred to as drug-diagnostic-co-development, also called companion diagnostic (CDx), to significantly define the appropriate patient population for treatment. A companion diagnostic test is essentially a biomarker test that enables better decision making on the use of a therapy. In other words, they are often developed concurrently with a therapeutic, but can also be developed to optimize treatment with a therapeutic that has already been approved (Simoncelli, 2013). Pharmaceutical and device sponsors have become increasingly interested in pursuing "co-development" strategies for the development of a therapeutic product and an accompanying IV-D companion diagnostic device. As described earlier, the concept of co-development was first applied in 1998, when the approval of the therapeutic Trastuzumab (Herceptin), was paired with approval of an immune histochemical IVD companion diagnostic device (HercepTest™) that measures expression levels of human epidermal growth factor receptor 2 (HER-2) in breast cancer tissue (Elkin et al., 2004).

The goal here is to increase the safety and the efficacy of the drug (Buyse et al., 2007). CDxis increasingly important tools in drug development because they have following advantages:

1. Reduced costs through pre-selected (smaller) patient population;
2. Improved chances of approval through more focused therapies;
3. Offer better outcomes, less toxicity, and fewer treatment delays;

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4. Significantly increased market uptake;
5. Added value for core business (late phase);
6. Regulatory trend to have CDx mandatory.

Tafinlar/Mekinist/THxID BRAF test: In May 2013, FDA approved Tafinlar (dabrafenib) and Mekinist (trametinib) for patients with advanced or unresectable melanoma, the leading cause of death from skin disease. The FDA approved Tafinlar and Mekinist with a genetic test called the THxID BRAF test, a companion diagnostic that will help determine if a patient's melanoma cells have the V600E or V600K mutation in the BRAF gene. Approximately half of melanomas arising in the skin have a BRAF gene mutation. Tafinlar is intended for patients whose tumors express a single BRAF gene mutation, V600E. Mekinist is intended for patients who express that mutation or the V600K mutation (Simoncelli, 2013).

A list of companion diagnostics that have been approved to date can be viewed athttp://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm.

But actual co-development seems to be a rare phenomenon until now because of following reasons:

1. Clinically useful biomarkers are usually established late in the drug validation process because it is quite difficult to find clinically useful predictive biomarkers early on in a drug development program, simply because they can only be determined on the basis of the patients' responses to the drug. A number of biomarkers, such as KRAS and EGFR mutations, could only be established after a sufficient number of patients for a Phase III trial had elicited a better understanding of differential drug response. And still 30% of the drugs fail during Phase III, diagnostic manufacturers will not be able to afford the huge investment in the development of companion diagnostics (Collins, 2013).
2. The worlds of drug development and diagnostics, although both part of health care, are parallel universes in many ways. In general, they have different development timelines, product lifecycles, return on investment, customers, and regulations. Drugs are valued and reimbursed as products, typically of high value. Diagnostics are valued and paid for as services, typically at a much lower value. Drugs are protected by patents, but in the companion diagnostic arena, biomarkers are considered to be within the public domain and there is less emphasis on intellectual property. It is even debated whether biomarkers should be patented at all because biomarkers are not invented but already exist in cells.
3. The challenge of timing and alignment of the development strategies of the two products: if the diagnostic is going to be used to select patients for the trial, an analytically validated test should be available at the time of initiation of the trial. This can be challenging since sometimes the need for the companion diagnostic may not be evident until late in the development of the drug, or the need to change the test might arise during the course of the trial. The purpose of the trial is not only to assess the safety and effectiveness of the drug but also to investigate the performance of the diagnostic in that specific therapeutic context. A test that does not perform adequately may negatively impact the outcome of the trial and harm patients. Changes made to the test after initiation of the trial can make it difficult if not impossible to interpret the study results (Simoncelli, 2013).
4. Lastly, is the design of the trial itself. Identifying patients at the beginning who are most likely to benefit from a drug or biological product (or excluding those likely to suffer toxicities) can allow for smaller, faster, and less expensive clinical trials with a higher likelihood of success. However, there are some challenges associated with designing very small trials, such as being able to build insufficient statistical power to yield convincing results. Designing a trial to test whether a drug

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is effective for a subpopulation of patients also sometimes raises complex technical and ethical questions about whether to include marker-negative patients (i.e., those that are note expected to benefit from the drug) in the trial (Simoncelli, 2013).

Improvised treatment strategies and healthcare expenditure may reduce up to a greater extent with successful biomarker discovery and validation. For example, the American Society of Clinical Oncology estimates that routinely testing people with colon cancer for mutations in the K-RAS oncogene would save at least US $600 million a year (Drucker & Krapfenbauer, 2013). On the other side, thousands of projects in the course of potential biomarker discovery projects have been proposed, but only a few clinically useful biomarkers have been successfully validated for routine clinical practice (Poste, 2011). The following are the major pitfalls in the translation from biomarker discovery to clinical utility:

1. Lack of making different selections before initiating the discovery phase.
2. Lack in biomarker characterization/validation strategies.
3. Robustness of analysis techniques used in clinical trials.
4. The lack of characterization and validation of technologies such as protein chip, multiplex, etc. Besides technical characterization, it also needs quality requirements for correct characterization of the predictive value of biomarkers.
5. **Regulatory Hurdles:** Each of these details is hardly documented and can dramatically affect the predictive response of biomarker results. However, the selection of useful biomarkers must be carefully assessed and depends on different important parameters, such as on sensitivity (it should correctly identify a high proportion of true positive rate), specificity (it should correctly identify a high proportion of true negative rate), predictive value etc. Unfortunately, biomarkers with ideal specificity and sensitivity are difficult to find. One potential solution is to use the combinatorial power of different biomarkers, each of which alone may not offer satisfaction in specificity or sensitivity. Besides traditional immunoassays such as ELISA, recent technological advances in protein chip and multiplex technology offer a great opportunity for the simultaneous analysis of a large number of different biomarkers in a single experiment, which has expanded at a rapid rate in the last decade.

NANOBIOTECHNOLOGY IN PERSONALIZED MEDICINE

Nanobiotechnology is also an important contributor in the refinement of various molecular diagnostic technologies, biomarkers identification and other diagnostic and therapeutic technologies and useful field of science for personalized medicine.

Nanobiotechnology and Biomarkers

As already discussed in the previous chapter what biomarkers are, how important they are in personalized medicine approach and how important it is to identify novel biomarkers, Nanomaterials can be used for bio-labeling for identifying and characterizing biomarkers. Various new composites have shown high potential for cell labeling. One of such composite is made of biocompatible, water-soluble having fluorescence and being stable is nature silver dendrimernanocomposite for labeling cells in various *in-vitro*

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studies. Trials using quantum dots for molecular labels for detecting early signs of tumors and tracking drug efficacy and efficiency have shown significant results.

Nanoproteomics which is the application of Nanobiotechnology in proteomics helps in improving available protocols/procedures and analyzing less abundant proteins. Using Nanobiotech, we may design and build devices that can be implanted in the body to detect and identify biomarkers, thus enhancing the diagnosis of various diseases. It is now possible to mold certain known nanoparticles to selectively attach to a small set of biomarkers and help in sequestering them (Geho, Jones, Petricoin, & Liotta, 2006).

NanoDiagnostics

It refers to the appliance of nanotechnology in molecular diagnostics. It is believed that the use of nanotechnology in molecular diagnostics will enhance the sensitivity (Emerich, & Thanos, 2006). Nanopore technology has already been available to sequence single molecule of DNA providing higher sensitivity and offers correlation between gene and its expressed proteins with specific disease using not reusable and portable nanodevices. Molecular diagnostic tests used to detect and measure the activity of biomarkers will become speedier and reliable. Tagging of biomolecules with nanoparticles will provide an additional edge. It is viewed that nanotech has potential application in Point of Care (POC) diagnostics and thus will be helpful in personalized treatments.

Nanobiotechnology in Discovering and Delivering Personalized Medicine

Nanobiotechnology is making a noteworthy contribution to the pharmaceutical industry in developing personalized medicine. Nanobiosensors and Nanobiochips are being used for improving drug development process. Drug delivery to the targeted tissue/organ with precision is important for personalized medicine. Using nanocarriers in conjugation with ligands and aptamer allows precise targeting and improved clinical efficacy (Debbage, 2009). Nanobiotech will improve detection of cancer biomarkers. Scientists have applied Alpha-mu-beta 3 targeted paramagnetic nanoparticles for safely finding very minute regions of angiogenesis associated with cancer (Schmieder, Winter, Caruthers, Harris, Williams et al., 2005).

The contrast of MRI scans was enhanced by using particle filled with thousands of molecules of metal. The surface of each particle is coated with a substance that could connect to newly emerging blood vessels at the site of cancer, thus enabling detection of sparse biomarkers. Additionally, nanoparticles used for detection could also be used for precisely delivering doses of anti-cancer medication directly at the target site avoiding side-effects, thus posing another advantage of using nanoparticles. Furthermore, it would be easy for the physicians to evaluate MRI scans, thus fulfilling certain important criteria of a personalized therapy such as timely detection and amalgamation of diagnostics with therapeutics.

Similarly polyvalent dendrimers can be conjugated with several biofunctional moieties for targeting cancer cells. One such example is conjugating dendrimers with folic acid using complimentary DNA oligonucleotides to produce clustered molecules for targeting folate receptor overexpressing cancer cells (Choi, Thomas, Kotlyar, Islam, & Baker, 2005).

Investigations on multifactorial and multiplex nanoparticles linked with antibodies or other small molecules are speeding up for being using them to target malignant tumors with higher sensitivity and affinity (Cho, Wang, Nie, Chen, & Shin, 2008; Wang, Shin, Simons & Nie,2007).

Nanobiotech, when applied to the nervous system, will help us in improving our understanding of neurological disorders and help us design drug delivery systems for them (Jain, 2005). The first and

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foremost thing in applying nanobiotechnology to neurological disorders is to understand the pathomechanisms of it. The development of targeted drug delivery systems to brain that crosses blood-brain barrier is another important component of personalized treatment (nanobiotechnology-based drug delivery to the central nervous system).

NanoBiotechnology has already started establishing its foothold in the area of cardiovascular disorders diagnosis and treatment using targeted drug delivery (Wickline, Neubauer, Winter, Cauthers, & Lanza, 2006; Iverson, Plourde, Chnari, Nackman, & Moghe, 2008). For example, using perfluorocarbonnano particles for visualization and targeted drug delivery in cardiovascular disease. Such nanoparticles will ensure that the drug reaches the target and a subsequent molecular influence is taking place, a vital component of personalized treatment. For instance, with the technological improvement, it would be possible to screen and target biomarkers associated with atherosclerotic lesions expansion.

The main aim to personalized medicine is to improve healthcare and nanobiotechnology is surely all set to play an important role in it. Advancement in technology and availability of cheaper nanomaterials will surely help in cheaper and reliable molecular diagnostic technologies and personalized treatments. Both nanomedicine and personalized medicine will continue to advance and will play an important role in the development of healthcare industry towards personalized medicine. Safety and regulatory issues along with ethical and economic issues are being resolved. It is believed that nanotechnology will pace up the development of personalized medicine by integrating diagnostics with therapeutics.

BIOINFORMATICS TOOLS AND DATABASES AIDING PERSONALIZED MEDICINE

Systems biology along with the key components of personalized medicine i.e., pharmacogenomics, pharmacogenetics, and pharmacokinetics are developing as one of the most promising fields that give a complete understanding of diseases and enable targeted therapy. Although, there are a lot of hurdles that has to be overcome while preparing for personalized medicine (Yan, 2010). Few of the challenges are:

- The scientific challenges, such as determining which genetic markers have the most clinical significance, limiting the off-targeting effects of gene-based therapies, and conducting clinical studies to identify genetic variants that are correlated with a drug response.
- The policy challenges, such as finding a level of regulation for genetic tests that both protects patients and encourages innovation.

In order to make progress, the NIH and the FDA will invest in advancing translational and regulatory science, better define regulatory pathways for coordinated approval of co-developed diagnostics and therapeutics, develop risk-based approaches for appropriate review of diagnostics to more accurately assess their validity and clinical utility, and make information about tests readily available.

Translation of scientific discoveries into better therapeutic outcomes requires better information and workflow management, efficient literature and resource retrieval, and communication improvement. Translational bioinformatics is a powerful method to bridge the gap between systems biology research and clinical practice which provides biological and medical information to allow for individualized therapy. Such computational approaches encompass the development and application of informatics techniques in the biological sciences (Chang, 2005). It serves following purposes:

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- It would enable the identification of biomarkers based on systemic analyzes.
- It enables researchers to search online biological databases and use the biological information in their medical practices. For instance, the data obtained from a microarray is extremely complicated and bioinformatics aids in selecting appropriate software to analyze the microarray data for medical decision making.
- It can improve the understanding of the correlations between genotypes and phenotypes.
- It would enable novel insights of interactions and interrelationships among different parts in a whole system.
- In translational bioinformatics biological databases have been managed in EHR to improve health care and clinical practice moves toward personalized medicine. Bioinformaticians hope to pull all available genomic data into electronic health records (EHRs) that also consider the effects of genetic mutations. The EHRs will allow researchers to assess the contribution of genomic variations to disease (Schmidt, 2003). A virtual patient system has been developed and used to model obesity, diabetes, and asthma.
- Methods based on data integration, data mining, and knowledge representation can provide decision support for both researchers and clinicians.

Moreover, advances in molecular-based information have led to a deeper understanding of the complexity of life. Translational bioinformatics using the high volume of biological information will contribute to changes in practice standards in healthcare systems. It is believed that bioinformatics tools will provide potential benefits to patients by means of improved healthcare, disease prevention and health maintenance as we move towards the era of personalized medicine.

IMMNUNOINFORMATICS AND PERSONALIZED MEDICINE

Immunoinformatics refer to the use of bioinformatics to study immunology. The immune system plays a critical role in the precision medication advance for some chronic, infectious and inflammatory immune-mediated diseases. The genetic capability to generate a diversity of adaptive immune responses is critical to fighting pathogens and differentiates between self and non-self-components. This diversity will also aid in defining patient subgroups for individualized vaccine or drug development. The accumulation of huge amount of immunological relevant information requires efficient informatics methods for analyzing data and its management.

Research areas of Immunoinformatics are:

- Immune system response modeling and simulation of laboratory experiments.
- T-cell and B-Cell epitope prediction.
- Host-pathogen interaction.
- Identify novel functions of existing genes.
- Performing NGS and RNA-Seqin healthy and diseased states.
- Epigenetic Studies (Allergy prediction)
- Design and engineering of immune therapeutics and diagnostics.
- *In silico* vaccination

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Table 1. List of bioinformatics tools, databases used in different fields of personalized medicine

S.No.	Program/Databases	Description
Software for Analysis of Microarray Data		
1	Cluster	For clustering, SOM
2	TreeView	Graphically browse and analyzes results of clustering
3	ScanAlyze	Processes fluorescent images of microarrays
4	ArrayMiner	Set of analysis tools
5	Expression Profiler	Analysis & clustering of gene expression data
6	GeneX-Lite	Integrated toolset, provides an interface to RDBMS
7	BASE	Microarray database and analysis platform
8	dChip	Analysis of oligonucleotide arrays
9	ArrayDB	Mining and analysis of microarray data
Proteomics Strategy Tools		
1	ExPASy (Expert Protein Analysis System)	Analyzes protein sequences and structures
2	PROSITE	Protein database describing protein domains, families, and functional sites
3	Swiss-Prot	A manually curated protein sequence database
4	SWISS-3DIMAGE	An image database provides high-quality pictures of biological macromolecules with known 3D-structure
5	ENZYME	Repository of information relative to the nomenclature of enzymes
6	Protein Explorer	Software to display the protein structure
7	Cn3D	A structure viewer and a helper application
Pharmacogenomics Strategy Tools		
1	dbSNP (The database of Single Nucleotide Polymorphisms)	Free public archive for studying genetic variations.
2	OMIM (Online Mendelian Inheritance in Man)	Catalogues all the known diseases with a genetic component
3	dbGaP (The database of Genotypes and Phenotypes)	Archives the results of the studies of interaction of genotype and phenotype
4	PharmGKB (Pharmacogenomics Knowledgebase)	Encompasses clinical information including dosing guidelines and drug labels, potentially clinically actionable gene-drug associations
Systems Biology Strategy Tools		
1	KEGG (The Kyoto Encyclopedia of Genes and Genomes)	A knowledge base for systematic analysis of gene functions, linking genomic information
2	AfCS (Alliance for Cellular Signaling)	A large-scale collaboration designed to answer global questions about signaling networks
3	STKE (Signal Transduction Knowledge Environment)	Combines the traditional, albeit electronic, publishing of articles, such as reviews, perspectives, and protocols, with tools for organizing and collating information in the cross-disciplinary field of signal transduction

Out of above-mentioned research areas, the most studied is the T-cell and B-cell epitopes prediction because they play a crucial role in disease understanding, host-pathogen interaction, antimicrobial target discovery and vaccine design (Bette, Montiago & Karina, 2006). Epigenetic studies provide the understanding of how environmental changes can affect complex immune diseases such as allergy.

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Table 2. Immunoinformatics databases and tools for epitope analyses

Name	URL
Immune Epitope Database and Analysis Resource (IEDB)	http://www.immuneepitope.org
AntiJen Database	http://www.jenner.ac.uk/antijen/
Epitome	http://cubic.bioc.columbia.edu/services/epitome/submit.php
MHCPred	http://www.jenner.ac.uk/MHCPred/
Macrophages.com	http://www.macrophages.com/content/macrophages/home/
Inflenza Research Database	http://www.fludb.org/brc/home.do?Decorator=influenza

Table 3. Databases and tools for analysis of genetic variations of the immune system

Name	URL
IPD	http://www.ebi.ac.uk/ipd/_
MHC Haplotype Project	http://www.sanger.ac.uk/HGP/Chr6/MHC
dbSNP	http://www.ncbi.nlm.nih.gov/SNP/_
Allele Frequencies Database	http://www.allelefrequencies.net/_
International HapMap Project	http://snp.cshl.org/

With the help of Immunoinformatics, we can develop personalized medicine for chronic, infectious and inflammatory immune-mediated diseases.

DISEASE-BASED CASE STUDIES ON PERSONALIZED MEDICINE

Several genes have been found responsible for variations in drug response and metabolism among different individuals. The most important and common among them are the *Cytochrome P450* (*CYP*) genes. These genes encode enzymes involved in regulating in one way or the other the metabolism of about three-fourth of the prescribed drugs. Individuals carrying variations in certain *CYP* genes often differ in their ability to metabolize the drug. There are a number of other genes that affect drug response such as the ones encoding the regulatory molecule`s receptors, growth hormones, structural proteins, transcription factors and some cellular proteins. Diminished drug response and occurrence of adverse drug reactions (ADR) can often be due to polymorphism in these genes. Polymorphism in these genes can either hyperactive or inactive their protein proteins; or disable or interfere with the functioning of their protein, thus forcing physicians to discontinue the medication. A list mentioning drugs, and genetic variations affecting their response or safety, is available at U.S. Food and Drug Administration website for physicians, researchers and individual`s reference (Pharmacogenetics, FDA)

Also, it has been observed that certain drugs can lead to changes in the phenotype of individuals and influences the metabolizing capacity of enzymes by mimicking the effect of genetic variations. Quinidine is a known inhibitor of CYP2D6 activity. In case if an individual is prescribed quinidine, then he/she will be a poor metabolizer of CYP2D6, compared to someone who carries a loss-of-function polymorphism in *CYP2D6*. In those patients, drugs requiring the activity of CYP2D6, such as atomoxetine, will not

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be metabolized at the same rate as in most people. Mimicking the effect of genetic variations is also a known phenomenon in certain foods. For example, grapefruit juice, an inhibitor of CYP3A4 can lead to poor metabolism of drugs like diazepam, requiring the activity of CYP3A4.The following three case studies illustrate three categories for which pharmacogenomic knowledge can help take better decisions for medication respectively.

Case Study 1: In Predicting and Preventing Adverse Drug Reactions

Aman, a 30-year-old man has recently been diagnosed of HIV infection. Before initiating Abacavirantiretroviral therapy, his physician decided to go for genetic testing to determine whether he carries any relevant variation. The test confirmed the presence of the *HLA-B*5701* allele.

How did genetic testing help Aman and his physician?

Abacavir (Ziagen) marketed by GlaxoSmithKline is a nucleoside reverse transcriptase inhibitor often used in amalgamation with other antiretroviral medication to treat HIV infection. An immunologically-mediated hypersensitivity reaction is known to occur in about 5–8 percent of patients taking Abacavir, usually during the first six weeks after initiation of therapy. (GlaxoSmithKline. Ziagen, product labelling. 2010)

Knowing that Aman carried the *HLA-B*5701* variation and he would likely experience a hypersensitivity reaction and would develop fever, rash, nausea, and fatigue if he is given Abacavir, thus, the physician would avoid prescribing him Abacavir.

Case Study 2: To Predict Effectiveness

JS, a 55-year-old man recently had an acute myocardial infarction. The physician recommended him anti-platelet therapy and prescribed clopidogrel. 6-months following the anti-platelet therapy, JS surprisingly suffered another acute myocardial infarction. The physician suspected that clopidogrel therapy may have been ineffective or less effective. Following genotyping it was found to carry a variation on *CYP2C19*.

How would genetic testing help JS and his physician?

Clopidogrel is a prodrug and it is converted to an active metabolite by cytochrome P450 enzyme, CYP2C19 to exert its anti-platelet properties. Individuals carrying certain variations in CYP2C19 are considered poor metabolizers and show decreased ability to convert clopidogrel into its active metabolite (Shuldiner, O'Connell, Bliden, Gandhi, & Ryan, 2009). Further, these patients are more likely to have an ischemic event following clopidogrel therapy (Mega, Close, Wiviott, Shen & Hockett, et al., 2009). It was found that JS carried a variation in its *CYP2C19*. This variant has been known to be associated with the diminished antiplatelet effect of clopidogrel. The physician would have to re-examine him and consider giving him some other anti-platelet therapies, thus decreasing his chance of getting another cardiac attack.

Case Study 3: In Predicting Optimal Dose

Mitchell, age 66, has been recently diagnosed with atrial fibrillation. The physician recommended her warfarin therapy in order to reduce the risk of further getting any stroke and leading to other thrombotic events. In order to estimate the optimal dose, the patient has to undergo INR monitoring every day until a stable dose is determined, and then every few weeks thereafter for maintenance monitoring.

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How would genetic testing help Mitchell`s physician to determine the optimal dose for her?

The most commonly prescribed anti-coagulant is Warfarin (Coumadin), to treat and prevent thrombosis. CYP2C9 enzyme metabolizes it and further action by the enzyme VKORC1 mediates its anticoagulant effect. It is observed that polymorphism in the *CYP2C9* gene causes some individuals to slowly metabolize warfarin and increasing the half-life of the drug, resulting in higher than usual blood concentrations of warfarin and greater anticoagulant effect. On the other hand, certain variation in the VKORC1 gene reduce the activity of the enzyme encoded by it and subsequently decreases the production of coagulation factors. The blend of reduced warfarin metabolism caused by *CYP2C9* gene polymorphism and subsequent decreased coagulation due to *VKORC1* gene variations has been found to increases the chances of bleeding during warfarin therapy. Warfarin has a narrow therapeutic index; variations in *CYP2C9* and *VKORC1*, in addition to several other patient characteristics, make it difficult to predict the effective dose. (Reider, Reiner, Gage, Nickerson, & Eby, et al., 2005) (Bristol-Meyers Squibb Company. Coumadin (warfarin sodium) product labeling 2010). If Mitchell carries any mutation on *CYP2C9* and *VKORC1* genes then it would be difficult to optimize the dosage for warfarin but surely it would surely reduce the risk of bleeding. This would help thephysician to decide more appropriately decide the initial dosage.

The following case study which will demonstrate the application of personalized medicine/genetic testing in efficiently treating dreadful diseases.

Case Study 4

A clinician sends two samples for molecular analysis of the patients suffering from Chronic Myeloid Leukemia (CML) and been administered a drug called Imatinib, showing clinical signs of drug resistance. The samples are sent to the laboratory to confirm drug resistance and investigate the reason for drug resistance. A standard laboratory test called "IRMA" was performed to detect a point mutation in chromosome 9, as it is a known prognostic factor for drug resistance. One of the samples showed a positive result for IRMA and other showed a negative result.

How would one go through the rest of the process for personalized medicines for these 2 patients?

First of all, we have to identify all the other biomarkers present in the patient`s genome and understand their effect on the drugs available. For CML, not all the biomarkers are known yet, so it calls for studies regarding biomarkers. One such biomarker has been identified as single nucleotide polymorphisms of ABCB1. Also, one marker is surrogate for the other. Depending upon the biomarkers or the variants present in the patient we can go by tailoring a treatment specific to that patient only.

For more information on pharmacogenomics, visit the following websites:

- UC San Diego Skaggs School of Pharmacy and Pharmaceutical Sciences.
- Pharmacogenomics Education Program (http://pharmacogenomics.ucsd.edu/home.aspx).
- American College of Clinical Pharmacology.
- The Future of Medicine: Pharmacogenomics (http://user.accp1.org/Sample_Home.htm).
- Indiana University School of Medicine, Division of Clinical Pharmacology.
- Defining Genetic Influences on Pharmacologic Responses (www.drug-interactions.com).
- U.S. Food and Drug Administration Genomics (www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/default.htm).
- Pharmacogenomics Research Network (www.nigms.nih.gov/initatives/pgrn).

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ELSI (ETHICAL, LEGAL, AND SOCIAL IMPLICATIONS) OF PERSONALIZED MEDICINE

Introduction to Ethical Issues

Ethical issues of personalized medicine are based on PG, genetic information and impact on healthcare. We need to take care how the personal information of the patient, genetic information and information important for pharma companies flows during the implementation of personalized medicine approach. It is recognized that there are a number of barriers that need to be addressed before PGx will be part of routine clinical care.

Ethical Aspects of PG

Some of the ethical issues that are bound to rise by PG in implementation of personalized medicine are:

- When the geneticists can predict which patients are likely to benefit from the available pharmacotherapy and which are less likely, the issue of inequality may arise. We have to ensure that equality remains in medical healthcare.
- Confidentiality of patient`s Genetic information. Protecting patient privacy is one of the most important concerns that must be addressed before implementation of individualized medicine in clinical practice and just about everyone agrees that patients have a right to keep details about their health private from most people (even if not from, say, their insurance company or in some cases state or local governments).
- Another issue is to protect the right of a medical practitioner or scientist to reject a patient the available therapy on the basis of its genetic makeup.
- Cost is also a significant ethical issue as well. Still millions of people are without health insurance today, and many more have insurance plans that cover only the most basic things. How can 'payers' provide access to personalized medicine to everyone?

Ethical Aspects of Genetic Information

It covers the following aspects:

- **Ethical Issues of Whole Genome Analysis**: A large amount of data generated by genetic studies and their analysis predicting the patient`s response to different disease and the diseases they are likely to develop can have ethical issues as to what information is to be revealed to the patient and what is to be concealed.
- **Privacy Issues in Personalized Medicine:** As personalized medicine approach involves dealing with the complete information about a person, privacy issues are bound to rise. The patient might not be comfortable in sharing his/her personal information either due to social issues or some personal/moral issues. The information generated from genetic analysis of patient`s data may not be emotional good for the patient and physician might try to conceal it for better treatment of the patient. Sometimes the patient is reluctant to reveal his information even before the diagnosis is done.

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Molecular tests challenge two aspects: How comprehensive these tests are and how the access to this information is regulated:

- Accessibility of diagnostic tests and targeted treatments within and across countries with companies seeking orphan status for new targeted treatments.
- Patients' understanding and patients' role in future decision making.
- **Human Dignity:** Potential for stigmatization and discrimination.
- **Human Integrity:** How this affects moral convictions, preferences, and commitments
- **The Input-Output Problem:** Though personalized medicine aims at segregating populations into small subgroups based on their genotype and response to drug or susceptibility to a disease but in all these studies racial and ethnic minorities have been left unexplored. Even the GWAS studies take in account only European or in some cases Asian populations. The proportion of GWAS conducted with minorities suffered from health disparities. The input–output problem arises because allele frequencies and environmental exposures tend to vary among population groups (Fullerton, 2011). Because of this, the assumptions that inform personalized medicine practice in well-studied populations are not necessarily generalized to poorly studied populations.

Regulatory Aspects

All the above ethical issues need to be resolved for efficient implementation of personalized medicine approach in the healthcare industry. Regulatory authorities have to set some standards for this approach and ensure that the rights of the patient, physician, and Pharma companies are protected as well as ensuring that personalized medicine approach does not create any social differences (King, Casavant, & Lang, 2013).

Role of Regulatory Authorities

- Sometimes the patient is reluctant to reveal his information even before the diagnosis is done, in that case, it is needed to be ensured that patient is properly supported and informed about the personalized medicine approach.
- Medical products should be carefully evaluated to find out their benefits and risks.
- Regulatory authorities should remain updated about the new technology and must keep the pharma companies on the toes to inform the author about each and every aspect of drug development.
- Provide clarity to the pharma industry and the consumer/patient about the recent therapies available in the market.
- They should address "pipeline" problems about the drugs by identifying the opportunities for streamlining the processes.
- Scientific barriers, regulatory issues and ethical issues have to be tackled beforehand so that there is the smooth implementation of personalized medicine in clinical practice.

The need of an hour is to expand the amount of work and effort to address ELSI. This requires expanding our current list of experts in the field of bioethics and clinical policies along with collaborations with quality control experts, health economists, and strategists. It is also clear that new approaches towards

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informed consent, intellectual property, protection of individual`s privacy and data required to be established for facilitating the suitable and active application of new technologies to personalized medicine (Brothers, & Rothstein, 2015).

CHALLENGES IN THE JOURNEY TOWARDS PERSONALIZED MEDICINE

As every coin has two sides, personalized medicine too has another side to it. There is a need to take care how the personal information of the patient, genetic information and information important for pharma companies flows during the implementation of personalized medicine approach.

Whenever a new technology or a model is found, there are always some concerns in its implementation in the initial phase. Personalized medicine model`s implementation also have some concerns and limitations:

Economic Limitations

- Personalized medicine could raise issues for those who pay for treatment, people in developing countries like India. The cost of new assessment tests and personalized medication is more and predictive potential of personalized medicine could add more to the expense after the disease has been detected. Though health insurance is not a trending business in India, as the insurance premium are based on actuarial statistics that apply to large and predictable population, insured people might have to end up paying more for getting an insurance.
- It is difficult to convince pharma companies to manufacture drugs according to a group of people carrying a certain genotype. Pharma companies see more benefits in producing traditional drugs that work on 50-60% of the population and still being prescribed to patients who do not respond to it or have side effects after consuming these medicines.
- Genome-wide association studies (GWASs) have been used to assess the SNPs with many important common diseases. But only a limited number of variants have been characterized, and understanding the functional relationship between associated variants and phenotypic traits has been difficult. SNP Prediction methods are not to be solely relied on as they don`t provide much information about the pathophysiology of the diseases. So we have to switch to the wet lab and conduct experimental tests to validate genetic predictions. As we know Laboratory validations are always expensive and time-consuming and so there is a need for fast and accurate methods for gene prioritization.

Limitations in Identifying Disease Biomarkers

- To unravel DNA variants so as to predict common, complex diseases that result from a combination of genes and environmental factors will require cost-effective, high-throughput genotyping; large, well-characterized patient populations; sophisticated computational methodologies; and a detailed understanding of the biological pathways of disease.
- Uncovering mRNA and protein markers for application in screening, diagnosis & prognosis and monitoring of disease will have its own set of challenges. Access to optimal relevant tissues might not be possible for many diseases. Proteomic technologies still need further development, because

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computational approaches for analyzing massive amounts of gene and protein profile data. To realize the vision of personalized medicine, the agenda for medical and pharmaceutical research must include the assembly and integration data from many sources on large numbers of patients. Clinical investigations should incorporate genotyping and molecular profiling technologies along with traditional clinical data collection and should establish a repository of patient samples where possible.

- New molecular markers might face many hurdles before they can be implemented in patient care. The issues range from FDA regulation and acceptance of these new markers to developing tractable assay platforms, to resolving issues around the ethical, legal and social implications of obtaining highly sensitive genetic information. Foremost among these, in our opinion, is the education and engagement of physicians and patients in the paradigm shift to objective, quantitative marker-based clinical care. If appropriate patient management systems, integrated databases, educational tools and genetic counseling are not in place, then it will be difficult to realize the significant benefits forecast from this approach.

Bioinformatics Challenges

- **Processing Large-Scale Robust Genomic Data:** Sequencing technologies are becoming affordable and are replacing the microarray-based genotyping methods, which were limited to interrogating regions of known variation. Now a whole genome can be sequenced in less than 15 days with an error rate of ~ 1 error per 100 kb. Even such low error rates can lead to a significant number of errors, which is around ~30 000 errors in a 3 GB human. The error rate from these technologies is a source of significant challenges in applications, including discovering novel variants (Fernald, Capriotti, Daneshiou, Karczewski, & Altman, 2011). Whenever a novel variant is identified, it will still have to be verified due to this false positive rate. Even high-quality sequence reads must be placed into their genomic context to identify variants, which is an active area of research since, for example, different mapping and alignment algorithms often yield different results. Because de novo assembly (Shendure & Ji, 2008) is slow and complicated by repetitive elements, sequences are usually mapped to a genomic reference sequence instead. Algorithms such as BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) or Smith–Waterman (Smith & Waterman, 1981) have been traditionally used, but their execution speed depends on the genome size. While individual queries may only take seconds per CPU, aligning 100 million of them would require more than 3 CPU years.
- **Interpretation of the Functional Effect and Impact of Genomic Variation:** After genomic data has been processed, the functional effect and the impact of the genetic variations must be analyzed. Genome-wide association studies (GWASs) have been used to assess the statistical association of SNPs with many important common diseases. These methods are providing new insights, but only a limited number of variants have been characterized, and understanding the functional relationship between associated variants and phenotypic traits has been difficult (Frazer, Murray, Schork & Topol, 2009).The important resources for obtaining SNP data are adbSNP database, Online Mendelian Inheritance in Man, Human Gene Mutation Database. But such prediction methods do not provide any information about the pathophysiology of the diseases and so experimental tests are required to validate genetic predictions. Laboratory validation is expensive and time-consuming and so there is a need for fast and accurate methods for gene prioritization.

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- **Integrating Systems and Data to Capture Complexity:** Given the complex phenotypes involved in personalized medicine, the simple 'one-SNP, one-phenotype' approach taken by most studies is insufficient. Most medically relevant phenotypes are thought to be the result of gene–gene and gene–environment interactions (Manolio et al., 2009). For example, drug response often depends on multiple pharmacokinetic and pharmacodynamic interactions, which form a robust and tolerant system with highly polymorphic enzymes and many interaction partners (Wilke, Reif & Moore, 2005). As a result, of this complexity, a drug–response phenotype of interest is likely to depend on many genes and environmental factors. Basic GWAS approaches for pharmacogenomics have given some success. There is concern that pharmacogenomics GWAS themselves are susceptible to many limitations: insufficient sample size, selection biases for genetic variants, environmental interactions that may affect the outcome measures and multiple gene–gene interactions that may underlie unexplained effects (Motsinger-Reif et al., 2010).
- **Making it All Clinically Relevant:** The ultimate challenge for this research is to apply the results for improved patient care. Much of this research has yet to be translated to the clinic. In fact, many physicians are unprepared to incorporate personal genetic testing into their practice and it is unclear how to best apply research results to improve patient care (McGuire & Burke, 2008). Bioinformatics also translates discoveries to the clinic by disseminating discoveries through curated, searchable databases like PharmGKB, dbGaP, HGMD, OMIM (Mailmanet al., 2007; Thorn, Klein, & Altman, 2010). A major bottleneck for these databases is manual curation of the data. Biologically and medically focused text mining algorithms can speed the collection of this structured data, such as methods that use sentence syntax and natural language processing to derive drug–gene and gene–gene interactions from scientific literature (Garten, Coulet, & Altman, 2010). These databases and methods need to be developed and used carefully. All these data sources are susceptible to errors and so validation of data is essential, especially before the information is applied in the clinic. Finally, there are challenges and opportunities for bioinformatics to integrate with the electronic medical record (EMR) (Busis, 2010). For example, the BioBank system at Vanderbilt links patient DNA with a de-identified EMRs to provide a rich research database for additional translational research in disease–gene and drug–gene associations (Denny et al., 2010; Roden et al., 2008).

Other Limitations

- **Limited Understanding of the Intrinsic Biology of Disease:** The tools of the last two decades have left us overflowing in data, yet we still have a relatively limited understanding of what it all means. Its implementation in clinical practices remains in its infancy. Scientific understanding will likely remain the most important limiting factor for the momentum of this field. Physicians will have to be well equipped with the knowledge of genomics and proteomics to understand the new data.
- **Common Conditions Involving Multiple Genes/Biomarkers:** Common conditions are often influenced by multiple genetic, as well as environmental and social factors, in ways that are not yet well understood. Realization of the benefits of personalized patient management for common conditions affected by multiple genes will be a complex process that will depend on substantial investment in clinical research well beyond the initial demonstration of gene-disease correlations (Simoncelli, 2013).

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- **An Outdated Disease Classification System:** Currently used disease classification systems define diseases primarily on the basis of their signs and symptoms. These systems do not easily accommodate emerging information about disease mechanisms, particularly when it is at odds with traditional physical descriptions. As a result, many disease subtypes with distinct molecular causes are still classified as one disease, while multiple, different diseases that share a common molecular cause are not properly linked. The failure of our outdated disease classification systems to incorporate optimally new biological insights serves as a fundamental barrier to progress in personalized medicine. The National Academy of Sciences has called for the creation of a "New Taxonomy" of disease that is designed to advance our understanding of disease pathogenesis and improve health and that defines and describes diseases on the basis of their intrinsic biology in addition to traditional signs and symptoms. (National Research Council: Committee on a Framework for Developing a New Taxonomy of Disease. (2011). Toward Precision Medicine: Building a Knowledge Network for Biomedical Research and a New Taxonomy of Disease. Washington, DC: The National Academies Press).
- **Lack of Infrastructure:** Costs of genetic sequencing have plummeted over the past decade, resulting in an explosion of information. Yet, while information is becoming easier and easier to obtain, the infrastructure to collect, analyze, integrate, share, and mine that information remains lacking.
- **Clinical Implementation of New Diagnostics:** Many clinicians have been reluctant to use new diagnostics. Part of this reluctance may be due to the ongoing controversy over clinical utility and the fact that biomarker clinical utility can often be a moving target. Clinicians also commonly face the general problem of "information overload," making adoption of new tests difficult without decision-support tools in place that could be accessed to help the clinician to identify, order, and interpret the appropriate tests.
- We don`t know whether it will be a good idea to inform an individual that he/she is likely to develop a disease or is suffering from a disease for which there is no cure.
- Realization of personalized management in diseases with multiple genes affecting the diseased state is a complex process and will ask for higher financial input as well as in-depth clinical research.
- *Poor sequence quality* from sequencing technologies is a challenge in discovering novel variants. Moreover, it is difficult to detect INDELS, Copy number variants, and structural variants. The cost of sequencing is another major concern. Efforts are being made to reduce the cost of sequencing the whole genome under $1000.

CURRENT STATUS AND FUTURE IMPLICATIONS OF PERSONALIZED MEDICINE

Personalized medicine has just moved out of its infant shell and it is expected that it will enter its teen years by the end of this year. It is of outmost important that we design and device strategies for efficient implementation of the personalized medicine model in clinical practice. Currently, personalized treatment is employed in only few of the diseases such as Cancer, cardiovascular disorders, respiratory syndrome and psychiatric maladies. Most research and success has been achieved in case of anti-cancer therapies. But to reciprocate the success in other disorders, we need to work on areas such as lowering

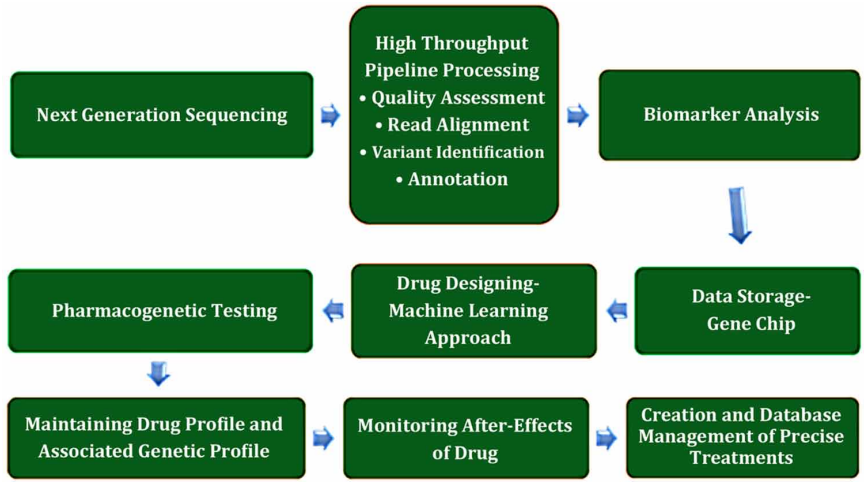
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down the cost of sequencing, applying exome sequencing for better diagnosis and treatment, improving the available infrastructure for the implementation of personalized medicine in clinics and all these is to be supported by new regulatory policies. New technology, new models require new regulatory policies to ensure safer and smoother implementation. Government and regulatory authorities like FDA have to draw in more stern regulations for personalized medicine thus ensuring the availability of safe and effective diagnostics. The regulatory science has to catch up with all that is happening in relation to personalized medicine and help in its faster, safer and regulated implementation. Drug labeling provides important information about the drug which is useful for both clinician and the patient. As personalized medication will be designed for a group of population, product labeling becomes important. Integration of nanobioscience and high throughput proteomics techniques with personalized medicine and development of point of Care Medication.

A good amount of work on electronic health records is being carried out in the USA along with an additional emphasis on the storage of genetic data and developing point-of-service prompts to assist busy clinicians in identifying and utilizing relevant genomic data at the proper time in diagnostics or treatment planning. A 10 years down the line we could reach a point where the analytical validity of sequencing technologies are high, with the easy ability to access and clinically interpret genomic information, and knowledge about patient responses to genomic information.

Owing to recent developments with respect to personalized medicine, it is expected that this model will eventually be turned into clinical practice by the end of this decade. Besides the application of PG and PGx, pharmacoproteomics will gain more application in identification of novel biomarkers, designing new drugs in accordance with the protein profile and understanding disease pathophysiology. By combining the three key components of personalized medicine, it would be possible to strictly define responsive populations and effectively monitor drug responses. Protein chips are expected to make it to the clinics within next five years thus aiding point-of-care diagnostics.

Figure 4. Flow chart for the case study 4



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Efficient Next Generation Sequencing (NSG)

1. High-Throughput Pipeline Processing. Better and efficient bioinformatics tools for quality assessment, alignment, and variation calling.
2. Database for identifying biomarkers.
3. Storing the patient`s data in a Gene Chip. It will give easy an access to the Clinician.
4. Designing a probable drug molecule through machine learning.
5. Virtual Biological systems that will analyze the drug path, molecular interactions, signal transduction pathways and some other parameters.
6. A complete profile of the drug will be generated.
7. Store the Drug profile in association with a genetic profile in a database or some records.
8. Administer the drug, if found suitable for a group of patients.
9. Analyze the After effects. A Chip will keep the record of the patient`s physiological parameters.
10. Create and manage a database of such personalized treatments.

CONCLUSION

This chapter has tried to present the very model of personalized medicine from different angles, emphasizing on its key components, its potential applications and trying to identify issues that may arise during implementation of such a model in clinical practice. It also summarizes the current developments and future potential of personalized medicine.

We believe it would be fair enough to say that personalized medicine has started spreading out its wings. It has the potential to re-define the approach we have about identifying and managing health problems. It has already shown it significant impact on the healthcare industry. Since personalized medicine has the potential of revolutionizing patient care targeting patient population; avoidance of drug-related toxicities and optimization of costs in non-responder patients; reduction in the cost of drug development, and reduction in patients to be tested in clinical trials. There lies a huge potential in the application of precision medicine to significantly improve the clinical outcomes in terms of treatments, prevention strategies, medication types and dosages- that may have inter-individual variability. However, the wider impact hasn`t been achieved yet despite of being increasingly recognized in literature and many disease cases in humans. High sequencing cost, lack of infrastructure support for scientists and physicians, ELSI, lack of regulatory measures and economic factors additionally are hindering the translation of this model into reality. In short, we could say that though catering medicine specifically to an individual`s requirement is a farfetched but certainly possible reality following more research, high financial investments and establishing more systematic guidelines.

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KEY TERMS AND DEFINITIONS

Biomarker: It is measurable indicator for a biological state or physiological condition.

Personalized Medicine: An approach followed for disease prevention and treatment that takes into account differences in people's genes, environments and lifestyles.

Pharmacodynamics: The study of the biochemical and physiological effects of drugs on the body and the mechanisms of drug action and the relationship between drug and response.

Pharmacokinetics: It refers to the study of drug pathway inside the body. It involves studying absorption, metabolism, distribution and excretion of a drug.

Pharmacoproteomics: It refers to the use of proteomic technologies in drug development and molecular diagnosis. It helps more clearly demarcate patient-to-patient variations as compared to genotyping.

Risk Assessment: It refers to identifying sensible measures to control the risks associated with different trials especially where live organisms are involved.

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Chapter 13
Role of Resveratrol (RES) in Regenerative Medicine

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ABSTRACT

Within the last quarter century, technology has been a major catalyst of the advancement in various fields of scientific knowledge, particularly medical research. This new enlightenment has spurred the exploration of alternative treatment methods to some of society's most problematic diseases. One such innovative treatment is the use of Resveratrol (RES) to treat a number of pathophysiological conditions. RES is a natural polyphenolic compound found in the skin(s) of blueberries, red grapes (a major constituent of red wine), some vegetables, and even peanuts. The compound has a number of potent regenerative properties, which include: anti-aging, anti-inflammatory, and antioxidative. Research has confirmed both in vivo and in vitro RES's beneficial applications to numerous diseases. This chapter centers on its unique healing powers and beneficial applications against myriad debilitating conditions.

INTRODUCTION

Recent scientific studies have revealed that conditions affecting the circulatory, muscular, skeletal, and other organ systems such as multiple sclerosis, heart disease, and diabetes have their origins rooted in a low-nutritious western diet. This emerging problem has led to the advancement in medical ingenuity such as regenerative therapeutics (Hubmayr 2013). For example, researchers have implemented the use of stem cells harvested from umbilical or fetal cord blood in an attempt to generate newly functional tissues (Hubmayr 2013; Bouros and Laurent 2012 ; Kolios and Moodley 2013; Liu et al. 2013; Viswanathan and Joshi 2013). Despite its potential benefits many take issue with this new technology due to the perceived unethical nature in which the cells are acquired. Therefore, research into alternative life-restoring compounds such as resveratrol (RES) and its conjugation with novel nanoparticles have presented the

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scientific community with a viable alternative to the mainstream medicines. RES is a naturally occurring polyphenolic compound found in the skin of red grapes, blueberries, select vegetable foods, and even peanuts (Zunino and Storms 2009; Sha et al. 2009; Kao et al. 2009; Trincheri et al. 2008; Sun et al. 2008; Shakibaei et al. 2008; Roccaro et al. 2008; Juan et al. 2008; Martin et al. 2004; Larrosa et al. 2003; Radhakrishnan et al. 2011). It is a powerful and versatile compound with potent regenerative properties including anti-oxidative, anti-inflammatory, and anti-aging. Combining the many beneficial properties of RES with unique nanotechnology, such as carbon nanoparticles, could give scientists the ability to enhance RES's inherent regenerative powers by increasing the stability of the compound *in vivo*, allowing it to deliver therapeutic effects over an extended period of time. Nanodiamonds (NDs) have been incorporated into the research of revolutionary drug-delivery system(s). These microscopic carbon compounds are harvested from the residue of explosive mixtures such as trinitrotoluene (TNT) and dynamite. The raw material is chemically refined and functional groups such as carboxyl (COOH), hydroxyl (-OH), and ketones (C=O) are incorporated onto the surface of the ND. This gives researchers the flexibility to conjugate a wide variety of therapeutic agents such as RES, creating an effective drug-delivery vehicle. This carrier system when introduced *in vivo* allows RES to remain intact and be released in a time-dependant manner. The agent conjugated to the surface of the ND is released only in response to a change in physiological conditions, specifically a drop in pH from basic to acidic [Hubmayr 2013; Figueiro et al. 2013; Li and Ye 2006]. This pioneering technology would allow physicians to decrease both the dosage and amount of times required to treat patient(s) suffering from debilitating conditions such as cancer. Previous clinical studies have demonstrated RES's impressive therapeutic capabilities and minimal side effects because it is a natural chemical (Mohanty et al. 2013; Liang et al. 2013; Borriello et al. 2014; Lachenmeier et al. 2014; Cottart et al. 2014). RES's natural regenerative properties and negligible toxicity makes it an extremely attractive option in the arduous pursuit for health and longevity. Many who have worked closely with RES are fascinated by its ability to operate through a variety of cellular biochemical pathways and mediate positive immune responses. Clinical data has shown that the administration of RES has restored significant functionality and vitality to target organs and tissues (Zhang et al. 2007; Sareen et al. 2007; Lee et al. 2007; Horvath et al. 2007; Hambrock et al. 2007; Gill et al. 2007; Ahmad et al. 2007).

(RES) RESTORES GROWTH OF CARDIAC STEM PROGENITOR CELLS (CSPC)

There have been numerous studies documenting RES's incredible ability to regenerate tissues. One such study investigated the effects of the application of RES to replenish degenerating cardiac tissue. As in the case of many pathophysiological conditions the common underlying cause for its manifestations is the quality of diet. The debilitating condition of diabetes mellitus is a result, among other mitigating factors, of a poor diet high in processed sugars and foods with little to no nutritive value. The onset of Diabetes can cause an imbalance in other organ systems via metabolic derangements that accompany the primary condition. Diabetic cardiomyopathy (DCM) is characterized as a disorder in the muscles of the heart which can lead to poor circulation and other complications (Delucchi et al. 2012). Evidence shows that high glucose levels leads to the hindered differentiation, migration, and adhesion of cardiac stem progenitor cells (CSPC) for the rejuvenation of worn or dying mature cardiac cells (Delucchi et al. 2012). This produces a net loss of cardiac cells because not enough new progenitor cells are able

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under stressful diabetic conditions to replace the cells that are lost over time. RES has been documented to restore positive growth back to the subpopulation of CSPC and improve cardiac tissue function and other model systems (Delucchi et al. 2012; Figueiro et al. 2013; Jana et al. 2009; Busanello et al. 2012; Schlachterman et al. 2008).

RES INCREASES MATRIX MINERALIZATION OF ADIPOSE STEM CELLS (ADSC)

In a separate study investigators examined the proliferative effects of treating both rat and human adipose derived stem cells (rADSC and hADSC, respectively) in a 3-D environment (polycaprolactone, PCL) with RES. The work revealed that the pre-treating or pre-conditioning of ADSCs significantly increased the osteogenic potential in both cell lines as compared to that of untreated ADSCs. However, variables such as species type, specific dosage(s), and various time-points all factored into the degree of cell mineralization/differentiation caused by RES (Dosier et al.). According to the study, increased matrix mineralization was observed at 12.5 μM RES concentration at four weeks. Concentrations of RES higher than 12.5μM proved to have an overall toxic effect on both rADSCs and hADSCs; as a result decreased matrix mineralization was observed (Dosier et al.). Surprisingly, the low concentration of 6.25μM RES yielded the greatest amount of mineralized matrix suggesting that lower concentrations/doses are more effective at sustaining the model cells and producing mineralized matrix over a prolonged period of time. Higher doses (i.e. 12.5μM RES) proved to be more efficient at shorter time-points. The results confirm RES's proliferative/life generating capabilities (Dosier et al. 2012).

RES EXHIBITS CYTOPROTECTIVE PROPERTIES OF LIVER CELLS

The liver is the second largest organ in the human body and plays a critical role in maintaining the metabolic homeostasis of an organism. Important functions related to immune system response, the neutralization of many toxic elements, metabolite(s)/nutrient synthesis, and digestion are managed directly or indirectly by the liver (Matsumoto and Nakamura 1992; Bouwens et al. 1992). In fact, one can consider liver as the "processing plant" of the body in most organisms. Many diseases and toxins that target/affect the liver will have a negative impact on the overall health and vitality of the affected organism(s). These conditions can be fatal, for example, cirrhosis of the liver due to the ingestion of toxins or pollutants can cause a condition where viable liver tissue is replaced by nonfunctional scar tissue. However, recent research has shown that among the many other beneficial properties of RES it also demonstrated the ability to prevent and even reverse liver damage. The administration of RES to murine model cells have shown to protect against oxidative stress and exhibit hepatoprotective activity against agents of acute and chronic liver damage. At low doses RES decreases ischemia-reperfusion-induced liver injury and protects hepatocytes against oxidative complications (Roy et al. 2011). Quantitative data showed that the liver and body weights of rat specimens treated with RES showed comparable weights with that of specimens in the normal control group. Furthermore, RES displayed protective properties against certain histopathologies caused by carbon tetrachloride (CCL4) in the liver. The numbers of hepatocyte nodules (aggregates of liver cells enclosed by collagenous fibers) were significantly less in specimens treated with RES than the group induced with the agent CCL4 (Roy et al. 2011). These nodules are prominent

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in damaged livers and replace the normal architecture of the organ, and RES both decreased the number and development of these nodules. RES mitigated the toxic effects of CCL4, the liver of specimens treated with RES exhibited fewer necrotic zones and the arrangements of liver cells were similar to that of the normal control group. Additionally, RES inhibited the secretion of pro-inflammatory cytokines of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6); therefore, eliminating complications resulting from inflammatory immune responses (Roy et al. 2011; Cerqueira et al. 2013).

RES REVERSES THE PROGRESSION OF AGE-RELATED MACULAR DEGENERATION (AMD)

Additional research data further substantiates RES's versatility and wide-spectrum mediatory capabilities. One such study implemented a RES based nutritional supplement named Longevinex (L/RV) to treat individuals afflicted with age related macular degeneration (AMD). Those of the sample test group were United States veterans of one of three major conflicts (World War II, Korean, or Vietnam wars). AMD is a debilitating condition characterized by the progressive deterioration of the macula of the eye, which can lead to a significant loss of sight at the center of one's vision. The results of this investigation show that the L/RV supplement reversed the symptoms of AMD as well as other systemic co-morbidities. Octogenarians observed improvements in subjects' hearing, blood pressure, and vitality after treatment with the resveratrol-based supplement. Conversely, after cessation of treatment with L/RV investigators observed a regression in the improving conditions of the subjects (Richer et al. 2013). In a normally active eye the retina, photoreceptors, and other important cells that are worn are regularly replenished with new cells. The tissues of the eye are continuously supplied oxygen and nutrients by blood vessels. However, the pathogenesis of AMD is characterized by inflammatory and oxidative conditions which hinder the regenerative capabilities of the eye. In mammalian species damage to photoreceptors and internal neurons of the eye are rarely replaced or regenerated. However, this was not the case when L/RV was administered to patients. Investigators recorded growth of glial cells and improvement in visual functionality (Richer et al. 2013). RES was shown to neutralize AMD disease progression and produced moderate improvements in the condition of many of the subjects. Although more extensive research is needed to confirm these preliminary results one cannot deny the beneficial impact of RES treatments (Richer et al. 2013).

RES ADMINISTRATION PREVENTS CELLULAR SENESCENCE

Endothelial progenitor cells (EPCs) are critical to the restoration and upkeep of endothelial tissues (Krautkramer et al. 2014; Kaur and Bajwa 2014; Sommese et al. 2014; Basile and Yoder 2014). Systemic metabolic abnormalities caused by chronic conditions such as hypertension and diabetes are major contributing factors to the impairment of EPC proliferation and cellular activity. As we will continue to see in this chapter, RES can initiate a wide range of beneficial physiological effects. RES possesses the ability to protect and regenerate many tissues of the body including cardiovascular, liver, and bone. Clinical data have revealed RES's ability to neutralize and reverse complications associated with condi-

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tions such as cardiovascular disease and diabetes both of which diminish the vitality of EPCs. Additionally, the dose and time dependent administration of RES *in vitro* have been documented to increase the proliferation, migration, and cellular adhesion of progenitor cells. Research has revealed that there are many causative agents to cellular aging (senescence); one of the more widely investigated causes of senescence is the decrease in telomerase activity. Telomerases are ribonucleoproteins that incorporate DNA sequence repeats to the 5' ends of eukaryotic chromosomes (Zappulla and Cech 2006; Li and Ye 2006; Hamma et al. 2005; Lee et al. 2003; Blackburn 2000; Collins 2000). These incorporated regions of DNA repeats are noncoding and prevent the loss of DNA from chromosome ends during replication (Xia et al. 2008). Many scientists proclaim that the malfunction of telomerases is a key initiating factor that triggers cellular senescence. Recent publications have shown RES to inhibit EPC senescence in a dose dependent manner with the greatest efficiency observed at 50μM. However, cellular senescence increased at high concentrations of RES, namely 100μM hinting at cytotoxic effects of RES at high doses, which confirms the findings of related research (Xia et al. 2008). Through the analyzation of various experimental assays RES demonstrated the ability to increase the proliferation/migration of EPCs and upregulate telomerase activity (Delucchi et al. 2012; Xia et al. 2008; Lefevre et al. 2007).

RES INCREASES MYELINATION OF NERVE CELLS

The myelin sheath (a plasma membrane that encases the axon of nerve cells) originates from the Schwann cells (SC) of the peripheral nervous system (PNS) (Payne et al. 2012; Zhang et al. 2012; Baird and Furek 2012). It is critical for a properly functioning nervous system. Information received from external stimuli is processed rapidly via the sheath along the axon of the nerve cell and initiates an appropriate response. SC health and vitality is fundamental in the production and maintenance of the myelin sheath. Pathologies of the PNS can lead to the deterioration of SCs and by default the myelin sheath resulting in the manifestation of clinical symptoms associated with malfunctioning nerves (Payne et al. 2012; You et al. 2013; Chen et al. 2013; Ho et al. 2012). Therefore, research into compounds that possess the ability to enhance the proliferation and regeneration of both of these structures are of the utmost importance. Scientific have researched the effects white and red wine, the latter which consist of RES, on the generation of SCs using an embryonic murine tissue model. According to various publications, red wine contains an approximate tenfold higher concentration of phenolic compounds as compared to white wine. Compounds such as catechin, epicatechin, gallic acid, anthocyanins, and RES were not even detectable in white wine (Stettner et al. 2013). The addition of both wines to model nerve cells resulted in the increase of myelin synthesis, but cells treated with red wine experienced significantly higher levels of myelination. This result is mostly likely due to the higher prominence of phenolic compounds, most notably RES, found in red wine. Additionally, investigators administered red wine exclusively to murine model cells and analyzed the effectiveness of different RES concentrations within red wine. The work revealed that RES introduced at higher doses in fact decreased myelination, "High RES concentrations (>mg/1) lead to a significant decrease of myelin synthesis, whereas RES administered in a lower concentration (~0.5 mg/1) resulted in a significant increase of myelin production (Stettner et al. 2013). These results confirm data from previous studies, which is that the administration of RES in moderate to low doses is most effective in myelin synthesis (Stettner et al. 2013; Wu and Ren 2009).

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CONCLUSION

The information expounded upon in this discussion and other documents not covered in this chapter well establish RES's regenerative capabilities. RES is truly an amazing compound fashioned by Mother Nature that offers many curative properties with little to no side effects which makes it such an attractive alternative to traditional medical treatments (Zunino and Storms 2009; Sha et al. 2009; Sun et al. 2008; Shakibaei et al. 2008; Larrosa et al. 2003; Alkhalaf et al. 2008; Ma et al. 2007; Li et al. 2007; Garvin et al. 2006; Uguralp et al. 2005; Su et al. 2005; Rigolio et al. 2005; Pozo-Guisado et al. 2005; Ito et al. 2003; Pozo-Guisado et al. 2002; Morris et al. 2002). Research into the healing powers of Natural compounds such as RES could usher in a new era of medical research and play a major role in revealing some of the root causes of chronic diseases ailing populations worldwide. Future research into exactly how RES mediates such powerfully positive biological effects will endow the scientific community with the knowledge of how to most effectively leverage this amazingly versatile compound against systemic health problems within society.

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KEY TERMS AND DEFINITIONS

Cytokine: Various proteins secreted by cells that elicits effects/actions on target cells.

In Vitro: Within a controlled experimental system.

In Vivo: Within a live organism/specimen.

Nanodiamonds (NDs): Carbon nanoparticle (diamond allotrope) harvested from explosive mixtures such as trinitrotoluene (TNT) and dynamite.

Physiological: Of or pertaining to the normal biological functioning(s) of an organism.

Regenerative: The tendency to reform, renew, or replace.

Resveratrol: Polyphenolic compound found in the skin of red grapes, blueberries, select vegetable foods, and peanuts. Used medicinally as an anti-oxidant and anti-inflammatory.

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Chapter 14
Strategies for Expanding Access and Improving the Quality of Pharmaceutical Services

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ABSTRACT

Pharmaceutical services are among the most accessible healthcare assistance systems worldwide, being provided generally in enterprises like Drugstores and Compounding Pharmacies. Pharmacists are highly accessible healthcare professionals considering also the availability, geographic distribution and location of pharmaceutical enterprises. However, there are several challenges for providing these services for patients with limitations such as low education, difficulties on reaching the Pharmacist, and the need for individualized monitoring (due to the complexity of therapy). Reports of low quality services are growing worldwide, and in order to expand access and improve the quality of pharmaceutical services, Pharmacists must move from being medication dispensers with focus in administrative management to a clinically-oriented practice with a humanistic view. The aim of this chapter is to make an approach on the implementation of effective strategies and ways to improve the quality of Pharmacists' work as specialized healthcare providers.

INTRODUCTION

If Pharmacists do not take their place in science and in professional practice, our future is condemned to be like inflammation and apoptosis. (Personal communication with Professor Flávio Figueiredo, Pharm.D, MSc).

Perception and satisfaction of patients with healthcare services are increasingly being considered important parameters to assess the quality and competence of care providers and the outcomes and impacts of their services in patients' health and in society. The World Health Organization (WHO) defines quality of care as the coverage to which the care provision achieves the most favorable outcomes when balancing risks

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and benefits in a given financial setting, and because of the worldwide rise in medication use in recent years, this subject has gained more attention among Pharmacists and pharmacy practice research (Schulz et al., 2001; Kheir et al., 2004; Patwardhan et al., 2014; Sakharkar et al., 2015; Shemeikka et al., 2015).

Pharmacy profession has improved to include the provision of services beyond the traditional role of medication dispensing. Pharmacists are highly accessible healthcare professionals considering also the availability, geographic distribution and location of pharmaceutical enterprises like drugstores and compounding pharmacies. Historically, the main role of pharmacy is to dispense medications and provide patients enough instructions regarding their correct use. The Pharmacist is usually the first professional addressed by a patient with minor or complex health issues, commonly due to the scarcity of physicians, or even due to financial barriers to private clinical services when public healthcare services cannot address patients' needs. Conversely, in spite of this context, differently from other health professionals, providing patient care is not an imperative activity for Pharmacists, as several technical fields of the profession (comprising both privative and non-privative activities) do not necessarily involve direct contact with patients (Hepler, 1996; Schulz et al., 2001; Kheir et al., 2004; Wiley et al., 2014; Penm et al., 2015).

The World Health Organization has pointed that more than half of the world population cannot access the medication they demand. Prices are often too high for people or health systems to afford, and therefore, spends on medicines are a major part of the total healthcare budget. Governments must control the cost of drugs through specific policies and make sure that essential medicines are not overpriced. Also, policies for pharmaceutical services must be included, given that the Pharmacists are the most appropriate professionals to provide the correct and safe use of medications, and to analyze, consider and include pharmacoeconomic and pharmacoepidemiological factors in the organization of drug dispensing (Malone et al., 2001; Murray et al., 2009; Penm et al., 2015).

In some countries, in addition to counseling patients on appropriate drug administration, dosage, side effects, storage and interactions, Pharmacists are allowed to prescribe over-the-counter and other types of medications in several countries, such as phytotherapics and homeopathic products, what can help patients to take decisions on self-treatment with medications. This helps to explain in parts why patients often seek advice in pharmacies, and frequently do not consider a physician consultation, which are also, sometimes, not accessible for patients, generally due to the scarcity of professionals. Nevertheless, there are several challenges for providing these services for patients with limitations such as low education, difficulties on reaching the Pharmacist, the need for individualized monitoring (due to the complexity of therapy), and reports of low quality services are growing worldwide. These facts have been requiring Pharmacists to rethink the way in which pharmacy practice is performed (Lewin, 2013; Sakthong et al., 2013; Sakharkar et al., 2015; Fisher et al., 2015; Malewski et al., 2015).

Pharmacy is a very dynamic profession, given that patients' and society needs regarding drug therapy are changing continually. Evidence-based pharmacy practices are the key to provide effective pharmaceutical services and to improve the quality of healthcare as a whole. In this sense, Pharmacists must move from being medication dispensers with focus in administrative and logistics management, to be patient-focused and clinically-oriented care providers, with more responsibility and commitment to improve their technical knowledge, but with a humanistic view of practice. Thus, the aim of this chapter is to discuss the implementation of effective strategies to expand the access of patients to pharmaceutical services focusing in PC, and ways to improve the quality of such services. Data regarding patients' views and satisfaction with Pharmacists' performance as a specialized healthcare provider, and Pharmacists' perception of receptiveness and evolution of patients will be also discussed in light of evidence-based practice (Straus, 2007; Sakharkar et al., 2015; Cho et al., 2015; Malewski et al., 2015).

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BACKGROUND

Pharmaceutical Services: In Way of Changes

Pharmaceutical services are generally designed in agreement to medicines policies in order to ensure access, affordability, and delivery of essential medicines to the population. Usually, they comprise medication dispensing, counseling on rational use, and logistic organization of dispensing services accordingly to local epidemiologic data. Strategies for the rational use of medications are than planned to promote safe and cost-effective treatments, considering regulatory and quality standards defined by local laws. Pharmacists should, therefore, develop mechanisms for regular review or new proposals of formularies, guidelines and policies regarding drug dispensing and prescribing; therefore, developing trained human resources for this field is of paramount importance, and efforts have been made in several institutions to provide this improvement in different levels of pharmaceutical education, such as PharmBSc (Bachelor of Science in Pharmacy), PharmMS (Master of Science in Pharmacy) and PharmD (Doctor of Pharmacy) (Vijayanarayana et al., 2014).

Pharmaceutical services are among the most accessible healthcare assistance systems worldwide, being quite often provided without additional charges (beyond medication prices) by Pharmacists in enterprises like Drugstores and Compounding Pharmacies; yet, some of these services can be charged, for instance, pharmaceutical consultations, the time spent in blood pressure measurements or preparation of healing bandages. The published experience on the traditional way that pharmaceutical services are established and offered to the population has shown that they have historically been working relatively well for patients in conditions such as: a) common and controlled acute or chronic diseases, b) use of not many medications (like two or three formulations), c) simple therapeutic schemes, and d) adequate education level to understand pharmaceutical advising on rational use of medications (Straus, 2007; Citrome, 2011; Fisher et al., 2015; Gabr & Shams, 2015).

However, what can Pharmacists do to patients with limitations such as low literacy, difficulties on reaching the Pharmacist, or unusual needs like individualized care and monitoring for complex diseases and/or drug therapies?

The answer is neither simple nor easy, and varied barriers can be mentioned. However, these difficulties can be overcome with adequate strategic planning, training and management. In this chapter, I will present different strategies for improving and expanding pharmaceutical services which have provided encouraging results that strongly indicates their effectiveness in providing pharmaceutical services with equity, justice, and addressing patients' needs.

PHARMACEUTICAL CARE

Overview of the Implementation Process

Since the 1990s, Pharmaceutical Care (PC) has been described as an important service and element of pharmacy practice and healthcare, in which Pharmacists are responsible for the quality. Because of the extension of this subject, the approach of this chapter is limited to demonstrate how PC can influence pharmaceutical services to become more accessible and with a broader coverage of patients.

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PC is a pharmaceutical service designed to achieve definitive therapeutics outcomes through preventing or solving drug related problems, and is generally defined as a direct provision of drug related care by the Pharmacist through health education, in order to improve patients' quality of life when using medication. It represents a shift of practice in pharmacy from being drug product-oriented to a patient-oriented view (Hepler & Strand, 1990; Penna, 1990; Cipolle et al., 2004; Dias-Souza & Barros, 2012c). As described by Hepler (1996), PC services comprise:

1. Recognition and correct identification of clinical conditions requiring safe and efficient drug therapy;
2. Prescribing for definite outcomes;
3. Dispensing and patient counseling, involving the patient or family/caregiver in all decisions;
4. Monitoring and management of outcomes;
5. Documentation of all actions.

PC activities also include medication dispensing, providing education on drug use through patient counseling, drug use monitoring, preparation of formulations for parenteral nutrition, monitoring adverse drug reactions, development of drug use guidelines, and cooperation with the healthcare team; these are offered in a mutually beneficial exchange way, in which the patient asks and consents to be supported by the Pharmacist regarding pharmacotherapeutics needs, and the Pharmacist accepts the responsibility to provide drug therapy support with competence and commitment. The Pharmacist provides this service for one patient each time, but groups can also be reached by PC services, in consultations that vary in time and frequency according to the patient's needs (Dias-Souza & Barros, 2012a; Alturkia et al., 2013; Cheng et al., 2015).

The steps of pharmaceutical consultations include (but are not limited to):

1. Collection and organization of patient-specific information (such as demographic data, body mass index and blood pressure);
2. Identification of health problems;
3. Analysis of all the medication being used, in order to identify potential drug-related problems (DRP) like dosage form, therapeutic schemes, administration, and drug-drug and drug-food interactions;
4. Patient counseling on pharmacological and non-pharmacological management of the disease;
5. Monitoring of the desired outcomes through clinical investigations.

These steps will be discussed in the following paragraphs.

In PC services, the Pharmacist work in concert with the patient and other healthcare professionals to promote health, prevent diseases, and to assess, monitor, and when necessary, modify medication use in order to assure that drug therapy regimens are adequate, safe and therefore, effective. PC services should optimize quality of life regarding drug therapy, and achieve positive and definitive clinical outcomes within realistic financial expenditures (Malone et al., 2001; Wait, 2005; Straus, 2007).

The implementation of PC depends essentially on data collection and documentation systems that register patients' information, such as demographic data and clinical history, communications with other professionals like nutritionists, physician in charge, dentist, and also for scientific research purposes, like the study of medication use profile. Moreover, specific equipments are needed to assess drug therapy adherence and effectiveness, and also patient education materials, as described in the previous section.

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Interactions between the Pharmacist in charge and the patient (and the family and/or caregivers, when necessary) should occur to assure efficient mutual communication and cooperation, and that the decision making process on drug therapy is shared by the Pharmacist and the patient. The Pharmacist uses technical knowledge and skills to optimize drug therapy and the patient agrees to supply personal information and actively participate in the process of PC (Pispati, 2003; Cohen et al., 2009; Schmelz et al., 2010).

Nevertheless, varied barriers have been described for the implementation and success of PC services (Schulz et al., 2001; Lada and Delgado, 2007; Maher et al., 2011; Kaufmann et al., 2015; Kuhmmer et al., 2015; Pauwels et al., 2015; Srivastava & McGuire, 2015), and some of them will be briefly presented:

- **Access to Effective Drug Information Resources:** Some professionals have scarce training in searching for evidence-based data in articles, for example. This can be overcome with specific training in articles search platforms;
- **Access to Full Patient Information:** This happens especially when the Pharmacist is dealing with the caregiver and not directly with the patient, or when the patient has some type of limitation that affects this parameter;
- **PC Courses in Graduate and Undergraduate Curricula:** Many pharmacy faculties still do not give attention to social activities of Pharmacists;
- **Comprehensive Understanding to Care Concepts:** PC is still often regarded as pharmaceutical assistance and vice-versa;
- **Continuity of Quality Improvement:** The lack of interest and motivation for quality improvement is very common when the Pharmacist is not properly valued;
- **Counseling Guideline or Plan for Effective Patient Counseling:** This is a critical point in PC: although patients have different needs, all steps in PC must be standardized when possible, to guarantee that care will be delivered to all patients in a similar way;
- Disparity of cultures and believes, often affecting the use of medication;
- **Poor Documentation of the Provided Care:** For an effective follow-up, all actions must be registered and properly saved;
- **Fear of Change to New Profession and Poor Education in Communication Skill:** The lack of training in these is the most critical issue regarding human resources in PC. However, it is possible that trainings with strategies like the simulated patient may help to improve this ability;
- Education in skills needed to deal with technology;
- Financial capital to start the service;
- Improper utilization of registration formularies;
- Inadequate private counseling area;
- **Lack of Insurance Assistance:** Most insurance services world-wide do not cover pharmaceutical services at all;
- **Legislation Barriers:** In some countries, pharmacy clinical practice is still poorly regulated, what may cause doubts when implementing PC;
- **Pharmacy Location:** Sometimes, the location makes it difficult for patients to reach the Pharmacist;
- **Professional Resistance to Interactions with the Pharmacist:** Often, some prescribing or care professionals do not interact with the Pharmacist. A good alternative is to try a personal contact and provide scientific literature as an ice breaker;

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- **Patient Interest:** Not all patients will be interested in PC. When the patient is adult and in his own, there is not much to do but to show the advantages of PC. When the patients demands family or caregiver support, it is important that the Pharmacist clearly shows how much the service can help the improvement of quality of life, promote financial savings and adequations on the use of medication;
- **Patient Literacy and Socioeconomic Status:** This is also critical for the patient that demands instructions. Memorizing is not always possible because of the load of information. However, some alternatives will be shown further in this chapter;
- **Profession Fees to Reward Care Services:** PC services are charged in some countries. Although it is a fair practice, not all patients with real need of PC services can afford them;
- **Software for Medication Assessment Support:** To avoid handwritten formularies, softwares for registration of the activities are necessary;
- Space and layout within the pharmacy;
- Trained Staff.

Pharmacists must collect subjective and objective information regarding the patient's general health status, past medical history, medication use history, social habits history (like alcohol use, smoking), diet, physical activities routine history, history of the current clinical condition, financial and insurance status, when necessary. To perform this, Pharmacists should ask patients for access to medical prescriptions, charts and reports, and should create health/physical assessment instruments. Some validated instruments have been published elsewhere, and often, adaptations are not required. Furthermore, interviews with family members or the caregiver are important, mainly for pediatric or senior patients. Other healthcare providers including physicians, nurses, and mid-level practitioners should also be contacted when necessary. All this information will form the basis for decisions on the development and modification of therapeutic schemes. Therefore, patient data must be updated, accurate, complete, organized and recorded with operational standard procedures, to assure that they are readily retrievable and recorded confidentially (Stewart et al., 1995; Hepler, 1996; Cipolle et al., 1998; Isetts et al., 2003; Dias-Souza et al., 2012a; Holtzman et al., 2015; Kaló et al., 2015).

Pharmaceutical consultations have professional limitations such as providing diagnosis; however, the experience of patients in pharmaceutical consultations generates opinions and views for both patients and Pharmacists, which are important tolls to evaluate and optimize the quality of services and to assess the need for new services. To perform PC services, Pharmacists must have characteristics like being a caregiver, communicator, decision-maker, teacher, researcher, leader, and manager, in order to offer individualized care to patients (Hepler, 1996; Cipolle et al., 1998). Conversely, the experience reported in the literature describes lack of training in such abilities during academic life (Sakharkar et al., 2015).

All the information is then evaluated and a drug therapy plan is developed with the patient. Based on a thorough understanding of the clinical picture of the patient and the treatment options available and affordable, the Pharmacist plans an outcomes-oriented drug therapy plan. For this, the Pharmacist considers not only clinical aspects of the disease, but also psychological and social consequences, including the complexity of therapy and alternatives to improve patient adherence (Hepler & Strand, 1990; Cipolle et al., 2004; Weiss et al., 2015).

Regarding medication use, the patient should be instructed about side effects, important negative or positive interactions with food and other medication, and eventually, of other therapeutic options and non-pharmacological complementary alternatives. The therapeutic therapy plan must be documented in

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the patient's pharmacy record and communicated to the patient's other healthcare providers when necessary. The therapeutic plan must be completely explained to the patient, including the responsibilities shared with the Pharmacist. Information should be provided to the patient at a level that the patient will understand; thus, appropriate language should be used, avoiding, for example, detailing drug mechanism of action. At the end of the consultation, the patient must know how to take the medication, why it is being administered, for how long it will last, risks and benefits of the use and what other health-related behaviors can increase quality of life (Seybert et al., 2008; Dias-Souza & Barros, 2012b; Dias-Souza & Barros, 2012c; Ross et al., 2015).

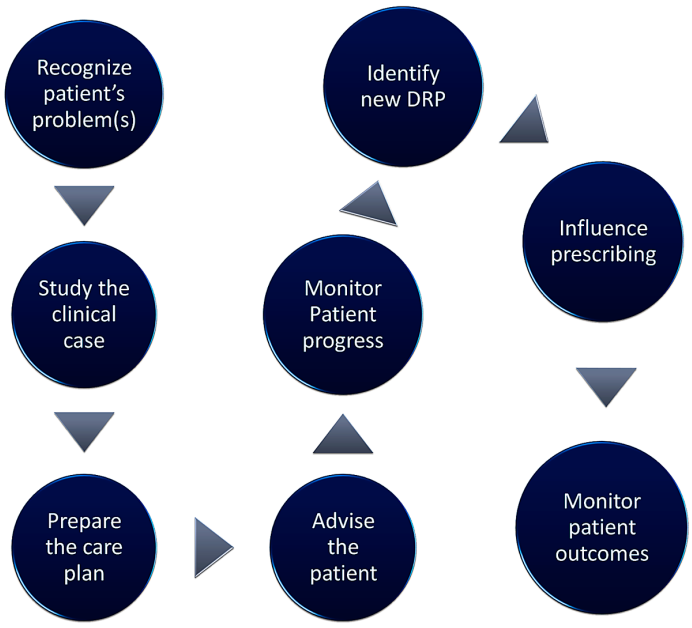
A short guide on how to start a PC consultation will be described as follows, and will be summarized in Figure 1:

Data Collection

An initial interview should be conducted with the patient/family/caregiver for establishing a professional working relationship and to open the patient's record, with confidentiality and privacy. In this first interview, all patient's needs concerning drug therapy should be met, therefore, sufficient time (often 30 to 60 minutes) is spent to assure that questions and answers are provided without embarrassment or hurry. The interviewee should be comfortable with the questions, and for scientific and professional protection of both Pharmacist and patient, a consenting form approved by an Ethics committee should be signed by them (Penna, 1990; Kroenke, 2001; Cadogan & Ryder, 2015).

Standard procedures should be used to systematically record patient-specific subjective information, including health and activity status, past medical history, medication history, social history (including economic situation), family history, and history of present illness. Patients' perceptions, thoughts, beliefs

Figure 1. PC consultation flow summary. Modified from Hepler (1996).



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and feelings of the diseases should be included on the formulary. When necessary, physical assessment techniques like blood-pressure monitoring, capillary measurements of lipids and sugars, should be appropriately performed to obtain patient-specific objective information (Toubro et al., 1999; Schwartz et al., 2008; Sancar et al., 2011; Safaeian et al., 2015).

The Pharmacist must guarantee that patients' records are properly organized, kept updated, and with all important points discussed in all consultations.

Evaluation of the Information Obtained at the Interview

After the patient left the consultation office, the Pharmacist begins an evaluation of the subjective and objective information gathered from the patient and 1) investigates what drug-related problems are happening or can happen in the current clinical picture; 2) analyses opportunities for optimizing the safety, effectiveness, and financial aspects of the drug therapy; 3) discuss the conclusions with the patient in the second consultation. The discussions with the patient, as necessary and appropriate, should guarantee an appropriate understanding of the clinical picture, treatment strategies and administration schemes, and what will be necessary to do for an effective management (Hepler, 1996; Cipolle et al., 1998; Cipolle et al., 2004; Cheng et al., 2013; Al-Quteimat & Amer, 2014).

Preparation of the Care Plan

This preparation happens during step b. The Pharmacist identifies, evaluates and then chooses the most appropriate actions to improve and/or assure the safety, effectiveness, and/or cost-effectiveness of current or planned drug therapy, and to minimize the existing or potential drug-related problems. The care plan is prepared according to the desired outcome. The plan may include work with the patient as well as with other health professionals to provide therapy protocol adjustments or to modify prescribed drug therapy, implement clinical monitoring, recommend alterations on diet, add non-prescription medications or non-pharmacological treatments, or recommend a visit to a specific professional (Spencer et al., 2005; Montgomery et al., 2008; Petrillo et al., 2011; Vijayanarayana et al., 2014).

For each identified problem, the Pharmacist should consider each of the patient's needs and determine the desirable and mutually agreed outcome and incorporate these to the care plan. The plan may include specific disease state and drug therapy endpoints and monitoring endpoints. The Pharmacist reviews the plan and desirable outcomes with the patient and with other healthcare professionals when it is necessary (Odedina & Segal, 1996; Campbell & Saulie, 1998).

Implementing the Care Plan

The Pharmacist take the steps necessary to implement the plan, which include contacting other health professionals when necessary for any modifications on drug therapy, counseling the patient and/or the caregiver about the correct use of the medication and all problems associated to the incorrect use, coordinate the acquisition of medications and related supplies and begin the clinical monitoring. The Pharmacist should maximize patients' understanding and involvement in the care plan, guaranteeing that the parameters for drug therapy monitoring like blood pressure, molecular markers, blood glucose and cholesterol testing, are made and understood by the patient, family and the caregiver (Moore, 1998; Spitzer et al., 2001; Diez-Quevedo et al., 2001; Ojasalo, 2001).

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The patient should be instructed on how to properly use all medications and related products when necessary. Information should be provided to the patient in an easy way to understand. All this information must be registered in the pharmacy record, as well as the steps taken to implement the plan including the appropriate baseline monitoring parameters (Knapp et al., 2004; Spencer et al., 2005; Austin et al., 2006, Nkansah et al., 2008).

Monitoring Outcomes and Modifying the Care Plan

The Pharmacist should review subjective and objective monitoring parameters in order to determine if the desired outcomes will be achieved as outlined in the drug therapy plan. If any problems are detected, the Pharmacist must make the required modifications with the patient, family and caregiver. As progress towards outcomes is achieved, the Pharmacist should provide positive reinforcement and update the care plan. The Pharmacist uses appropriate professional judgment to assess the need to notify other healthcare professionals involved in the patient's case about the registered evolutions (Classen et al. 2002; Brown et al., 2003; Cranor & Christensen, 2003; Malone et al., 2003; Osterberg & Blaschke, 2005; Scuffham et al., 2008; Fiz et al., 2011)

Establishing evidence-based practice is important for effective, updated and relevant PC services. Furthermore, the implementation of PC is relevant to patients because not only drug therapy will be optimized, but complex pharmacological regimens prescribed may be restructured or simplified by the Pharmacist in order to make it more understandable to the patient. This becomes even more important when we consider that a single patient may visit more than one physician in a given clinical condition and get different prescriptions, and the communication among them about drug prescription is often insufficient. Additionally, medication costs often consume a significant proportion of patients' and public health financial resources, what makes PC services economically relevant. Different pharmacoeconomics analysis described that significant financial savings could be achieved by patients, institutions and health insurances through PC services (Isetts et al., 2003; Kiel & McCord, 2005; Morello et al., 2006; Nkansah et al., 2008; Dias-Souza & Barros, 2012b; Lewin, 2013).

Different methodologies of PC have been used worldwide, and the most explored (and published) will be presented briefly:

A. **PWDT (Pharmacists Workup of Drug Therapy):** In this method, patients are assessed by a single Pharmacist for the all drugs used for any health problems that the patient might be affected by, and the DRP are analyzed for each formulation used. Therefore, if the patient has migraine and diabetes, the same Pharmacist is responsible for optimizing the drug therapy of both diseases (Isetts et al., 2003; Kiel & McCord, 2005).
B. **TOM (Therapeutic Outcomes Monitoring):** In this method, patients are assessed by more than one Pharmacist for the drugs used for each health problem that the patient might be affected by, and the DRP are analyzed for each formulation used. Therefore, if the patient has asthma and arthritis, a specialist Clinical Pharmacist in asthma and another, specialist in arthritis, shall be visited (Dias-Souza & Barros, 2012b; Dias-Souza & Barros, 2012c)
C. **Dáder:** This method was developed by a Spanish group of PC headed by Maria José Fáus-Dáder, and has similarities with PWDT in its organization and coverage of diseases, but DRP are analyzed in a different way. This method is widely used also in Latin America (Amariles et al., 2012).

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Other methodologies for PC services include FARM (Findings, Assessment, Resolution and Monitoring), DOT (Direct Observed Treatment), and SOAP (Subject, Objective, Assessment, Planning) (Amariles et al., 2012).

Providing Written Information in PC

A critical point regarding PC is that most of the counseling offered by Pharmacists to patients is provided orally, and hardly ever the patient or the Pharmacists take notes of the information in a standardized and validated way. As a result, patients may misunderstand or forget important details like drug onset of action and side effects, leading to poor compliance problems and negative results. Doubts regarding the need for therapy may arise and complex therapeutic schemes, usually difficult to be managed by patients without professional support, also contributes to poor compliance. The use of written and/or pictorial aids and other educational approaches is still scarce in pharmacy practice, and here, I would like to encourage the reader to explore this resource to improve the access of patients to pharmaceutical services. In this sense, previous works of our group provided evidence of the relevance of written information for patient counseling and education on drug administration (Worley-Louis et al., 2003; Worley et al., 2007; Seybert et al., 2008; Dias-Souza & Andrade, 2010; Dias-Souza & Barros, 2012c, Khan et al., 2013).

A clinical case of chronic pain in an elderly patient had drawn our attention at first: because of the use of several medications, the patient had difficulties in administrating them correctly, and several interactions were detected in the first pharmaceutical consultation. We reorganized the therapeutic scheme and provided handwritten information in a simple and accessible language such that the elderly patient could easily understand. As the clinical monitoring followed, the evolution of the patient was positive and pain was finally under pharmacological control. We recommended physical therapy sections for the patient, and advised for a clinical-functional assessment by a Physiotherapist (Dias-Souza & Andrade, 2010).

Nevertheless, all this was directed for a single patient. How to deal with several people that simultaneously demand specific written information like this patient? And how to do this for so many possible medications – isolated or combined – in compounding pharmacies or drugstores? A possible answer comes in a study developed by our group. The development of drug use instructional cards (DUIC) for different pharmacological groups and its use in compounding pharmacies was described in 2012 for the first time (Dias-Souza & Barros, 2012c).

The DUICs were developed due to a rising demand of basic advising on the correct use of medications on the pharmaceutical setting where the work was developed (a Brazilian compounding pharmacy located at the state of Minas Gerais). Using patients' complaint formularies (PCF) – a document that the good manufacturing practices (GMP) laws from the National Sanitation Regulatory Agency (ANVISA, an agency equivalent to the FDA in the U.S.A.) obligate compounding pharmacies to have in order to register any complaints from patients or clients – we have explored the most frequent drug-related problems to delineate how the DUICs should be designed. The parameters we considered were:

1. Drug-drug interactions
2. Drug-food interactions
3. Correct way and time of administration
4. Storing the dosage form
5. Experienced side effects and potential toxicity

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After analyzing these data, we performed an extensive literature review for collecting technical information for correct drug using, regarding:

1. Most common drugs and indication;
2. Pharmacokinetics and metabolism;
3. Drug-drug interactions (obtained at the drug interaction checker in Medscape website);
4. Drug-food interactions;
5. Basic Nutritional Support;
6. Best time of administration;
7. Pregnancy, lactation and fertility impairment warnings;
8. Contraindications;
9. Common Clinical Adverse Reactions.

DUICs were then developed for the following groups:

1. Antihistamines;
2. Antimicrobials;
3. Anti-hypertensives;
4. Anti-lipemics;
5. Proton pump inhibitors
6. Non Steroidal Anti-inflammatory Drugs (NSAID);
7. Psychotropic drugs

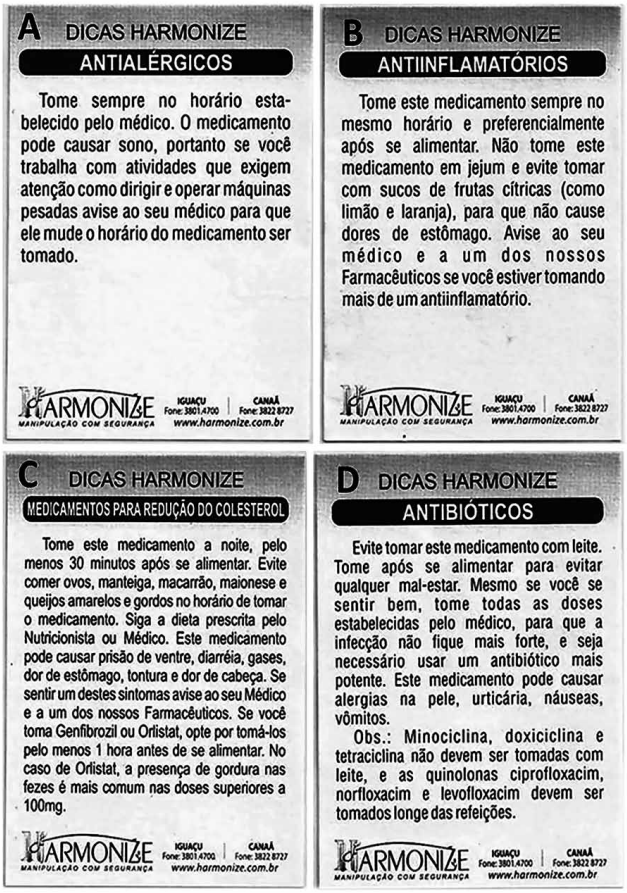
After, the design was created and adjusted for the size of the document, such that it could be attached to the medication that was dispensed through the delivery service or at the counter. DUICs samples (in Portuguese) are shown in Figure 2.

We showed through our data that the DUICs not only were effective in counseling patients in a standardized way, but also improved telepharmacy services: the basic instructions provided in the DUICs could be assimilated by non-Pharmacists attendants, and some basic information about drug use could be rapidly provided for patients. Although the DUICs were planned (and are still being used) for compounded medication, the same instructional material can be adapted for industrialized medication, for which, information commonly found on package inserts has been described as poorly designed by professionals and not completely understood by patients. Also, patients often describe that written information on prescription drug labels are confusing or incomplete.

To overcome these problems, Kripalani et al. (2007) developed illustrated medication schedules for helping low-literacy patients to administrate medications correctly. The development of the cards design began by raising the needs of the patients through surveying local Pharmacists by telephone and posted emails on national pharmacy list servers, asking about the use of medication aids, visuals, or other patient education tools. Physicians with expertise in medication adherence, patient education, and literacy helped to draft the cards, using pictures to illustrate instructions to follow drug therapy schedules. A multidisciplinary team of healthcare providers then reviewed the content and format of the design draft. Twelve patients of different age and educational attainment reviewed sample pill cards to provide input into the types of pictures used, the size of the text and overall document, and the desired content. The instrument could maximize comprehension across levels of health literacy, providing only the most es-

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Figure 2. DUIC samples (available only in Portuguese) developed by Dias-Souza & Barros (2012c): a) antihistamine drugs; b) NSAID; c) antilipemic drugs; d) antimicrobials. DUICs are still being distributed for patients that are costumers of the compounding pharmacy where the study was conducted



sential medication instructions like name, purpose, appearance, and time of administration. Patients with inadequate or marginal literacy skills, less than high school education, or cognitive impairment were most likely to refer to the card on a regular basis initially and after 3 months. Patients found it easy to understand and helpful for remembering important medication information, such as the name, purpose, or time of administration.

Kassardjian et al. (2008) validated a Pictorial Representation of Illness and Self Measure (PRISM) as a measure of suffering in patients with chronic non-cancer pain. Patients were recruited from 2 hospital pain clinics, and the majority of them were middle-aged women, in whom chronic pain is more prevalent and also more treated with medication. For that task, patients were shown a letter size white paper with a fixed yellow circle glued in the bottom left-hand corner of the piece of paper. Then, they were asked to imagine that the paper represented his/her life and that the yellow disk represented his/her concept of self. They were given a red paper disk representing their pain and asked to glue it in a location related to the "self" disk, reflecting the impact of pain into their lives. The same request was made regarding work, family, spouse/partner and recreation. Patients glued the disks onto the page in whatever order

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they wanted. Blind patients also completed the task, indicating that it also behaved as a tactile tool. Eight main parameters were assessed: Physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, and mental health. Higher scores in a scale of 0–100 indicated better health-related quality of life. Different situations were identified using this PRISM system: Patients reported that their close relatives were a negative influence to the treatment because they were overly controlling, or did not believe in the state of disability due to pain. Patients with high scores felt controlled by partners or family members, or emotionally distant from them. It was also reported by group of patients that their partner and family have either very little influence over their lives, or a significant negative influence. Patients found PRISM thought-provoking, and it was described by the authors as a quick, simple, and manageable tool for patients with chronic pain to complete.

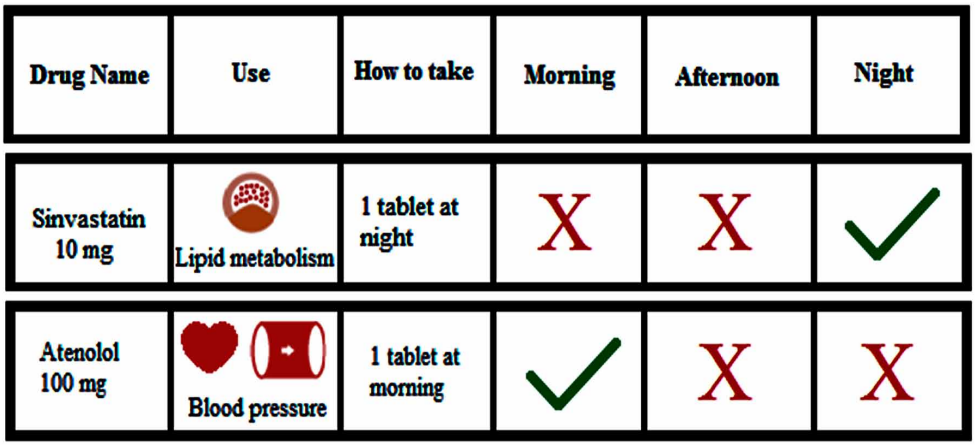
A randomized blind trial comparing picture-based versus prose-based information pamphlets was conducted by Thompson et al. (2010). The authors compared memory, comprehension, and preference for medication information presented in a prose-based medication information pamphlet versus a pictorial-based information pamphlet. Specific information regarding methotrexate was used in this study. A total of 100 participants aged 18–65 years of age, who had not completed high school, could read English, and had no prior knowledge about methotrexate were included. The (better) effects of pamphlet type were assessed using immediate free recall, cued recall and comprehension. In the end, each participant was shown a card displaying both the picture and prose-based pamphlets and asked to decide which pamphlet they felt to be most visually appealing, which they would rather receive, which looked more useful, and which was easier to read. According to the statistical analysis, no differences were detected between picture and prose pamphlets in free recall, cued recall, and comprehension either immediately or after a 1-week interval, and the pictorial pamphlet was preferred over the prose-based pamphlet.

Mohan et al. (2013) conducted research with focus groups and interviews with Latino patients with diabetes at two safety net clinics in the USA to understand medication taking practices and perceptions of illustrated medication instructions. They explored PictureRx™, a software platform that generates illustrated, patient-centered medication lists in a daily medication schedule. The following essential information for patients is included: name, dose, frequency, purpose, and special instructions when needed. The design of PictureRx cards (Figure 3) was evidence-based, drawing on research and best practices for the creation of patient education materials with illustrations for medication instructions and other health-related information. Patients reported confidence in being able to take medications and expressed preference for illustrated medication instructions, which could overcome patients' difficulties on administrating the medication.

Pictorial representations of clinical scenarios for asthmatic patients and of commonly used inhalers devices were developed by Roberts et al. (2010). Initial images were developed, designed and reviewed in consultation with medical and nursing respiratory members of staff before testing it with patients. A computerized software package was developed for the preparation of personalized asthma action plans during a consultation: the health professional should select the options according to the patient's needs. Following a series of sequential screens, the professional identifies appropriate images and scenarios to build a plan. In the end, a pictorial action plan is generated, which should be printed and provided to the patient. The software package automatically checks dosages used, and maximizes inhaled therapy. When the patient uses peak flow for self-monitoring, the program calculates the personalized action levels based upon the reported best peak flow. Users can print out a picture only plan, a words only version or a plan containing pictures and words. According to the authors, the pictorial care plans are

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Figure 3. Adapted schematic sample of how the charts for drug information are disposed on the works developed by Kripalani et al. (2007) and Mohan et al (2013)



quickly prepared to be printed for the patient to take away in several formats, and the data can be stored in the patient's record.

A recent work evaluated the efficacy of a pictorial medication sheet to improve adherence in patients with heart failure and cognitive impairment (Hawkins & Firek, 2014). A total of 36 patients were enrolled. Four study visits spread over approximately four months were performed. At visit one, subjects were asked to bring in all of their regularly prescribed medication for a direct count of administered tablets. After 30 days, in visit two, another tablet count was performed, and adherence was estimated. The subject then received a customized pictorial medication sheet with brief instruction on how to use the pictorial medication sheet that represented their actual prescribed medication regimen. Subjects were asked to use the new sheet in place of any prior medication instruction sheet they may have been using. The medication sheet included of images of the subject's current medications printed in full color and dose range, arranged in columns for morning, noon, and evening administrations. Descriptions of the medication name, indication and dose were also included. Authors reported that although medication adherence was poor, statistically significant improvements (described in tablets counting) were detected after the interventions.

Nevertheless, written information must be used properly, and should be used regardless of problems not related to the patient. A negative example in this sense was described. Hamrosi et al. (2013) examined and compared the use and provision of written medicine information in Australia, focusing in Consumer Medicine Information (CMI) - available for medicines as package inserts, computer prints, or leaflets - by Pharmacists and consumers. The reasons surrounding readership and provision of the CMI and comparisons on its use by both consumers and pharmacists were also investigated. A total of 1000 consumers and 1100 Pharmacists were interviewed by postal or telephone surveys. The authors found that 66% of consumers usually read the CMI looking for information regarding side effects and clinical use of the medication. Reasons mentioned for not reading the CMI comprised previous experience with the medications, enough information was provided verbally from the Pharmacist, and also that the document was too long or contained too much information. Interestingly, the disparity detected by the authors amongst the types of written medicine received by patients and provided by pharmacists is strongly suggestive of incomplete/inadequate (or both) provision of pharmaceutical services. Almost

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90% of pharmacists reported providing package inserts, but 88% of consumers reported receiving them when provided with written medicine information. Personalized computer-generated CMI was provided by 99% of pharmacists, but only 37% of consumers reported receiving it. Around 40% of pharmacists and 25% of GPs (n = 45) also reported providing loose leaflets/brochures yet only 7% (n = 47) of consumers reported receipt.

Telepharmacy Services

In this section, I would like to introduce the need and the context of telepharmacy services, a powerful tool for expanding pharmaceutical services when used correctly. According to the World Health Organization (WHO, 2010), telemedicine is defined as the delivery of healthcare services, where distance is a critical factor using information and communication technologies for the exchange of information for diagnosis, treatment and prevention of diseases, research and for the continuing education of healthcare providers, all in the interests of advancing the health of individuals and their communities. Telepharmacy, following the example of telemedicine, offers pharmaceutical services at a distance, i.e., with the Pharmacist and patient communicating through telephone, videoconferencing systems such as Skype®, or any online communication system such as WhatsApp® and social networks. Online chat services available at the website of the pharmacy are also a good alternative.

And why this is important? Telepharmacy technologies can improve patient access to pharmaceutical services beyond remote medication dispensing, including PC. This sort of communication is especially important when the patient cannot reach the Pharmacist due to several possible limitations like mobility issues (the patient does not have access to a vehicle), or if the patient is affected by a disease that impairs the patient to meet the Pharmacist (Garrelts et al., 2010).

Nevertheless, to use this sort of service, a Pharmacist must work exclusively on it, and must have trained people on using the system for technical support or as workmates, to make the service work in a normal flow. In this service, patients can be advised about any aspects of the medication. Also, as some are not comfortable on going to the pharmacy for several reasons (embarrassment on talking to the Pharmacist face-to-face about the health problem, embarrassment on talking about it in public), the service may make the patient more comfortable on talking to the Pharmacist through typing or by phone.

Bynum et al. (2001) assessed the effect of telepharmacy counseling and using interactive compressed video, on how to use the asthma inhaler and patient satisfaction among adolescents with asthma. The telepharmacy service provided accessible education regarding asthma inhaler techniques for adolescents.

Moser et al. (2004) found 4136 documents using the search term “telemedicine” in MEDLINE from 1964 to 2003. They reported significant correlations between publications per million inhabitants and GDP per capita. Fatehi and Wootton (2012) examined the occurrence of the terms “telemedicine”, “telehealth”, and “e-health” in the Scopus database and a total of 11.642 documents were found in 2012. They found that “telemedicine” was the most common term with 8.028 documents.

Stubbings et al. (2005) described a telepharmacy call center service that was developed at a multidisciplinary health service through collaboration among the health professionals and the health plan to care for patients with medication-related questions. Pharmacists work close to physicians, nurses, and service associates of the call center. The team answered approximately 1.200 calls daily, regarding their transition into the health insurance plan and drug therapy questions. The service improved the quality and cost-effectiveness of drug therapy regimens and significant cost savings were registered.

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The group of Lam et al. (2009) reported telepharmacy-related services and outcomes in western Washington State (USA) from 2007 to 2008. Five network clinics without in-house pharmacies were remotely connected to a central pharmacy to provide telepharmacy services, and automated drug dispensing system (ADDS) units, webcams, and electronic medical charts were used to provide remote medication order processing/dispensing, patient counseling, refill authorizations, and medication assistance referral services. Patient demographics, numbers of dispensed medications, patient consultations, and refill authorizations were registered, and service referrals, Pharmacist feedback and workflow changes as well. Antimicrobial drugs were the most frequently dispensed medication, and Pharmacists described webcam-enabled interviews as an excellent method for better privacy and longer counseling.

Garrelts et al. (2010) implemented a telepharmacy service at five hospitals from a Catholic, nonprofit, integrated delivery network health system. Telepharmacy services were provided by a team of Pharmacists employed by the health system. Using a virtual private network or terminal server, Pharmacists directly accessed hospital servers and information systems to conduct their work. The time spent in routine orders of medication reduced significantly after telepharmacy services were implemented. The number of clinical interventions documented increased and annual savings reached 1 million dollars.

Sankaranarayanan et al. (2014) documented remote Pharmacist interventions for patients at eight rural hospitals in the Midwestern United States during prospective prescription order review/entry from 2008 to 2011. Remote Pharmacists' interventions were divided in three broader-level definitions: (a) interventions in interactions and potential interactions, contraindications, adverse effects, drug product selection, drug regimen and recommendations, (b) patient medication management regarding therapy review and action, and (c) health system-centered medication use process, regarding medication prescribing, transcribing and documenting, administering, and monitoring clinical results. Frequencies of intervention levels were compared by year, hospital, remote Pharmacist, and hospital pharmacy status. Frequency of interventions significantly increased each year, and differed significantly across the hospitals, suggesting that patients' needs were addressed particularly.

Service Quality Perspectives

The quality of healthcare services, included the ones provided by Pharmacists, is a topic of intense discussion. The aim of this section is to briefly present important aspects of the services that should be focused such that patients' perspectives are built on positive experiences.

As mentioned, Pharmacists are key professionals to improve medication compliance among users, given their availability of contact with patients, specific knowledge and skills. The burden of several diseases can be reduced through improving medication compliance, and the implementation of clinical pharmaceutical services has positive impacts on patient care, satisfaction and perspectives on these services. Patients' perceived quality of interaction with the Pharmacist, service facilities and outcomes have a significant effect on a patient's choice to use the medication or not (Khan et al., 2013; Al-Quteimat et al., 2014; Fisher et al., 2015).

Managing quality is clearly not a simple task, given that not only technical and regulatory points of clinical services are involved, but values, knowledge and behavior of the Pharmacists regarding patient care are included. Although there is some agreement on how Pharmacists can manage patients' therapy-related needs, there is less agreement on whether the pharmacists are able to meet those needs. Services are generally designed to be broad enough to meet several clinical needs of patients - such as the so called cognitive services (characterized by direct interaction between patient and pharmacist, making

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use of the pharmacist's knowledge), however, due to the exposed reasons, patients' perspectives of the services are variable. Also, standards to evaluate services vary among patients, once previous experiences are the starting point. All this data, however, is essential for understanding patients' interaction, satisfaction and trust in the Pharmacist (Cho et al., 2015; Malewski et al., 2015; Gabr & Shams, 2015).

Understanding the intricate communication between these features helps us to improve the quality of services. Clinical effectiveness, safety, positive outcomes, patient experiences (self-rated health), proximity, availability and access are the main items to build patients perspectives of the services. However, the pressures for the development of adequate and efficient services can sometimes present divergent logics considering the economic aspects of an enterprise. This technical difficulty should be overcome and negotiated in a way that economic needs of productivity are in line with professional and patients' values regarding high quality health services. This is not a simple endeavor, but in a middle-term perspective, these actions support the growth of the enterprise, as more people tend to look for the service after patients spread the word about the experiences with the service (Dias-Souza & Barros, 2012a; Kaufmann et al., 2015; Kuhmmer et al., 2015).

When patients are engaged in the development of services, it is more likely that they will use these services more frequently (whenever it is necessary) and will be more compliant to recommended treatment plans, leading to the expected health outcomes. The existence of high quality patient-centered services based on reliable and safe patient-Pharmacists relationships, the convenience and ease of access to services and the responsiveness to their therapeutic needs - and when adequate, the quick and safe provision of medication supply, are elements of paramount importance for patients' compliance and development of positive perspectives. These are also important requirements of the patient and caregivers as clients of the pharmaceutical enterprise, especially if the clinical services are charged. Young, highly educated consumers and recently diagnosed patients tend to consider these items with even more importance (Ross et al., 2015; Safaeian et al., 2015).

CONCLUSION

Strategies on expanding and improving pharmaceutical services have been presented in opportunities that are still poorly explored in daily practice: the provision of written information for patients and telepharmacy services. Written information may help patients to remember and reinforce how medication should be correctly taken, and in case of low literacy, alternatives of pictorial support were discussed. Telepharmacy services may help patients and health professionals to quickly reach the Pharmacist and solve drug-related problems, especially when there mobility issues. The implementation of these services in PC context is not a simple endeavor; however, to overcome the technical difficulties, the Pharmacist must work together with patients, families and caregivers, generating solid services, and along with other health professionals. This team work is the key for improving and expanding pharmaceutical services.

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KEY TERMS AND DEFINITIONS

Drug-Related Problems: Any problem on drug therapy, such as side effects, high cost, allergies, poor adhesion to therapy, etc.

Pharmaceutical Care: A model of pharmacy practice in which the Pharmacist takes the responsibility of the pharmacological treatment of a patient and optimizes it, preventing drug-related problems.

Pharmaceutical Consultations: An ambulatorial service provided by the Pharmacist to conduct clinical interviews and assessment; however, no clinical diagnosis of diseases is made.

Pharmaceutical Counseling: A pharmaceutical service to provide patients the most relevant information on the best way of drug use.

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Pharmaceutical Enterprise: Any place where a Pharmacist works with medication or subjects related to medication, like manufacturing industries, compounding pharmacies or drugstores.

Pharmaceutical Services: Activities developed by the Pharmacist for the wellbeing of a patient, considering (or not) drug use.

Phytotherapics: Plant-based natural products.

Side Effects: Adverse reactions due to incorrect or even correct use of medications.

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Chapter 15
An Overview of Therapeutic Applications

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ABSTRACT

Over the last few years' great advances have been made on the development drug delivery systems for different purposes for targeting the diseased conditions. Novel drug delivery originates from polymers or associated with some devices is generally related with the emergence of novel characteristics. These changes are what eventually comprise the value of drug delivery system and Novel drug delivery system. Novel properties become existed without making new materials. Novel drug delivery system comparable to traditional system, following Targeted Drug Delivery System (TDDS) is also called targeting drug system. A new drug delivery system makes the drugs densely gather pathological-change structures, and has an improved healing effect and less toxic side effects. The drugs can improve the strength of pharmacological action and reduce the bad effect all over the body, for they release in the target organs.

INTRODUCTION

The method of treating infection and illness as we enter in next era twenty first century includes the subsequent forms of treatment a. surgery, b. psychotherapy, c. physical therapy, d. radiation and e. chemo or pharmacotherapy. The most commonly used is treatment method (Banker, Siepmann; Rhodes, 2002).

Novel drug delivery system whether it originates from polymers or associated with some devices is generally related with the emergence of novel characteristics. The most extensive results of their minute size, dosage form or targets are without distrust the change in their final properties. Next in significance are changes in other properties, both physical and chemical. These changes are what eventually comprise the value of drug delivery system and Novel drug delivery system. Novel properties become existed without making new materials.

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Role of Targeted Drug Delivery System (TDDS)

Novel drug delivery system comparable to traditional system, following Targeted drug delivery system (TDDS) is also called targeting drug system'. A new drug delivery system makes the drugs densely gather pathological-change structures, and has an improved healing effect and less toxic side effects. The drugs can improve the strength of pharmacological action and reduce the bad effect all over the body, for they release in the target organs (Chaturvedi et al., 2011).

Carrier-mediated drug delivery has emerged as a powerful line of attack for the action of various pathologies. The therapeutic index of conventional and novel drugs is enhanced via the increase of specificity due to target of drugs to a particular tissue, cell or intracellular segment, the control over release kinetics, the protection of the active agent or a combination of the above. Nanoparticles (NPs) were proposed as drug carriers over 30 years ago and have received increasing attention since, mainly due to their stability, improved loading capabilities and control over physicochemical properties. The unique pathophysiology of solid tumors allows passive accrual of NPs at these sites upon intravenous injection. Furthermore, stealth NPs with long circulation times is better organized in reaching tumor tissue.

Drug activity is a result of molecular interaction(s) in certain cells; it is therefore easily deduced that it is essential for the drug to reach one way or another site of action following administration (oral, intravenous, local, transdermal, etc.) at sufficient concentrations. The systematic field dealing with this issue is known as drug delivery and has fundamentally the following aim: to deliver the drug at the exact place, at the right concentration for the right period. This is not possible by simply selecting a suitable administration route, or if such administration causes patient uneasiness, strategies based on the involvement of the drug with a carrier. Additional motivations for such approaches include the reduction of required resources for therapy, accomplished by an increase of the drug's therapeutic index and the prevention of frequent, unpleasant or expensive treatments.

Role of Drug Delivery System Other than TDDS

Drug delivery systems, ranging from implantable electronic devices to single polymer chains, are required to be well suited with processes in the body (biocompatibility) as well as with the drug to be delivered. DDS alter the biodistribution and pharmacokinetics of the associated drug: that is the time-dependent proportion of the administered dose in the different organs of the body. Furthermore, obstacles arising from little drug solubility, degradation (environmental or enzymatic), fast clearance rates, non-specific toxicity, in ability to cross biological barriers, just to mention a few, may be dealing by drug delivery system. Overall, the challenge of increasing the therapeutic outcome of drugs, with a simultaneous minimization of side effects, can be tackled from side to side-appropriate design and engineering of the DDS, in a case-to-case manner (Kelner, 2005; Mann et al, 2001; Pathak et al, 1992).

Even the most difficult part in all Drug delivery systems development actions, the accomplishment of release of the drugs, can be controlled suitably. This is true of both Drug delivery systems and novel drug delivery systems. Because of the extensive history of development, there has been profusion of time to develop perfect Drug delivery systems to novel one. One decisive finding opened up completely novel strategies for both traditional systems and Drug delivery systems the make use of polymers, frequently consisting of organic molecules. Such interesting molecules not only support the delivery systems by their kinetic and size-limiting function; they also make the drugs soluble in solvents, depending on the chemical character of the delivery systems.

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Role of Novel Drug Delivery Systems (NDDS)

However, novel drug delivery systems have also had a notable revival after a hundred years' sleep. This is in particular true of delivery systems. Apart from leading to an increase in our information of delivery systems, these offer unimagined application possibilities. Although novel delivery systems are currently, being used in numerous industrial and medical applications, organized research into the novel delivery systems of drugs needs some attention.

It is apparent, for any variety of chemical approach, that dependence on the present-day pattern of information exchange in logic fundamentals or memory devices will entail defect-tolerant computer architectures with suitable, intellectual software. On the other hand, it may be promising to develop other routes which require more complex architectures and unlike principles of information exchange where the problems associated with idleness, current density, recital speed, and especially contact confrontation and charge redistribution at the lead-to-molecule interface are less relevant (Schmid, 2011).

Role of Novel Drug Delivery Systems with Its Different Application

Most of the anti hyperlipidimic drugs have reduced water solubility hence poor dissolution, which lead to reduced bioavailability. Therefore, when these drugs are given in predictable dosage forms they do not meet the therapeutic requirements therefore are given in large doses, which show the various side effects. Therefore, it is the essential to formulate that type of dosage form in which the drug shows better bioavailability and not as much of side effects (Farooq et al, 2013). Researchers are going on in this field and a variety of novel drug delivery systems are formulated and evaluated which have exposed the intensify the bioavailability of these drugs. Some of the novel drug delivery systems for antihyperlipimic drugs are:

1. **Self-Micro Emulsifying Drug Delivery System:** Self-emulsifying formulations enhance readily in the GI tract, and the digestive motility of the stomach and the intestine make available the agitation essential for self-emulsification. Lovastatin is inadequately soluble in gastric fluid when administered throughout predictable dosage forms like tablet, so SMEDDS are formulated to enhance the solubility of drug in gastric fluid and therefore improving the bioavailability by rising gastrointestinal absorption through passive diffusion. Fenofibrate is a (BCS) Class II drug with a high dose number. Thus, has little oral bioavailability due to its solubility and dissolution limits. An optimized SMEDDS formulation consisting of Labrafac CM10 (31.5% wt/wt), Tween 80 (47.3% wt/wt), PEG 400(12.7% wt/wt), and fenofibrate (8.5% wt/wt) has been developed with an improved dissolution rate, increased solubility therefore, improved bioavailability of a poorly water-soluble drug. Supersaturated drug delivery systems include the drug in a high-energy or rapidly dissolving form, which allows the solution to have drug concentrations higher than the solubility limit. (Cowan-Lincoln)
2. **Ternary Solid Dispersions:** Solid dispersions have been generally used to enhance the solubility, dissolution rate, and bioavailability of poorly soluble drugs. There are different types of solid dispersion systems categorized according to the physical states of the drug and the mover in the systems. It may be a molecular solid solution, a dispersion of amorphous or crystalline drug particles in an amorphous carrier matrix or an arrangement of a solution and distribution of solids. Fenofibrate compound is almost insoluble in water and has high lipophilicity. Therefore, the dissolution rate

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of fenofibrate is likely to limit its absorption from the gastrointestinal tract. The solid dispersion of the fenofibrate was prepared by spray drying method using Poloxamer 188 as carrier and TPGS as surfactant shows highest solubility improvement of fenofibrate. Solid dispersion along with an adsorption method that employs a water-soluble adsorbent can be used to improve the dissolution of Ezetimibe, which may effect from the combined effect of hydrophilic carriers and improved surface area. Many drugs can be given in such form which will improve the efficiency of the drug. (Bhise, 2011; Parmar et al, 2011)

3. **Nano Suspension:** Nano suspensions not only increase the surface area but also at the same time enhance the saturation solubility and the solution velocity by raising the vapor pressure of the particles. Here the dissolution velocity slowly is improved due to decrease in the diffusional distance on the surface of drug nanoparticles. The saturation solubility gets affected through in situ generation of energies throughout alter of drug microcrystals to nanoparticles. Fenofibrate is a Nano suspension currently marketed in the name of Tricor. Nanosuspensionsmake use of some technique to decrease the particle size to the nano level which help to enhance the solubility of the drug suitable to small particle size. (Selvamuthukumar et al, 2012)
4. **Ion Exchange Resins:** Cholestyramine resin USP, when used as an active ingredient, binds bile acids; this leads to replacement of bile acids; through improved metabolism of serum cholesterol resultant in lowered serum cholesterol levels. Ion exchange resins no uncertainty are very effective in improving the bioavailability of the drugs but due to the use of some polymers it also enhances the charge of the delivery system. (Pande et al, 2011; Srikanth et al, 2010)
5. **Nanosponge Drug Delivery:** Nanosponges are a new class of materials and prepared of microscopic particles with few nanometers wide cavities, in which a huge variety of substances can be encapsulated. These particles are capable of carrying equally lipophilic and hydrophilic substances and of enhancing the solubility of poorly water-soluble molecules. This technology offers entrapment of ingredients and is supposed to decrease side effects by controlling the release, develop stability, enhance elegance and enhance formulation flexibility. These complexes can be also used to enhance the dissolution rate and solubility, to cover unpleasant flavors and to convert liquid substances to solids. By calculating the ratio of polymer to the cross-linker the particle size and release rate can be modulated. Nanosponges facilitate the insoluble drugs, protect the active moieties from physicochemical degradation, and controlled release. Atorvastatin, Fenofibrate, Glibenclamide, Glipizide, Lovastatin can be formulated into nanosponges. Nanospongesdevelop the solubility of the drugs efficiently but the technology employed in this process is not cost effective as this technique utilizes some polymers which are costly in nature. (Sharma & Pathak, 2010)
6. **Nanoparticle Formulation:** Nanoparticulate drug delivery as the biological dimension of nanotechnology has been developed, which may help generate useful formulations of OA for clinical applications. Nanoparticulate drug delivery system increases the dissolution rate and bioavailability of OA, providing a practical formulation method for clinical applications. (Chen et al, 2011)
7. **Mucoadhesive Microcapsules:** Simvastatin (SV) is a cholesterol-lowering agent that is resulting synthetically from a fermentation product of *Aspergillus terreus* and is widely used to treat hypercholesterolemia. Microcapsules of Simvastatin were prepared by complexation with HPBCD and by this means including this complex in the polymeric matrix by make use of orifice gelation method resulted in more improved drug delivery. (Bal et al, 2012)
8. **Pulsatile Drug Delivery System:** Pulsatile Drug Delivery System is helpful in the diseases in which the drug release is timed to counterpart rhythms of the disease to optimize the beneficial

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effect and reduce the side effects. Circadian rhythm occurs during hepatic cholesterol synthesis, which is higher during the night than daylight and diurnal synthesis represent upto 30-40% of daily cholesterol synthesis. The maximal production occurs in the morning. So there is a regular require for new delivery systems that can offer improved therapeutic benefits to the patients by delivering drug at the right time, right place & in right amounts to correspond with circadian rhythm of body. These dosage forms offer several advantages, such as almost stable plasma drug level without any fluctuations, decrease in dose of drug, decreased dosage frequency, least side effects, and enhanced patient compliance. Various systems like capsular system, osmotic system, and single multiple unit system based on the use of soluble or erodible polymer coating and use of repturable membrane. (Rajput et al., 2012; Rasve et al, 2011)

Ternary solid dispersion and ion exchange resins are the systems which improve the solubility of drugs largely and hence the bioavailability but it utilizes the variety of polymers which are costly in nature hence they may enhance the cost of the medication. Gastric floating drug delivery system is helpful in improving the solubility of those drugs, which are less soluble in acidic pH, and the drug waste is much more. This system is helpful in prolongs the gastric retention of the drugs and therefore increases the solubility of the drugs. While as pulsatile drug, delivery system is the system, which responds to the circadian rhythms of the body, and as a result, this system is very useful because cholesterol synthesis depends on circadian rhythms. SMEDD Scan is used to improve the solubility of the drugs by lessening the particle size in the emulsion base, which will efficiently enhance the bioavailability of the drug. Among all the systems above SMEDDS seems to be the most successful delivery system for antihyperlipidemic drugs because it is very easy to formulate and does not require any costly polymers, which make it effective in therapy as well as in cost. Many other novel drug delivery systems can be used to enhance the efficiency of these drugs.

Role of Novel Drug Delivery Systems in Cancer

Over the past several years, great advances have been made on expansion of novel drug delivery systems of anticancer drug (NDDS). The selection of novel formulations like polymeric nanoparticles, nanocapsules, liposomes, phytosomes, nanoemulsions, microsphere, hydrogels has been reported using bioactive and plant extracts. The novel formulations are reported to have extraordinary advantages over conventional formulations of anticancer, which include improvement of solubility, bioavailability, and protection from toxicity, improvement of pharmacological activity, improvement of stability, better tissue macrophages distribution, sustained delivery, and protection from physical and chemical degradation. (Kushwaha et al, 2012).

Role of Polymers in Drug Delivery Systems

Multiple synthetic and natural biodegradable polymers have been investigated for these purposes, with polyesters, polyether's, poly amino-acids, polysaccharides, and proteins (Medina et al, 2004). These polymers are employed as injectable drug delivery system, and especially as injectable drug delivery system for cancer chemotherapy, and have been investigate dynamically so as to reduce the toxic side effects and increase the carcinostatic pharmaceutical effects (Heller & Hoffman, 1996).

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Methods of local administration of drug delivery system, nanoparticles (Barraud et al., 2005; Brannon-Peppas & Blanchette, 2012; Panyam & Labhasetwar, 2003), microspheres(Freiberg & Zhu, 2004; Kakinoki et al., 2003), polymeric micelles (Hrubý et al., 2005; Nishiyama et al, 2005; Rapoport et al, 2003), liposomes (Abraham et al., 2005; Goyal et al., 2005; Kaneda, 2000; Saul, et al., 2003), and hydrogel systems (H. He et al, 2004; Ruel-Gariepy & Leroux, 2004)for targeting and controlled release have been investigated with non-biodegradable polymers. However, the targeting drug delivery system has not been acceptably achieved. Accordingly, the injectable in situ formulating a drug delivery system with hydrogel system, that demonstrates a sol–gel transition in a physiological environment, has many uses (Packhaeuser et al, 2004). For example, water-soluble polymers with stimuli responsiveness have been widely used as injectable drug delivery system.

For the last few years, novel drug delivery systems have been investigated to conquer the control of the conventional systems. Cancer chemotherapy and DNA-based vaccines have been well known as special areas of need for improved drug delivery. Lipoproteins as drug delivery systems have turn out to be an attractive area of research and they are considered admirable candidates as novel drug delivery systems. Herbal drugs have massive therapeutic impending, which should be explored through some significance additional drug delivery systems. Lipid solubility and molecular size are the major restraining factors for drug molecules to pass the biological membrane to be absorbed systematically follows oral or topical administration. Several plant extracts and phytomolecules, despite having exceptional bioactivity in vitro show fewer or no in vivo actions due to their poor lipid solubility absorption and reduced bioavailability. Standardized plant extracts or mainly polar phytoconstituents like flavonoids, terpenoids, tannins, xanthones when administered through novel drug delivery system show much improved absorption profile, which enables them to cross the biological membrane, resulting enhanced bioavailability. Hence, extra amount of active constituent becomes present at the site of action (liver, brain, heart, kidney, etc.) at similar or less dose as compared to the conventional plant extract or phytomolecule. Therefore, the therapeutic action becomes improved, more detectable and prolonged. Several excellent phytoconstituents have been successfully delivered using NDDS. Hence, there is a great potential in the development of novel drug delivery systems for the plant actives and extracts.

Previously used for simple encapsulation of cells or drugs, in homogenous materials, today's hydrogels are new complex smart polymers with different types of ligands and cross-links allowing for highly regulated structures and different bio responsive functionalities. Hydrogels that undergo swelling changes in response to specific biomolecules have become ever more important because of their potential applications in the development of biomaterials and drug delivery systems and the biomedical applications of the hydrogels. We concentrate on the bio responsive hydrogels and sensors, also introduces the physical background of the special properties of stimuli-responsive hydrogels; use of the variety of transducers describing changes in physical properties of the hydrogels. It considers the opportunity of using artificial neural network models to identify model for swelling behavior as new techniques.

Molecular imprinting involves forming a pre-polymerization complex between the template molecule and functional monomers or functional oligomers (or polymers) (Lim & Sun, 1980) with detailed chemical structures interrelate with the template either by covalent (Wulff, 1995), non-covalent chemistry (Kirsch, et al, 2000). Formerly the pre-polymerization complex was formed, the polymerization reaction occurs in the presence of a cross linking monomer and an appropriate solvent which controls the overall polymer morphology and macro porous structure. Once the template is removed, the product is a hetero polymer matrix with specific known elements for the template molecule. It is explaining the upcoming

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field of molecular imprinting and designed molecular recognition (Takeuchi & Haginaka, 1999). The macromolecular architecture must be planned in a different way than more traditional dense network and must contain a spatially varying cross-linking density (micro and macro-porous regions). A density fluctuation in the polymer network generates regions or microgels of localized higher cross-linking, which contain an effective imprinting structure and suitable rigidity to produce enough specificity (areas or patches of recognition).

Hydrogel Applications (Kalshetti, et al, 2012)

1. Biological molecules can be covalently built-in into hydrogel structures.
2. Hydrogels has mechanical properties, which are highly elastic, and elasticity can be tailored by modifying cross-link densities.
3. Hydrogels provide appropriate semi wet and three-dimensional environments for molecular-level biological interactions.
4. Many hydrogels shows inert surfaces that prevent nonspecific adsorption of proteins, known as antifouling.
5. Hydrogels can be designed to alter properties due to externally useful factors, such as temperature, ionic strength, solvent polarity, electric /magnetic field, light, or small biomolecules.

The objective of every drug delivery system is to give a therapeutic amount of drug to the proper target site in the body and then maintain the desired drug concentration. A well-designed controlled drug delivery system can overcome some of harms of conservative therapy and improve therapeutic efficacy of the given drug. There are various approaches in delivering therapeutic substance to the target site in sustained and controlled release fashion. One such approach is using microspheres as carriers for drug. Microspheres are characteristically free flowing powders consisting of proteins or synthetic polymers, which are biodegradable in nature ideally, have particle size less than 200μm.Various synthetic and natural materials are used for the preparation of microspheres. These are prepared by techniques like Single emulsion, Double emulsion, Polymerization, Phase separation co-acervation, Emulsion solvent evaporation and solvent diffusion. Microspheres are having wide range of applications because of controlled and sustained release. Most important use is that it is used for targeting tumours usinganti-cancer drugs. It is important carrier for safe and effective in vivo drug delivery (see Table 1). (Kotta et al., 2012; J. Silva et al., 2014; Thiagarajan, 2011)

Role of Microencapsulation in NDDS

Microencapsulation is used to alter and delayed drug release of pharmaceutical dosage forms. A well-planned controlled drug delivery system can overcome the problems of traditional therapy and improves the therapeutic efficacy of a drug. It is the dependable way to deliver the drug to the target site with specificity, if modified, and to retain the desired concentration at the site of interest without untoward effects. Microspheres received much attention not only for extended release, but also for targeting of anticancer drugs to the tumor. The objective is to draw attention to the potential of microencapsulation technique as a vital method in novel drug delivery. Polymerization is a relatively new microencapsula-

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Table 1. Microsphere applications

Type of Microspheres	Applications
Bio-adhesive microspheres	Buccal, oral, ocular, nasal, colonic drug delivery Nasal - Gentamicin, Insulin (Farraj et al.,1990), GI –Glipizide (Kyyrönen et al., 1992) Colonic – Insulin (Geary & Schlameus, 1993), Ocular - Methyl prednisolone (J. Patel et al., 2004)
Magnetic microspheres	Used in DNA analysis, cell isolation, protein purification and targeting drugs to tumour sites(Doxorubicin) (Pawan& Hemchand, 2009)
Floating microspheres	Carriers for drugs like antiviral, antifungal and antibiotic agents (so called absorption windows), non-steroidal anti-inflammatory drugs, Prednisolone, Lansoprazole (Garima Chawla et al., 2013; Shirwaikar et al., 2006)
Radioactive microspheres	For diagnostic purpose - Diagnostic radio embolization: 99mTc-macroaggregated human serum albumin (MAA) (Ercan, 1992), Thrombus imaging in deep vein thrombosis: 99mTc-sulfur colloid (Knight, 1986) For therapeutic purpose – Radio embolization of liver and spleen tumours: 90Ymicrospheres, Local radiotherapy: 212Pb-sulfur colloid (Russell et al., 1988).
Polymeric microspheres Vaccine delivery:	Hepatitis, Influenza, Pertussis, Diphtheria toxoid (Haefeli et al., 1992), Oral drug delivery of easily degraded drugs: Gene therapy with DNA plasmids; delivery of insulin, LHRH Controlled drug delivery after local application: Release of proteins, hormones and peptides over extended times

Prasanth, et al., 2011; Ramteke et al., 2012.

tion technique utilizes polymerization technique to from protective microcapsule coatings in situ. The methods involve the reaction of monomeric units located at the interface existing between a core material substance and a continuous phase in which the core material is dispersed. The continuous or core material supporting phase is usually a liquid or gas, and therefore the polymerization reaction occurs at a liquid-liquid, liquid-gas, solid-liquid, or solid-gas interface (Venkatesan et al., 2009). Microspheres are typically free flowing powders consisting of synthetic polymers which are biodegradable in nature and ideally having particle size less than 200 μM (N. Jain, 2002). There are many reasons why drugs and related chemicals have been microencapsulated (N. K. Jain, 1997; Lachman et al., 1986).

The technology has been used widely in design and proposes of controlled release and sustained release dosage forms.

1. To mask the bitter taste of drugs like Paracetamol, Nitrofurantoin, etc.
2. Many drugs have been microencapsulated to reduce gastric and other G.I. tract irritations. Sustained release Aspirin preparations have been reported to cause significantly less G.I. bleeding than conventional preparations.
3. A liquid can be converted to a pseudo-solid for easy handling and storage. e.g. Eprazinone.
4. Hygroscopic properties of core materials may be reduced by microencapsulation e.g. Sodium chloride.
5. Carbon tetra chlorides and a number of other substances have been microencapsulated to reduce their odor and volatility.
6. Microencapsulation has been employed to provide protection to the core materials against atmospheric effects, e.g. Vit. A. Palmitate.
7. Separation of irreconcilable substance has been achieved by encapsulation.

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Role of Nanoemulsions in NDDS

Nanoemulsions are novel drug delivery systems containing emulsified oil and water systems with mean droplet diameters ranging from 50 to 1000 nm. Usually, droplet size is between 100 and 500 nm and can exist as oil-in-water (o/w) or water-in-oil (w/o) form, where the core of the particle is either oil or water, respectively. Nanoemulsions are made from pharmaceutical surfactants that are generally regarded as safe (GRAS) (Chime et al., 2014).

The major advantages of nanoemulsions as drug delivery carriers comprises of increased drug loading, improved drug solubility and bioavailability, reduced patient variability, controlled drug release, and protection from enzymatic degradation (Kotta et al., 2012). A range of novel strategies are currently being developed for capable delivery of poorly water-soluble drugs, such as the formulation of amorphous solid form, nanoparticles, microemulsions, solid dispersions, melt extrusion, salt formation and formation of water-soluble complexes. Among all, the most accepted approach is the lipid-based formulation approach (Dixit & Nagarsenker, 2008; Shafiq et al., 2007).

Recently importance of nanoemulsions have become increasing as good vehicles for the controlled delivery of cosmetics and for the optimized dispersion of active ingredients in particular skin layers. Nanoemulsions are suitable in cosmetics because there is no chance of creaming, sedimentation, flocculation or coalescence, which is observed within microemulsions. The incorporation of potentially nauseating surfactants can be avoided by using high-energy equipment during manufacturing process. PEG free nanoemulsions for cosmetics have also been developed and formulations shown good stability (Chime et al., 2014; Lovelyn & Attama, 2011; Thiagarajan, 2011).

A characteristic of the nanoemulsions is their highly selective toxicity to microbes at concentration range that are non-irritating to skin or mucous membrane. The safety range of nanoemulsions is because of the minute amount of detergent in each droplet, so far when acting in concert; these droplets have enough energy and surfactant to destabilize targeted microbes without affect healthy cells. Nanoemulsions can get a level of current antimicrobial activity, which can only be formerly achieved by systemic antibiotics.

The US Army (RestOps) checked the broad-spectrum nanoemulsions on surfaces in Dec 1999 for decontamination of Anthrax spore. It was checked again by RestOps in March 2001 as a chemical decontamination agent. This tool has been tested on gangrene and clostridium botulism spores, and can even be used on infected wounds to salvage limbs. The first applications as vaccine, an influenza vaccine and an HIV vaccine, can proceed to clinical trials. The nanoemulsion causes proteins useful to the mucosal surface to be adjuvant and it help uptake by antigen presenting cells. This results in the significant systemic and mucosal immune response due to that the production of specific IgG and IgA antibody as well as cellular immunity. Research has been ongoing to complete the evidence of concept in animal trials for other vaccines including Anthrax and Hepatitis B. The University of Michigan has licensed this technology to NanoBio (Chime et al., 2014).

Another interesting application, which is experiencing an active development, is the use of nanoemulsion formulations, for controlled drug delivery and targeting (Wang et al., 2007). The development of magnetic nanoemulsions is an innovative approach for cancer therapy. These can transport photosensitizers as ifFoscan® to deep tissue layers crossways the skin thereby inducing hyperthermia for subsequent free radical generation. This method can be used for the treatment of cancer in the type of photodynamic therapy (Primo et al., 2007).

Among the possible delivery systems, the use of nano based carriers hold a great promise to protect the biomolecules, promote nanocarrier interface with mucosa and to direct antigen to the lymphoid tis-

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sues. As a result, the use of nanoemulsions in intranasal drug delivery system is set to transport about important results in targeting drugs to the brain in treatment of diseases correlated to the central nervous system (Clark et al., 2001). Major clinical and pre-clinical trials have therefore been carried out with parenteral nanoemulsion based carriers. The advances in these novel drug delivery systems have been reviewed by Patel and Patel (R. Patel & Patel, 2010). Nanoemulsions overloaded with thalidomide have been synthesized where a dose as low as 25mg leads to plasma concentrations which can be therapeutic (Araújo et al., 2011). However, a significant reduce in the drug substance of the nanoemulsion was observed at 0.01% drug formulation after two months' storage which could be overcome by the addition of polysorbate 80. Chlorambucil, a lipophilic anticancer agent has been used against breast and ovarian cancer. Lipid formulations are used to treat cardiovascular diseases since they accrue in the walls of injured vessels, transporting the drug to the place of vascular injury and to protect it from rapid inactivation by the lungs (Hoshi, 1996; Igarashi et al., 2001; Momma, 1996; Scheffler et al., 1996). Nanoemulsions are proposed for numerous applications in pharmaceuticals as drug delivery systems because of their ability to solubilize non-polar active compounds. Future perspectives of nanoemulsion are very hopeful in diverse fields of therapeutics or application in development of cosmetics for hair or skin. One of the versatile applications of nanoemulsions is in the part of drug delivery where they act as efficient carriers for bioactive, facilitating administration by various routes.

Role of Microbubbles in NDDS

Ultrasound-mediated microbubbles destruction has been planned as a novel method for noninvasive delivering of drugs and genes to different tissues. Microbubbles are used to carry a drug or gene until a specific area of importance is reached, and then ultrasound is used to burst the microbubbles, causing site-specific delivery of the bioactive materials. In addition, the capacity of albumin-coated microbubbles to adhere to vascular regions with glycocalix damage or endothelial dysfunction is another probable mechanism to deliver drugs even in the absence of ultrasound. Microbubbles that give those therapeutic properties and some important aspects of ultrasound parameters that are known to control microbubble-mediated drug delivery (Majumdar & Chowdhury, 2010).

In addition, current studies relating this novel therapeutic use of microbubbles (Majumdar & Chowdhury, 2010; Tsutsui, Xie, & Porter, 2004) have shown that microbubbles targeted to AlphaV, BetaIII can be used to measure the temporal expression of this integrin in association with angiogenesis. A contrast agent targeted to endothelial- based markers of angiogenesis might be used to develop diagnosis and treatment of disorders affected by angiogenesis. Other endothelialbased targets such as P-selectin might be shown to develop targeted imaging agents for detecting inflammation. Vulnerable plaques are those that have been infiltrated by macrophages, and are undergoing inflammation (Majumdar & Chowdhury, 2010). Inflammation can show the way to rupture of vulnerable plaque and formation of thrombus, as in stroke and myocardial infarct. Vulnerable plaques may lie unknown as unseen threats, liable to cause morbidity and sudden death.

Microbubbles accelerate the rate of SonoLysis. Improvement is greater for the targeted molecular ultrasound contrast agent, MRX-408, than for the non-targeted agent. Hynynen has shown (O'Reilly & Hynynen, 2012) that intravenous doses of microbubbles and transcranial function of ultrasound can be used to reversibly open the blood brain barrier. Laser-induced bubble formation around nanoparticles may play a crucial role in selective laser nanophoto thermolysis of cancer cells targeted with nanoparticles. Progress in cardiovascular gene therapy has been vulnerable by concerns over the safety and practicality

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of viral vectors and the ineffectiveness of current nonviral transfection techniques. Ultrasound exposure (USE) enhances transgene expression in vascular cells by up to 10-fold after naked DNA transfection, and enhances lipofection by up to three-fold. The presence of gas in the gene-filled microbubble allows ultrasound energy to "pop" the bubble. An energetic wave is then created which allows the genetic material to enter surrounding cells (Lawrie et al., 2000).

Microbubbles are presently used clinically as contrast agents in ultrasound (US) diagnostics and experimentally as drug or nucleic acid carriers. The protective blood-brain barrier blocks many drugs from acting on brain cells. Now, a new technique for delivering drugs directly to the affected area may enhance the effectiveness of chemotherapy in brain tumors and reduce its toxic effect on healthy cells. Researchers at the University of California are developing a novel technique that uses ultrasound and drug-laden "microbubbles" to deliver concentrated chemotherapy drugs to the inner lining of blood vessels. Doctors already use ultrasound to recognize tumors and leads to biopsy procedures. Ultrasound pulse sequences can also guide micro-packaged medications to specific parts of the body (Shortencarier et al., 2004).

The purpose of nanotechnology in the field of health care has come under great attention in recent times. There are many treatments today that take a lot of time and are also very costly. Using nanotechnology, quicker and much cheaper treatments can be developed. By performing further research on this technology, cures can be found for diseases that have no cure today. We could make surgical instruments of such accuracy and precision that they could operate on the cells and even molecules from which we are made - something well beyond today's medical technology. (Bhowmik et al., 2009)

Role of Vesicular Systems in NDDS

Novel drug delivery attempts to either sustain drug action at a fixed rate, or by maintaining a relatively constant, effective drug level in the body with related minimization of undesirable side effects. The vesicular systems are highly ordered assemblies of one or several concentric lipid bilayers formed, when certain amphiphillic building blocks are confronted with water. The vesicular system such as liposomes, niosomes, sphingosomes, ethosomes, transferosomes and pharmacosomesare used to develop the therapeutic index of both existing and new drug molecules by encapsulating an active medicament inside vesicular structure in one such system. It prolongs the existence of the drug in systemic circulation and finally reduces the toxicity. Such different systems are widely used in gene delivery, tumor targeting, oral formulations, in stability and permeability problems of drugs. Now a day's vesicle as a carrier system have become the vehicle of choice in drug delivery and lipid vesicles were found to be of value in immunology, membrane biology and diagnostic technique and most recently in genetic engineering (Jadhav, et al., 2012).

Different types of pharmaceutical carriers such as particulate, polymeric, macromolecular and cellular are present. Particulate type carrier also known as colloidal carrier system, includes lipid particles (low and high density lipoprotein-LDL and HDL, respectively), microspheres, nanoparticles, polymeric micelles and vesicular like liposomes, sphingosome, niosomes, transferosomes, pharmacosomes, virosomes (Sezaki & Hashida, 1985).

The cells of RES preferentially take up the vesicles. The uptake of niosomes by the cells is also by circulating serum factors known as opsonins, which mark them for approval. Such limited to a small area drug accumulation has, however, been exploited in treatment of animal tumors known to metastasize to the liver and spleen and in parasitic infestation of liver (Malhotra & Jain, 1994).

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Niosomeshave been used for studying the nature of the immune response provoked by antigens. Brewer and Alexanderhave reported niosomes as potent adjuvant in terms of immunological selectivity, small toxicity and stability (Brewer & Alexander, 1992).

Transferosomes improve the site specificity, overall drug safety and lesser the doses quite a lot of times than the currently available formulations for the treatment of skin diseases. Because of their excellent penetration power and flexibility, transferosomes formulations are used for useful delivery of non-steroidal anti-inflammatory agents like ibuprofen and diclofenac. Transferosomes not only enhance the penetration of diclofenac through intact skin, but also transfer these agents directly into the depth of the soft tissues under the application site (Cevc, 1996).

Ethosomes have high penetration rate through the skin as compared to liposomes therefore these can be used commonly in place of liposomes. Although, the exact mechanism for better penetration into deeper skin layers from ethosomes is still not clear. The synergistic effects of combination of phospholipids and high concentration of ethanol in vesicular formulations have been recommended to be responsible for deeper distribution and penetration in the skin lipid bilayers. The size range of ethosomes may differ from tens of nanometers to microns (Dayan & Touitou, 2000; S. Patel, 2007). Ethosomesare mainly used as replacement of liposomes mainly for the transdermal route of drug delivery.

Sphingosomes solve the major disadvantage of vesicle system (liposomes) like less stability, less in vivo circulation time, low tumor loading efficiency in case of cancer therapy. Sphingosomes are clinically used delivery system for chemotherapeutic agent, biological macromolecule and diagnostics. Due to flexibility in size and composition, different types of sphingosomes have been developed (Saraf, Gupta, Kaur, & Saraf, 2011). Sphingosomal products such as Marqibo(TM) (sphingosomal vincristine) are loaded with active, cell cycle-specific anticancer agents that may help from increased targeting and longtime drug exposure at the tumor site. Vincristine, vinorelbine and topotecan are approved cancer therapies which have been chosen for sphingosomal formulation specifically for their ability to help from this novel encapsulation.

Pharmacosomes are the colloidal dispersions of drugs covalently bound to lipids and may be present as ultrafine vesicular, micellar or hexagonal aggregates, depending on the chemical structure of the drug–lipid complex (Semalty, Semalty, Rawat, Singh, & Rawat, 2009). Because the system is formed by involving a drug (pharmakon) to a carrier (soma), they are called pharmacosomes. Any drug possessing a free carboxyl group or an active hydrogen atom (–OH, NH2) can be esterified (with or without a spacer group) to the hydroxyl group of a lipid molecule, thus generating an amphiphilic prodrug, which will facilitate membrane, tissue, or cell wall transport, in the organism. Any drug with a definite cut-off molecular weight may be formulated as pharmacosomes provided it has active functional groups to integrate with the vesicle-forming amphiphilic molecule.

Various amphipathic liponucleotideprodrugs, 1-β-D-arabinofuranosylcytosine 5'-Diacylglycerols (Ryu et al., 1982) (containing either dimyristoyl, dipalmitoyldistearoyl free fatty acid side chains) and determined the aggregational and morphological character of their sonicated dispersions in relation to alter in temperature. The sonication at low temperature gave turbid solutions containing big bilayer sheets. On increasing the temperature, a transition temperature (tg) was reached at which a stable three dimensional cross-linked network of small interlocking bilayer stacks was formed. Sonication at the temperature close up to tg produced small disc-shaped micellar structures. These micelles were shown to be present in another aggregational equilibrium consisting of stacking-destacking process. In contrast, the prodrug containing unsaturated fatty acid chain gave multilamellar liposomes. Improved biological activity after incorporation of drug into pharmacosomes was found in case of Taxol and Cytarabin.

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Role of Nanocarriers in NDDS

Nanocarriers with optimized physicochemical and biological properties are in use up by cells more easily than larger molecules, so they can be effectively used as delivery tools for currently existing bioactive compounds (Suri, Fenniri, & Singh, 2007). It is usually accepted that nanoparticles with a hydrodynamic diameter of 10–100 nm have best possible pharmacokinetic properties for *in vivo* applications. Smaller nanoparticlesare subjects to tissue extravasations and renalclearance whereas larger are rapidly opsonized and removed from the bloodstream *via* the macrophages of the reticuloendothelial system (Cole et al., 2011).

Once the drug-nanocarrier conjugates reach the diseased tissues, the therapeutic agents are released. A controlled release of drugs from nanocarriers can be achieved during changes in physiological environment such as temperature, pH, osmolality, or *via* an enzymatic activity. Nanocarriers used for medical applications have to be biocompatible (able to integrate with a biological system without eliciting immune response or any negative effects) and nontoxic (harmless to a given biological system) (Wilczewska et al., 2012).

SLN (solid lipid nanoparticles), NLC (nanostructured lipid carriers) and LDC (lipid drug conjugates) are types of carrier systems based on solid lipid matrix, i.e., lipids solid at the body temperature (Wissing et al., 2004). They have been exploited for the dermal (Abdel-Mottaleb et al., 2011), peroral (Muchow et al., 2008), parenteral (Nayak et al., 2010), ocular (Attama et al., 2007), pulmonary (Liu et al., 2008), and rectal (Sznitowska et al., 2001) delivery.

The application of biodegradable nanosystems for the development of nanomedicines is one of the most successful ideas. Nanocarriers composed of biodegradable polymers undergoes hydrolysis in the body, produces biodegradable metabolite monomers, such as lactic acid and glycolic acid. Kumari et al. (Kumari et al., 2010) reported a minimal systemic toxicity related with using of PLGA for drug delivery or biomaterial applications. Such nanoparticles are biocompatible with tissue and cells (Panyam & Labhasetwar, 2003). Drug-biodegradable polymeric nanocarrier conjugates used for drug delivery are stable in blood, non-toxic, and non-thrombogenic. They are also non-immunogenic as well as non-pro inflammatory, and they neither activate neutrophils nor affect reticuloendothelial system (des Rieux et al. 2006).

Silica materials used in controlled drug delivery systems are classified as xerogels (Czarnobaj, 2008) and mesoporous silica nanoparticles (MSNs), e.g., MCM-41 (Mobil Composition of Matter) and SBA-15 (Santa Barbara University mesoporous silica material) (Wei, Hu, & Zhang, 2010). They exhibit several advantages as carrier systems, including biocompatibility, highly porous framework and an ease in terms of functionalization (Amato, 2010). Among inorganic nanoparticles, silica materials are the carriers which most often are chosen for biological purposes (Slowing, Trewyn, Giri, & Lin, 2007). Xerogels used in controlled drug release (Quintanar-Guerrero et al., 2009). Phenytoin (Fidalgo et al., 2009), doxorubicin (Prokopowicz et al., 2010), cisplatin (Katarzyna et al., 2007), metronidazole (Katarzyna et al., 2012), nifedipine (Maver et al., 2007), diclofenac (Katarzyna Czarnobaj & Czarnobaj, 2008), and heparin (Ahola et al., 2001) are examples of drugs which have been loaded into xerogels using this technique.

The best known types of mesoporous silica nanomaterials are MCM-41 with a hexagonal arrangement of the mesopores and SBA-15 with a well-ordered hexagonal connected system of pores. The MSNs, in comparison with xerogels, possess more homogenous structure, lower polydispersity and higher surface area for adsorption of therapeutic or diagnostic agents (Di Pasqua et al., 2009). The mechanism of drug loading into mesoporous silica material is a chemical or physical adsorption. By these processes, diverse

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types of drugs, including anticancer drugs (Q. He et al., 2011), antibiotics (Li et al., 2010), and heart disease drugs (Popovici et al., 2011) have been embedded into MNSs.

Carbon nanocarriers used in DDS are differentiated into nanotubes (CNTs) and nanohorns (CNH). CNTs are characterized by unique architecture formed by rolling of single (SWNCTs – single walled carbon nanotubes) or multi (MWCNTs – multi walled carbon nanotubes) layers of graphite with an enormous surface area and an excellent electronic and thermal conductivity (Beg et al., 2011). Drug release from carbon nanotubes can be electrically or chemically controlled. To prevent the unwanted release of the drug, the open ends of CNTs were sealed with polypyrrole (PPy) films [129]. Homing devices, i.e., folic acid [130] and epidermal growth factor (Bhirde et al., 2009), were attached to improve selectivity of such drug delivery systems. Newer studies have pointed out the similarity in carcinogenic potential between CNT and asbestos. (Poland et al., 2008). Carbon nanotubes have been shown to cause necrosis or apoptosis of macrophage cell lines and changes in cell morphology (Jia et al., 2005). Radomski et al. (Radomski et al., 2005) studied the effects of engineered carbon nanoparticles (MWCNT and SWCNT) on human platelet aggregation *in vitro* and rat vascular thrombosis *in vivo*. Incubation of platelets with carbon nanomaterials caused platelet aggregation with little or no granular release.

Therapeutic activity of diverse drugs incorporated into iron oxide nanocarriers have been tested and reported. Magneic Nano Particals have been examined for multitasking treatment as biosensors (diagnosis) and drug carriers (therapy) simultaneously. Concomitant use of magnetic resonance or magnetofluorescent imaging and targeted therapy (*via* conjugation of targeting moieties) can enhance effectiveness of cancer therapy (Chomoucka et al., 2010). Magnetic nanoparticles promote activation of phagocytotic and cytokine-release functions of macrophages (Dobrovolskaia& McNeil, 2007). Gas vesicles were observed in MNPs treated cells with increased granularity of the cells (Mahmoudi et al., 2010). Magnetite nanoparticles have the potency of causing oxidative DNA lesions in cultured A549 cells (the human lung epithelial cell line) (Karlsson, Cronholm, Gustafsson, & Moller, 2008). They also cause transient increase of ALT (serum alanine aminotransferase), AST (aspartate aminotransferase), and ALP (alkaline phosphatase) levels. (Jain TK et al., 2007)

Most of the applications of nanotechnology in medicine are still under development nanocrystalline silver is used already as an antimicrobial agent in the treatment of wounds. Qdots will be useful to identify the location of cancer cells in the body. Use of nanoparticles that can deliver chemotherapeutic drugs directly to cancer cells which minimizes damage to the healthy cells. Nanoshells concentrate the heat from infrared light to destroy cancer cells with minimal damage to surrounding healthy cells. Nanotubes used in the broken bones to provide a structure for newbone material to grow. Nanoparticles that can attach to cells infected with various diseases and permit a doctor to identify, in a blood sample, the particular disease (Bhowmik et al., 2009).

Nanomedicine refers to future developments in medicine that will be based on the ability to build nanorobots. In the future, these nanorobots could actually be programmed to repair specific diseased cells, functioning in a similar way to antibodies in our natural healing processes. Nanomedicines would be able to eliminate of bacterial infections in a patient within minutes as an alternative of using treatment with antibiotics over a period of weeks, also able to perform surgery at the cellular level, removing individual diseased cells and even repairing defective portions of individual cells. Significant lengthening of the human lifespan by repairing cellular level conditions that cause the body to age.

Nanoparticles can be planned with a structure very similar to the bone structure. An ultrasound is performed on existing bone structures and then bone like nanoparticles are created using the results of the ultrasound (G. A. Silva et al., 2004). The bone-like nanoparticles are inserted into the body in a

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paste form (Adhikari, Kundu, & Rao, 2013). When they arrive at the fractured bone, they accumulate themselves to form an ordered structure which later becomes part of the bone.

Another key use for nanoparticles is the treatment of injured nerves. Samuel Stupp and John Kessler at Northwestern University in Chicago have made tiny rod like nano-fibers called *amphiphiles*. They have kept capped with amino acids and are known to spur the growth of neurons and prevent scar tissue formation. Experiments have shown that rat and mice with spinal injuries recovered when treated with these nano-fibers.

The use of RNA interference (RNAi) to down regulate several targets has emerged as a treatment modality of exceptional promise for cancer treatment (Fire et al., 1998). The RNAi approach uses small RNA molecules which bind to messenger RNAs (mRNA) by complementary base pairing, to induce degradation of the mRNA and/or block protein synthesis. The small RNA molecules are included and processed into cellular RNA processing machinery to induce their inhibitory effects in one RNAi-based therapy modality, a 21-27 base pair double stranded RNA (siRNAs) is introduced into the cells, where it binds to its specific complementary mRNA sequence and inhibits protein synthesis (this effect is commonly called RNA silencing) (Elbashir et al., 2001). SiRNAs are designed to target only one gene which is normally over expressed in cancer cells compared with normal cells. As a result, it is highly desirable to use siRNAs targeting key genes involved in cancer cell migration, invasion, and metastasis. Before RNAi discovery, sequence-specific inhibition of gene expression was possible using nucleic acid-based antisense technology. One of the major advantages of RNAi over antisense based approaches is that the RNAi depends on the cellular machinery to target complementary transcripts resulting in efficient and potent down-regulation of gene expression (Aagaard & Rossi, 2007). Furthermore, the use of the RNAi strategy is highly specific when modulation of a particular target is desired. In this scenario, lower side effects are expected compared to conventional chemotherapy.

CONCLUSION

In this chapter we explore different therapeutics applications of different Novel Drug Delivery System which will be providing complete set of information about drug delivery systems are being of great concern due to their sole purposes. The significance of different drug delivery and targeted drug therapy is to carry a drug directly to the centre of the disease under physiological condition and thereby treat it purposely, with neither effect on the body. This is particularly significant in cancer chemotherapy and enzyme substitute treatment. Drug targeting is the delivery of drugs to receptors or organs of the body to which one hopes to deliver the drug entirely. The targeted delivery of drugs is truly a very inviting aim because these supply one of the most potential ways to develop therapeutic index of the drugs. Previous work done between late 1960s and the mid-1980s stressed the need for drugs carrier system originally to alter the pharmacokinetics of the before proven drugs whose efficacy might be improved by changing the rates of metabolism in liver or clearance by the kidneys. These approaches commonly were not focused to achieve site specific or targeted delivery such as getting a cytotoxic drug to cancerous tissue while sparing other normal, though equally sensitive tissue with the progress in the carrier technology the issue of delivering either single or the entire carrier to the specific site has been addressed during the last few years. The effective use of applications in targeted drug delivery relies on a number of different factors all play a key role in determining the effectiveness of magnetic drug delivery.

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Chapter 16
Bioinformatics and Its Therapeutic Applications

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ABSTRACT

Bioinformatics has emerged as a major element in contemporary biomedical and pharmaceutical region. Bioinformatics deals with growth in biological data and has led to development of many databases. Bioinformatics deals with collection of data that is relevant clinically and these days separate term clinical information has come up. Data mimics are another field which is gaining importance. This chapter shall deal with introduction of bioinformatics and its applications in medicine and health care.

INTRODUCTION

Bioinformatics is an applied science including computer science and Biological related research area. There is model change in biological research approach used in computer application (Bayat, 2002) many software tools and computational mathematics model in different field experiment in a high labeled (Cohen, 2004). Bioinformatics introduces the exponential development in life science including chemical and biological data development of primary database and secondary database protein sequencing, nucleic acid sequencing and many structures (Altman, 1998). Some of the known database including Protein database, gene database, zinc database, Swissprot database, CATH database, SCOP database and PIR database etc. These databases are easily available in many private internet Server Company and government domain information. Essential research and protein modeling is completed using these database with helpful for the protein sequencing analysis tool like FASTA, BLAST,CLUSTALW etc. and the protein modeled structure are visualized in using (Russo, 2000) Bioinformatics work as im-

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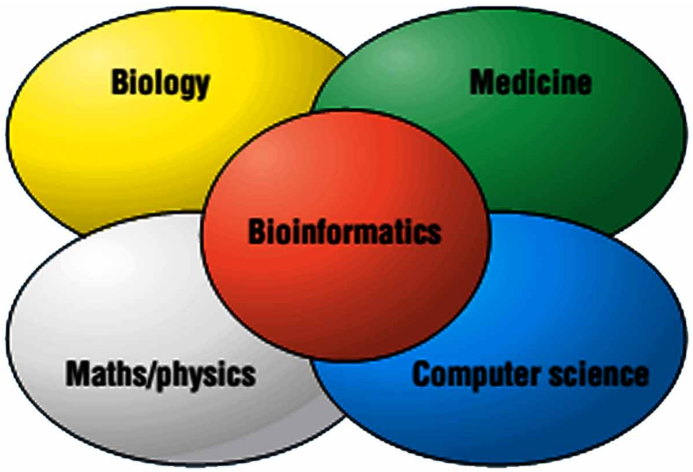
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portant role in mixture of broad disciplines of biological organ/tissue to understand the organ complex mechanisms of the body cell. Bioinformatics is also the way in which Pharmaceutical investigation, biomedical investigation and health science use the information technology in their testing proof. The complete process of biological and information data collection to analysis of the proof result such testing may be classified under the specific area named –cheminformatics, Pharma co-informatics and clinical information (Wooley, 1999) (Figure 1).

WHAT IS BIO-INFORMATICS

Bioinformatics research and development studies two important information technology steps in modern biology. The first is the step of proteomic &genomics information from the DNA and RNA of an individual *Homo sapiens* organism up to the characteristics of a population growth of such organisms (Altman, 1998). The second is the steps of practical information from observed biological activity phenomena to models that explain them and then to new experiments in order to test these models. The discipline of bioinformatics has its roots in a number of activities, including the body organization of DNA & RNA sequence and protein three-dimensional structural data collections in the 1960's and 1970's (Langridge, 1974). It has become a booming academic and industrial enterprise with the introduction of biological experiments that rapidly produce massive amounts of data (such as the multiple genome sequencing projects, the large scale analysis of gene expression, and the large scale analysis of protein-protein interactions). Basic biological science has always had an impact on clinical medicine& pharmaceutical (information systems), and is creating a new generation of epidemiologic, diagnostic, prognostic, and treatment modalities. Bioinformatics efforts that appear to be wholly geared towards basic science are likely to become relevant to clinical informatics & pharmaco informatics in the coming decade. For example, DNA sequence information and sequence annotations will appear in the medical chart with

Figure 1. Interaction of disciplines that have contributed to the formation of bioinformatics (source- Science, medicine, and the future: Bioinformatics, a new interdisciplinary science, is essential to managing, understanding, and harnessing clinical benefit from new genetic data.)



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increasing frequency. The algorithms developed for research in bioinformatics will soon become part of clinical information and chemo informatics systems.

Bioinformatics is a discipline/sub discipline has been recognized for less than 10 years. The first efforts in bioinformatics can be traced back to the early application of computers to biology in the 1950's and 1960's. The early applications of computers to molecular biology were for graphical rendering of three-dimensional molecular structures and the creation of databases of molecular sequence and three-dimensional structure information. Bioinformatics can generally be defined as the study of how information technologies are used to solve problems in biology. The precise definition of bioinformatics is a matter of some debate. The most narrow usage of the term refers to the creation and management of biological databases in support of genomic sequences. The most broad usage includes essentially all applications of computers and information sciences to problems in biology. Bioinformatics is based on the fundamental example of molecular biology: genomics and proteomics information is stored in sequences of DNA bases. Finally this concept of molecule sequencing including molecule storage and analysis is important and central biological data and does not occur in many other area of information technology.

Bioinformatics and Its Scope

Bioinformatics uses modern approaches in the area of life science, computer science and information science including communication technology, computer technology and information technology to problem defined in one or more area in pharmaceutical chemistry, biotechnology and life science. Bioinformatics is data stored in big amount and find out data analysis in data mining have occur main issues for pharma analyst and biological scientists due to fast development in quantitative data in life science such as complete proteomic and genomics of life science species including homo sapiens (human) genome, protein 3-D structure, metabolic pathway and protein sequencing database related information. Advances in bioinformatics particularly the internet source and approach ever increasing information in life science and biotechnology. Functional genomics and proteomics development of new pharmaceutical target and vaccine other pharmacogenomics and biochemical molecule diagnostic kit are many of the field in which bioinformatics has developed in essential part of research and development. The knowledge of multi database, tool to carry out data analysis and design of pharmaceutical molecule and life science system in computer work place as well as in network atmosphere has occur important for any candidate of bioinformatics. Bioinformatics is the multi-disciplinary field and has developed so more than one divides it into Biochemical molecular bioinformatics, sepsis bioinformatics and organelle bioinformatics. Therefore bioinformatics research and development unit are being founded in many pharmaceutical industry, biotechnology laboratory and even in other traditional company introduced in life science (biological). Bioinformatics is thus determined in many numbers of opportunities in this field.

Bioinformatics is introduced with database design, data modeling and data analysis. Data creating is done not only for copy material but also from networking source. Database in life science are mainly multi organized in affiliated database development.

Bioinformatics Tool

The main tools of bioinformatics function are online various related server and computer software like Schrodinger, Auto dock. The fundamental action of molecule sequencing of protein and DNA molecule using different types of software program and biological database easily available on the web server.

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Bioinformatics is a developed science and every day there is improvement in every branch of life science and computer science, and today bioinformatics expert use in various complex software programs for recovering, molecule prediction, molecule analysis, molecule protein and DNA sequencing data (NCBI, http://www.ncbi.nlm.nih.gov).These are major class of bioinformatics tool they are using data Ming software and genomics & proteomics sequencing database tool, homology and protein functional analysis tool:

- Homology and Similarity Tools
- Protein Function Analysis
- Structure Analysis
- Sequencing Analysis

Some Example of Bioinformatics Tool

- BLAST (Basic Local Alignment Search Tool)
- FASTA
- EMBOSS (European Molecular Biology Open Software Site)
- crustal W
- RasMol
- Prospect
- Pattern Hunter
- COPIA (Consensus Pattern Identification and Analysis)

Application of Programmers in Bioinformatics

- Bio Java
- Bio Perl
- Bio XML
- Bio cobra
- Ensemble
- Bio Perl-db
- Bio Python & Bio Java

CONVERSION OF 2D TO 3D

Chemsketch and CORINA are easy conversion software's and biological data based program system. They create natural data base library in SDF File format convert into 3D for screening which is taken as input. And hydrogen atoms are added (e.g. Ions in salts) and all molecules are neutralized. Structures that couldn't be converted are excluded from the 3D output file but written to a find out error file. The output file is also formatted in MDL SDF File format.

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DOCKING TOOL

Schrodinger Software

Schrödinger software arrangement in drug design is software applied for ligand molecule and structure molecule based methods. Schrödinger deliver actual, creditable and high achievement computational dependent technology to solution in life science and other applied science research. Maestro is the unified interface for all Schrödinger software products. Schrodinger software are Supplier L.L.C., US. Following software products of Schrödinger are usually installed.

- Glide
- Desmond
- Q Site
- Maestro
- Site Map

Glide

Glide is all about the ligand and receptor complex docking studies. Glide tool response has good speed and accuracy for virtual screening of compounds. Virtual screening is performed by the help of various phases such as HTVS, SP and XP. Glide tool is most common advanced scoring energy function.

Desmond

Desmond tool performs molecular dynamic simulation for ligand complex molecule of proposed ligand and protein. Molecular simulation dynamics provides natural environment and finds out biological system movement at atmospheric condition. Technical and Pharmaceutical industries take up molecular dynamic simulations from time to time to study computational simulations for knowing stability of bound protein.

Q-Site

Q Site is applied for Quantum mechanism studies, to demonstrate the interaction point of a target, active site and protein molecule interaction function to the comfort of the system. Classical molecular mechanism (MM) process cannot be defined by the electrostatic force change condition of reaction and finding out ligand receptor interaction. Quantum mechanism (QM) is expected to study active chemical molecule affect of transition metal with a protein in atmospheric conditions.

Maestro

Maestro tool is the undivided interaction for all Schrodinger tool and most commonly selected analysis tool. Maestro tool has been developed for chemical molecule simulation and is used in pharmaceutical company and research institute. Maestro tool is computational technology visualization function. Maestro tool is used to find out chemical molecule properties with intramolecular interaction. Maestro tool are used in advanced computational science in drug discovery.

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Site Map

Site map is a tool for identification of binding site and characteristic visualization tool. It is easy to use and finds out good ligand binding site corresponding to that area of drug design. Site map is based on proven algorithm for active bind position identification and assessment and can help in research associated to actual point of binding site.

ORGANISM INFORMATION BIOINFORMATICS DATA

The concept of under based many research development procedure in bioinformatics is that big database can be created side by side depending on chemical and biological analogs, for example proteomic and genomic DNA and protein sequence gene (Pedersen, Jensen, Brunak, Staerfeldt, & Ussery, 2000).An essential aspect of management of big amount of database is in developing procedure for doing comparative study between different molecules in biological data and identifying related bimolecular data. We introduced major data base that provide primary source of bioinformatics and introduced in secondary data base in order to colony the data. These are classified correlation between genomic, proteomics and its derivative product (Luscombe, Greenbaum & Gerstein, 2001). There are common term to define the correlation between pair of protein sequence and gene sequence. The two classes can be some time difficult to different especially if the relation between the two proteins is distance (Russell, Saqi, Sayle, Bates & Sternberg, 1997, 1998). Protein that are relation between gene duplication and genome (Fitch, 1970). Normally orthologs contain the same work (Tatusov, Koonin, & Lipman, 1997).The important notation of these area observations is limited for different organism (Gerstein & Hegyi, 1998; Skolnick & Fetrow, 2000) (Table 1).

MOLECULE INFORMATION BIOINFORMATICS DATA

The type of bioinformatics data that are analysed in range of subject and that are consider to relative to that area. We also give near about value introduced in size of biological data base being discussed. Source of information in bio informatics may be defined to raw material in DNA sequencing, genome molecule sequencing, macromolecule protein structure and all other genome bioinformatics data. Basic DNA molecule sequencing is initial stage four base character comparing gene base pair, each generally more than 1000 base pair large. The gene bank stock of nucleic acid sequence latest holds a 9.5 million base pair data base. The protein sequencing strings of 20 amino acids. At time presented 300,000 known protein sequence, with a typical bacteria protein contenting near about 300 amino acids. Macro molecular data represents more information. Latest the big data base for bacteria has made near about 20 times analysis for 6000 gene (Holstege et al., 1998) (Table 2).

ACCOMPLISHMENTS OF BIOINFORMATICS

There are two categories of accomplishments of bioinformatics that can be credited to bioinformatics. The first categories are an empirical principal that have become accepted by this field and generate context

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Table 1. List of URLs for the Bioinformatics databases that are cited in the review.

Bioinformatics Data Base	URL
Protein sequence (primary) SWISS-PROT PIR-International	www.expasy.ch/sprot/sprot-top.html www.mips.biochem.mpg.de/proj/protseqdb
Protein sequence (composite) OWL NRDB	www.bioinf.man.ac.uk/dbbrowser/OWL www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein
Integrated databases Inter Pro Sequence retrieval system (SRS) Entrez	www.ebi.ac.uk/interpro www.expasy.ch/srs5 www.ncbi.nlm.nih.gov/Entrez
Genome sequences Entrez genomes Gene Census COGs	www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome bioinfo.mbb.yale.edu/genome www.ncbi.nlm.nih.gov/COG
Protein sequence (secondary) PROSITE PRINTS Pfam	www.expasy.ch/prosite www.bioinf.man.ac.uk/dbbrowser/PRINTS/PRINTS.html www.sanger.ac.uk/Pfam/
Macromolecular structures Protein Data Bank (PDB) Nucleic Acids Database (NDB) HIV Protease Database ReLiBase PDB sum CATH SCOP FSSP	www.rcsb.org/pdb ndbserver.rutgers.edu/ www.ncifcrf.gov/CRYS/HIVdb/NEW_DATABASE www2.ebi.ac.uk:8081/home.html www.biochem.ucl.ac.uk/bsm/pdbsum www.biochem.ucl.ac.uk/bsm/cath scop.mrc-lmb.cam.ac.uk/scop www2.embl-ebi.ac.uk/dali/fssp
Nucleotide sequences Gen Bank EMBL DDBJ	www.ncbi.nlm.nih.gov/Genbank www.ebi.ac.uk/embl www.ddbj.nig.ac.jp

(source-What is bioinformatics? An introduction and overview" Yearbook of Medical Informatics 2001")

which guides both training and research development. The second categories are particular biological database and algorithm that have been evolved and which are service to Biological science.

1. The molecular structure of protein in evolution contains more changes like mutation, dilation and insertion of building blocks amino acid protein to the sequence. There are many similar chemical and biological structures with many different sequences (Lesk & Chothia, 1980).
2. Chemical and biological sequence can be aligned using algorithm based dynamic program. These mathematically choice alignment are most reliable when the similarity between sequences is high (>30% identical), but become less reliable in the range below 25% (Chung & Subbiah, 1996).
3. Probabilistic model of protein sequencing model known as hidden markov model can sensitively characterize a class of related sequence (Durbin, Eddy, Krogh, & Mitchison, 1998).
4. Different in both host and DNA pathogen genetic information can be used to comprehend which proteins are involved in diseases.

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Table 2. Sources of data used in bioinformatics, the quantity of each type of data that is currently

Biological Data Source	Biological Data Size	Bioinformatics Topics
Raw DNA sequence	8.2 million sequences (9.5 billion bases)	Separating coding and non-coding regions Identification of introns and exons Gene product prediction Forensic analysis
Protein sequence	300,000 sequences (~300 amino acids each)	Sequence comparison algorithms Multiple sequence alignments algorithms Identification of conserved sequence motifs
Macromolecular Structure	13,000 structures (~1,000 atomic coordinates each)	Secondary, tertiary structure prediction 3D structural alignment algorithms Protein geometry measurements Surface and volume shape calculations Intermolecular interactions Molecular simulations (force-field calculations, molecular movements, docking predictions)
Genomes	40 complete genomes (1.6 million – 3 billion bases each)	Characterization of repeats Structural assignments to genes Phylogenetic analysis Genomic-scale censuses (characterization of protein content, metabolic pathways) Linkage analysis relating specific genes to diseases
Gene expression	largest: ~20 time point measurements for ~6,000 genes	Correlating expression patterns Mapping expression data to sequence, structural and biochemical data
Other data Literature Metabolic pathways	11 million citations	Digital libraries for automated bibliographical searches Knowledge databases of data from literature Pathway simulations

(2000) available, and bioinformatics subject areas that utilize this data.
(Source-What is bioinformatics? An introduction and overview" Yearbook of Medical Informatics 2001")

The effect of bioinformatics has also been made with the improvement of algorithm and biological database that are generally used some of which were developed by bioinformatics developer/researcher and other important bioinformatics resources and modern bioinformatics tool. The physician is not decided which symptom the patient may have and conformed to go on the web site server to investigate the known genetic syndrome related with disease. Today physician cross web site server to gather biological information in the following manner.

1. The physician first goes to the NCBI (National center for Biotechnology Information) web site server page. Today NCBI maintains a large number of biological database and molecular medicine database.
2. The molecule genetic gen data bank is then approach forms the molecule protein sequence to see the detailed molecular sequence of DNA base that encoded for the gene.

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THERAPEUTIC APPLICATION

Bioinformatics has several therapeutic applications. Some of which are discussed below. The application of bioinformatics is in - Preventative medicine, Gene therapy, Antibiotic resistance, Evolutionary studies, Waste cleanup, Biotechnology, Climate change Studies, Alternative energy sources, Crop improvement, Forensic analysis, Bio-weapon creation, Insect resistance, Improve nutritional quality, Development of Drought resistant varieties and Vetinary Science.

BIOINFORMATICS USED IN DATA MINING

In bioinformatics there is huge amount of data to deal, in order to deal with this huge amount of data some techniques are required to sustain this huge amount of data properly. In this sense DATA MINING software provide huge contribution.

Some of the applications are as follow –

1. Finding the gene in large DNA molecule.
2. Domain Finding
3. Protein and gene interaction recognition
4. Motif Detection

For example Microarray technology is used to predict the patient health behavior. On the basis of genotypic behavior of patient, the survival time of the patient and the disease in the patient is recognized i.e. very useful in disease estimation or analyzing. The data mining tool provide machine learning technology for analyzing massive amount of data i.e. peptide identification of mass spectroscopy.

These are main task of data mining:

- **Classification:** provide tools for classification of data based on requirement of experimental purpose i.e. Classes are predefined in it.
- **Estimation:** Given some input data, it helps in estimating the value of some unknown parameter.
- **Prediction:** it is similar to the classification and estimation but the records are classified based on the behavior of future.
- **Association Rules:** this rule helps to determine the dependency between the records so that the similar thing can come up together.
- **Clustering:** is a process of dividing the large population into small-small group containing the similar feature?

Description & visualization the above classified data is now is shown by using the some of visualization technique.

Learning by using these tool fall out under two categories

- Supervised learning
- Unsupervised learning

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The classification, estimation, prediction are the example of supervised learning. Supervised means there is guideline to perform the task.

The next three task – association rules, clustering and description & visualization comes under the the categories of unsupervised learning.Unsupervised learning means there is no any definite guideline to perform the task.

BIOINFORMATICS AND DRUG DISCOVERY

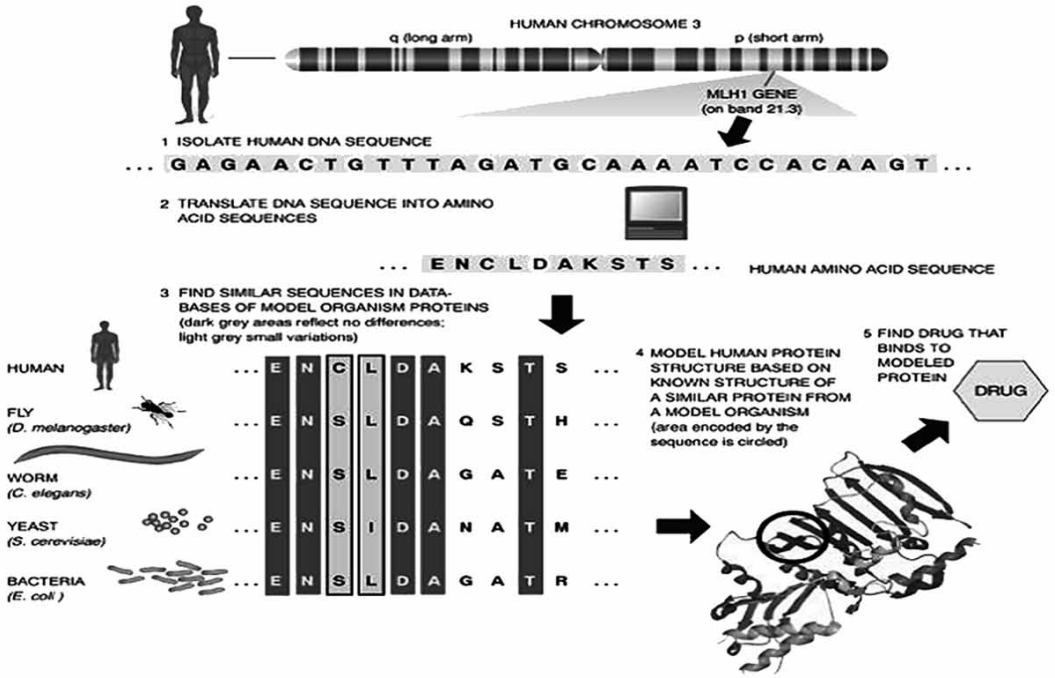
Today various type of disease is now known. Today more than 14 to 15 million death every year occur in developing countries as per WHO report. Most death from infectious disease occurs in developing countries like Ebola, malaria, H1N1 etc. (Clark & Pickett, 2000). Such drugs are high in cost and not easily available for developing world. The drug development of low cost and good quality drug for a different type of diseases is one of the most common problems faced by pharmaceutical industry or research institute. The solution to problem drug design is use of bioinformatics. Pharmaceutical company research and development of new drug using bioinformatics tool and various techniques reduces time and cost of pharmacological agent. Computational procedure are used for the forecast of drug similarity which is nothing but the molecules identification of hit molecule that will probably prove to be drug in later stage of drug discovery and development (Shortliffe & Blois, 2000). The pharmaceutical research institute and pharma industry‘s point of view bioinformatics is a key to drug design. It reduces the number of molecule trial in the screening of drug molecule and identifies potential drug receptor for the specific diseases using computational server workstation and bioinformatics tool. The application of bioinformatics in genomic and proteomic sequence has opened up a new area in pharma co informatics, pharmacogenomics which is the study of molecular genetics basis for the different type of drug design (Figure 2).

BRIEF HISTORY OF COMPUTER AIDED DRUG DESIGN

2000’s: Vast information handling technology (pharmacogenomics)
1990’s: First Computer based genomic assembly or Genomics based target selection
1900: Human Genome Project, Bioinformatics, Combinational chemistry and HTVS (High throughput virtual Screening)
1900: Receptor based and locks and key principal [(1909) P. Ehrich and (1894) E. Fisher]
1980: Beginning of Computer aided drug design, Computer graphics, X-ray crystallography, and Multidimensional NMR, Molecular Modeling and Molecular computational biology.
1980’s: First computer based docking
1980’s: Data based combinatorial libraries including information technology
1970: QSAR (Quantitative Structure Activity Relationship) including 2-Dimentional and retrospective analysis.
1960’s: Review the target or drug interaction

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Figure 2. Above is a schematic outlining how scientists can use bioinformatics to aid rational drug discovery.
(Source-What is bioinformatics? An introduction and overview "Yearbook of Medical Informatics 2001")



COMPUTER AIDED DRUG DESIGN

Computer based drug design including Structure or ligand based drug design; rational drug design and intelligent drug design are very typical and longtime processes developed in last 12to15 years (Richards, 1994). Today computer aided drug design (CADD) works on various types of algorithm based software and working technology in a very short time for drug development and gives a hypothetical result. And it has led to development of a branch called Bioinformatics (applied science). (Clark & Pickett, 2000). Today entire scientific community, most pharmaceutical research and development companies including biotechnology research development laboratories and research institute use computational based design software tools. Because of low cost involved by their use in drug development (Zeng, 2000). Computer aided drug design is of two types: structure dependent drug design and ligand based drug design. Structure based drug design; main hypothesis is by this influence the potent molecule's capability to interact by hydrogen bond with special protein pulls a required biological impact which depends on its capability to favorably interact with a specific binding site on that molecular protein. And other ligand based computer aided drug design depends on ligand and involve the analysis of ligand molecule known/unknown to interact by hydrogen bond with target ligand and active molecule. Compounds found by ligand based virtual screening method are again and again more potent molecule (Klebe, 2000). The latest development is human immunodeficiency virus (HIV) potent inhibitor which has convincingly demonstrated the impact and the relevance of structure-based approaches to the discovery of new drugs (Lipkowitz & Boyed, 1996).

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ADMET Properties

The pharmacological activity in our body or tissue, pharmaceutical compound has to pass through physiological barriers such as the BBB (blood brain barrier), gastrointestinal barrier, microcirculatory barrier and plasma member barrier before passing to blood circulatory system. Pharmaceutical compound are subsequently transported to effective receptor site for distribution into organs and tissue by specific pocket site and other wastage material out of the body this process is called excretion. ADMET (Absorption, Distribution, Metabolism, Excretion and toxicity) properties check out biological activity of pharmaceutical compound (Song, Lim, & Tong, 2009). Drug development for novel pharmaceutical agent is filtering out one compound out of several screened compounds, and this reduces long time and cost for research and development. Today pharmaceutical industry and biotechnology laboratory develops several potential ligands and checks out ADEMT properties at every stage of drug development (Veselovsky & Ivanov, 2003).

Pre-Filtering

Initial step in ligand virtual screening is screening the generated library by the Lipinski's Rule of Five (Lipinski, Lombardo, Dominy, & Feeny, 2001; Kerns, & Carte, 2009). Lipinski's rule work depends on the analysis of profiling the calculated physical property-

- Maximum five hydrogen bond donor nature
- Molecular mass less than 500Da
- Octanol water partition coefficient less than five
- More than 10 Oxygen and Nitrogen atom accepter
- No. of rooted bond less than ten

Molecule chemical structure characteristic eligible of optimization and designed by combinatorial ligand and medicinal chemistry-

- ADME properties is good
- No toxic substructures

Thus lead molecule chemical structures are the starting point for drug development. The meaning of leads molecule structure requires at least one marketed preparation drug design based on this particular lead molecule structure (Clark, & Pickett, 2000).

Design of Combinatorial Natural Compound Library

Virtual high through screening use used in high performers computational to virtual screening large ligand molecule data base compound for most potent inhibiter screen. The performers a how ever virtual combinatorial library must be possible for natural library screening. Virtual screening can be earned in variation of molecule size and drug design inclusive of general virtual screening library that can be used to screen data base against proposed protein, selected library this are designed structure for a data base of

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related proposed target. And proposed target data base libraries that are specific structure designed for a single potent target. At last finally proposed data base methods is called generated data base (Sliwoski, Kothiwale, Meiler & Lowe, 2014).

Virtual Screening

Virtual screening is a standard process in drug discovery and selection of most potent chemical molecule structure this process involved in computer aided drug design. VS basically meninges data base library screen again proposed target protein molecule high energy scoring function in selection of data base library. This time Meany drug rejected in clinical trial phase because the drug is not potent for receptor. And pharmacokinetic including absorption, distribution metabolism, excretion and toxicology based blamed and reject clinical trial phase. The term virtual screening has been define in process computational based large data base chemical compound analysis and best compound selected. Virtual screening following the principal of Lipinski rules used in drug deployment. The selection of an applicable virtual screening process depends on much point e.g. on the availability of structure based biological information of the proposed target protein molecule and other selected ligand molecule the size of data base used for virtual screening and computation based resources. Virtual screening method can be categorized in two parts first part is ligand based and second part is protein structure based (Clark & Pickett, 2000) (Figure 3).

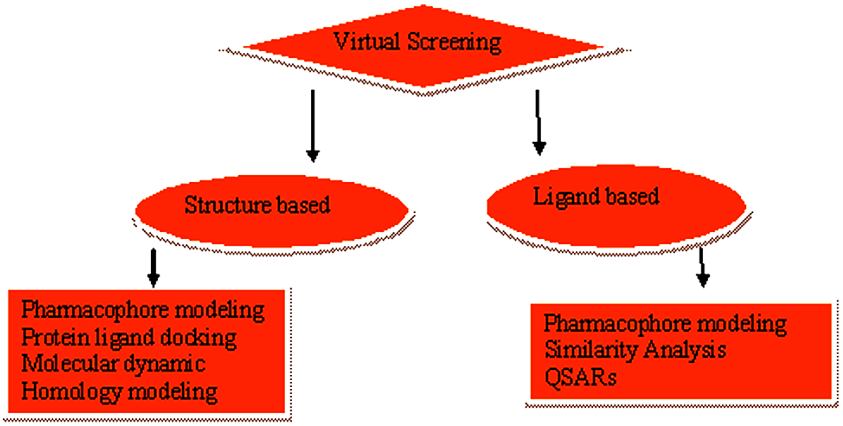
Ligand-Based

Ligand based drug development ligands bind to specific biological target. Mainly ligand based virtual screening procedure are QSARs, pharmacophore based modeling and similarity analysis.

Ligand Base Pharmacophore Modeling

Pharmacophore modeling introduced by Paul Ehrlich time duration the late 1800s (Schueler, 1960). The word pharmacophore coined by Schueler introduced in his book Drug design and Chemo biodynamics, a molecular structure that carry the essential features responsible for drug pharmacological activity

Figure 3. Virtual screening method application in drug development.



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(Schueler, 1960).International Union of Pure and Applied Chemistry (IUPAC) introduced the term pharmacophore in 1997, Pharmacophore is the ensemble of electronic and steric features that is necessary to ensure the optimal supramolecular interactions with a specific pharmacological target and to block its pharmacological activity. Computational pharmacophore structure is generally involved to generate novel initials point for drug development research center (Wermuth, Ganellin, Lindberg, & Mitscher, 1998). Many category of pharmacophore modeling based ligand development, such as Discovery of studio/Catalyst, phase (Leach, Gillet, Lewis, & Taylor, 2010). Ligand Scout and MOE (Dixon et al., 2006). These chemical ligand based can be labeled hydrogen bond accepter or donor, cation, anionic, hydrophobic or aromatic and any probity aggregate (Wolber, & Langer, 2005) (Figure 4).

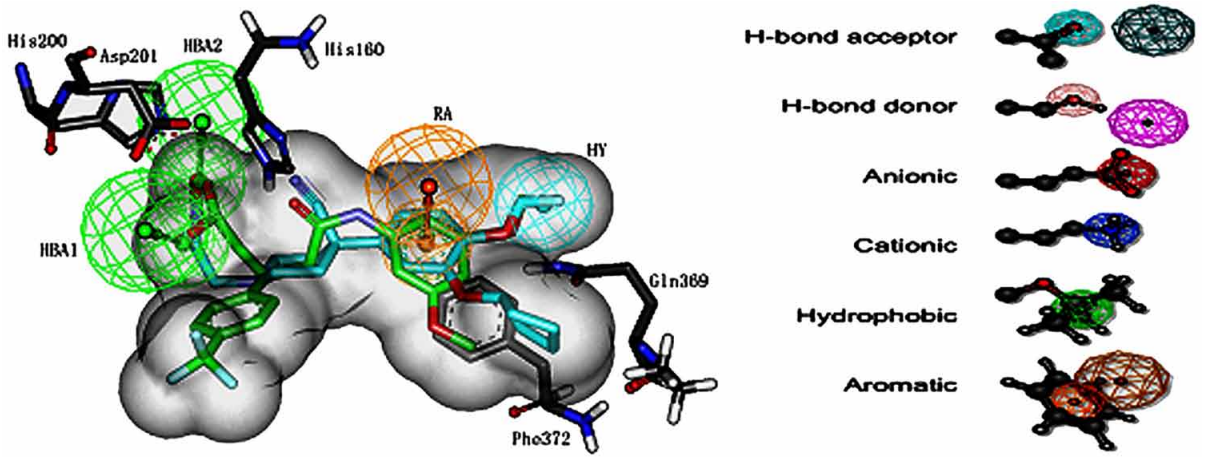
Similarity Analysis

Similarity search based algorithms use full techniques are finger print similarity analysis (2D finger print) or Electrostatic similarity analysis (3d shape) to find out similarity of a pharmacological activity query ligand with a biological data base. Fundamental developed for ligand chemical structure search, finger print similarity analysis very popular technique for conclusion of chemical molecular similarity between ligand based chemical compound (Gund, 2000). Finger print of data molecule similarity performed used in a coefficient score (Leach & Gillet, 2003).most popular finger print similarity analysis algorithms include ECFPs (Scitegic Extended Connectivity Fingerprints), 2D mol print and daylight finger print (Duan, Dixon, Lowrie, & Sherman, 2010).

Quantitative Structure Activity Relationship (QSAR)

Quantitative Structure Activity Relationship (QSAR) defined the mathematical correlation between structure characteristic and pharmaceutical target properties of ligand database (Perkins, Fang, Tong, & Welsh, 2003; Verma, Khedkar, & Coutinho, 2010) (Table 3).

Figure 4. Pharmacophore mapping of ligand and compound predicted by molecular docking.
[Source-Pharmacophore Modeling and Virtual screening for the Discovery of New type 4 cAMP Phosphodiesterase (PDE4) Inhibitors]



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Table 3. QSAR software and source of information link.

QSAR Software	Software Source
Topological index software (MolconnZ)	http://www.edusoft-lc.com_
CODESSA descriptor software	http://www.semichem.com/codessa.html
Dragon descriptor software	http://www.disat.unimib.it/chm/Dragon.htm
Pomona College QSAR site	http://clogp.pomona.edu/medchem/chem/qsar-db
CoMFA 3-D QSAR method	http://www.tripos.com/software/qsar.html
QSAR and Modelling Society	http://www.ndsu.nodak.edu/qsar_soc/index.htm
Summaries of QSAR studies in literature	Journal Quantitative Structure-Activity Relationship

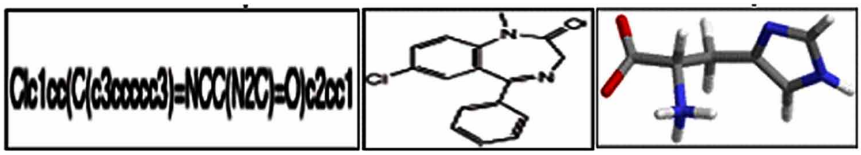
Hence in contrast to hit drug development technique such as pharmacophore based modeling, QSAR are commonly used in the optimization phase of drug development (Fischer, 2008). QSAR technique 1st time introduced Corwin Herman Hansch (father of QSAR concept) in 1860 (Hansch, 1969) and this technique improved by two scientists Crum Brown and Fraser in 1865 expressed the idea chemical structure and relationship activity(Crum –Brown & Fraser,1969; Richet, 1893; Meyer, 1899; Overton, 1901).

Different type molecule structure like one dimension, two dimension, three dimensions and multidimensional Quantitative Structure Activity Relationship (QSAR) based have been design time period the past separate decades (Lill, 2007).one dimensional QSAR defined biological activity by correlation of the pharmaceutical ligand physicochemical properties eg.log P value. And two dimensional QSAR structure character of pharmaceutical ligand compound relation between structures based eg.2D-Pharmacophore (Lill, 2007).In three dimensional Quantitative structure activity relationship (QSAR) congeniality is correlated with the three dimensional structure of pharmaceutical ligand eg- CoMFA. CoMFA (Comparative molecular field analysis) is defined the 3Dimenstional visualization of electrostatic field and steric of pharmaceutical ligand molecule bioactive conformation and protein ligand binding (Cramer, Patterso, & Bunce, 1989). Three dimensional Quantitative Structure Activity Relationship used tool CoMSIA (comparative molecular similarity indices analysis) procedure calculation pharmaceutical ligand field characters like hydrogen bond accepter, hydrogen bond donor and hydrophobicity to created 3-dimenstional Quantitative structure activity relationship models (Klebe & Abraham, 1999) (Figure 5).

QSAR Modeling Process

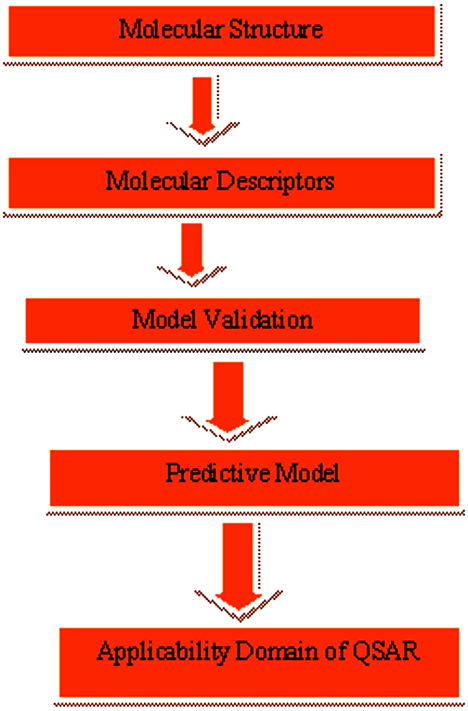
Quantitative structure activity relationship modeling process mainly five phases (Figure 6).

Figure 5. 1D, 2D, 3D structure



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Figure 6. Flow chart of QSAR modeling process.



Structure Based Virtual Screening

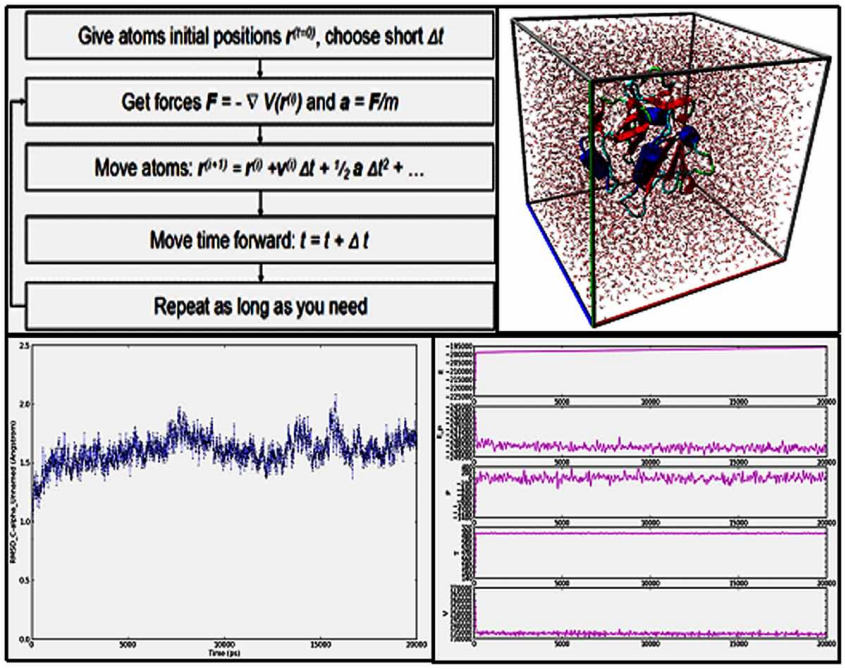
Structure based Virtual screening applied for drug development process pharmacological ligand target is available for three dimension ligand structure data. Structure based virtual screening interaction on ligand and protein and tool for optimization and hit drug development (Waszkowycz, 2008).

Molecular Dynamics Simulation

Molecular dynamic simulation is docking complex computational simulation method. Molecular dynamic come up of the first simulation method form the search function to the molecular dynamic of liquid phase introduced by Alder, Rahman and Wainwright in the later 1950s and early 1960s. There are two main category of molecular dynamics simulation process. In the classical working output to molecular dynamics simulation partials are treated as classical object, homologues much the ball and stick model. Molecule atom approach to soft ball and elastic stick corresponding to bond (Sandeep, Priyadarshini, Pradhan, Munikumar, & Umamaheswari, 2012). The rule of classical work defines the dynamic of the method. The first principal of molecular dynamic simulation started in 1980 with the seminal work of car and Parinello accepted finally in to account the quantum nature of the chemical bond. Quantum molecular dynamics simulation performs an important correction over the access and they are used in given information on a number of biological problem. Computer molecular dynamic simulation act as a pool between microscopic distance and time parameter and microscopic earth sphere of the laboratory we provide a conjecture at the interaction between molecule particles and receive correct prediction of high amount characteristic. A molecular dynamic simulation is illustrated with figure (Figure 7).

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Figure 7. A simplified description of molecular dynamics simulation.



Protein Ligand Docking

The Docking method is calculation of energy in complex including ligand and protein molecule. There are two role of docking research first is protein structure modeling and second is prediction information of biologically activity of ligand molecule. Docking is selection of scoring faction energy interact between ligand and protein ranking. Molecular characteristic such as hydrophilic nature, hydrogen bonding and electro static force as well as weight combination have been obtain a completely automated arrangement of molecule. Docking is basically work for accurate proposed structural modelling and correct prediction of the biological activity (Virtanen, 2013). The protein ligand docking development manly divided in two phase first know pocket site of pharmaceutical ligand docking development process and other randomly pharmaceutical ligand binding and using scoring faction of ligand docking energy (Kirchmair, Markt, Distinto, Wolber, & Langer, 2008).

Structure Based Pharmacophore Modeling

Structure based Pharmacophore modeling used in the geographical information related to the pharmaceutical ligand protein target to create a topologically characterization of pharmaceutical ligand protein interaction (Leach, Gillet, Lewis, & Taylor, 2010).Three dimensional structure information on the pharmaceutical ligand protein is mainly got from multidimensional or X-ray crystallography spectroscopy and NMR. It is necessary to secure the credibility of the ligand bind site residues and pharmaceutical ligand co-ordinate by visually check out theirs degree of fitness to the corresponding electron density graph available for illustration at the Uppsala Electron Density Server (Kleywegt et al., 2004).Structure

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based pharmacophore models example for the optimization and generation found in literature (Markt, Schuster, Kirchmair, Laggner, & Langer, 2007 and Barreca, 2007). Salam et al. (2009) defined a different procedure for creating structure based pharmacophore using active analysis. Software programs that allow for the manual construction of pharmacophores from protein-ligand complexes include Schrodinger's Phase, Accelrys Discovery Studio, MOE by the Chemical Computing Group and Inte:Ligand's LigandScout (Table 4).

Homology Modeling

The large scale different between the number of possible sequence and number of laboratory proof protein molecular sequence, which is limitation by time, product cost and laboratory objection built-in to the development of ligand structure determination could likely may be decide using homology modeling(Eswar, Eramian, Webb, Shen, & Sali, 2008). In non-attendance of laboratory proof structure homology modeling play important role of structure based drug development process.homology modeling is performed in seven phases (Krieger, Nabuurs, & Vriend, 2003).

1. Template recognition and initial alignment
2. Alignment correction
3. Backbone generation
4. Loop modeling
5. Side-chain modeling
6. Model optimization
7. Model validation

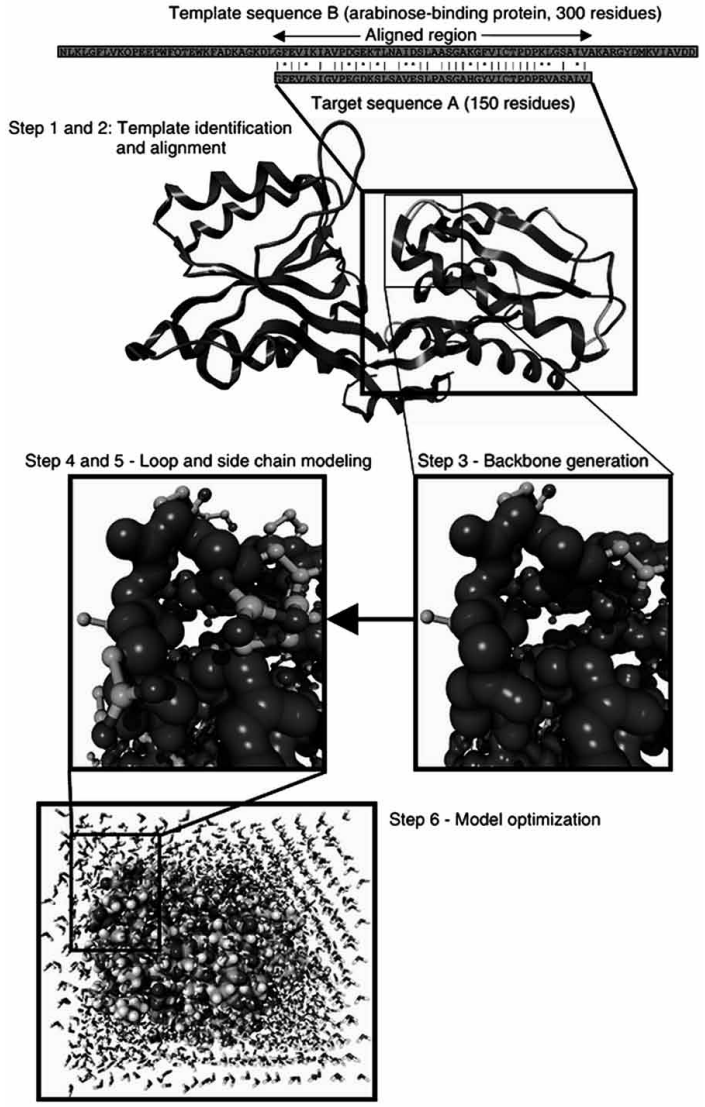
These phase may be continue until a good model is assembled (Cavasotto & Phatak, 2009). Although homology structure model are description of the known three dimension protein ligand structure, their property for virtual screening moment has been proved (Miguet, Zhang, Barbier, & Grigorov, 2006) (Figure 8).

Table 4. Structure based Pharmacophore modeling using various software.

Source	Software	Recent Published Use Cases
Accelrys	Catalyst (Discovery Studio)	http://dx.doi.org/10.1007/s00894-011-1105-5 http://dx.doi.org/10.1016/j.bmcl.2010.12.131
Schrödinger	Phase	http://10.1111/j.1747-0285.2011.01130.x http://cstest.ias.ac.in/cs/Volumes/100/12/1847.pd
Tripos	GASP	http://dx.doi.org/10.1016/j.jmgm.2010.02.004
Tripos	GALAHAD	http://dx.doi.org/10.1016/j.bmc.2011.09.016 http://dx.doi.org/10.1016/j.ejmech.2010.09.012
Inte:ligand	Ligandscout	http://dx.doi.org/10.1016/j.eplepsyres.2011.08.016
Chemical Computing Group	MOE	http://dx.doi.org/10.1007/s10822-011-9442-0 http://dx.doi.org/10.1016/j.ejmech.2010.07.020

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Figure 8. Steps of homology modeling
(Source- HOMOLOGY MODELING. Structural Bioinformatics)



Drug Discovery Process (Drug Cycle)

Approach for computer aided drug design deployment depend on the molecular structure and other biologically data information helpful regarding the propose target (Receptor/Enzyme) and screen ligand. Present time used in drug design deployment process two approach modeling design of drug directly and indirect design. In the direct strategies drug design based on relative analysis of the stretcher based characteristic know active and passive molecular compound. In direct drug design the three dimensional characteristic by the propose target (inhibiter) are directly consider (Frenking et al., 1996).

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The strategy of drug discovery process has remained evasive despite important analysis. Like the biological approach bioinformatics on which the drug designs discovery process is based (Cavagnaro & Lewis, 1987). Discovery process strategies for drug development depend on following factors-

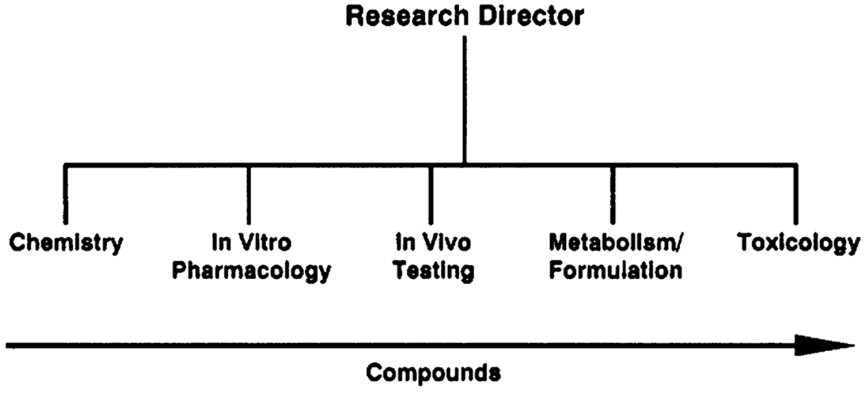
- Corporate sector, research, and marketing department cultures
- Individual scientists working within a company
- Synergies among various research disciplines
- Good quality of research management R&D leadership
- Individual and corporate experience
- Extent to which the management of a company is accustomed to risk Serendipity
- A particular company's market franchise (the therapeutic areas in which it markets drugs)

These processes are completed in 12-15 years (Figure 9)

Transmembrane Ion Channels

Voltage gated ion channel are a class of transmembrane ion channels that are activated by changes in electric potential difference. Sodium channels are integral membrane proteins that form ion channels which conducting sodium ions (Na^+) through a cell's plasma membrane (Jessell & Sanes, 2000).Voltage Gated Sodium channels (VGSCs) are members of the ion channel protein superfamily consisting of a large core protein, the α-subunit (220–260 kDa, ≈2000 amino acids), associated with one or more smaller regulatory β-subunits (22–36 kDa). VGSCs are transmembrane complexes play an essential role in neuronal and non-neuronal function, being responsible for the initiation and propagation of action potentials in excitable cells by allowing the influx of sodium ions. The α-subunit is sufficient for functional expression, forming the pore, determining the biophysical properties of the channel, and containing the ion selectivity filter. B-Subunits can modify the kinetic and voltage dependence properties of the channel and are involved in channel localization and interaction with cell adhesion molecules, extracellular matrix, and intracellular cytoskeleton. Voltage-dependent calcium channels (VDCC) are a group of voltage-gated ion channels found in excitable cells (e.g., muscle, glial cell, neurons etc.) with a permeability to the ion Ca^{2+}(William, Goldin, & Stephen, 2005). These channels are slightly perme-

Figure 9. Functional organization graph



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able to sodium ions so they are also called Ca^{2+}-Na^{+} channels, but their permeability to calcium is about 1000-fold greater than to sodium under normal physiological conditions(Yamakaqe & Namiki, 2002). A physiological or resting membrane potential VDCCs are normally closed. They are activated (i.e., opened) at depolarized membrane potentials and this is the source of the "voltage-dependent" epithet Activation of particular VDCCs allows Ca^{2+} entry into the cell, which, depending on the cell type, results in activation of calcium-sensitive potassium channels, muscular contraction (Michael & Walsh, 1982),excitation of neurons, up-regulation of gene expression or release hormones or neurotransmitters. On the other hand, K+ channels are membrane proteins that allow rapid and selective flow of K+ ions across the cell membrane, and thus generate electrical signals in cells. Voltage-gated K+ channels (Kv channels), present in all animal cells, open and close upon changes in the transmembrane potential. Kv channels are one of the key components in generation and propagation of electrical impulses in nervous system. Voltage-gated K^{+} channels are selective for K^{+} over other cations such as Na^{+}. Sodium channels are integral membrane protein that form ion channels which conducting sodium ions Na^{+}through a cell's plasma membrane. In excitable cells such as neurons, myocytes and certain types of glia, sodium channels are responsible for the rising phase of action potentials. Sodium channels can be of two types: voltage-gated called VGSCs and the other ones that respond upon binding by a ligand and thus are called as ligand-gated sodium channel. The sequence set of DNAs for VGSC, VGKc, VGCC and its Docking sites with various neurotoxins can help us to understand the structure and function of these ion channels. Further, neurotoxins that bind can be a potent Analgesics, Also this can be useful in knowing the molecular basis of the diseases which are the result of disfunctioning or the alteration in their normal rhythmicity of the ion channels.

Calcium Channel Sub Unit

Calcium channel biochemical properties are ligand protein complex composed of near about four or five separate encoded subunit by multiple genes (William, 2000) (Figure 10).

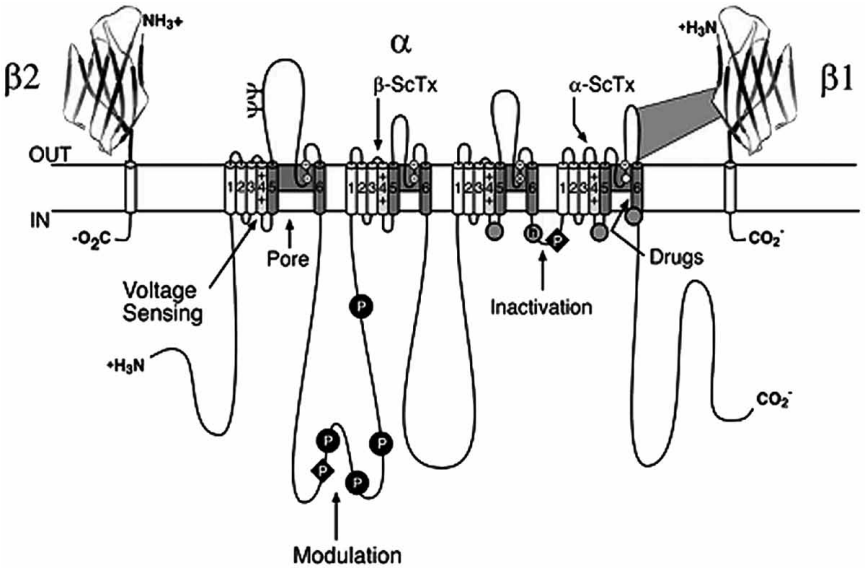
Sodium channel are tetrodotoxin resistant degree due to changes in amino acid protein sequence a indusial position of domain and more expressed in root ganglion neurons and heart pace maker(Fozzard & Hanck, 1996) (Table 5).

Table 5. Receptor site target on sodium channels.

Drug	Receptor Site	Domains
Tetrodotoxin, **Saxitoxin, μ-Conotoxin**	Neurotoxin receptor site-I	IS2–S6, IIS2–S6 IIIS2–S6, IVS2–S6
Veratridine, Batrachotoxin **Grayanotoxin**	Neurotoxin receptor site-II	IS6, IVS6
α-Scorpion toxins, Sea anemone toxins	Neurotoxin receptor site-III	IS5–IS6, IVS3–S4, IVS5–S6
β-Scorpion toxins	Neurotoxin receptor site-IV	IIS1–S2, IIS3–S4
Brevetoxins, Ciguatoxins	Neurotoxin receptor site-V	IS6, IVS5
δ-Conotoxins	Neurotoxin receptor site-VI	IVS3–S4
Local anesthetic drugs Antiarrhythmic drugs **Antiepileptic drugs**	Local anesthetic receptor site	IS6, IIIS6, IVS6

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Figure 10. Transmembrane organization of sodium channel subunits. The primary structures of the subunits of the voltage-gated ion channels are illustrated as transmembrane-folding diagrams. Cylinders represent probable α-helical segments. Bold lines represent the polypeptide chains of each subunit, with length approximately proportional to the number of amino acid residues in the brain sodium channel subtypes. The extracellular domains of theβ1 and β2 subunits are shown as immunoglobulin-like folds. ψ, sites of probable N-linked glycosylation; P, sites of demonstrated protein phosphorylation by protein kinase A (circles) and protein kinase C (diamonds); shaded, pore-lining S5-P-S6 segments; white circles, the outer (EEDD) and inner (DEKA) rings of amino residues that form the ion selectivity filter and tetrodotoxin binding site;+ +, S4 voltage sensors; h in shaded circle, inactivation particle in the inactivation gate loop; open shaded circles, sites implicated in forming the inactivation gate receptor. Sites of binding of α- and β- scorpion toxins and a site of interaction between αand β1 subunits are also shown.



BIOINFORMATICS AND MEDICAL SCIENCES (CLINICAL SCIENCES)

Bioinformatics has a deep effect in Medical sciences (Clinical Application). The molecular biological data base is very helpful for physicians to diagnose the disease and research strategies for therapy. Consider a situation where a patient with a genetic disease meets physicians. The physicians is not classified symptom casing disease physicians go to web information resource available at http://www.ncbi.gov. in which available detail gens related information on symptom. The information on the protein crystal structure can be acquired by following the link to web-protein data bank given in swiss port database. Following the other link to the DNA molecule sequence in the molecular genetic database also called gene bank the nucleotide sequencing of the gene is accrued along with data of gene asymmetry. Thus the Doctor/Physicians used in the biological database to collection of biological information about the symptom which disease diagnose and device approach for therapy (Cote & Robboy, 1980; Slee, 1978 and Current procedural terminology, 1970).

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The human genome project policy to complete the human gene sequencing process a gene database of all the dissimilarity in gene sequencing. And other bioinformatics tool is already acquired in certain clinical problem in diagnoses and treatment plan like PubMed etc (Markowitz & Ritter, 1995).

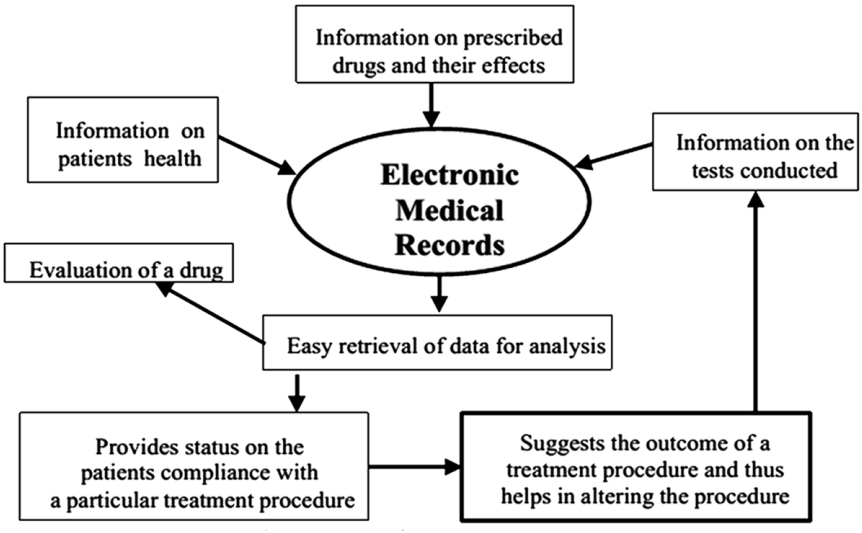
Most important of EMR (Electronic Medical Record) in medical science the information can be very easily acquired and shared in comparison to Conventional medical record. The EMR data expected for a study can be obtained directly from the electronic medical record thus producing research biological data collection for medical analysis a side effect of generally clinical data record keeping. The electronic medical record helpful for research protocol, point out to a physician when a eligible for a study, or when the protocol for a study calls for a specific management plan given the currently available data about that patient (Figure 11).

GENE THERAPY

Gene therapy is the therapeutic delivery of nucleic acid polymers into a patient's cells as a drug to treat disease. Gene therapy is a way to fix a genetic problem at its source level. The polymers are either expressed as proteins, interfere with protein expression, or correct genetic mutations. Biomedical helps to build up a therapeutic method to treat genetic diseases. The most common form uses DNA that encodes a functional, therapeutic gene to replace a mutated gene. The polymer molecule is caged within a carrier or vector, which carries the molecule inside affected cells.

Gene therapy was conceptualized in 1972, by authors who urged caution before commencing human gene therapy studies. The first gene therapy experiment approved by the US Food and Drug Administration (FDA) occurred in 1990, when Ashanti DeSilva was treated for ADA – SCID. (Sheridan, 2011), Bioinformatics has led to identification of several genes and development of databases where large data is stored which can be seen for conducting gene therapy.

Figure 11. Heterogeneity of Information in EMR and its advantages in designing better treatment procedures.
(source-www.mrc-lmb.cam.ac.uk/genomes/madanm)



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MICROBIAL GENOME APPLICATION

Microbial genome is ubiquitous and microorganisms are found in every place and any time. They have been found living and growing in crown of heat, cold emaciation, mineral acid, basicity and pressure. They are present in atmosphere, in human body and water, air and food. Generally use has been built of a different of microbial characters in the fermentation, bakery and food Technology Company. The coming of the whole genome sequence and their atmosphere, energy, healthy and food technology application. Introduced in 1994 department of energy (DOE) US proposed the microbial genome project (MGP) to sequencing gene of bacteria produced in energy formation, clean atmosphere, company processed food and harmful waste material.

Disease Causing Microorganisms

- Bacillus anthracis
- Brodetella pertusis
- Chlamydia trachomatis
- Clostridium botulinum
- Clostridium perfringens
- Clostridium tetani
- Corynebacterium diphtheriae
- Escherichia coli
- Gardinerella vaginalis
- Helicobacter pylori
- Haemophilus influenzae
- Klebsiella pneumoniae
- Legionella spp.
- Listeria monocytogenes
- Borrelia burgdorferi
- Anthrax
- Lyme disease
- Trachomas (blindness), etc.
- Botulism
- Gas gangrene & food poisoning
- Tetanus
- Diphtheria
- Typhoid fever
- Vaginitis
- Stomach ulcer
- Lung, ear infection, meningitis
- Atypical pneumoniae
- Legionnaire's disease
- Damage to fetus
- Whooping cough (pertusis)

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Understanding of specialized organism system used on microbial host to live in climate environment with other host. (http://microbialgenomics.energy.gov/) (Figure 12)

MOLECULAR MEDICINE

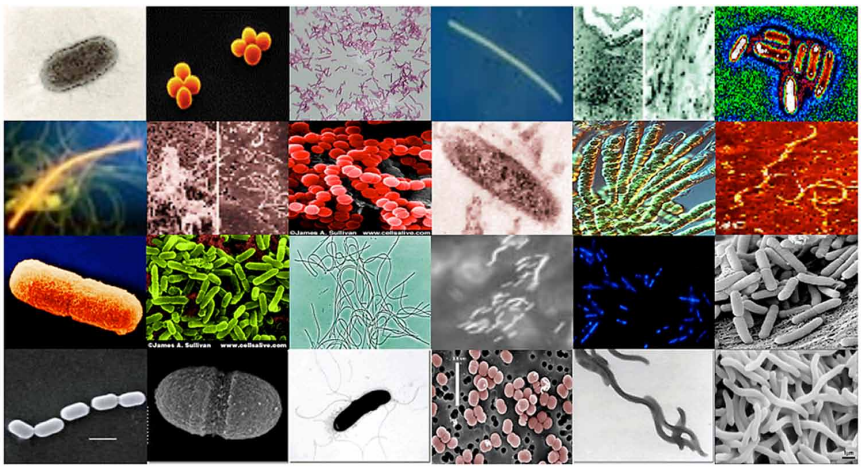
The human genome study has profound effect on the area of modern clinical research and bio medical science. This may be due to genetic response to environmental pressure which results in change in genome like-Diabetes, cancer, cardiac disease etc. The new knowledge of the molecular mechanism properties of causing disease will offer potentially best treatment and develop specific tool for disease diagnosis, treatment and prevention (Muniba, 2010) (Figure 13).

PERSONALIZED MEDICINE

Personalized medicine is a medical system which points out the systematic use in information technology about clinical medicine and patient information is collected and this has created a new area i.e. pharmacogenomics (Indiana University Division of Clinical Pharmacology, 2010). This field studies affected body genetic character response to medicine. Some medicine have been rejected in market (development phase) because of a less presence of the personalized medicine patient population, displays advance effect to a medicine due to gene sequencing variant in their DNA molecule. Personalized medicine can be defined by this field and nature of human physiology change that permit the targeting of medical medicine treatment which may include cell or tissue therapeutics (Muniba, 2010) (Figure 14).

To date, the labeling of more than 100 approved drugs contain information on genomic biomarkers (including gene variants, functional deficiencies, expression changes, chromosomal abnormalities, and others). Some, but not all, of the labeling include specific actions to be taken based on genetic information. Pharmacogenomic information can appear in different sections of the labeling (e.g., Therapeutic Indications, Warnings and Precautions) (Table 6).

Figure 12. Bacteria Morphological Diversity: fm http://ag.arizona.edu/plp/courses/plp329/Micd invitro



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Figure 13. Integration of these disciplines molecular medicine.

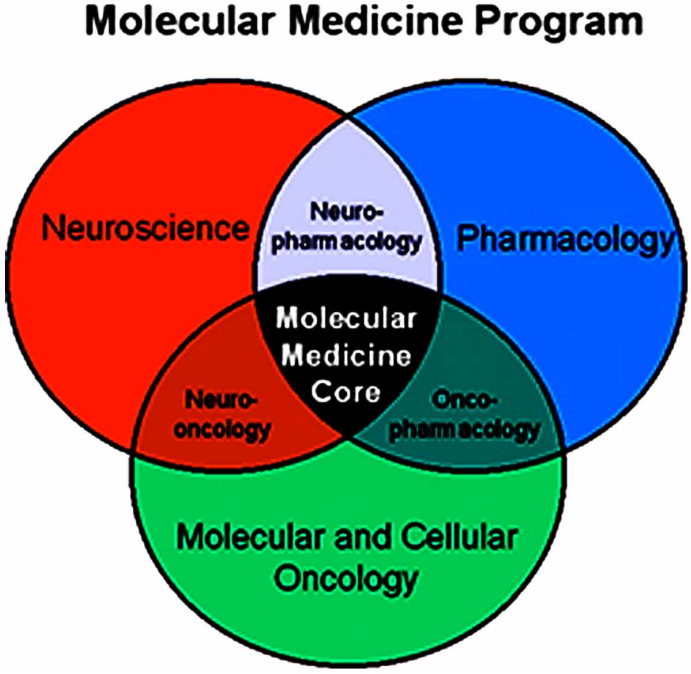
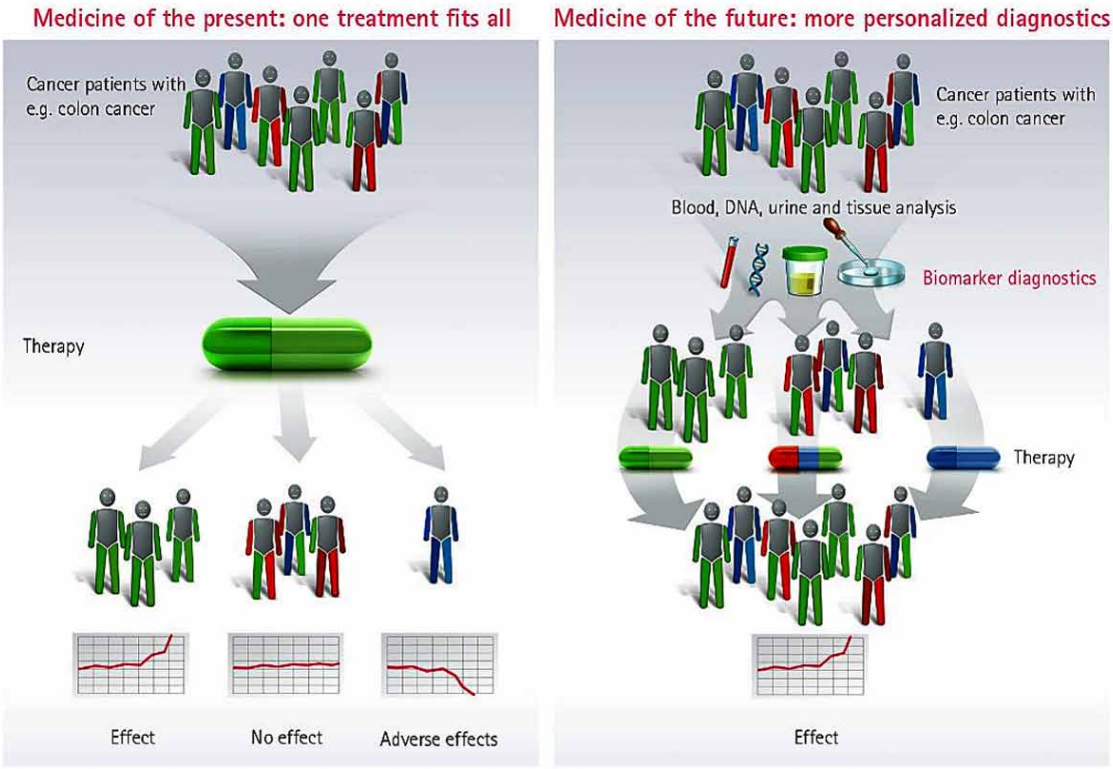


Figure 14. Different people respond differently to the same therapy.



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Table 6. Selected drugs with specific actionable guidance in labeling (arrange).

Biomarker	Therapeutic Range	Medicine (Drug)	Approval Time
PML/R ARα	Oncology	Arsenic Trioxide Tretinoin	2000 1995
CD30	Oncology	Brentuximab Vedotin	2011
DPD	Oncology	Capecitabine Fluorouracil	1998
EGFR; KR AS	Oncology	Cetuximab Panitumumab	2004 2006
ALK	Oncology	Crizotinib	2011
CD25/IL2	Oncology	Denileukin Diftitox	1999
ER/PR	Oncology	Letrozole Exemestane Fulvestrant	1997 1999 2002
C-Kit, PDGFR, FIP1L1	Oncology	Imatinib Lapatinib	2003 2007
HER 2	Oncology	Trastuzumab Everolimus Pertuzumab	1998 2009 2012
Ph Chromosome	Oncology	Imatanib Dasatanib Nilotinib	2003 2006 2007
G6PD	Oncology	Rasburicase	2002
CD20 antigen	Oncology	Tositumomab	2003
BRAF	Oncology	Vemurafenib	2011
UCD	Psychiatry	Valproic Acid	1978
CY P2C19	Psychiatry	Citalopram	1998
CYP2D6	Psychiatry, Neurology	Thioridazine Pimozide Aripiprazole Tetrabenazine Iloperidone	1962 1984 2002 2008 2009
CFTR	Pulmonary	Ivacaftor	2012
CYP2C9	Analgesics	Celecoxib	1998
CCR5	Antiviral	Maraviroc	2007
Chromosome 5qdeletion	Hematology	Lenalidomide	2005

Medical product (drug) characteristic must be arranged with adequate information about the drug molecule and its application. Food and drug administration (FDA) desire medical product arranged to be adequately (U.S. Food and Drug Administration., 2013). Scientifically accurate, tricky and finally information must be communicated to health care practitioner for drug prescription (Margaret & Hamburg, 2013).

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BIOINFORMATICS IN AGRICULTURE

Application of bioinformatics in agriculture plays most important role in fast generation society, world economy and environment. Mainly crop is important part of agriculture. Today crop percentage yield has increased since last century. And will continue to increase as agronomy relies on new breeding technology and biotechnological approach (Xue, Zhao, Liang, Hou, and Wang, 2008).

FUTURE RESEARCH DIRECTIONS

Today large scale of commercial and academic enterprise such as pharmaceutical industry research & development and Quality control employ bioinformaticians to work, perform and analyse. There is large scale and complete bioinformatics demand from the academia and industry. Today increasing demand of Bioinformatics expert has arisen from most of the pharmaceutical laboratory and biomedical department. Bioinformatics growth has been global due to developed computer network allowed for easy access to molecular biological data and development of various type of software program for easy analysis.

CONCLUSION

Rapid advances in the field of computers coupled with increasing computer literacy among professionals favor the implementation of computer applications in medical practice. Further, the availability of numerous databases on the Internet has revolutionized the way by which a physician devices a strategy for treatment. Projects like the Human Proteomics Initiative is a classic example to show the necessity of integrating Bioinformatics to predict structures and functions of proteins, Medical Sciences - to identify proteins that are important in metabolic or other disorders and Pharmacology (drug discovery) - to identify novel drugs against the predicted targets. Thus it is apt to conclude that all the three areas must work in concert to achieve the ultimate goal of understanding the basis of life process and apply it for the betterment of human lives.

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KEY TERMS AND DEFINITIONS

Bioinformatics: As an interdisciplinary field of science, bioinformatics combines computer science, statistics, mathematics, and engineering to analyze and interpret biological data.

Biomedical: Is the application of engineering principles and design concepts to medicine and biology for healthcare purposes (e.g. diagnostic or therapeutic).

Cheminformatics: Use of computer and informational techniques applied to a range of problems in the field of chemistry.

Database: A database is an organized collection of data.

Genomics: Is a discipline in genetics that applies recombinant DNA, DNA sequencing methods, and bioinformatics to sequence, assemble, and analyze the function and structure of genomes.

Pharmaceutical: Is a drug used to diagnose, cure, treat, or prevent disease.

Pharmacoinformatics: Drug discovery and development requires the integration of chemistry, biology, pharmacology, pharmaceutical technology and extensive use of information technology.

Proteomics: Is the large-scale study of proteins, particularly their structures and functions.

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Chapter 17
A Perspective on the Phytopharmaceuticals Responsible for the Therapeutic Applications

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ABSTRACT

The word phytopharmaceutical deals with a complex mixture of compounds derived from the plant source that is used as a medicine or drug. Primitive human societies have been depending on plants and plant products for various remedies. Several plants in the different forms have been reported in traditional medicine and to find a rational for the treatment of various diseases than to isolated single compounds. Many of the single compounds isolated from the plant origin are effectively used in the medicine. The search of natural products in drug discovery has been greatly enhanced in the last few years. The impetus to use phytopharmaceutical agents for the treatment of disease, most of the plant based drugs are quite safe and have lesser adverse effects and are claimed that it works as synergistic effects.

INTRODUCTION

Primitive human societies have been depending on plants and plant products for various remedies. In certain areas, these folk medicinal prescriptions are endemic and have survived through ages from one generation to next through word of mouth. They do not exist as written knowledge. Generally, these systems of medicines depend on old people's experiences. The person, prescribing these medicines has so-called scientific knowledge about the diseases. Indigenous systems of medicine are specially conditioned by cultural heritage and myths. In India, the reference to the curative properties of some herbs in the Rig Veda seems to be the earliest records of use of plants in medicine. The identity of several plants referred to in Suktas of the Rig Veda can be fixed with reasonable certainty, e.g. of *Semal, Pithvan, Palash,* and *Pipal.* A more detailed account is available in the Atharva Veda. The period of Rig Veda is

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estimated to be between 3500 and 1800 B.C. After the Vedas, there is no information on the development of this science in India for a period of about 1,000 years. Then appeared the two most important works on Indian system of medicine, the works of Charak and Susruta, namely the Charak-Samhita and Susruta-Samhita. The latter deals with about 700 drugs, some of these are not indigenous to India. In the modern era, the medicinal plants are being replaced more and more by synthetic drugs. But of late it is being realized that synthetics, though useful as life saving drugs, are responsible for many hazardous side effects. This has led to the revival of interest in herbal medicine with the reawakening of efforts to promote indigenous system of medicine.

Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies. For example, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections have been reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) are described as broad-spectrum antimicrobial agents (Rios& Recio, 2005).

Variety of plant extracts, such as an extract of the root of *Sophora flavescens*, an extract of stem bark of *Magnolia obovata*, and oil of *Melaleuca alternifolia*, have been used as traditional medicines for microbial infections, strongly suggests that natural products are the major source of important antimicrobial agents (Sohn et al., 2004). Several essential oils and their isolates have been found to exhibit strong antibacterial and antifungal activity. According to Wan et al. (1998), the majority of the essential oils assayed for their antibacterial properties showed a more pronounced effect against the Gram positive bacteria. The resistance of Gram negative bacteria to essential oil has been ascribed to their hydrophilic outer membrane, which can block the penetration of hydrophobic compounds into target cell membrane (Wan et al. 1998). The essential oils of the medicinal and aromatic plants rather than their extracts that have had the greatest use in the treatment of infectious pathologies in the respiratory system, urinary tract, gastrointestinal and biliary systems, as well as on the skin (Rios & Recio, 2005).Various essential oils viz., *Plectranthus mollis* (Joshi, 2014a), *Ocimum basilicum* (Joshi, 2014b), *Artemisia absinthium* (Joshi, 2013a), *Ocimum gratissimum*, *Ocimum sanctum* (Joshi, 2013b),*Cyathocline purpurea* (Joshi, 2013c), *Senecio belgaumensis* (Joshi, 2012a), *Lantana camara* (Joshi, 2012b), *Tridax procumbens* (Joshi& Badakar, 2012), *Phlomis bracteosa* (Joshi et al., 2011), *Feronia elephantum* (Joshi et al., 2011), *Erigeron floribundus* (Joshi et al., 2011), *Craniotome furcata* (Joshi, 2010) and *Anaphalis nubigena* var. *monocephala* (Joshi et al., 2009) have been reported promising *in vitro* antimicrobial activity.

The essential oils seem to have no specific cellular targets. As typical lipophiles, they pass through the cell wall and cytoplasmic membrane, disrupt the structure of their different layers of polysaccharides, fatty acids and phospholipids and permeabilize them. Cytotoxicity appears to include such membrane damage. The cytotoxic property is a great importance in the applications of essential oils not only against certain human or animal pathogens or parasites but also for the preservation of agricultural or marine products (Bakkali et al., 2008). Cytotoxic activities of essential oils or their major components, sometimes activated by light, have also been demonstrated in the mammalian cells *in vitro* by short-term viability assays using specific cell staining or fluorescent dyes including NRU (Neutral Red Uptake) test, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) test, Alamar Blue (resazurin) test, Trypan Blue exclusion test or Hoechst 33342 and propidium iodide test (Bakkali et al., 2008).

Essential oils have a calming, stimulating, pain alleviating and mood enhancing effect. They improve the functioning of internal organs like heart, lungs etc. Till now they were on the periphery of pharmacological preparations, inflammatory and flavouring agents. This is because they have multiple

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beneficial effects than just being pain relievers (Shukla & Shukla, 1997). Aromatherapy by essential oils and their isolates have been studied as an emerging trend of treatment for some physiological disorder (Buchbauer & Jirovetz, 1994). Pharmacology, pharmaceutical botany, medical and clinical microbiology, phytopathology, and food preservation are some fields in which the essential oils can be applied (Daferera et al., 2000).

Terpenoids are also used as chemical messengers. In the communication between different parts of the same organism, the messenger is referred to as a hormone. Chemicals that carry signals from one organism to another are known as semiochemicals, and grouped into two main classes. If the signal is between two members of the same species, the messenger is called a pheromone. Chemicals which carry message between members of different species are known as allelochemicals (Devon& Scott, 1972).

The ancient medical literature reports that surgery was performed but that physicians also recommended the use of some natural, and especially plant products, which represent an interesting point of comparison with current knowledge (Nobili et al., 2009). Curcumin has been widely studied because of its antioxidant and anti-inflammatory properties. It inhibits growth and induces apoptosis in a range of different cell types. Curcumin has ability to inhibit signaling through NF-kB and reduce expression of COX-2, LOX, NOS, MMP-9, uPA, TNF-a, cyclin D1, downregulates growth factor receptors EGFR, HER2, inhibits Bcl-2, Bcl-XL, IAP, Inhibits activity of protein kinases (EGFR, HER2, ERK, JNK, PKB/Akt, PKC, CYPs, GSTP1-1 and Inhibits VEGF production (Manson et al., 2005). Epigallocatechin-3-gallate, a polyphenol found in green tea, also affects growth, inducing G0/G1 cell cycle arrest and apoptosis in many cell types, and appears to modulate a range of signalling pathways and target molecules. Epigallocatechin-3-gallate inhibits the activity of 5-cytosine-DNA methyltransferase, p38, TNF-α- release, urokinase, LOX, COX. angiogenesis and VEGFR phosphorylation and VEGF induction. It also downregulates signalling through Her2 neu, PDGFR, transcription factors AP-1 and NF-kB (Manson et al., 2005).

Podophyllotoxins have a long therapeutic history. Podophyllotoxins have been extracted from the root of the Indian podophyllum plant (*Podophyllum peltatum*). Podophyllotoxin was used as a remedy by the American Indians for its emetic, cathartic, and anthelmintic effects. From a wide program of chemical synthesis (about 600 derivatives from 1950s to 1960s), two active compounds, etoposide and teniposide, emerged. Unlike other podophyllotoxin derivatives, etoposide and teniposide have no effect on microtubular structure or function at concentrations used in the clinic (Nobili et al., 2009). Psoralen is a natural photodynamic compound belongs to the family of furocoumarins, whose chemical structure consists of a furan ring fused with coumarin. It is produced by a variety of plants as a natural defence against pest. It occurs in *Psoralea corylifolia*, from which the natural compound derives its name, as well as in the common fig, celery, parsley, and parsnips. Its extended π-electron system, psoralen displays UV light-induced photosensitizing activity, useful in photodynamic treatment of various diseases, including psoriasis, vitiligo and other skin conditions like cutaneous T-cell lymphoma (Nobili et al., 2009). Natural products play a relevant role in cancer therapy today with substantial numbers of anticancer agents used in the clinic being either natural or derived from natural products from various sources such as plants, animals and microorganisms (also of marine origin) (Nobili et al., 2009).

Use of plants as a source of medicine has been an ancient practice and is an important component of the health care system in India (Pandey et al., 2013). In India, about70 percent of rural population depends on the traditional Ayurvedic system of medicine. Most healers/practitioners of the traditional systems of medicine prepare formulations by their own recipes and dispense to the patients. In the Western countries, approximately 40 per cent of people are using the herbal medicine for the treatment of various

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diseases (Pandey et al., 2013).The results of the herbal treatments led to their increased acceptance by the local people of Thailand. In a recent scientific studies that supported treatment with herbal preparations at the Kabchoeng Hospital of Thailand, where the efficacy and safety of *Cinnamon stomachic* mixture has been reported. The research suggested that this preparation was suitable for the treatment of patients with functional dyspepsia, in clinical randomized controlled trials, compared with simethicone (Chotchoungchatchai et al., 2012). The folk healers maintained knowledge regarding the proper use of indigenous plants for healing diseases specific to that area. Various preparations have been derived from folk medicine knowledge that consisted of medicinal plants that were available in the local area and were used for symptoms that related to diseases of the local people of Thailand (Chotchoungchatchai et al., 2012). For implementation of herbal medicine into hospitals, some uncertainty exists about the safety, effectiveness, and cost-effectiveness of complementary and alternative medicine methods, expanding their use, where reasonable evidence of their effectiveness and good evidence of their safety exists, might yield health, social, and economic benefits (Pandey et al., 2013).

Nature has gifted varieties of plant species occurring from different regions of world, whereas some species are found in particular zones. The alpine flora has their own properties to produce secondary metabolites compared to temperate region plants and vice versa. A plant can be considered as a system where many biochemical reactions occur. The secondary metabolites of plant or phytopharmaceutical are the product of biosynthesis where several factors alter the synthesis route. These factors are soil, climate, rainfall, altitude, grazing frequency, sunshine length etc, where plant produced different metabolites. As per the geoclimatic distribution of the flora, botanicals are consumed by the people as their own localities to cure the diseases. Some plants are frequently used in the food preparation almost as their daily recipes. A single plant is used in the treatment of various diseases or as a food supplement can varies phytochemicals as per the geoclimatic distribution. As the development of technologies more efforts are utilized to search of new chemical entities for treatment of the diseases which are safe from adverse effects. Now a day's various bioactive compounds are investigated from those where the plants are traditionally used for treatment of particular disease.

The isolated compounds which are found to be potent biological activities have been synthesized in laboratories as a modern medicine to fulfill the requirements of patients. A multidisciplinary approach to drug discovery, involving the generation of truly novel molecular diversity from natural product sources, combined with total and combinatorial synthetic methodologies, and including the manipulation of biosynthetic pathways (so-called combinatorial biosynthesis), provides the best solution to the current productivity crisis facing the scientific community engaged in drug discovery and development (Newman & Cragg, 2007). The exploration of Nature as a source of novel active agents that may serve as the leads and scaffolds for elaboration into desperately needed efficacious drugs for a multitude of disease indications (Newman & Cragg, 2007). For this purpose plant cell can be cultured as an alternative potential renewable source for sustainable production of phytopharmaceuticals. Though several studies on such aspects have been published in peer reviewed journals of national and international repute, the amount of research transferred from laboratory to published papers are more as compared to apply research findings to field or real ground is very less (Hurakadle et al., 2010; Pai et al., 2008). The greatest discovery in this field was made by Haberlandt (1902), where he showed un-natural successful growing of plant tissue and further use of plant growth regulators to control the organogenesis phenomenon. Since then it come across many successes stories in using this technique to grow, multiply and transfer number of plant species belonging to above mentioned categories. The only success being in agriculture industry wherein, varieties of banana, sugarcane have been widely used commercially apart from floriculture crops

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frequently using this technique. Medicinal plant industry is a booming cell which has several branches such as nutraceuticals, cosmetics, health medicines and several others. Most of today's herbal industries rely on the natural plant population as it being easy and less cost bearing in comparison to the costlier technologies in biotechnology field.

The discovery of fungal endophytes that produce novel compound has significant biological and commercial implications. Commercially, the fungal culture can be scaled up to provide adequate production to satisfy new drug development and patient treatment needs. This production reduces the need to harvest wild populations of the source plants, preserving these species for the future (Eyberger et al., 2006). Endophytic fungi from herbs could the sources of new drugs for treatment of various diseases in humans, animals, and plants. Endophytes are microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease (Bacon & White, 2000). The fungi in the plant appear to "peacefully coexist" with each other in the nutrient-rich tissues of the host plant. This coexistence may actually be more of a "chemical truce" (Rahman, 2000). Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance (Strobel et al., 2004). Distinctly from plants, endophytes can be cultured quickly and the biomass can be accumulated by large scale fermentation. Production of bioactive compounds can be increased by using biotechnology on endophytic fungi in order to meet demands while keeping biodiversity and sustainable ecosystem (Onifade, 2007).

A successful account of the necessity of other source like endophytes from *Taxus brevifolia* for production of taxol has been described by the Stierle and Strobel, (1995). According to Stierleand Strobel, (1995), the highly functionalized taxol a diterpene was isolated primarily from the inner bark of the relatively rare and slow growing Pacific yew tree, *Taxus brevifolia*, and a few related species, in extremely small quantities (<0.02% dry wt). The emergence of taxol as an effective anticancer agent created a difficulty viz., how to ensure an adequate supply of a natural compound of non-microbial origin. Although the pharmaceutical potential of taxol elevated the status of the yew from a nuisance weed to a precious commodity and natural resource, it did not alter the underlying problem; there were simply not enough trees to supply the growing demands for taxol. Advanced preclinical and phase I clinical development of taxol required several collections ranging in size from 5,000 to 15,000 pounds of dry bark. A mature Pacific yew (100 years old) yields approximately 10 pounds of dry bark, so each collection required the sacrifice of 500 to 1500 trees. As the efficacy of the compound became more apparent, the demand for additional taxol was increased. In 1987-1988 a 60,000-pound bark collection was undertaken with little controversy. The need for a second 60,000-pound bark collection in 1989, however, sparked concern about the impact such collection sizes may have on the continued existence of the species.

Even with this estimate, however, it was clear that additional sources of taxol would be necessary. In 1991-1993, a single course of clinical treatment was 125-300 mg of taxol, and typical treatments extended for ten or more courses. Treatment of the 12,000 women who die annually of ovarian cancer alone would consume as much as 36 kg of the drug. Isolation methods yielded 1 kg of taxol from 25,000 pounds of dried bark, or the bark of 2,500 yews. Simply treating ovarian cancer over a one-year period would consume 90,000 mature yews. The recent approval of taxol for the treatment of breast cancer will triple the projected demand. Should taxol prove as effective against other refractory cancers (head and neck and non-small cell lung cancers) as clinical trials have indicated, it is not unreasonable to assume that the demand for taxol may exceed 300 kg, or 750,000 trees per year. This represents an enormous demand on a limited resource. There are simply not enough yews growing in North America to satisfy projected needs of this drug over the next twenty years (Stierle & Strobel, 1995).

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Several research groups have addressed the supply problem in a variety of ways. Total syntheses from simple precursors were published virtually simultaneously by Holton et al., (1994) and Nicolaou, et al., (1994) and their co-workers. Although these syntheses are chemical masterpieces, they will probably not answer the supply question (Stierle & Strobel, 1995). To fulfill the requirement of the taxol, a novel endophytic fungus isolated from the inner bark of the Northwest Pacific yew tree was able to synthesize taxol in de novo fashion (Stierle & Stierle, 2000).

The clinically-active agents, topotecan (Hycamtin) and irinotecan® (Camptosar®; CPT-11), are semi-synthetically derived from camptothecin, isolated from the Chinese ornamental tree *Camptotheca acuminata* Decne (Cragg & Newman, 2009). Camptothecin has also been reported to be produced by an endophytic fungus of the family Phycomycetes, subsequently identified as *Entrophospora infrequens*, isolated from the inner bark of *Nothapodytes foetida* (Cragg & Newman, 2009). Despite being dropped from clinical trials by NCI in the 1970s due to severe bladder toxicity, extensive research on the structural modification of camptothecin led to the development of the more effective derivatives, topotecan (Hycamtin®) and irinotecan (CPT-11; Camptosar®). Topotecan is used for the treatment of ovarian and small cell lung cancers, while irinotecan is used for the treatment of colorectal cancers. This class of agents acts through inhibition of topoisomerase I, another important enzyme involved in the replication pathway of DNA during cell cycle progression and, to date, remains by far the most important class of topoisomerase I inhibitors (Cragg & Newman, 2004).

Many endophytes have the potential to synthesize various bioactive metabolites that may directly or indirectly be used as therapeutic agents against numerous diseases (Strobel et al., 2004; Staniek et al., 2008). In recent years, substantial efforts to discover effective inhibitors of α-glucosidases from natural sources have been made (Miller et al., 2012; Rivera-Chavez et al., 2013). Endophytic fungi are thus a rich source of novel organic compounds with interesting biological activities and a high level of biodiversity (Hussain et al., 2009). Novel antibiotics, antimycotics, immunosuppressants, anticancer, anti-diabetic compounds are few natural products have been obtained from endophytic fungi (Dompeipen et al., 2009). A non-peptidal fungal metabolite (L-783,281) was isolated froman endophytic fungus *Pseudomassaria* sp. collected from an African rainforest near Kinshasa in the Democratic Republic of the Congo (Zhang et al., 1999). This compound acts as an insulin mimetic and unlike insulin, is not destroyed in the digestive tract and may be given orally. Oral administration of L-783,281 to two mouse models of diabetes resulted in significant lowering of blood glucose levels. These results may lead to new therapies for diabetes (Zhang et al., 1999). The compounds thielavins A, J and K have been isolated from an endophytic fungus from *Hintonia latiflora*. All these compounds showed in vitro α-glucosidase inhibitory action which was higher than that of acarbose. The a-glucosidase inhibitory properties of thielavin K were corroborated in vivo since it induced a noted anti-hyperglycemic action during an oral sucrose tolerance test (3.1, 10.0 and 31.6 mg/kg) in normal and nicotinamide–streptozotocin diabetic mice. In addition, at a dose of 10 mg/kg, it provoked a moderate hypoglycemic activity in diabetic mice (Rivera-Chavez et al., 2013). Fungal endophytes have begun to be recognized as a rich and diverse source of natural products. Podophyllotoxin, an aryltetralin lignan, is a valuable natural product as a precursor to three anticancer drugs, etoposide, teniposide, and etoposide phosphate. Destruction of wild populations of the primary source plant (*Podophyllum emodi* Wall. Ex Royle) and difficulties of total synthesis of the compound have led many laboratories to search for alternative sources. Endophyte fungi are widespread and perhaps ubiquitous in their occurrence in higher plants (Eyberger et al., 2006).

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BACKGROUND

The word phytopharmaceutical deals with a complex mixture of compounds derived from the plant source that is used as a medicine or drug. Plants are the "Sleeping Giants of Drugs Development" as they form a reservoir of potential useful untapped source of drugs that have been serving mankind since the dawn of civilization.

The plant chemicals used for these latter purposes are largely the secondary metabolites, which are derived biosynthetically from plant primary metabolites (e.g., carbohydrates, amino acids, and lipids) and are not directly involved in the growth, development, or reproduction of plants. These secondary metabolites can be classified into several groups according to their chemical classes, such alkaloids, terpenoids, and phenolics. The functions of secondary metabolites in the plants are not understood, but it is believed that these compounds are utilized by the plants for self-defense against microbes or by other external predators. Moreover, the quantitative and qualitative divergence of secondary metabolites may be due to the geographical, climatic, and soil conditions, which in turn may affect the composition and/or synthesize new secondary metabolites\chemotypes from the same plant species (Joshi, 2013d; 2014c). It was observed that *Ritu* or season influences secondary metabolite concentration in the plants or its parts. The ability to synthesize and accumulate the compounds in plants varied greatly throughout the seasons. Consequently, to obtain optimum utility of the plant as a source of medicine, the screening for presence of the secondary metabolites during different season is useful (Ketkar et al., 2015). Several plants in the different forms have been reported in traditional medicine and to find a rational for the treatment of various diseases than to isolated single compounds. The phytopharmaceuticals or extracts are under pipeline in clinical trials for treatment of diseases. The efficacy of phytopharmaceutical agents used in different forms by the people for centuries was validated in many cases by pre-clinical and clinical studies. The impetus to use phytopharmaceutical agents for the treatment of disease, most of the plant based drugs are quite safe and have lesser adverse effects and are claimed that it works as synergistic effects. Synergy effects of the mixture of phytopharmaceuticals and their byproducts contained in plants are claimed to be responsible for improved effectiveness. The multi-drug theory is already being practiced worldwide in the treatment of AIDS and other infectious diseases, hypertension, numerous types of cancer and rheumatic diseases. However, many of the single compounds isolated from the plant origin are effectively used in the medicine.

MAIN FOCUS OF THE CHAPTER

The search of natural products in drug discovery has been greatly enhanced in the last few years. In recent years many of the natural products of medicinal value are the source of drugs, which have been used effectively for centuries in traditional medicine. All the chemical structures are depicted in Figures 1, 2 and 3 wherein the compounds are numbered in parenthesis. In 1805, morphine (1) became the first pharmacologically active compound to be isolated in pure form from a plant. Morphine is profusely found in dried latex of unripe seedpods of *Papaver somniferum* commonly known opium poppy. Morphine is belongs as a narcotic analgesic drug which directly acts on the central nervous system of the body to relieve pain. Codeine (2) or 3-methylmorphine is also found in *Papaver somniferum* in low quantity but it can be derived from morphine. Codeine is used mainly as an antitussive agent, but also as a non-narcotic

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analgesic. It has been said that it is useful in treating malaria patients. Its use in the treatment of diarrhoea, however, is out of date, because more effective substitutes are now available (Theuns et al., 1986).

Ephedrine (3) is a sympathomimetic alkaloid, isolated from traditional Chinese herb *má huáng*known as *Ephedra sinica.* The ephedrine is used for therapeutic purpose, primarily as a bronchodilator and decongestant. Its pharmacological applications are related to its sympathomimetic properties, which in turn occur due to its stimulating action on α-, β1-, and β2-adrenergic receptors through direct and indirect effects. Nowadays, there is a concern about the indiscriminate use of ephedrine, since it started to be used to weight loss and enhance athletic performance (Limberger et al., 2013).

The drug atropine (4) is a tropane alkaloid isolated from the plants *Atropa belladonna* of the family Solanaceae. In general, atropine counters the "rest and digest" activity of glands regulated by the parasympathetic nervous system. This occurs because atropine is a competitive antagonist of the muscarinic acetylcholine receptors (acetylcholine being the main neurotransmitter used by the parasympathetic nervous system). Atropine dilates the pupils, increases heart rate, and reduces salivation and other secretions (http://en.wikipedia.org/wiki/Atropine) (see Figure 1).

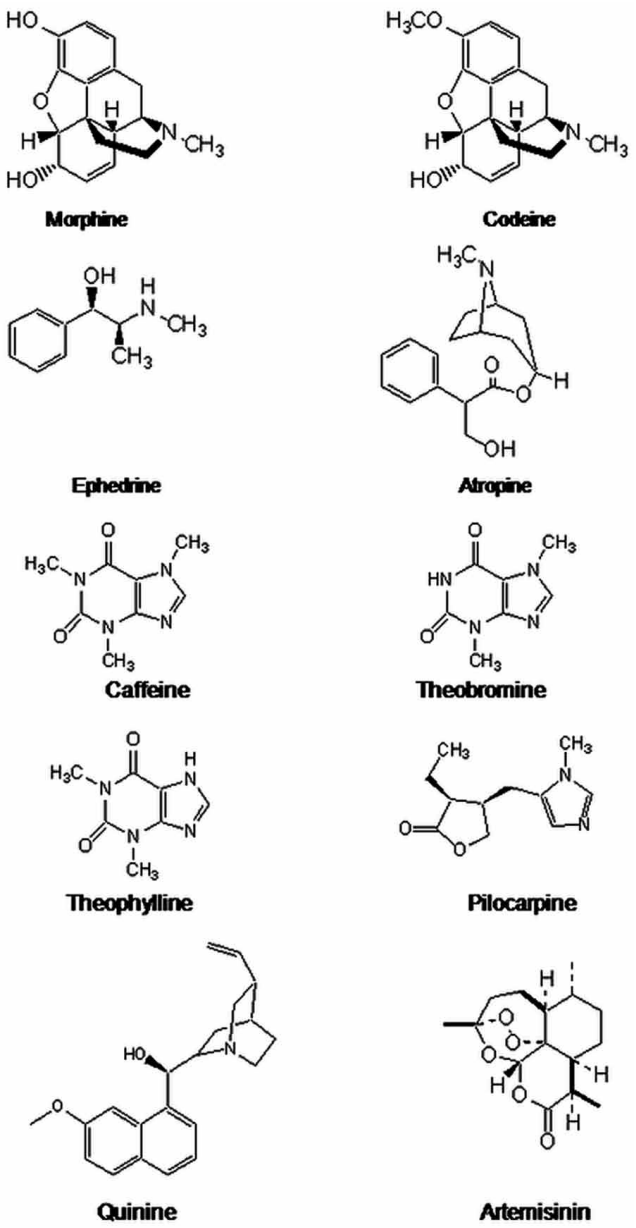
Caffeine (5) (1,3,7-trimethylpurine-2,6-dione), is a bitter white crystalline central nervous system stimulant psychoactive (Nehlig et al., 1992) alkaloid and has mild diuretic property (FDA, 2010). The chief source of caffeine is a plant *Coffea arabica* of the family Rubiaceae commonly known as coffee. The beans, leaves, and fruit of more than 60 plants contain varying quantities of caffeine (Kovacs, 2008; Andrews et al. 2006). In those plants caffeine serves as a pesticide, killing or paralyzing insects that feed on the plants. Coffee, tea, and cocoa are the major plants containing caffeine that are cultivated. Guarana, kola nuts, and yerba maté are also cultivated for use as sources of caffeine in energy drinks and snacks. The FDA says that caffeine is both a drug and a food additive. Caffeine is used in both prescription and over-the-counter medicines to treat tiredness or drowsiness and to improve the effect of some pain relievers. People with heart problems shouldn't use caffeine because it makes their hearts work too hard, and people with anxiety problems or panic attacks may find that caffeine makes them feel worse (http://www.fda.gov/downloads/UCM200805.pdf). Caffeine is used for prevention and treatment of bronchopulmonary dysplasia which is a chronic pulmonary condition that usually evolves after premature birth and respiratory distress syndrome (Kugelman & Durand, 2011; Schmidt, 2005). Caffeine therapy for apnea of prematurity improves weight gain (Schmidt et al., 2007), neurodevelopmental disability in infants, with very low birth weight. Caffeine is an adenosine-receptor blocker that inhibits adenosine induced vasodilatation by blocking these receptors used to treat orthostatic hypotension due to autonomic failure (Schmidt et al., 2006).

Theobromine (6) (3,7 dehydro3,7 dimethyl-IH-purine-2,6-dione) also known as 3,7-dimethylxanthine is a purine alkaloids. It is widely distributed in nature in a variety of plants such as *Commelia thea* (tea leaves), *Theobroma cacao* (cocoa), *Cola acuminata* (cola), *Paullinia cupana*, *Ilex aquifolium* and other *Ilex* species including Theaceae, Sterculiaceae, Sapindaceae (Eteng et al., 1997). Theobromine is used as a diuretic, central nervous system stimulants (Leonard et al., 1987), myocardial stimulants, vasodilators and smooth muscle relaxants (Windholz, 1983).

Theophylline (7) is the typical alkaloid in mate´ and green tea. Only the slightest of traces has been established in cacao (Matissek, 1997). Theophylline has been used in the treatment of asthma for over 70 years, but its use has recently declined, as inhaled corticosteroids have become the mainstay of asthma control and inhaled β_2-agonists are more effective bronchodilators (Ito et al., 2002).Asthma is a chronic inflammatory disease of the airways characterized by reduced airway patency, which is regulated by bronchodilators such as β-agonists, and by the infiltration of inflammatory and immune cells, which

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Figure 1. Chemical structures of: (1) Morphine; (2) Codeine; (3) Ephedrine; (4) Atropine; (5) Caffeine; (6) Theobromine; (7) Theophylline; (8) Pilocarpine; (9) Quinine; (10) Artemisinin.



is treated by corticosteroids (Ito et al., 2002). There is increasing evidence that theophylline has anti-inflammatory or immunomodulatory actions in asthma (Barnes & Pauwels, 1994).

Pilocarpine (8) is an imidazole alkaloid found large amount in the plant *Pilocarpus microphyllus* (Pinheiro, 1997). This plant is a shrub and found in the under storey of the pre-Amazonian rain forest and in the state of Maranhão (Sandhu et al., 2006). The pilocarpine is a cholinergic parasympathomimetic agent with predominantly muscarinic action, it may stimulate cholinergic receptors on the surfaces of

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the exocrine glands, causing a reduction of the symptoms of xerostomia, includes dry mouth, mucosal burning, and fissured lips and/or tongue, with difficulty in eating, swallowing, conversing, and wearing removable prosthetic devices such as dentures and even in patients with no measurable salivary flow at rest (Gornitsky et al., 2004). Xerostomia represents a frequent and significant radiation-induced side effect in the treatment of head and neck cancers. It leads to increased incidence of dental caries, oral mucositis, and impaired mastication (Gornitsky et al., 2004). Moreover, various studies reported that pilocarpine improves the overall condition of xerostomia when prescribed after radiotherapy (LeVeque et al., 1993; Johnson et al., 1993).

Quinine (9) is cinchona alkaloid isolated from the bark of several species of cinchonatrees. The high yield obtained from the plant *Cinchona ledgeriana* (Song, 2009). Quinine had a profound influence on modern colonial history and a number of historians have highlighted the importance of this single drug as one of the "tools of empire" (Meshnick & Dobson, 2001). Quinine was used as the antimalarial drug caused by *Plasmodium falciparum*. As per the recommendation of World Health Organization (WHO) quinine should be used when artemisinins are not available (WHO, 2006).

Artemisinin (10), having very strong potential as the non-conventional antimalarial drug, is a sesquiterpene lactone with endoperoxide bridge and has been isolated from *Artemisia annua*, a pervasive wormwood with a long history of use, known as 'Qinghao'. The Chinese scientist extracted a crystalline compound named "Qinghaosu" (QHS) from 'Qinghao', *Artemisia annua*(Kinghorn, 1987) and this stable peroxide emerged as a potent antimalarial (Luo & Shen, 1987).

Artesunate (11), a derivative of artemisinin isolated from *Artemisia annua* is recommended by the WHO in preference to quinidine for the treatment of severe malaria and has been used worldwide for many years (http://www.cdc.gov/malaria/diagnosis_treatment/artesunate.html). In laboratory models artesunate has demonstrated cytotoxic effects against a variety of cancer cells. However, there is little available information about the antitumor effects of artesunate on human gastric cancer cells (Zhang et al., 2015).

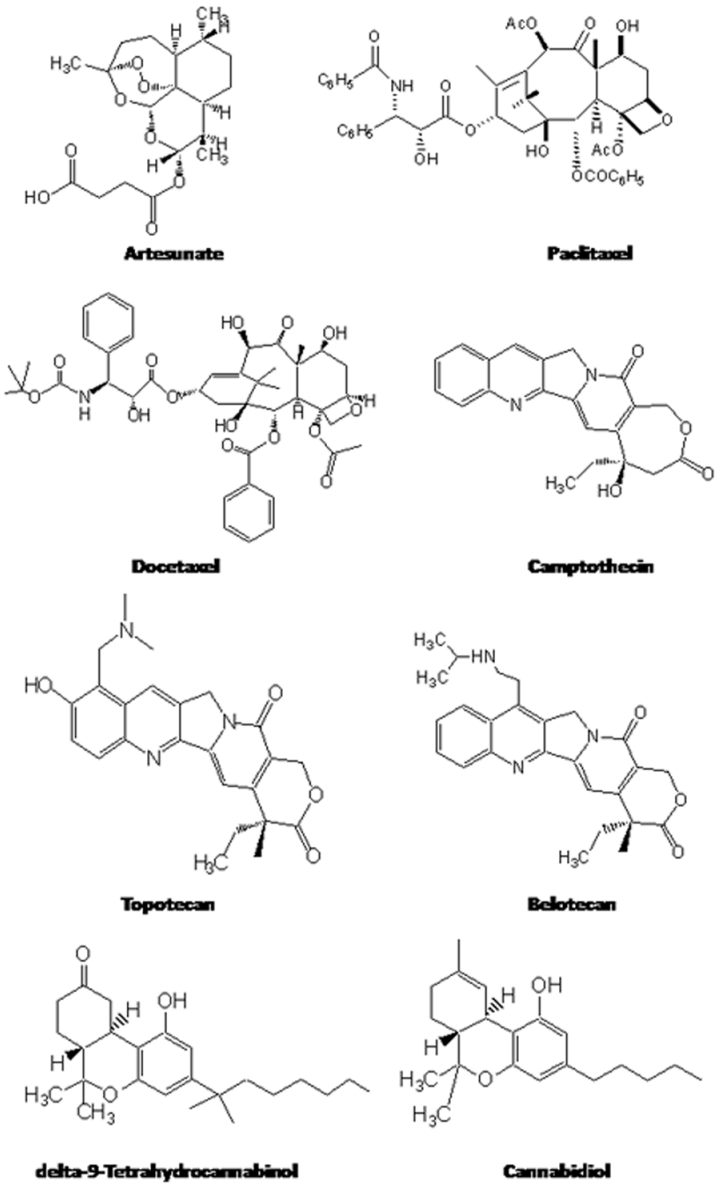
The taxane class agents (paclitaxel and docetaxel) are one of the most important cancer chemotherapeutics in the past several years. Both drugs are very similar in mechanism of action, preclinical and clinical activities. Paclitaxel (12) (Taxol), one of microtubule-stabilizing anticancer agent, was first obtained from the bark of Pacific yew (*Taxus brevifolia)* in 1971. The Pacific yew is a highly toxic plant but it was employed medicinally by several native of North American Indian tribes who used it to treat a variety of complaints (Moreman, 1988). Paclitaxel is used for the treatment of metastatic pancreatic cancer, non-small cell lung cancer and breast cancer (http://www.cancer.gov/about-cancer/treatment/drugs/fda-nanoparticle-paclitaxel) (see Figure 2).

Docetaxel (13) is semi-synthetically derived compound from 10-deacetylbaccatin III extracted from the needles of the European yew (*Taxus baccata)*. Docetaxel is used for treatment of various diseases viz., head and neck cancer, gastric cancer, breast cancer, prostate cancer, and non-small cell lung cancer (http://www.cancer.gov/about-cancer/treatment/drugs/fda-docetaxel). The laboratory studies showed that the paclitaxel a chemotherapy drug, can be used in the treatment of renal fibrotic diseases. Mechanistically, paclitaxel treatment blocks the STAT3 activity by disrupting the association of STAT3 with tubulin and STAT3 nucleus translocation (Zhang et al., 2015).

The compound camptothecin (14), a planar pentacyclic ring system containing a pyrrol (3,4-b) quinoline and α-hydroxy lactone was isolated from the plant *Camptotheca acuminata* Decne (Nyssaceae), a tree that is native to China, growing in relatively warm areas of southeastern provinces of Szechwan, Yunnan, and Kwangsi (Perdue et al., 1970). Topotecan(15) is a chemotherapeutic agent that is a topoisomerase

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Figure 2. Chemical structures of: (11) Artesunate; (12) Paclitaxel; (13) Docetaxel; (14) camptothecin; (15) Topotecan; (16) Belotecan; (17) delta-9-Tetrahydrocannabinol; (18) Cannabidiol.



inhibitor. It is a water-soluble derivative of camptothecin. It is used in form of the hydrochloride to treat ovarian cancer and lung cancer, as well as other cancer types (http://www.ebi.ac.uk/chebi/searchId.do?chebiId=CHEBI:63632. In addition, a semi-synthetic camptothecin derivative, belotecan (16), was launched in Korea for treatment of small-cell lung and cervical cancer (Butler, 2005).

Two FDA-approved drugs, dronabinol and nabilone, contain delta-9-tetrahydrocannabinol (THC) (17). They treat nausea caused by chemotherapy and increase appetite in patients with extreme weight loss caused by AIDS (NIDA, 2015). THC is the main ingredient of the plant marijuana, and is principally

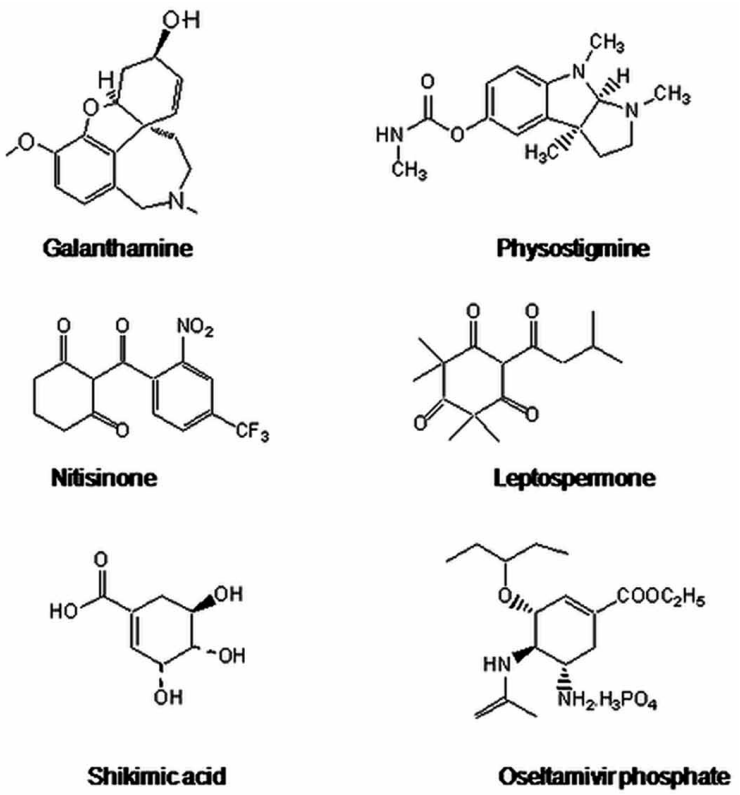
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responsible for the psychoactive effects (Cooper & Haney, 2009). Marijuana is the crude drug derived from the plant *Cannabis sativa*, belongs to the family Cannabaceae that has only one genus *Cannabis* with only one species *sativa* that is highly variable (Elsohly & Slade, 2005). Cannabis has had a long history of use (over 5000 years) starting in Central and Northeast Asia with current use spreading worldwide as a recreational drug or as a medicine albeit unauthorized. The cannabis plant (*Cannabis sativa*) and products thereof (such as marijuana, hashish and hash oil) have a long history of use both as a medicinal agent and intoxicant. Over the last few years there have been an active debate regarding the medicinal aspects of cannabis. Currently cannabis products are classified as Schedule I drugs under the Drug Enforcement Administration (DEA) Controlled Substances act, which means that the drug is only available for human use as an investigational drug (Russo, 2001; 2002; Elsohly, 2002; Elsohly & Slade, 2005). Cannabidiol (18) (CBD) another compound of marijuana exhibited prominent anticancer activity (Scott et al., 2013).The United Kingdom, Canada, and several European countries have approved nabiximolssynthetic cannabinoids, a mouth spray containing THC and CBD. It treats muscle control problems. The United States is conducting clinical trials for its safe use in treating cancer pain (NIDA, 2015*).In vitro* studies of THC and CBD both alone and in combination with radiotherapy in a number of glioma cell lines (T98G, U87MG, and GL261) inhibit glioma growth as well as neutralize oncogenic processes such as angiogenesis. High-grade glioma is one of the most aggressive cancers in adult humans and long-term survival rates are very low as standard treatments for glioma remain largely unsuccessful. These studies suggested a potential clinical benefit for glioma patients by using these treatment modalities.

Alzheimer's disease (AD) was first described by the German psychiatrist, Alois Alzheimer, in 1907. Alzheimer's disease is a neurological disorder in which the death of brain cells causes memory loss and cognitive decline. It is a neurodegenerative disease, which means there is progressive brain cell death that happens over a course of time (National Institute of Neurological Disorders and Stroke (NINDS), 2013).The total brain size shrinks with Alzheimer's - the tissue has progressively fewer nerve cells and connections (NINDS 2013; Alzheimer's Association, 2013a). The most recent census has enabled researchers to give estimates that in 2010, 4.7 million people of 65 years of age and older were living with Alzheimer's disease in the US (Hebert et al., 2013). In 2013 statistical report from the Alzheimer's Association gives a proportion of the population affected - just over a tenth of people in the over-65 age group have the disease in the US. In the over-85s, the proportion goes up to about a third (Alzheimer's Association, 2013b). The development of galanthamine (galantamine) (19) a drug commonly used today in the treatment of AD. It is a natural product known from several members of the Amaryllidaceae and the idea for developing a natural product from these species seems to be based on ethnobotanical information. This alkaloid was first isolated from snowdrop (*Galanthus* spp., most notably *G. woronowii*), but today it is obtained from *Narcissus* spp. and *Leucojum* spp. (esp. *L. aestivum*) as well as synthetically (Heinrich & Teoh, 2004). Galantamine has a unique, dual mode of action. It is a reversible, competitive inhibitor of acetylcholinesterase, and proven activity as an allosteric modulator of nicotinic acetylcholine receptors (nAChRs). This latter activity is thought to be particularly important since decreases in the expression and activity of nAChRs make a large contribution to the reduction in central cholinergic neurotransmission in patients with AD (Lilienfeld, 2002). Physostigmine (20) or serine is a parasympathomimetic alkaloid, widely regarded as the antidote to anticholinergic toxicity, is a naturally occurring cholinesterase inhibitor found in the Calabar bean (Watkins et al., 2014) and short span of action has rendered it useless for treating Alzheimer's disease (Scheindlin, 2010).The Calabar bean or ordeal bean (*Physostigma venenosum* Balf. of the family Leguminosae) is the seed of a leguminous plant, a native of tropical Africa, poisonous to humans (Lloyd, 1897) (see Figure 3).

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Figure 3. Chemical structures of: (19) Galanthamine; (20) Physostigmine; (21) Nitisinone; (22) Leptospermone; (23) Shikimic acid; (24) Oseltamivir phosphate



Tyrosinemia type 1 is a rare genetic metabolic disorder characterized by lack of the enzyme fumarylacetoacetate hydrolase (FAH), which is needed to break down the amino acid tyrosine. Failure to properly break down tyrosine leads to abnormal accumulation of tyrosine and its metabolites in the liver, resulting in severe liver disease. Tyrosine may also accumulate in the kidneys and central nervous system (McKiernan, 2006). Tyrosinemia type 1 affects males and females in equal numbers. It is estimated to occur in one of every 100,000-120,000 births in the United States. In one region of the Quebec province of Canada, the disorder is estimated to occur in approximately one in 1,850 births. The birth prevalence is also higher in Norway where it is estimated to be one in 60,000 (NORD, 2012). The triketones, an important new class of herbicides, are reported to be derivatives of the plant-produced phytotoxin leptospermone, derived from the bottlebrush plant (Callistemon spp.) (Duke et al., 2000). *Callistemon citrinus* commonly known as Bottlebrush belongs to a family Myrtaceae and comprises over 30 species that has a great medicinal importance (Mabberly, 1997). *Callistemon* spp. are also used as weed control (Wheeler, 2005) and as bioindicators for environmental management (Burchett et al., 2002). Nitisinone (21) is a derivative of leptospermone (22), a new class of herbicide isolated from the *Callistemon citrinus*. Both the compounds nitisinone and leptospermone inhibit 4-hydroxyphenyl pyruvate dioxygenase (HPPD), the enzyme involved in plastoquinone and tocopherol biosynthesis in plants (Duke et al., 2000). In humans, inhibition of HPPD prevents tyrosine catabolism, leading to the accumulation of tyrosine metabolites, 4-hydroxyphenyl pyruvic acid and 4-hydroxyphenyl lactic acid,

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which can be excreted through the urine (Hall et al., 2001). The drug nitisinone was approved by the FDA for the treatment of hereditary tyrosinemia type 1 diseases.

Shikimic acid (23) was first isolated in 1885 by Eijkman from the fruit of the Japanese plant *Illicium religiosum* Sieb. The elucidation of its structure nearly 50 years later, and the discovery of shikimic acid, the starting material in the commercial synthesis of the antiviral agent oseltamivir under the trade name Tamiflu (Enrich et al., 2008). Oseltamivir phosphate (24) (Tamiflu) is an orally active neuraminidase inhibitor developed for the treatment and prophylaxis of influenza viruses A and B. The starting material for the oseltamivir synthesis is (−)-shikimic acid, an important biochemical intermediate in plants and microorganisms.

One of the compound fulyzaq (crofelemer), the first anti-diarrheal drug for HIV/AIDS patients has been recently approved by U.S. Food and Drug Administration (FDA). The compound crofelemer is a proanthocyanidin oligomer, isolated from red latex of the plant *Croton lechleri* (Euphorbiaceae) found in the western Amazonian regions of South America (Orozco-Topete et al., 1997). Native populations of South America have used the sap of the plant for self-treatment of secretory diarrhea for many years (Crutchley et al., 2010). Crofelemer is a first-in-class agent that may be useful for different types of secretory diarrhea, since it prevents chloride and fluid secretion into the bowel by directly inhibiting 2 distinct intestinal chloride channels (Crutchley et al., 2010). Crofelemer possesses a unique mechanism of action through inhibition of both cyclic adenosine monophosphate (cAMP)–stimulated cystic fibrosis transmembrane conductance regulator (CFTR) and calcium stimulated (CaCC) chloride intestinal Channels (Crutchley et al., 2010). The drugcrofelemer has been investigated for the treatment of several types of secretory diarrhea, including traveler's diarrhea (DiCesare et al., 2002), AIDS-associated diarrhea (Holodniy et al., 1999) and infectious diarrhea such as cholera (Bardhan et al., 1999).

Some of the botanical compounds, which have been demonstrated to have positive effects in cancer therapy, have a long history behind them. It was recently demonstrated that the green tea antioxidant epigallocatechin-3-gallate significantly slowed breast cancer growth in female mice: its use is attested in ancient Japanese texts (Nobili et al., 2009). The juice, peel and oil of *Punica granatum* has also been shown to possess anticancer activity, including interference with tumour cell proliferation, cell cycle, invasion and angiogenesis (Lansky & Newman, 2007). Promising and selective anti-cancer effects have been observed with Saffron (stigmata of *Crocus sativus* L.) *in vitro* and *in vivo*, but not yet in clinical trials (Nobili et al., 2009). Homoharringtonine was isolated from the Chinese tree *Cephalotaxus harringtonia* var. *drupacea* (Sieb and Zucc.), and a racemic mixture of harringtonine and homoharringtonine has been used successfully in China for the treatment of acute myelogenous leukemia and chronic myelogenous leukemia. Purified homoharringtonine has shown efficacy against various leukemias, including some resistant to standard treatment and has been reported to produce complete hematologic remission in patients with late chronic phase myelogenous leukemia (Cragg & Newman, 2009). Vinca alkaloids are isolated from the periwinkle plant *Catharanthus roseus*, also known as *Vinca rosea*. The extracts of *Vinca rosea* possess many therapeutic effects including anti-tumour activity. The compounds vincristine, vinblastine and vindesine are the first Vinca alkaloids with anti-tumour activity to be identified from the plant *Vinca rosea* (Nobili et al., 2009).

The compound vinflunine, a bis-fluorinated vinorelbine derivative, has been synthesized by superacid chemistry. Due to its favourable preclinical anti-tumour activities, including microtubule dynamics disruption, anti-angiogenesis and prolonged multidrug resistance development, vinflunine is now being widely studied in phase I–III clinical trials (Nobili et al., 2009).There are many flavonoids in the plant kingdom which may possess cancer chemopreventive efficacy. The isoflavone genistein from soya and

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the flavone quercetin a constituent of onions, are examples of flavonoids which prevent cancer in rodent models of carcinogenesis. The compound genistein is currently in clinical trial for the prevention of recurrent localized prostate cancer subsequent to radical prostatectomy (Manson et al., 2005). Rohitukine, isolated as the constituent responsible for anti-inflammatory and immunomodulatory activity from *Dysoxylum binectariferum* Hook. f. (Meliaceae) which is phylogenetically related to the Ayurvedic plant *D. malabaricum* Bedd.used for rheumatoid arthritis. It is currently in phase I and phase II clinical trials, either alone or in combination with other anticancer agents, against a broad range of tumors, including leukemias, lymphomas and solid tumors (Cragg& Newman, 2009).

The plants have provided humans with many of their essential needs, including life-saving pharmaceutical agents from the antiquity. A number of terrestrial as well as marine flora are available and many of them have not yet been screened for biological activities and it need to explore the active constituents in the search for new medicines, particularly with the development of highly sensitive and versatile analytical methods are available.

ISSUES, CONTROVERSIES, PROBLEMS

Plants have been the mainstay of the oldest known systems of medicine in the world and have survived the ravages of time spanning thousands of years. Existing biological resources can be exploited in the search for new molecules, because while there are many compounds already under investigation for their chemopreventive properties, we have only begun to scratch the surface in terms of the number of possible efficacious agents in the plant kingdom (Manson et al., 2005).

The advent of next generation sequencing technology has led to the discovery of rare private mutations which has revolutionized our understanding of the genetics of complex diseases, with major focus on Cancer. The development of high-throughput platforms like Roche-454, Illumina Hiseq, Ion Torrent PGM etc., has provided the geneticists with large chunks of genomic data like never before, and its relevant bioinformatics pipelines contributing to uncover myriad cancer biological complexity and heterogeneity. Thus, there is a growing trend of studies undertaking whole genome and exome-sequencing from individuals affected with different subtypes of cancer thereby identifying and validating specific driver events and novel target genes by integrative approaches to understand their oncogenic potential and intratumoral diversity. The recent developments in diverse study designs and approaches in cancer genomics globally, viz., whole-genome, exome sequencing, transcriptome, methylome analysis, genome wide association studies, gene regulation analysis, cytogenetic analysis, DNA- Protein interaction analysis (CHIP-Seq), small RNA discovery to name a few have been inclusively studied. The focus on the future perspective of these genomic and epigenomic studies in developing important therapeutic targets and their subsequent validation by effective clinical trials that leads to cancer drug discovery, personalized medicines, painless and costless treatment, patients management and lifespan. Moreover, importance of dietary and natural resources as the medicine particularly phytochemicals for cancer prevention are required.

One of the major issue, more than 70% of all cancer patients are very interested in herbal drugs. However, many of them do not inform their oncologist about their use (Kelberet a., 2014). As 19 to 75% of all cancer patients suffer from sleep disturbances. The cancer patients often have difficulties to sleep and are restless, so that they can use herbal drugs such as valerian in order to avoid chemically defined

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sedatives and hypnotics as they fear becoming addicted. However, they state that warnings that valerian (*Valeriana officinalis*) may stimulate CYP 3A4 (Kelberet a., 2014).

Nutraceuticals are emerged as potential cancer preventive natural sources from food (Baichwal, 1999). It was hypothesized that this observation is related to diet or more specifically to nutraceuticals present in fruits, vegetables, common beverages, grains, marine products, medicinal plants, and herbs (Jankun et al., 2006). The nutraceuticals are natural bioactive chemical compounds and have value in health promoting, disease preventing or semi-medicinal properties. The nutraceuticals are found as natural products from (a) the food industry (b) the herbal and dietary supplement (c) pharmaceutical industry and (d) the newly emerged bioengineered microorganisms, agro products or active biomolecules. It may range from isolated nutrients, herbal products, dietary supplements and diets to genetically engineered "custom" foods and processed products such as cereals, soups and beverages (Dureja et al., 2003). Many dietary agents have extremely promising modes of action when investigated in cultured cells, but *in vivo* are much less effective. One of the reasons is bioavailability. Many purified dietary compounds have limited solubility, making it difficult to attain plasma or tissue concentrations equivalent to those showing activity *in vitro*. On a cautionary note, there may be a very good reason why many of these compounds are poorly available to tissues, so any attempts to use them in humans at higher doses than are available from the diet should be assessed for possible toxic consequences (Manson et al.,2005). Chemically the nutraceuticals may be classified as isoprenoid derivatives (terpenoids, carotenoids, saponins, tocotrienols, tocopherols, terpenes), phenolic compounds (couramines, tannins, lignins, anthocyanins, isoflavones, flavanones, flavanoids), and carbohydrate derivatives (ascorbic acid, oligosaccharides, non-starch polysaccharides) etc. (Sharma, 2009). The validation of nutraceuticals against cancer is under progress. In recent reports on cancer protection by nutraceuticals were evidenced for lymphoma (Russell et al., 2007), skin cancer (Hercberg et al., 2007), pancreatic cancer (Larsson et al., 2006), while vitamins and minerals were validated in chemoprevention trial of different cancers (Sandler et al., 2001). Two prominent nutraceuticals (curcumin and EGCG from green tea) have been implicated in anti-cancer activity (Manson et al., 2005). Natural product based drug discovery is increasing based on new technologies, such as combinatorial synthesis and high-throughput screening and their associated approaches. The vincristine, irinotecan, etoposide and paclitaxel are classic examples of plant-derived compounds; actinomycin D, mitomycin C, bleomycin, doxorubicin and l-asparaginase are drugs coming from microbial sources, and cytarabine is the first drug originating from a marine source. To date, new generations of taxanes, anthracyclines, vinca alkaloids, camptothecins, as well as the novel class of epothilones have been developed. Some of these are in clinical use, others in clinical trials. Other agents originating from marine sources (both plants and animals) (e.g. trabectedin-ET-743, bryostatin-1, neovastat) have also entered clinical trials (Nobili et al., 2009). Most *in vitro* studies investigating a new dietary agent initially look for indications of growth inhibition, cell cycle arrest and induction of apoptosis in tumour cell lines (Manson et al., 2005).

There is concomitant increase in the prevalence of type1 diabetes, the major driver of the epidemic is the more common form of diabetes, namely type2 diabetes which accounts for more than 90% of all diabetes cases. Nowhere is the diabetes epidemic more pronounced than in India as estimated that 32 million people had diabetes in the year 2000 (Wild et al., 2004). The International Diabetes Federation (IDF) estimates the total number of diabetic subjects to be around 40.9 million in India and this is further set to rise to 69.9 million by the year 2025 (Sicree et al., 2006). The most disturbing trend is the shift in age of onset of diabetes to a younger age in the recent years. This could have long lasting adverse effects on nation's health and economy (Mohan et al., 2007). Several plants are used for the management of the diabetes. Some example of the plants viz.,*Acacia arabica or nilotica* (Singh, 2011), *Cap-*

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paris sepiaria (*S*elvamani et al., 2008), *Catharanthus roseus* (Rasineni et al., 2010; Singh et al., 2001), *Barleria prionitis* (Geetha and Wahi, 2001), *Barleria lupulina* (Suba et al., 2004), *Balanites aegypticea* (Baragob et al., 2014), *Aegle marmelos* (Seema et al., 1996; Das et al, 1996), *Allium cepa*(Augusti, 1973; Mathew & Augusti, 1975; Gupta et al.,1977), *Artemisia pallens*(Subramonium et al., 1996); *Areca catechu* (Chempakam, 1993), *Beta vulgaris* (Yoshikawa et al., 1996), *Biophytum sensitivum* (Puri & Baral, 1998), *Brassica juncea* (Khan et al., 1995), *Caesalpinia bonducella*(Sharma et al., 1997), *Cajanus cajan*(Amalraj & Ignachimuthu, 1998), *Citrullus colocynthis* (Abdel-Hassan et al., 2000), *Eugenia jambolana, Tinospora cordifolia* (Grover et al., 2000), *Feronia elephantum* (Joshi et al., 2009a), *Coccinia indica* (Mukherjee et al., 1972), *Eugenia uniflora*(Arai et al., 1999), *Bambusa arundinacea* (Joshi et al., 2009b), *Andrographis paniculata*(Nugroho et al. 2012), *Gymnema sylvestre* (Thakur et al., 2012) and may more plant possessing anti-diabetic activity in *in vivo* models. Several compounds viz., S-methyl cysteine sulphoxide (sulphur containing amino acid) (Kumari et al., 1995), allicin (Mathew & Augusti, 1973), S-allyl cysteine sulphoxide (sulphur containing amino acid) (Sheela & Augusti, 1992), C-flavonol glucoside called as shamimin (Saleem et al., 1999), andrographolide (Nugroho et al., 2012), gymnemic acid, gymnemagenin (Persaud et al., 1999; Porchezhian & Dobriyal, 2003)and many more compounds have been isolated from various plants containing anti-diabetic activity.

Parthenin (a sesquiterpene pseudoguaianolide) isolated from *Parthenium hysterophorus* exhibits significant anti-malarial activity against a multidrug resistant strain of *Plasmodium falciparum*. Molecular matching of the oxygen atoms of parthenin and the new anti-malarial drug qinghaosu (Artemisinin) show that both molecules to be amphipathic with common areas for their oxygen atoms and hydrogen framework (Hooper et al., 1990). Parthenin also shows antiamoebic (Sharma & Bhutani, 1988) and allelopathic properties (Kanchan, 1975; Patil &Hegde, 1988). Forskolin (also called Coleonol) is a labdane diterpene that is produced by the Indian Coleus plant (*Coleus forskohlii*). Forskolin is commonly used to raise levels of cyclic AMP (cAMP) in the study and research of cell physiology. Forskolin activates the enzyme adenylyl cyclase and increases intracellular levels of cAMP. cAMP is an important second messenger necessary for the proper biological response of cells to hormones and other extracellular signals. It is required for cell communication in the hypothalamus/pituitary gland axis and for the feedback control of hormones (Mulhall et al., 1997). The flavonoids are known to exhibit several biological activities including anti-HIV activity (Vlietinck et al., 1998). The anti-HIV prenylated flavonoids as 6, 8-diprenylaromadendrin, 6, 8-diprenylkaempferol and lonchocarpol an isolated from the plant *Monotes africanus* exhibited HIV-inhibitory activity in the XTT-based, whole-cell screen. (Meragelman et al., 2001). *Ixeris chinensis* Nakai (Compositae) known as Siyekucai, is a perennial plant, and used as a folk medicine in China for the treatment of bronchitis, pneumonia, pharyngitis, dysentery and poisonous indigestion on the basis of its anti-febrile, antidotal and analgesic effects (Shiojima et al., 1996). Three new guaianolides, 10α-hydroxy-3-oxoguaia-4(15),11(13)-dieno-12,6α-lactone, 10α-hydroxy-3-oxo-4βH-guaia-11(13)-eno-12,6α-lactone, and 10α-hydroxy-3-oxoguaia-4,11(13)-dieno-12,6α-lactone have been isolated from the methanolic extract of the whole plant of *Ixeris chinensis* (Zhang et al., 2002). 3-Hydroxy-4-pyrone-3*α*-D-Glucopyranoside a novel glucoside has been isolated from aerial parts of *Erigeron karwinskyanus* DC (Mathela et al., 1984), and shows a strong biological activity against certain pathogens viz., *Aspergillus, Penicillium* and *Fusarium* species (Mathela et al., 1986).

The major issue for the plant based drug especially isolated compounds which are present in plant with low yield. It cannot execute the demand of pharmaceutical industries. For this reason biotechnology should be apply taking consideration into enhancement of the compound of interest. Plant tissue culture is an option to solve the purpose. Though this statement is controversial we must think over in

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the way to en-cash the amount of manpower and cost involved in such research. Here in this section it is attempting to highlight such issues in plant biotechnology especially related to tissue culture. Manipulation of DNA to alter its expression in medicinal plants is an area that is widely expanding, provided that the character of interest is present shelled to small number of genes. Although the primary target of trait for manipulation in medicinal plants is the content of active compounds, to which other traits such as development as crops, basic agronomic characters related to uniformity, stability, growth and development, and resistance to biotic and abiotic stresses, can serve as secondary also to be improved (Bajaj, 1998; Dubey & Guerra, 2002). The application of biotechnological approaches to medicinal plants does not start from a low baseline. There is already considerable interest in manipulating plant biosynthetic pathways to produce drug precursors, food components or pesticides; for example, through trichomes, specialized surface organs that manufacture, store and exude secondary metabolites (Stevenson, 2004).

In *Mentha* spp. (mints), biosynthetic pathways have been engineered to modify essential oil production in the trichomes and to enhance the resistance of the plant to fungal infection and abiotic stresses (Veronese et al., 2001). There is a long history of experimental and commercial production of high-value phytochemicals by tissue culture, an *in vitro* system for growing plant organs, explants, tissues and cells or protoplasts. Genetic transformation of cultures using bacterial vectors to transfer genes into the cultured plant DNA has been widely employed to improve product output in such systems. For example, hairy root cultures, transformed by infecting cells with the bacterium *Agrobacterium rhizogenes*, often sustain stable and high productivity in hormone-free culture conditions (Sevon & Oksman-Caldentey, 2002). An efficient transgene delivery system is thus already in place for several important medicinal plants, including *Papaver somniferum* (Park & Facchini, 2000), *Artemisia* spp. (wormwoods) (Chen et al., 1999), members of the family Solanaceae (Moyano et al., 1999) and *Taraxacum platycarpum* (Lee et al., 2004).

Tissue culture often promotes genetic disturbances, which result in somaclonal variation, greatly extending the range of useful variation available to the breeder (Kukreja & Dhawan, 2000). Tissue culture can also contribute to the conservation of valuable biodiversity (Augustine & D'Souza, 1997) and somatic embryogenesis. The micro-propagation of whole plants from cultured cells, has a potentially significant part to play in establishing breeding material from wild populations (Kintzios & Michaelakis, 1999) and in mass-producing material for selection or engineering (Lakshmanan et al., 2002).

The other option could be the plant endophytic fungi, where not only the possibilities to get plethora ofcompound of interest but also can be obtain new biologically active compounds in mass production. In this issue several plant endophytic fungi have been reported in the literature with varieties of the compounds which exhibited prominent biological activities. It must be considerable that genuine endophytic fungus producing the secondary metabolites likes signature constituent(s) or other compound(s) that produced by the plant itself, though in small quantity. Several research groups now a day are working in this area.

Herbs are natural products and their chemical composition varies depending on several factors, such as botanical species, used chemotypes, the anatomical part of the plant used (seed, flower, root, leaf, and so on) and also storage, sun, humidity, type of ground, time of harvest, geographic area; and merchandized products containing on the label the same product varying in their content and concentrations of chemical constituents from batch to batch; and even the same manufacturer can merchandize in different periods and products containing different substances although standardized to achieve a high pharmaceutical quality. This variability can result in significant differences in pharmacological activity involving both pharmacodynamic and pharmacokinetic issues (Firenzuoli& Gori, 2007).

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Currently, the major pharmaceutical companies have demonstrated renewed interest in investigating higher plants as sources for new lead structures along with development of standardized phytotherapeutic agents with proven efficacy, safety, and quality. Herbal medicinal preparations are normally very popular in developing countries because of being economical, backed with a long history of traditional use and also in some developed countries such as Germany, France, Italy and United States where appropriate guidelines for registration of such medicines (Sharma et al., 2010).Chemicals known to have medicinal benefits are referred to as "active ingredients" or "active principles" and their presence depends on a number of factors including the plant species, the time and season of harvest, the type of soil, the way the herb is prepared.The impact of variability of the constituents in herbs or herbal preparations due to genetic, cultural and environmental factors is a major issue in herbal medicines.

Standardization of active chemical constituents of herbal drugs as a finished product must be carried out using standard available protocols. Now a day various sophisticated techniques viz., high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC),gas chromatography (GC), gas chromatography–mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC-MS) are frequently used in identification of known phytochemicals. The qualitative and quantitative divergence of the compounds in herbal materials may be due to the climatic, which in turn may affect the composition and other secondary metabolites of the plants (Joshi, 2013d; 2014c) and can accumulate the compound at a particular period and environment. According to WHO (1996a and b, 1992), standardization and quality control of herbals is the process involved in the physicochemical evaluation of crude drug covering aspects, such as selection and handling of crude material, safety, efficacy and stability assessment of finished products, documentation of safety and risk based on experience, provision of product information to consumer and product promotion. Quality of herbal medicines indices such as: macro and microscopic examination, foreign organic matter, ash values, moisture content, extractive values, crude fibre, qualitative and quantitative chemical evaluation, chromatographic examination and toxicological studies. The name of marker constituent(s) along with percentage of each herb either single preparation or multidrug formulation should be mentioned in the final and market product and must be under the strict surveillance of drug regulatory authority.

Various problems appeared during the clinical trial of the crude raw herbmaterial viz., chemical variations as per geographicallocations, climatic conditions, environmental hazards, harvesting methods, collection protocols, etc. which makes it a difficult task to standardize the end product for are producible quality (Bauer& Tittel, 1996). Adverse and side effects is another open problem, because in citizens still prevail the respect for everything that is natural tout court, more as a cultural-fashion based choice than thinking that the patient is introducing in his/her body chemical substances of vegetal origin; not knowing that salicylic glucosides and lactonic sesquiterpenes of many Compositae are often responsible of allergic reactions. Some constituents of the plants are cancerogenic like safrole, bergapten and pyrrolizidines alkaloids. Not of minor importance especially for the old patient using contemporary more synthetic drugs is the problem of drug interferences. Some plants reduce or improve the bioavailability of some drugs due to induction or inhibition of cytochromes (Firenzuoli & Gori, 2007).

In the case of wild harvesting, continual selection of the largest wild-growing individuals or those with the traits considered desirable from a medicinal point of view, if it involves destruction of the whole plant or its reproductive organs, will inevitably lead to a degradation of the wild population. It cannot use medicinal plants on a large scale without modifying the characteristics of the plant populations available to us. If medicinal herbs are brought into cultivation, then we can at least attempt to do this in a controlled manner and at the same time can attempt to conserve wild populations. A particular challenge for me-

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dicinal plants is the degree to which synergistic effects can be not only conclusively demonstrated but also realistically defined for biotechnological intervention. Otherwise, increasing understanding of what the active components in herbs are and how they work will simply lead to their isolation as plant-derived drugs, and biotechnological interest in the plants and whole extracts from them will not be justifiable.

NOTE

In 2004, the FDA created a pathway for drug makers to get approval of drugs derived from plants. While it has received about 500 applications, it approved only one prescription botanical drug in the 8 years since, until today. There are lots of opportunities for developments of phytopharmaceuticals. Undoubtedly a very few molecules from plant origin have been characterized in detail as a drug candidates. One of the question arises taking into consideration of the yield of compound in the plant, if the compound showing prominent biological activity. Can it supply the demand of the drug based on the plant itself? The plant origin drug can only utilized for the research purpose and may not complete the demand of plant based drugs for disease like diabetes or other life style diseases. Otherwise alternate sources viz., plant tissue culture and endophytes can be the option for potential renewal sources for sustainable production of phytopharmaceuticals.

Quality control using modern analytical tools for testing the various qualitative parameters of herbal medicine as a final market product must be emphasized as per the allopathic medicine norms adopted by the various government agencies of the globe and WHO guidelines must be strictly implemented universally.

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Chapter 18
Resealed Erythrocytes as Drug Carriers and Its Therapeutic Applications

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ABSTRACT

In this pharma innovative world, there are more than 30 drug delivery systems. Today's due to lacking the target specificity, the present scenario about drug delivery is emphasizing towards targeted drug delivery systems. Erythrocytes are the most common type of blood cells travel thousands of miles from wide to narrow pathways to deliver oxygen, drugs and nutrient during their lifetime. Red blood cells have strong and targeted potential carrier capabilities for varieties of drugs. Drug-loaded carrier erythrocytes or resealed erythrocytes are promising for various passive and active targeting. Resealed erythrocyte have advantage over several drug carrier models like biocompatibility, biodegradability without toxic products, inert intracellular environment, entrapping potential for a variety of chemicals, protection of the organism against toxic effects of the drug, able to circulate throughout the body, ideal zero-order drug-release kinetics, no undesired immune response against encapsulated drug etc. Resealed erythrocytes are rapidly taken up by macrophages of the Reticuloendothelial System (RES) of the liver, lung, and spleen of the body and hence drugs also. Resealed erythrocytes method of drugs delivery is secure and effective for drugs targeting specially for a longer period of time. This chapter will explain the different method of drug loading for resealed erythrocytes, their characterization, and applications in various therapies and associated health benefits.

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INTRODUCTION

The emerging new drug delivery technologies are likely to have significant and lucrative impact on drug industries. Drugs delivery assembly suffers various limitation including the amount of molecule delivered and parallel adverse effects. To improve the targeting of drugs a number of attempts has been performed through engineering the properties of the drugs carrier system (Dinda, 2013). The present scenario about pharmaceutical research is aimed to develop drug delivery system with lower side effects and maximum therapeutic benefit. The major desirable properties of a drug carrier is being selective to a target tissue as well as to protect the drugs from premature bio-inactivation. New emerging drug targeting strategies through improving carrier towards tissue exhibit maximal therapeutic index with minimum adverse effects. In diverse drugs carrier, the cellular carriers fulfill maximum criteria desirable in clinical applications. Various carrier are being uses as drug targeting; however, cellular carriers like erythrocytes, platelets, leukocytes, fibroblasts, hepatocytes etc. evidenced themselves to have greater potential over other cellular carriers (Hamidi & Tajerzadeh, 2003, Rossi et al., 2005). Erythrocytes are most common type of blood cells travel thousands of miles from wide to narrow pathways to deliver oxygen, drugs and nutrient during their lifetime (Pandey & Rizvi, 2010). Erythrocytes have good potential carrier capabilities for varieties of drugs and could efficiently deliver various drugs to selected target cells (Gothoskar, 2004). Drug-loaded carrier erythrocytes or resealed erythrocytes are promising for precise drugs targeting.

Resealed erythrocytes as carrier for drugs provides secure and more effective drug targeting for longer period of time and have advantage over several drugs carrier models regarding the biocompatibility, biodegradability without toxic products, inert intracellular environment, entrapping potential for a variety of chemicals, protection of the organism against toxic effects of the drug, able to circulate throughout the body, ideal zero-order drug-release kinetics, no undesired immune response against encapsulated drug etc. (Gothoskar, 2004). Loading of drugs in erythrocytes are performed through process of collecting blood, isolating erythrocytes from blood, entrapping drug of interest in the erythrocytes then followed by resealing the resultant erythrocytes carriers (Green & Widder, 1987). Drug loading process in erythrocytes and sealing of them is based on the response of erythrocytes under osmotic condition (Jain & Jain, 1997). Drug loaded erythrocytes or resealed erythrocytes are biocompatible with large drug loading capacity, biodegradable, non-immunogenic which could circulate for long periods of time (months) through circulation of blood and can be easily targeted to macrophages. In clinical trial, use of resealed erythrocytes was extensively investigated *in vitro* and recently validated *in vivo* also.

BLOOD

Blood is mobile connective tissue composed of fluid called plasma and cells have been extensively uses in pathologies and research laboratory. Blood have several functions including transport of oxygen to cell for aerobic respiration, nutrients, drugs, and excretes toxic substances generated endogenously. Circulating nature of blood makes it universal and primary sample for assessing several organ functions test. Blood have two major components as cellular component (erythrocytes, white blood cells and platelets) and the solvent of cellular component called plasma. Cellular component are again fractionated major cell RBCs and WBCs and platelets (Guyton & Hall, 2006). Blood is a tissue of mesenchymal origin with liquid matrix. The matrix in which the cells are suspended is termed as plasma. This plasma holds the

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blood cells in form of a suspension. As whole plasma consists of 55% of blood volume and rest 45% is made up of formed elements (blood cells). The cellular component of blood are categories as red blood cells (RBCs) or erythrocytes, white blood cells (WBCs) and platelets.

Plasma

Plasma, the non-living fluid portion of blood is slightly alkaline (pH 7.40) containing an immense number of ions, inorganic molecules and organic molecules. Plasma transits to various parts of the body with several substances. It consists up to 5% of body weight, or roughly 3500 mL in a 70-kg man (Guyton & Hall, 2006). Generally, plasma clot in the absence of anticoagulant, and after clotting the remaining fluid is called serum. Serum has essentially the same composition as plasma except fibrinogen and clotting factors II, V, and VIII, and has a higher serotonin level. Plasma consists of 90-92% water, 0.9% mineral salt, sodium bicarbonate, nutrients (glucose, fatty acid, amino acids, nucleosides, etc.), 7 -8% plasma proteins, defence compound immunoglobulin, anticoagulant, vitamins, enzymes, dissolved gasses and excretory substances (Guyton & Hall, 2006).

The plasma protein consist albumin, globulin, and a fraction of fibrinogen. The globulin fraction is subdivided into α1, α2, β1, β2, and γ globulins and fibrinogen. Circulating antibodies in the γ globulin fraction of the plasma proteins are manufactured in the plasma cells. Most of the other plasma proteins are synthesized in the liver. Plasma protein provides 15% buffering capacity of blood due to presence of weak ionization of their substituent COOH and NH_2 groups. Albumin serves as a carrier for metals, ions, fatty acids, amino acids, bilirubin, enzymes, and drugs despite antioxidant potential (Guyton & Hall, 2006). Plasma protein used as marker of oxidative stress via measuring protein hydroperoxides, protein carbonyl (PCO), and advance oxidative protein products (AOPP) (Rizvi & Pandey, 2011, Singh & Rizvi, 2015).

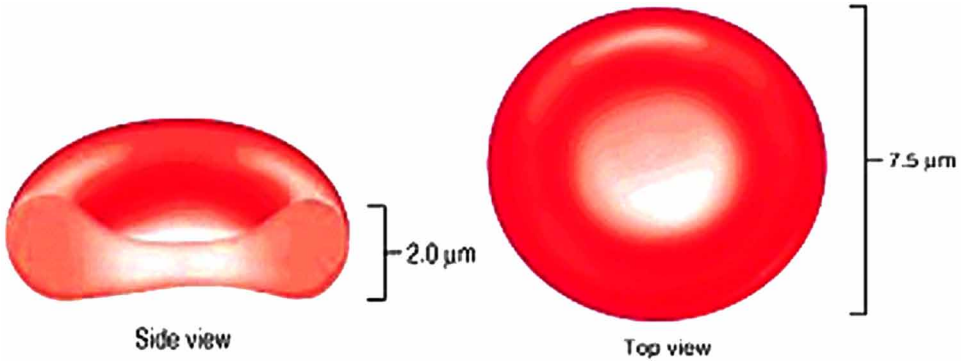
Plasma lipid includes cholesterol, low density lipoprotein (LDL)-cholesterol, high density lipoprotein (HDL)-cholesterol, very low density lipoprotein (VLDL) cholesterol, triglycerides, phospholipids and free fatty acids. Phospholipids, LDL cholesterol and triglycerides are generally found to in higher amount and repeatedly used in diagnosis of fatty liver, atherosclerosis, or susceptibility for cardiac attack. Plasma proteins and lipids are good marker of oxidative stress implicated in several diseases (Mehdi & Rizvi, 2013, Singh & Rizvi, 2015). Carbohydrates content in plasma are mainly arise from the ingested food and dissociated glycan moiety form the cellular membrane. Plasma carbohydrates are presently uses to diagnose and evaluate various pathological conditions due to increasing the oxidative stress or with ageing (Mehdi et al., 2012).

Erythrocytes

Erythrocytes (Greek *erythros* for "red" and *kytos* for "hollow vessel", with -cyte translated as "cell" in modern usage) are unique circulating cells in the mammalian cells travels thousands of miles from wide to narrow pathways to deliver oxygen, drugs and nutrient during their life time. In normal men, the average number of RBCs per cubic millimeter is 5,200,000 (±300,000); in normal women, it is 4,700,000 (±300,000). Persons living at high altitudes have greater numbers of RBCs. RBCs have the ability to concentrate hemoglobin in the cell fluid up to about 34 grams in each 100 milliliters of cells. Millions of erythrocytes are produced every second, they developed in bone marrow and circulate for about 100-120 days in the human body however the mean erythrocyte lifespan of Wistar rats is 59.8 ±

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Figure 1. Morphology of Erythrocytes: Side view & Top view



2.2 days and fixed during the modeling process, 60 days (Guyton & Hall, 1996). In most mammalian blood, mature RBCs are oval and flexible biconcave disks with lacking cell nucleus and most organelles to accommodate maximum space for hemoglobin. Normal erythrocytes are approximately 5.7 µm in diameter and a thick 2.5 µm at the thickest point and 1 µm or less in the center, discoid, and biconcave in cross (Figure 1) section (Guyton & J.E. Hall, 1996).

Erythrocyte Membrane

The erythrocyte membrane is composed of cholesterol and phospholipids, and secured an elastic network of skeletal proteins via trans-membrane proteins (Mohandas & Gallagher, 2008). Membrane is bilayer in nature and the composition of outer and inner layer is according to requirement. Glycolipids, phosphatidylcholine and sphingomyelin are located in the outer half of the bilayer; however, phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine occur in the interior layer facing the cytoplasm (Mohandas & Gallagher, 2008). Due to membrane fluidity and flexibility erythrocytes can squeeze themself through much narrower than their diameter and can recover rapidly to their original shape. Phosphatidylcholine forms highly fluid lipid regions to induced fluidity, while sphingomyelin induces rigidity of membrane. Lipid domain controlling the fluidity is determined by the molar ratio of cholesterol to phospholipid, degree of unsaturation of phospholipid acyl chains and phosphatidylcholine sphingomyelin ratio (Mahmmoud, 2011).

An intact membrane skeleton is crucial for the mechanical and functional integrity of the RBCs because intact membrane skeleton determines RBCs viscoelastic properties to withstand the shear forces of the circulation and squeeze out through capillaries (Chassis et al., 1988). The membrane skeleton is constituted by a multi-protein network (ankyrin, anion exchanger (AE1 or band 3) and protein 4.1, 4.2 etc.) lying just beneath the plasma membrane (Lux & Palek, 1995). Band 3 and glycophorin A are most abundant glycated integral membrane protein whose concentration in the membrane varies according to the weather of living subject. Band 3 present as dimer, composed of two identical subunits in membrane and serves as a channel for the passive exchange of anions across the membrane like chloride shift. The tetrameric form of band 3 binds to ankyrin (Michaely & Bennett, 1995) and protein 4.2 (Rybicki et al., 1996) constituting the major attachment site of the RBCs membrane to the underlying cytoskeleton. A second site of attachment involves the glycophorin C complex with protein 4.1 and p55. Glycophorin

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A is 1TMS consisting carbohydrate cover 16 oligosaccharide chains and make up of 60 percent of the molecule's weight. Glycophorin have negative charge due to presence of sialic acid the sugar residue at the end of carbohydrate chain due to which RBCs repel each other and prevent the RBCs from clumping during circulation through the tiny capillaries (Mehdi et al., 2012).

Functions of Erythrocytes

In the 19th century, Hope Seyler identified hemoglobin and its crucial role in oxygen delivery to various parts of the body (Telen, 1993). In some lower animals, hemoglobin circulates as free protein in the plasma not enclosed in RBCs. The tetrameric hemoglobin molecule in turn is designed to unload oxygen cooperatively within the range of physiological tissue pO_2 (Sivilotti, 2004). Carbonic anhydrase, an important enzyme of erythrocytes catalyzes the reversible reaction between carbon dioxide (CO_2) and water to form carbonic acid (H_2CO_3) regulates the acid base balance in the blood. Despite these gaseous exchange, hormones, enzymes and nutrients transport function, blood actively remove toxic excretes of different cells, drugs of chemical inhaled, injected or ingested (Table 1) (Sivilotti, 2004).

When RBCs undergo shear stress during the circulation in constricted vessels, RBC release ATP which in turn relax and dilate the blood vessel to promote normal circulation of blood flow (Ristenpart et al., 2008). After deoxygenation of hemoglobin erythrocytes release S-nitrosothiols, which also acts as vasodilator for directing more blood in areas having less oxygen of the body (Diesen et al., 2008). Erythrocytes has also been found to synthesize nitric oxide is another vasodilator via using L-arginine as substrate (Kleinbongard et al., 2006). Shear stress on erythrocytes induced the nitric oxide synthase for nitric oxide synthesis and exportation of them is thought to involve in the regulation of vascular tonus (Ulke et al., 2009). Erythrocytes is also reported to produce hydrogen sulfide, a signaling gas which have similar effects like vasodilator through relaxing the vessel walls. It is believed that the cardio protective effects of garlic are due to erythrocytes converting its sulfur compounds into hydrogen sulfide (Benavides et al., 2007). For all these functions to run well, efficient and regulated transport of these molecules (O_2, CO_2 HCO^{3-}, Cl^-, S-nitrosothiols, nitric oxide) is very essential.

Table 1. Important biochemical reaction occurs in erythrocytes

Process	Chemical Reaction
Production of superoxide (by-product of various reactions)	$O_2 + e^- \rightarrow O_2^{\bullet -}$
NADPH-oxidase	$2\ O_2 + NADPH \rightarrow 2\ O_2^{\bullet -} + NADP + H^+$
Superoxide dismutase	$O_2^{\bullet -} + O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$
Catalase	$H_2O_2 \rightarrow 2\ H_2O + O_2$
Myeloperoxidase	$H_2O_2 + X^- + H^+ \rightarrow HOX + H_2O$ ($X^- = Cl^-, Br^-, SCN^-$)
Glutathione peroxidase (Se-dependent)	$2\ GSH + R\text{-}O\text{-}OH \rightarrow GSSG + H_2O + ROH$
Fenton reaction	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$
Iron-catalyzed Haber-Weiss reaction	$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^-$
Glucose-6-phosphate dehydrogenase (G6PD)	$G6P + NADP \rightarrow 6\ Phosphogluconate + NADPH + H^+$
Glutathione reductase	$G\text{-}S\text{-}S\text{-}G + NADPH + H^+ \rightarrow 2\ GSH + NADP$

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Life Span of Erythrocytes and Changes Associated with Erythrocyte Senescence

Worn-out erythrocytes are removed from circulation and destroyed in the spleen and liver, and the breakdown products are recycled. In human, erythrocytes are produced in red bone marrow under the regulation of a hemopoietic hormone erythropoietin by process called erythropoiesis (Torotra & Grabowski, 1993). RBCs are produced exclusively in the bone marrow until age of in younger age; however, most of cells continue to produce in marrow of vertebrae, sternum, ribs, and ilia. Blood cells are originated from pluripotential hematopoietic stem cells. Pluripotential cells differentiate in to committed stem cells which differentiate to form the other cells in the blood. Growth and differentiation of stem cells in to erythrocytes are controlled by multiple regulatory proteins as growth factor protein regulates the growth of stem cells and differentiation inducer induced the differentiation.

Erythropoiesis is process of production of erythrocytes from the committed stem cells through different process via different organs in about seven days. In healthy adult red bone marrow is the major site for the continuous synthesis of RBCs at a rate of 2 million per second. After the process of erythrocytes maturation steps, RBCs live in the blood for about 100-120 days (Guyton & Hall, 2006). Like animal aging erythrocytes passes through cellular aging as at the end of life erythrocytes become senescent and removed from the blood circulation. They become susceptible to selective recognition by macrophages and subsequent phagocytosis in the reticuloendothelial system (spleen, liver and bone marrow), thus removing old and defective cells and continually purging the blood. This process is termed eryptosis, or erythrocyte programmed cell death (Guyton & Hall, 2006).

Process of erythrocytes ageing induced changes in the membrane composition, fluidity and hence the activity of various enzymes and transport system (Rizvi et al., 2010). Progressive aging of erythrocytes increased the cellular density which could affect the properties of resealed erythrocytes. Aged cells shows a little decreases in the deformities index. Increases in the elastic modulus and surface viscosity in senescent erythrocytes has been observed as compare to young erythrocytes (Racca et al., 1999). There are studies which indicate a decrease in antigenic expression in the population of senescent erythrocytes of both blood group. One hypothesis that is related with erythrocytic senescence proposes that denatured/oxidized hemoglobin (hemichromes) arising late during an RBC's life span induces clustering of the integral membrane protein, band 3. In turn, band 3 clustering generates an epitope on the senescent cell surface leading to autologous IgG binding and consequent phagocytosis (Michael et al., 1999).

Oxidation of hemoglobin is caused by free radicals and ROS (reactive oxygen species) with progressive aging (Pandey & Rizvi, 2010). The progressive accumulation of oxidized hemoglobin covalently bound to the membrane skeleton not only disrupts membrane organization but also threatens eventual phospholipids oxidation via a calcium-promoted quasi-lipoxygenase activity. The emergence on the cell surface of a threshold concentration of oxidized phospholipids, principally phosphatidylserine, signals recognition by the CD36 macrophage receptor (Kiefer & Snyder, 2000).

Erythrocytes as Model Study

Erythrocytes have been extensively studied for their potential carrier capabilities for the delivery of nutrients, gasses, excretory products, toxic substances and drugs. Currently different types of mammalian erythrocytes have been used for research purpose, including erythrocytes of mice, cattle, pigs, dogs, sheep, goats, monkeys, chicken, rats and rabbits (Gothoskar, 2004). Erythrocytes are specialized cells

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perform critical functions in wide range of cellular environments. Due to absence of protein synthesizing machinery in erythrocytes they have additional impact on carrier capabilities for varieties of drugs.

RESEALED ERYTHROCYTES

Erythrocytes have water content 65% and rest of 35% are solid. Isotonic nature of plasma for erythrocytes protects them against osmosis. However, if medium is hypotonic erythrocyte get swelled and simultaneously lose their hemoglobin content. Erythrocytes without hemoglobin content are termed as ghost. There are three types of ghost has been recognized as (a) ghosts which reseal immediately after haemolysis, (b) resealed ghost after reversal of haemolysis by addition of alkali ions and, (c) erythrocytes ghost with leaky nature under different experimental conditions (Deloach et al., 1980, Ihler, 1983, Ihler & Tsang, 1987, Jain & Jain, 1997). During hypotonic shock and on resealing of erythrocytes hemoglobin is lost but cellular constituents are retained and erythrocytes called "resealed erythrocytes".

Encapsulation of adenosine triphosphate (ATP) in erythrocytes with was tried in 1953, by Gardos (Gardos, 1953). Ihler et al. and Zimmermann (1973) reported to use erythrocytes as drugs carrier; however, the term carrier erythrocytes was came in existence in 1979 which describe drug-loaded erythrocytes (Ihler, 1983, Zimmermann, 1973, Jain & Jain, 1997). It was found that several molecules leak rapidly from erythrocytes via membrane due to simple diffusion. However, principally drugs having nature of peptides, nucleic acids etc. may potential be entrapped in to erythrocytes. Erythrocytes have its own enzymatic activities which can be conveniently utilized to convert an inactive pro-drug into an active drug. These behavior of erythrocytes leads to design a number of pro-drugs with charged chemical groups making them non-diffusible or non-toxic. Red cells enzymes hydrolyze these chemical groups and converted pro-drug in to an active drug which can diffuse through erythrocytes membrane and released them in circulation or at specific sites. Hemoglobin free resealed erythrocytes with drugs serves as slow circulating depots to reticuloendothelial system (RES) (Zimmermann, 1983, Lewis & Alpar, 1984, Ropars et al., 1987, Jain & Jain, 1997).

Morphology of Resealed Erythrocytes

The survival and existence of resealed erythrocytes in biological system is affected by the membrane fluidity and biological behavior of resealed erythrocytes. The *in vitro* characterization of resealed erythrocytes is an important process to predict the biological properties of resealed erythrocytes in *in vivo*. Erythrocytes are flexible and they squeeze out themselves in narrow capillaries hence morphology of erythrocytes after loading of drugs and resealing decides their life span after their administration in body.

Morphological evaluation are usually performed through the microscope. Light microscopy has been reported to reveals no observable change in resealed cells; however, some spherocytes (spherical erythrocytes) has been detected (Talwar & Jain, 1992a, Jain et al., 1995, Jain et al., 1997). Increasing the resolution power, i.e. scanning electron microscopic (SEM) have shown that maximum drug loaded resealed erythrocytes are in their biconcave discoid shapes (Jrade et al., 1987), some form spherocytes with invagination in one point (stomatocytes) after the drug loading procedure (Garin et al., 1996). Smaller cells (microcyte) were also reported in some cases (Teisseire et al., 1987).

Deformities of erythrocytes has also been reported to modulate the life span of erythrocytes. Viscoelasticity of erythrocytes membrane, viscosity of the cell contents, and cellular surface-to-volume ratio

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are found to affects the rheological behavior of resealed erythrocytes in narrow capillaries and the RES (Jrade et al., 1987). Changes in the shape of resealed erythrocytes after drugs loading measured by the passage time of definite volume of resealed erythrocytes via a polycarbonate filter (pore size of 45 μm) or a capillary of 4 μm diameter (Jrade et al., 1987, Zanella et al., 1987). Turbidimetrically, chlorpromazine mediated shape changes is another approach to evaluate morphological changes (Hamidi et al., 2001).

PREPARATIVE METHODOLOGY

Source and Isolation of RBC

Erythrocytes isolated form mice, cattle, pigs, dogs, sheep, goats, monkeys, chicken, rats, and rabbits have been used for resealed erythrocytes or drugs encapsulated erythrocytes. Fresh erythrocytes isolated from whole blood has been found to have more encapsulation efficiency for the loading purposes of drugs. It has been also reported that young erythrocytes have higher capability then old erythrocytes. Fresh blood, collected from mammals chilled have to be store at 4 ºC and less than of two days. The erythrocytes are harvested and washed with phosphate buffer saline by centrifugation. Mammalian erythrocytes have advantage over the other species erythrocytes due to lacking the nucleus and various cellular organelles such as their mitochondria, golgi apparatus and endoplasmic reticulum in their mature state. Erythrocytes consist nucleus during the early phases of erythropoiesis but the gradually disappear during development in order to provide more space for hemoglobin. Due to absence of nucleus and cellular organelles erythrocytes they do not contain DNA, cannot synthesize any RNA and also used to depend on ATP by the glycolysis (Ihler & Tsang, 1987). These properties of erythrocytes ensures that no virus can target erythrocytes for growth and propagation (Vienken et al., 1978).

Carrier erythrocytes are prepared collecting blood samples, separating erythrocytes from plasma, entrapping desire drug in the erythrocytes, and finally resealing the carriers (Green & Widder, 1987). Venous blood from different healthy volunteers or subjects can obtained in heparin by venipuncture. The blood was centrifuged at 800 × g for 10 min at 4°C. After the removal of plasma, buffy coat and upper 15% of the packed erythrocytes, the packed erythrocytes were washed twice with cold phosphate buffer saline at 4°C (0.9% NaCl, 10 mM Na_2HPO_4, pH 7.4). The washed erythrocytes are diluted with phosphate buffer saline and stored at 4ºC until used (Singh et al., 2015, Singh & Rizvi, 2015).

Methodology of Drugs Loading

Various methods has been established to load drugs or other bioactive compounds in erythrocytes isolated from different mammals. Majorly includes physical method like electricalpulse method, osmosis-based systems, and chemical methods. Erythrocytes have capability to reverse the shape changes with or without accompanying volume change of erythrocytes and for reversible deformation under various stress.

Osmosis and Concept of Tonicity

Tonicity is a measure of the osmotic pressure of two solutions separated by a semi permeable membrane. Like osmotic pressure, tonicity is influenced only by solutes which cannot cross the semi permeable membrane. Higher the concentration of solute higher will be the tonocity of solution. Term is generally

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used when describing the response of cells immersed in an external solution. The process by which water moves across the semi permeable membrane from the region of higher solute concentration to the region of lower solute concentration is termed as osmosis. The process of osmosis is depended on erythrocytes response under the osmotic conditions. These methods exchanged the extra cellular solute having desired drugs with the cellular solute (hemoglobin) of erythrocytes before resealing. Hypo-osmotic methods are again classified in to hypotonic dilution methods, dialysis methods, Presswell method, and isotonic osmotic lyses.

Hypotonic Dilution

A hypotonic solution is that which contains a lesser concentration of impermeable solutes than the solution on the other side of the membrane. When a cell's cytoplasm is hypotonic than surrounding then water will be drawn out of the cytoplasm. If water molecules continue to diffuse into the cell, it will cause the cell to swell, and finally lysis will occur. Hypotonic dilution was the first method investigated for the encapsulation of chemicals into erythrocytes (Iher, 1973). In this methods, erythrocytes are exposed to hypotonic solution of 0.4% NaCl to escape of cellular contents in the exchange of hypotonic solution having desired drugs. This method was used to entrap β-glucosidase, β–galactosidase (Iher, 1973), asparginase (Updike & Wakamiya, 1983), and arginase (Adriaenssens et al., 1976), as well as bronchodilators salbutamol (Bhaskaran & Dhir, 1995). Hypotonic dilution methods is simplest and fastest (exchange of cellular content and hypotonic solution equilibrium is achieved with in one minute), but having lower capsulation efficacy (1 – 8%).

Dialysis

This methods are performed by erythrocyte dialyzer through which initially a desired haematocrit is achieved through suspending the washed erythrocytes with phosphate buffer (pH 7.4) having desired drug solution. This method provide good entrapment efficiency yet have disadvantages of time consuming and size distribution of loaded ghosts were not found to be homogeneous.

Press Well Dilution Method or Hypotonic Preswelling

This method was developed by Rechsteiner (1975) and again was modified by Jenner et al. (1981). Method is based on swelling the erythrocytes without the lyses due to suspending them in to slightly hypotonic solution (0.6% NaCl) and recovered the swollen erythrocytes by centrifugation at low speed. Then, a little volumes of drug solution are added to the point of erythrocytes lyses. Now cells placed in isotonic medium to make resealed erythrocytes. Press well dilution method are simple and fast with higher survival rate *in vivo* as long as native RBCs.

Isotonic Osmotic Lyses or Osmotic Pulse Method

Isotonic solutions contain equal concentrations of impermeable solutes on either side of the membrane. When cell cytoplasm is isotonic to surrounding no such change occur because there is no net movement of water across the cell membrane. Red blood cells are bound by a membrane which allows water to pass through while generally restricting the solutes. This process, causes cells to shrink due to loss of water (called exosmosis) when placed in hypertonic solutions. On the other hand, red cells absorb water (called endosmosis) when in a hypotonic medium. This results in swelling and ultimately hemolysis as the cell

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bursts. In isotonic osmotic lyses or osmotic pulse method erythrocytes are placed in high transerythrocytic membrane permeability solution which allow encapsulation of drugs through solution into erythrocytes via direct chemical potential gradient. Various chemical methods has adopted for isotonic hemolysis like ammonia chloride, urea solution, and polyethylene glycol (Davson & Danielli, 1970, Billah et al., 1977).

Electrical Breakdown/ Electro-Insertion/ Electroencapsulation

Zimmermann (1973) developed this method to encapsulate bioactive molecules. However, Kinosita & Tsong (1977a) suggested the use of transient electrolysis for required membrane permeability for drug loading. In this method optimum intensity of an electric field and optimal discharge time has been reported between 1–10 kW/cm and between 20–160 μs (Zimmermann et al., 1976, Kinosita & Tsong, 1977b, Zimmermann, 1983, Lewis & Alpar, 1984,, Tsong & Kinosita, 1985). Voltage (2kV/ cm for 20μ sec) mediated polarization induce electrical breakdown of erythrocytes membrane through formation of pore which helps in entrapment of drugs. Pore generation generally takes place at the lipid protein junction or lipoid regions in membrane and can be resealed by incubation in suitable solution at 37º C. In comparison with osmotic methods advantage of this method is the uniform distribution of loaded cells (Zimmermann, 1983). However, this process is very expensive.

Endocytosis

This method was reported by Schrier et al. (1975). In erythrocytes intracellular vesicles with desired drugs could be induce. This process can load virus (up to 1000 nm diameter), enzymes and small molecules. Intracellular vesicles derived from cytoplasm may release intracellular content in erythrocytes cytoplasm depending upon the nature of drugs. This method have been used to encapsulate primaquine and related 8-amino-quinolines, vinblastine, chlorpromazine and related phenothiazines, propranolol, tetracaine, hydrocortisone, and vitamin A (Tsong & Kinosita, 1977, DeLoach, 1983, Schrier, 1987).

Membrane Perturbation

This method is based on the ability of chemical to modulate membrane permeability of erythrocytes. Deuticke et al. (1973) has found that the erythrocytes membrane permeability increases when RBCs expose to polyene antibiotic such as amphotericin B. Antibiotics have ability to increase the permeability of erythrocytes membrane were exploited for loading of drugs in to erythrocytes e.g. Amphotericin-B interacts with the cholesterol in plasma membrane causing change in permeability for anti-leukemic drugs. Kitao et al., (1978) entrap the daunomycin (antineoplastic drug) in human and mouse erythrocytes. However, Lin et al. (1999) used halothane for the same purpose.

Lipid Fusion

Lipid vesicles are generally used to encapsulate the drugs and loaded to erythrocytes. Lipids vesicle tagged with specific phospholipids induced the drugs loading potential and functional properties of drugs loaded erythrocytes. This method was mainly used for entrapping inositol monophosphate which improve the oxygen carrying capacity of RBCs (Nicolau & Gersonde, 1997).

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Red Cell Loader

Red cell loader was developed by the Magnani and co-workers, a novel method for loading of non-diffusible drugs into erythrocytes (Magnani et al., 1998). The loading process takes place in two sequential steps of hypotonic dilutions of washed RBCs and then an isotonic process for resealing the erythrocytes. This process of drugs entrapment have 30% of drug loading efficiency with 35–50% cell recovery despite normal survival of erythrocytes *in vivo*.

IN VITRO CHARACTERIZATION OF RESEALED ERYTHROCYTES

Characterization of resealed erythrocytes with drugs provide the significances of methods used to prepare the drug encapsulated erythrocytes. The following process could be used to evaluate and characterized the resealed erythrocytes-

Measuring Erythrocyte Sedimentation Rate (ESR)

Erythrocyte sedimentation rate determine the sedimentation rate of blood cells in a standard tube. It measure the suspension stability, size and number of the RBC in plasma or relative concentration of plasma protein (fibrinogen and α, β globulins). ESR is standardized as 0 to 15 mm/hr. for normal blood, higher rate indicates active but obscure disease processes (Teisseire et al., 1987).

Measuring Drug Content

Drugs contents was evaluated in erythrocytes. Initially erythrocytes were depolarized with acetonitrile and then centrifuge the whole content at 2500 rpm for 10 mins. Now supernatant was used to evaluate the drug content (Gothoskar, 2004).

In Vitro Release of Drug and Hemoglobin

In this process normal and resealed erythrocytes are incubated at room temperature in PBS in metabolic rotating wheel incubator path. Now sample was depolarized to evaluate the drugs content and hemoglobin. Hemoglobin can be measured by correlating the hemolysate solution with standard hemoglobin graph (Gothoskar, 2004).

Drug Release Pattern

Drug release pattern is the most important parameters to evaluate the properties of resealed erythrocytes. Hemoglobin is also reported to invariably release with drug form erythrocytes due to loss of cell membrane integrity, but drug releasing potential of erythrocytes is considerably higher than hemoglobin, reflects readily diffuses of drug. Size and polarity of the drug molecule are two factors that determine the drug release pattern (Eichler et al., 1987). It was found that cell lysis is not essential for the release of lipophilic drugs like phenytoin, dexamethasone (Eichler et al., 1987), methotrexate (Lewis & Alpar, 1984), primpquin (Talwar & Jain, 1992), and vitamin B-12 (Eichler et al., 1985). However, cell lysis

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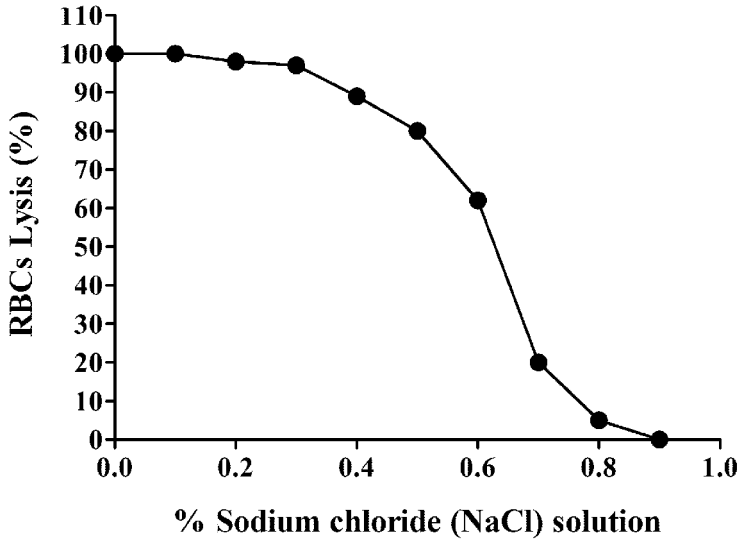
is essential for non-diffusible or polar drugs like heparin, gentamicin and enalaprilat, and enzymes asparginase, peptides urogasterone and l-lysine-l-phenylalanine (Gothoskar, 2004). Cross-linking cell membrane with gluteraldehyde is reported to reduce the drugs release rate (Jenner et al., 1981, Talwar & Jain, 1992). Complex formation of drug with dextran and albumin also reduced the release rate (Ihler, 1983). Entrapment of biodegradable prodrug (cortisol-21- phosphate, o-acetyl propranolol, o-pivaloyl propranolol, and cytosine arabinoside monophosphate) has similar effects on drugs release pattern (Gothoskar, 2004).

Osmotic Fragility

The osmotic fragility test is a measure of the resistance of erythrocytes to hemolysis by osmotic stress. Osmotic stress is produced by exposing cell to NaCl solution of tonicity lower than that of plasma. The test consists of exposing red cells to decreasing strengths of hypotonic saline solutions and measuring the degree of hemolysis calorimetrically at room temperature (Pandey & Rizvi, 2014a). The osmotic fragility test ultimately tries to determine the concentration of solute inside the cell subjecting it to the hemolytic effects of solutions of different concentrations. Erythrocytes were added to tubes containing increasing concentrations of phosphate-buffered sodium chloride (0%, 0.1%, 0.2%, 0.3%, 0.5%, 0.7%, 0.8%, and 0.9%) solution at pH 7.4 in a final volume of 10 ml and incubated for 30 min at 37 ºC. After centrifugation at 1500 x g for 10 min, absorbance of the supernatant was measured at 540 nm. Hemolysis in each tube was expressed as a percentage, using hemolysis in distilled water (0% NaCl) as 100% as shown below in Figure 2 (Pandey & Rizvi, 2014b).

In osmotic fragility test % hemolysis values are calculated and finally plotted against concentration to give and sigmoidal curve. This sigmoidal shape of curve indicates that normal RBC varies in their resistance to hypotonic solution. The osmotic fragility of resealed erythrocytes is affected by concentration, type and process of drugs loading. Osmotic fragility indicate the changes in cell membrane integrity and their resistance towards osmotic pressure of the suspension medium.

Figure 2. Osmotic fragility



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Based on integrity and resistance of cell membrane another method, turbulence fragility measure the resistance of drugs loaded erythrocytes against hemolysis from turbulent flow within circulation by vigorously shaking (Hamidi et al., 2001) or by passing them through needles with smaller internal diameter (e.g., 30 gauge) (Talwar & Jain, 1992a, Talwar & Jain, 1992b, Jain et al., 1995, Jain et al., 1997). In above both method released hemoglobin and drug were determined and found that turbulent fragility is higher than osmotic fragility of resealed cells (Talwar & Jain, 1992a, Talwar & Jain, 1992b, Jain et al., 1995, Jain et al., 1997, Hamidi et al., 2001).

Morphological Evaluation

The plasma membrane of RBC is closely associated with a proteinaceous skeleton. This cytoskeleton and other features like excess membrane for given volume of cell make RBC (Mehdi et al., 2012). Erythrocyte membrane consists of approximately equal amounts of lipids proteins. Phospholipids and unesterified cholesterol constitute major portion of membrane lipids. Phosphatidylcholine are more abundant in the outer leaflet whereas phosphatidylethanolamine are more concentrated on the inner leaflet of the lipid bilayer. Cholesterol molecules are intercalated between the phospholipid molecules. The relative amounts of cholesterol & phospholipids are responsible for the fluid properties of the RBC membrane (Ballas & Krasnow, 1980). The distribution of lipids in both leaflet is asymmetrical. This asymmetry is maintained by action of flippase proteins (Daleke & Lyles, 2003). Morphological evaluation including changes in shape due to changes in membrane biochemical composition or altering the proteinaceous skeleton modulates the cellular compatibility to survive in various solution or pressure of narrow capillaries.

ADVANTAGE OF RESEALED ERYTHROCYTES (GOTHOSKAR, 2004)

- Able to circulate throughout the body
- The biocompatibility of resealed erythrocytes and no immunological responses.
- It degraded biologically and do not release any toxic substances.
- Generally in produced uniform and drugs carriers.
- Intracellular environment are inert hence protection of encapsulated dugs by endogenous chemicals
- Have capability to entrap wide variety of chemicals.
- It also protects the organism against side-effects of drugs
- It check the immune response by organism against the loaded drug
- Ideal for deal zero-order drug-release kinetics (Eichler et al., 1986)
- Ensures the dose sufficiency as a large amount of drugs can be loaded in the resealed erythrocytes.
- Resealed erythrocytes have larger life span comparatively other synthetic drugs carriers
- Easily monitoring and control of resealed erythrocytes during life span ranging from minutes to months (Summers, 1983).

APPLICATIONS OF RESEALED ERYTHROCYTES

Erythrocytes as carrier for drugs cover pharmacological as well as therapeutic targets. Resealed erythrocytes with drugs has been reported to have slow releasing tendency of drugs when intravenously injected

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(Gothoskar, 2004). Erythrocytes is used as circulating carriers to release drug for a long period of times as well as target specific for liver, spleen, and lymph nodes. Despite these the following are the major application of resealed erythrocytes as drugs carriers.

Slow Drug Release

Due to long life span or erythrocytes, resealed erythrocytes as drugs carrier could be used as circulating depots of various drugs including anti-tumor, anti-parasitic, antibiotics, cardiovascular drugs, vitamins and steroids (Jenner et al., 1981, Pitt et al., 1983, Eichler et al., 1985, Magnani et al., 1998). Slow drugs release propensity of resealed erythrocytes arise when drugs or selected methods for drugs loading do not changes the morphological and physiological properties of erythrocytes.

There are various mechanism which explain the drugs releasing properties-

- Passive diffusion
- Specialized carrier transporter at the membrane
- Macrophages of RES do phagocytosis of resealed erythrocytes with drugs and finally accumulated drugs into the macrophage interior fallowed by slow release pattern of drug (Talwar & Jain, 1992a, Jain & Jain, 1997).
- The subcutaneous injection resulted an accumulation of erythrocytes in lymph nodes followed by hemolysis to release the drug (Deloach et al., 1987).

Drug Targeting

Modification on the surface of erythrocytes has been used to target the organs of mononuclear phagocytic system or reticuloendothelial system (Alvarez et al., 1998). Site-specific and target-oriented drug delivery system exhibit maximal therapeutic index with minimum adverse effects.

Targeting RES Organs

Kupffer cells in liver and spleen has been found to rapidly clear the damaged erythrocytes from circulation. Modification in erythrocytes membrane induces the targeting specificity. In presence scenario, various approaches has been performed to modify the surface characteristics of erythrocytes membrane with the help of antibodies, gluteraldehyde, carbohydrates such as sialic acid, sulphydryl, chemical cross-linking (Alvarez et al., 1998). Sialic acids has been responsible to induced negative surface on erythrocytes reduce in erythrocytes membrane with aging (Mehdi et al., 2012).

Targeting the Liver

Enzyme Deficiency/Replacement Therapy

This methods is used to prevent the accumulation of glucocerebrosides in the liver and spleen by glucocerebrosidase-loaded erythrocytes (Deloach & Ihler, 1977). This methods used to overcome the allergic reactions, and toxic manifestations in exogenous enzyme therapy because resealed erythrocytes with enzymes can be treated as native cell.

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Treatment of Hepatic Tumors

Antineoplastic drugs used in hepatic tumors like methotrexate (Zimmermann, 1983, Jaitely et al., 1996), bleomycin (Zimmermann, 1973), asparginase (Updike & Wakamiya, 1983, Jaitely et al., 1996), and Adriamycin have been found to delivered successfully by resealed erythrocytes (Benatti et al., 1987, Zocchi et al., 1988, Al-Achi & Boroujerdi, 1990, Jaitely et al., 1996).

Treatment of Parasitic Diseases

Parasitic infection involve the harboring of parasites in the RES organs which can be successfully treated by the drug loaded resealed erythrocytes due to selectively accumulation within RES organs. Results has been reported as favorable in animal models for anti-malarial (Summers, 1983), anti-leishmanial (Summers, 1983, Eichler et al., 1986, Berman, 1987), and anti-amoebic drugs (Talwar & Jain, 1992b, Jaitely et al., 1996).

Removal of RES Iron Overload

It was found that aged erythrocytes are destroyed in RES organs which in turn increased the accumulation of iron in these organs. Resealed erythrocytes with desferrioxamine induction has been found to reduce the excess iron accumulated in thalassemic patients (Berman, 1987, Jaitely et al., 1996,).

Removal of Toxic Agents

Resealed erythrocytes encapsulated with recombinant phosphodiestrase has been reported to reduce the organophosphorus intoxication (Pei et al., 1994). Cannon et al. (1994) also reported that carrier erythrocytes containing sodium thiosulfate and bovine rhodanase inhibited the cyanide intoxication.

Targeting Other Organs

Despite the RES targeting, resealed erythrocytes have been used to target other organs. Various method have been used for different organs like entrapment of paramagnetic particle with desired drugs, drugs with photosensitive material, attachment of antibody at erythrocytes membrane or use or ultrasound waves (Gothoskar, 2004).

Entrapment of paramagnetic particle in to erythrocytes could induced their localization under the influence of an external magnetic field to a particular location. Photosensitized erythrocytes are photo-triggered carrier use to deliver methotrexate in cancer treatment (Flynn et al., 1994). Targeted micro bubble destruction with ultrasound has also been used to deliver the colloidal particles and erythrocytes to tissue through rupturing of microvessel (Price et al., 1998).

Despite the above approaches following could also use for targeting different organs other than RES:

- Preparation of erythrocytes carrier having thermo responsive liposomes (localization could performed through an external thermal source)
- Intra-peritoneal injection for drug targeting to peritoneal macrophages

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- Resealed cells pre-treated with lectin and loaded with antineoplastic drugs for improving target specificity to tumor cells.

Delivery of Antiviral Agents

Resealed erythrocytes have been used to deliver recombinant herpes simplex virus type 1 (HSV-1), deoxycytidine derivatives (Ihler, 1983), azidothymidine derivatives (Benatti et al., 1989), fludarabine phosphate (Fraternale et al., 1996), glycoprotein B (Chiarantini et al., 1995) and azathioprene, acyclovir (Magnani et al., 1998). Resealed erythrocytes has been used to deliver to anti-viral agents because most of them are either nucleotides or nucleoside analogs. Nucleosides have tendency to rapidly transported across the membrane; however, nucleotides have not, and hence exhibiting a prolonged release profiles.

Enzyme Therapy

Various biochemical derangement has been associated with the altered or deficient enzymes activity. Direct injection of enzymes into the body has been reported to induce the immunological response or deactivation due to other enzymes activity. Resealed erythrocytes overcome the above problem and act as a "circulating bioreactors" in which substrates enter, interact with enzymes, and generate products (Ihler et al., 1975, Adriaenssens et al., 1976, Zimmermann et al., 1976, Zimmermann, 1983, Alpar & Lewis, 1985, Fiorelli et al., 1987, Magnani et al., 1989, Jaitely et al., 1996) In addition, it can also accumulate enzymes in RES after hemolysis (Iher, 1973, Deloach & Ihler, 1977, Lewis & Alpar, 1984).

Following enzymes are being uses with resealed erythrocytes:

- β-glucoserebrosidase for the treatment of Gaucher's disease (Beutler et al., 1977)
- Aasparginase for the treatment of pediatric neoplasms.
- β -aminolevulinate dehydrogenase (ALA-D) against lead poisoning

Despite these, urease (Zimmermann et al., 1976), galactose-1-phosphate uridyl-transferase (Harris, 1977), uricase (Ihler et al., 1975), and acetaldehyde dehydrogenase (Tan, 1977) has also been reported to use in resealed erythrocytes.

Oxygen Delivery to Tissues

Hemoglobin is the red pigmented protein responsible for the oxygen transporting capacity of erythrocytes. Under normal condition the major fraction of oxygenated hemoglobin is recirculated with venous blood to the lungs. Use of this bound fraction has been reported in oxygen deficiency. 2,3-Diphosphoglycerate (2,3-DPG) a side glycolysis product through Rapoport-Luebering cycle has been reported to changes the binding affinity of hemoglobin for oxygen. Affinity of 2,3-DPG towards oxygen has been reported to much higher than that of hemoglobin (Guyton & Hall, 1996). Despite these 2,3-DPG, inositol hexophosphate (IHP) is also one of the modulator of binding affinity of hemoglobin for oxygen. IHP or 2,3-DPG loaded erythrocytes could beneficial under high altitude conditions, patient having reduced number of alveoli, reduction in oxygen carrying capacity, mutation or chemical modification associated

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with decrease in oxygen affinity for hemoglobin, oxygen-delivery capacity of stored blood and ischemia of myocardium, brain, or other tissues (Zimmermann, 1983, Villareal et al., 1987, Teisseire et al., 1987, Baron et al., 1987).

CURRENT NOVEL APPROACHES

Microinjection

Macromolecules such as DNA, RNA, and proteins are currently exploited in every field of therapeutics and research. Various models are present today; however, erythrocytes lacking of complex cellular components (e.g., nucleus) with relatively simple structure make them good candidates for the entrapment of macromolecules (Furusawa et al., 1974). In this microinjection methods, erythrocytes are used as microsyringes for injection of biomolecule or drugs to the host cells (Zimmermann, 1983). Above process takes place *in vitro* as culturing host eukaryotic cells coated with fusogenic agent (Sendai virus, or its glycoproteins or polyethylene glycol) are suspended with biomolecule or drugs loaded erythrocytes in an isotonic medium (Furusawa et al., 1974, Zimmermann, 1983, Schlegel & McEvoy, 1987). The microinjection process has been performed for micoroinject of DNA fragments (Lewis & Alpar, 1984), ferritin, latex particles, arginase (Kruse et al., 1981, Kruse et al., 1987), bovine and human serum albumin, and thymidine kinase (Wille & Willecke, 1976) to various cultured eukaryotic cells.

Erythrosomes

It is engineered vesicular systems which are chemically cross-linked to erythrocytes by modified reverse-phase evaporation technique. Erythrosomes has been hypothesized for macromolecular drugs encapsulation in erythrocytes (Cuppoletti et al., 1981, Jung, 1987, Vyas &. Dixit, 1999).

Nanoerythrosomes

Extrusion of erythrocyte ghosts has been associated with the production of small vesicles (diameter 100 nm) known as nanoerythrosomes. Specially used for daunorubicin which was found to with covalently conjugated with the help of gluteraldehyde spacer to nanoerythrosomes. Covalently conjugated daunorubicin to nanoerythrosomes has been reported to more active than free daunorubicin alone in *in vtiro* model as well as *in vivo* model (Moorjani et al., 1996, Lejeune et al., 1997).

IN VITRO STORAGE OF RESEALED ERYTHROCYTES

Resealed erythrocytes activity also depends to a greater extent on the medium and duration of their *in vitro* storage. Drug encapsulated erythrocytes and maintaining their life with drug content can be regulated by using appropriate *in vitro* storage methods. Hank's balanced salt solution (Lewis & Alpar, 1984, Lewis, 1984, Lewis & Desai, 1987, Jaitely et al., 1996, Hamidi et al., 2001,) and acid–citrate–dextrose media are has been reported to frequently uses as storage media for resealed erythrocytes (Zimmermann, 1983). Erythrocytes with drug remain viable regarding the physiological and carrier properties when

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stored in above media for at least 2 weeks at 4ºC (Lewis & Desai, 1987). Cells circulation life span was found to improve after addition of the purine nucleosides (Alpar & Irwin, 1987, Lewis & Desai, 1987) or calcium-chelating agents (Alpar & Irwin, 1987). Membrane stabilizing agents (dimethyl,3,3-di-thio-bispropionamide, dimethyl sulfoxide, toluene-2-4- diisocyanate, gluteraldehyde) exposure to drug loaded cells followed by sintered glass filtration and lyophilization has been reported to significantly increase the resealed erythrocytes stability upon storage (Jain & Jain, 1997, Talwar & Jain, 1992a, Talwar & Jain, 1992b, Bhaskaran & Dhir, 1995). Lyophilized powder has been found to stable without any detectable changes for at least one month. The major drawback of this method is the lower circulation life span of resealed erythrocytes. Despite this method encapsulation of a prodrug (Talwar & Jain, 1992a), high glycerol freezing technique (Zanella et al., 1987, Fiorelli et al., 1987), and reversible immobilization in alginate or gelatin gels (Zimmermann, 1983, Lewis & Alpar, 1984, Lewis, 1984) are other progressive methods use to perform for increasing storage stability of resealed erythrocytes.

CONCLUSION

Resealed erythrocytes are promising for precise and safe drugs delivery. In presence scenario methodologies applied for drugs encapsulation and preparation of resealed erythrocytes need some optimization for making it usual drugs carriers. The concept of resealed erythrocytes can be also extended to increase the bioavailable of various molecule including biopharmaceuticals. Above chapter prompt us to conclude that resealed erythrocytes is an effective carriers to deliver the various drugs and bioactive molecule, and much remains to be explored regarding the potential of resealed erythrocytes.

CONFLICT OF INTEREST

None

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Chapter 19
Sedation and Analgesia

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ABSTRACT

Patients in the cardiothoracic intensive care unit (CTICU) are subject to numerous physical and mental stresses. While most of these cannot be completely eliminated, intensivists have many tools in their armamentarium to alleviate patients' pain and suffering. This chapter will consider the importance of analgesia and sedation in the CTICU and the relevant consequences of over- or under-treatment. We will examine the tools available for monitoring and titrating analgesia and sedation in critically ill patients. The major classes of medications available will be reviewed, with particular attention to their clinical effects, metabolism and excretion, and hemodynamic characteristics. Lastly, experimental evidence will be assessed regarding the best strategies for treatment of pain and agitation in the CTICU, including use of non-pharmacologic adjuvants.

INTRODUCTION AND BACKGROUND

The experience of being in the intensive care unit (ICU) can present many stresses, both physical and psychological. Up to 77% of patients in the ICU suffer pain (Van Gulik et al., 2010) and 15% may go on to develop posttraumatic stress disorder (PTSD) (Girard et al., 2007). Many of the daily necessities of critical care, such as endotracheal intubation, mechanical ventilation, placement and maintenance of lines, and use of restraints, cause pain and distress, to say nothing of the discomfort that can come from surgical wounds and interventions. Through the use of judicious analgesia and sedation, intensivists are able to attenuate these stresses.

Inadequate sedation and analgesia have been associated with elevated catabolism, immunosuppression, hypercoagulability, and sympathetic overactivity (Consales, Chelazzi, Rinaldi, & De Gaudio, 2006). These effects are deleterious for patients in the perioperative period, even more so for those with cardiovascular disease, in whom they may precipitate arrhythmias and myocardial ischemia. Conversely, heavy sedation can lead to more time spent undergoing mechanical ventilation, longer ICU stays, and increased needs for imaging and other studies to evaluate patients' neurologic status, all of which put patients at risk for complications (Kress, Pohlman, O'Connor, & Hall, 2000).

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As a result, it is critical to maintain a balance between providing adequate analgesia and sedation and limiting side effects. Several principles seem to logically follow from this:

1) Selection of medications should be tailored to the individual patient and situation.
2) There is growing evidence that the medications used in the ICU may impact long-term outcomes, so current best evidence and guidelines should be followed.
3) Titration of medications should be based on reproducible, validated measurements, and frequent reassessment is mandatory.
4) Use of non-pharmacologic measures should be optimized to reduce the need for medication.

While analgesics and sedatives can produce adverse effects, they have the potential to relieve suffering and improve patients' outcomes and quality of life. This is as true in the cardiothoracic population as in any other group of critically ill individuals. The goals of this chapter will be to discuss the assessment of pain and mental state in the cardiothoracic ICU (CTICU), review various options for providing analgesia and sedation, and provide a framework for evaluating the evidence and selecting the most appropriate agents and strategies for individual patients.

ASSESSMENT OF LEVEL OF ANALGESIA/SEDATION

Regardless of the sedative used, it is critical to monitor level of analgesia and sedation closely so as to avoid over- or under-dosing. For pain intensity, the gold standard is the patient's subjective report; when this is not available, clinicians are forced to estimate pain levels via observation of behavior. One simple and well-validated tool for measuring self-reported pain intensity is the Visual Analog Scale (VAS), which plots relative pain intensity along a line. An alternative is the Numerical Reporting Scale (NRS), which adds numerical increments to the VAS (Hawker, Mian, Kendzerska, & French, 2011).

When patients cannot report their pain intensity due to altered mental status or inability to communicate, an observational scale may be used, such as the Behavioral Pain Scale (BPS). The BPS relies on visual indicators of discomfort, assessed by a healthcare practitioner, and ranks pain intensity on a scale of 3 to 12 (see Table 1). This scale has been shown to have good reliability and validity in a broad population of critically ill patients (Cade, 2008). However, the BPS relies partially on increases in patient movement to indicate greater pain intensity, and some studies suggest that increased pain intensity is correlated with *decreased* movement, calling its results into question (Blenkham, Faughnan, & Morgan,

Table 1. The behavioral pain scale

Item	1 Point	2 Points	3 Points	4 Points
Facial Expression	Relaxed	Partially tightened	Fully tightened	Grimacing
Upper Limb Movements	No movement	Partially bent	Fully bent with finger extension	Permanently retracted
Compliance with Mechanical Ventilation	Tolerating movement	Coughing but tolerating ventilation for most of time	Fighting ventilator	Unable to control ventilation

(Adapted from Payen et al., 2001)

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2002). When the BPS has been compared to self-reported measurements, it indeed has been found to underestimate pain intensity, especially at higher levels of discomfort. Therefore, it is appropriate to utilize patients' self-report of pain intensity whenever feasible (Ahlers et al., 2007).

Similarly, quantitative tests of level of sedation provide for more uniform assessment and trending of data over time. One of the most commonly used sedation assessment tools is the Ramsay Sedation Scale, which uses a series of numbers to identify levels of arousal ranging from severe agitation to coma (See Table 2). It exhibits good inter-rater reliability and has been used for many years, making it familiar to clinicians (Sessler, Grap, & Ramsay, 2009). The Ramsay scale, however, has several problems, one of which being that patients may fall under more than one of its levels at the same time if, for example, they are suffering from a mixed form of delirium. Additionally, the scale only has one score corresponding to agitation.

Another tool to assess sedation is the Richmond Agitation-Sedation Scale, or RASS (See Table 3). The RASS has the advantage of having a full range of responses quantifying both sedation and agitation, and has been found to have excellent inter-rater reliability (Sessler et al., 2002).

Table 2. The Ramsay Sedation Scale

Score	Definition
1	Anxious and agitated or restless or both
2	Cooperative, oriented, and tranquil
3	Responds to commands only
4	Brisk response to a light glabellar tap or loud auditory stimulus
5	Sluggish response to a light glabellar tap or loud auditory stimulus
6	No response to a light glabellar tap or loud auditory stimulus

(Adapted from Sessler, Grap, & Ramsay, 2009)

Table 3. The Richmond Agitation-Sedation Scale

Score	Term	Description
+4	Combative	Violent, immediate danger to staff
+3	Very Agitated	Pulls on or removes tube(s) or catheter(s) or has aggressive behavior towards staff
+2	Agitated	Frequent nonpurposeful movement or patient-ventilator dyssynchrony
+1	Restless	Anxious or apprehensive but movements not aggressive or vigorous
0	Alert and Calm	
-1	Drowsy	Not fully alert, but has sustained (more than 10 s) awakening, with eye contact, to voice
-2	Light Sedation	Briefly (less than 10 s) awakens with eye contact to voice
-3	Moderate Sedation	Any movement (but no eye contact) to voice
-4	Deep Sedation	No response to voice, but any movement to physical stimulation
-5	Unarousable	No response to voice or physical stimulation

(Adapted from Sessler, Grap, & Ramsay, 2009)

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Since delirium is common in the ICU, it behooves clinicians to have an objective way of evaluating it and grading its severity. One of the most utilized tools for doing this is the Confusion Assessment Method for the Intensive Care Unit (CAM-ICU) (See Table 4). This instrument was the first developed to monitor for delirium in ICU patients receiving mechanical ventilation; it can also be used in patients who are not receiving ventilator support (Ely et al., 2001).

Unfortunately, visual observation and neurologic examination are not always adequate for evaluating a patient's level of sedation, especially if he or she is minimally responsive. An alternative method is to use physiologic variables, such as processed electroencephalography (EEG). The BIS monitor, a popular device used to assess depth of anesthesia in the operating room, analyzes the frequency spectrum of the EEG and produces an indexed value representing the patient's level of sedation. This device can assess states ranging from full alertness to deep anesthesia (Riker & Fraser, 2001). Unfortunately, the EEG signal can be degraded by artifact, especially in patients who are moving, but it can provide valuable information in those who appear to be deeply sedated or are receiving neuromuscular blockers.

ANALGESICS

Almost all patients in the CTICU will suffer from some degree of pain, whether it is due to surgical trauma or other sources. Risk factors for more severe postoperative pain include younger age, duration of surgery, and location of surgery; the thoracotomy incision is generally more painful than the median sternotomy

Table 4. Confusion assessment method for the intensive care unit (CAM-ICU)

Features and Descriptions	Absent	Present
1. Acute Onset or Fluctuating Course		
C. Is there evidence of an acute change in mental status from baseline? D. Did the abnormal behavior fluctuate during the past 24 hours as evidenced by fluctuations in the RASS or Glasgow Coma Scales?		
2. Inattention		
Did the patient have difficulty focusing attention (score of less than 8 on the visual or auditory components of the Attention Screening Examination)?		
3. Disorganized thinking		
Is there evidence of disorganized or incoherent thinking as evidenced by incorrect answers to 3 or more of the 4 questions and inability to follow commands? Questions: 5. Will a stone float on water? 6. Are there fish in the sea? 7. Does 1 pound weigh more than 2 pounds? 8. Can you use a hammer to pound a nail? Commands: 4. Are you having unclear thinking? 5. Hold up this many fingers. 6. Now do the same thing with the other hand.		
4. Altered level of consciousness		
Is the patient's level of consciousness anything other than alert, such as being vigilant or lethargic or in a stupor or coma?		
Overall CAM-ICU Assessment: Features 1 and 2 and either Feature 3 or 4		

(Adapted from Ely et al., 2001)

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(Cogan, 2010). Analgesia is crucial for these patients for multiple reasons. Inadequate analgesia may result in hypertension, tachycardia, and other hemodynamic changes, increasing the risk of myocardial ischemia, arrhythmias, and bleeding (Rouby et al., 1981). It may also predispose patients to atelectasis and respiratory compromise. Likewise, while many analgesics have some sedative properties, it is difficult to achieve adequate sedation without sufficient analgesia. Therefore, it is prudent to ensure that a patient's pain is treated before adding additional sedatives to their regimen. It has also been suggested that inadequate analgesia may increase the risk of delirium, especially in the elderly (Morrison, 2003).

Numerous approaches to analgesia are available for CT surgery patients. Multiple approaches can be used simultaneously as "multimodal analgesia," potentially improving pain control and decreasing side effects. This strategy has been shown to be effective in both cardiac (Rafiq et al., 2014) and thoracic (Bottiger, Esper, & Stafford-Smith, 2014) surgical populations. The medications and techniques used to achieve pain control depend somewhat on the individual patient and surgery. In many cases, however, standardized perioperative analgesic algorithms can be developed to improve the efficacy and efficiency of perioperative analgesia.

OPIOIDS

Parenteral opioids are the mainstay of pain management for CT surgery patients. This approach offers flexibility, relative hemodynamic stability, and a manageable side effect profile. Some commonly used opioids include fentanyl, morphine, and hydromorphone (See Table 5). All opioids act through stimulation of opioid receptors in the central nervous system (CNS), which belong to the G-protein-coupled receptor family, causing multiple physiologic effects, including reliable dose-dependent analgesia. Other effects include miosis, pruritus, sedation, decreased gastrointestinal motility, and suppression of hypercarbic respiratory drive. Opioid analgesia appears to be mediated primarily by inhibition of pain signals via pathways involving the midbrain, medulla, and dorsal root ganglion of the spinal cord, as well as possibly at other sites (Basbaum & Fields, 2004). The most significant receptor subtype mediating analgesia for the majority of opioids is the μ-opioid receptor (Jordan & Devi, 1998).

The most severe toxicity of opioids is respiratory depression due to inhibition of the central response to hypercarbia. Up to 1% of patients receiving postoperative opioid analgesia may experience respiratory depression (Shapiro et al., 2005), and such events can result not just in morbidity and mortality, but also increased hospital length-of-stay and costs (Oderda et al., 2007). Patients receiving potent IV opioids

Table 5. Comparison of typical opioids used in the CTICU

Drug	Typical IV Bolus Dose	Onset	Duration of Action After Bolus	Metabolism	Active Metabolites?
Fentanyl	25-100 mcg	1-4 min	30-60 min	Hepatic	No
Morphine	2-5 mg	5-10 min	2-6 hrs	Hepatic	Yes
Hydromorphone	0.2-1 mg	5-10 min	3-5 hrs	Hepatic	No
Sufentanil	5-50 mcg	1-4 min	30-60 min	Hepatic	No
Remifentanil	0.5-1 mcg/kg	1 min	5-10 min	Plasma esterases	No

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should be monitored closely for hypoxia and hypercarbia. The use of end-tidal CO2 (EtCO2) monitoring, "smart" infusion devices, and other interventions may mitigate such risks (Maddox, Oglesby, Williams, Fields, & Danello, 2006). Other opioid-related adverse effects include nausea, vomiting, pruritus, and constipation. Opioid administration is associated with development of tolerance and may necessitate dose escalation.

Some of these side effects can be treated with opioid antagonists. Naloxone, which is a non-selective opioid receptor antagonist, is useful in the management of respiratory depression. However, naloxone itself can induce rebound pain, tachycardia, and hypertension. It should therefore be administered in smaller, titrated doses (20-40 micrograms at a time) when the situation is less emergent. As an additional consideration, naloxone has an elimination half-life of roughly 30 minutes, so it may need to be redosed when used to counteract the effects of a long-acting opioid. Several new agents are being investigated to treat opioid-induced respiratory depression, such as serotonin agonists, ampakines, and minocycline, all of which help maintain respiratory drive via pathways that bypass opioid receptors (Dahan, Aarts, & Smith, 2010). While these agents are still being tested experimentally, they have the potential advantage of treating opioids' side effects without counteracting their analgesic effects. Nausea and vomiting may respond to 5HT-3 receptor antagonists and other antiemetics, and methylnaltrexone, a peripheral opioid antagonist, is useful in ameliorating adverse effects on gastrointestinal motility.

Hemodynamically, most opioids induce some degree of bradycardia and hypotension. This occurs due to a decrease in sympathetic and an increase in parasympathetic autonomic tone, mediated by the central nervous system (CNS). These changes can be potentiated by the use of other medications, like beta-blockers, that cause similar effects. Direct effects on cardiac contractility are variable between opioids but are probably relatively minor compared to other classes of sedative medications (Fukuda, 2010). Opioids may also exert protective effects against myocardial ischemia, although the clinical significance of this fact is unclear (Schultz, Hsu, & Gross, 1996).

Fentanyl

Fentanyl is a synthetic opioid that is highly lipophilic and has a relatively fast onset of action. It diffuses quickly in and out of the CNS. Fentanyl may be administered by bolus and can also be given via infusion at doses ranging from 25 to 200 mcg/hr. It is metabolized in the liver by CYP3A4 enzymes and has no active metabolites.

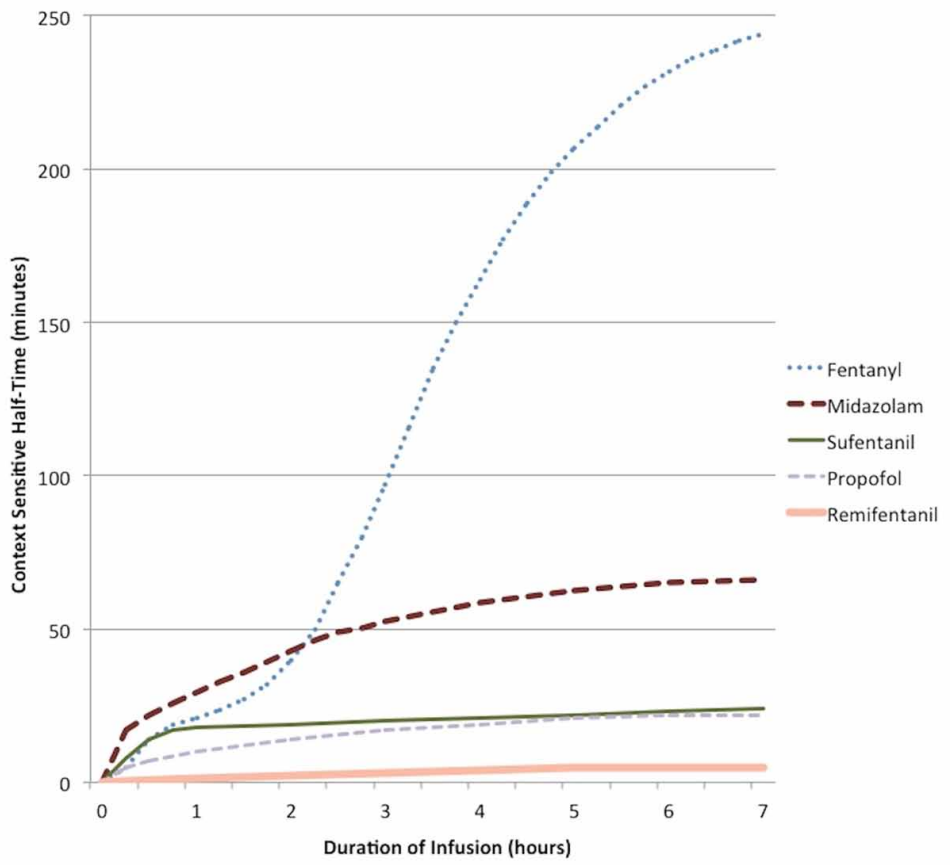
Although it has an elimination half-life of several hours, fentanyl's clinical effect is usually terminated via redistribution out of the CNS into other tissues, such as muscle and adipose tissue, rather than elimination. Therefore, it appears to have a shorter duration of action than would be expected based on its metabolism and elimination. However, fentanyl is notable in that its context-sensitive half-time increases substantially after 120-180 minutes of continuous infusion. At this point, non-CNS tissue compartments start to become saturated, and the drug's clinical effects will last much longer after discontinuing an infusion. Dosage may need to be adjusted accordingly (Hill, 2004). (See Figure 1)

Morphine

Morphine is the least lipophilic of the commonly used opioids. While morphine actually has a shorter elimination half-life than fentanyl, it diffuses more slowly out of the CNS once it has entered and thus appears to have a longer duration of action than fentanyl and other highly lipophilic opioids (Hug, Murphy,

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Figure 1. Context-sensitive half-times of common analgesics/sedatives
(Adapted From Hughes, Glass, & Jacobs, 1992, and Egan et al., 1993)



Rigel, & Olson, 1981). Morphine can cause prominent histamine release, particularly if given as a rapid bolus. Additionally, it is conjugated in the liver to morphine-3-glucuronide, which has neuroexcitatory properties, and morphine-6-glucuronide, which has properties similar to morphine itself (Christrup, 1997). Because these metabolites are partially excreted by the kidneys, repeated doses or prolonged infusion of morphine may have unpredictable effects in patients with renal insufficiency.

Hydromorphone

Hydromorphone, which has pharmacokinetic and dynamic properties similar to morphine, has a slightly more favorable side-effect profile. It appears to cause less histamine release and may have a more consistent duration of action; some pain specialists feel it may cause less nausea and constipation, although studies on this subject have produced mixed results (Felden et al., 2011; Wirz, Wartenberg, & Nadstawek, 2008). IV hydromorphone is roughly seven times as potent as IV morphine. Both morphine and hydromorphone are available in oral form although hydromorphone is less orally bioavailable than morphine.

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Sufentanil

Sufentanil is a synthetic opioid that has similar properties to fentanyl but is 5-10 times more potent. It is commonly used intraoperatively during cardiac surgery and provides similar hemodynamic control to fentanyl (Thomson, Harding, & Hudson, 2000). However, sufentanil exhibits much less increase in context sensitive half-time than fentanyl with longer infusions (see Figure 2). Some studies have found that sufentanil consequently facilitates earlier emergence and more rapid extubation than other opioids, particularly when high doses of opioids are administered as adjuncts to general anesthesia (Sanford, Smith, Dec-Silver, & Harrison, 1986).

Remifentanil

Another potent and very short-acting synthetic opioid is remifentanil, which is particularly notable because it is metabolized by nonspecific plasma esterases instead of by hepatic enzymes. Therefore, remifentanil's duration of action is unaffected by hepatic or renal dysfunction. Its context sensitive half-time is the shortest of the opioids and is affected minimally by duration of infusion (see Figure 2). As a result, remifentanil is an ideal choice when patients may need frequent neurologic assessments or must undergo procedures that are short but painful (Ruggeri et al., 2011). Because remifentanil is so potent, it can cause pronounced bradycardia and hypotension, especially when bolused; it can instead be administered by infusion at doses of 0.05-2 mcg/kg/min. It can also easily induce apnea and should be dosed carefully when patients are breathing spontaneously.

While remifentanil has several practical advantages, it can also have adverse effects. When used intraoperatively, remifentanil can induce rapid opioid tolerance, which may result in higher postoperative narcotic requirements (Guignard et al., 2000) and even hyperalgesia (Angst, Koppert, Pahl, Clarke, & Schmelz, 2003). These effects may be particularly problematic in patients with chronic pain disorders and remifentanil should be used judiciously in this population.

KETAMINE

Ketamine is a phencyclidine derivative that can act as both an analgesic and a sedative/anesthetic. It acts primarily via antagonism of the *N*-methyl-D-aspartate (NMDA) receptor, but likely has numerous other targets, such as opioid receptors, monoaminergic receptors, muscarinic receptors, and ion channels (Hirota & Lambert, 1996). Higher doses of ketamine (1-2 mg/kg) will produce sedation and a "dissociative" state, which is characterized by an appearance of alertness although the patient will generally be amnestic and minimally responsive. Dense analgesia is also a prominent feature of this state. Ketamine has a fast onset (1-5 minutes after a bolus). It can also be administered by infusion as a sedative with doses in the range of 10-30 mcg/kg/min.

At lower, subanesthetic doses (4-5 mcg/kg/min), ketamine has relatively little sedative effect, but acts as an analgesic. A Cochrane review found that low-dose ketamine given postoperatively decreases opioid requirements and leads to less nausea and vomiting without substantial side effects (Bell, Dahl, Moore, & Kalso, 2006). It also seems to attenuate acute opioid tolerance (Kissin, Bright, & Bradley, 2000). This effect is particularly helpful in patients with high postoperative opioid requirements, either due to chronic opioid use or the magnitude of surgery itself.

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Ketamine is metabolized in the liver, producing norketamine, a less potent active metabolite. Its clinical effect is terminated by redistribution, and it has an elimination half-life of 2 to 3 hours. Because of its active metabolite, the duration of action of ketamine may be prolonged in patients with liver or renal dysfunction or those who receive multiple doses.

Ketamine's major cardiovascular effect is stimulation of central sympathetic outflow. This results in increased heart rate, blood pressure, and cardiac output, as well as potentially increased pulmonary arterial pressures (Tweed, Minuck, & Mymin, 1972). These effects seem to be more prominent after bolus dosing than with infusions, and can be attenuated by premedication with benzodiazepines or other sedatives (Jackson, Dhadphale, & Callaghan, 1978). In some cases, this sympathetic stimulation may be beneficial, preserving hemodynamic stability. However, in critically ill patients, ketamine may occasionally cause hypotension, presumably due to its direct myocardial depressive effects, which are typically masked by increased sympathetic tone (Schwartz & Horwitz, 1975). Because of these diverse hemodynamic effects, use of ketamine in the CT surgery population requires careful monitoring.

An additional benefit of ketamine compared to opioids is its relatively benign respiratory profile. It has minimal effect on respiratory drive and airway reflexes, even with higher doses (Soliman, Brinale, & Kuster, 1975; Lanning & Harmel, 1975), and can act as a bronchodilator. However, ketamine can also cause an increase in salivation. Antisialogogues (such as glycopyrrolate) can be administered to avoid this effect.

Ketamine also has prominent neurologic effects. While it is sometimes cited as a proconvulsant and can induce epileptiform activity in deeper brain structures, it does not appear to induce generalized seizures (Myslobodsky, Golovchinsky, & Mintz, 1981). Likewise, while it was long thought to increase intracranial pressure (ICP), ketamine likely maintains or decreases ICP as long as ventilation is controlled and PaCO2 does not rise substantially (Mayberg, Lam, Matta, Domino, & Winn, 1995). Most prominently, ketamine can cause emergence delirium as its effect is wearing off. This state is characterized by hallucinations, vivid dreams, and confusion. Emergence delirium may occur in up to 30% of patients receiving higher-dose ketamine for anesthesia; risk factors include age > 15 years, female gender, total doses greater than 2 mg/kg, rapid administration, and a history of personality issues or frequent dreaming (White, Way, & Trevor, 1982). Premedication with GABA-ergic agents, particularly benzodiazepines, seems to lessen this effect, with midazolam being the preferred medication (Cartwright & Pingel, 2007). Interestingly, it seems that despite this effect, ketamine may not precipitate long-lasting delirium. In fact, low-dose ketamine administered intraoperatively may reduce delirium after cardiopulmonary bypass (CPB) via anti-inflammatory effects (Hudetz et al., 2009).

ADJUVANTS

Numerous other non-sedative medications may be used to augment the analgesic effect of opioids and ketamine. Ideally, this enables the reduction of a patient's analgesic dosage and thus reduces adverse effects. Multiple agents may be used together as part of a multimodal pain management strategy.

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDS act via inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). These enzymes participate in the formation of mediators such as prostacyclin, prostaglandins, and thromboxane, causing fever, inflammation, and pain. While inflammation often serves an adaptive purpose, it can be

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counterproductive if it increases post-surgical pain and impedes recovery. Numerous NSAIDs have been used in the management of postoperative pain, such as diclofenac and ketorolac. Ketorolac is available in intravenous form, facilitating perioperative use.

NSAIDs exert potent analgesic and anti-inflammatory effects. They improve pain control after sternotomy and allow for decreases in opioid dosage, resulting in decreased rates of prolonged intubation, desaturation, and gastrointestinal side effects when compared to therapy with opioids alone (Fayaz et al., 2004). As an additional (and perhaps unintentional) benefit, use of ketorolac has been suggested to improve graft patency after coronary bypass grafting and possibly improve postoperative survival (Engoren, Hadaway, Schwann, & Habib, 2011). Ketorolac has been shown to have similar efficacy in relieving post-thoracotomy pain, resulting in improved respiratory parameters (Carretta et al., 1996). Thoracic surgical patients frequently have limited pulmonary reserve and may thus be unable to tolerate the respiratory depression that can result from high doses of systemic opioids.

Unfortunately, NSAIDs do have adverse effects that must be taken into account. All NSAIDs can induce some decrease in renal function, potentially resulting in fluid retention, hyperkalemia, and other abnormalities. These effects are particularly likely in individuals whose renal function is already impaired and are dependent on prostaglandin production to maintain renal blood flow and glomerular filtration rate (GFR). Interstitial nephritis is also possible but rare. Most of the decrease in GFR due to NSAIDs is reversible when the medication is discontinued (Whelton & Hamilton, 1991).

Because they decrease production of prostaglandins, NSAIDs can induce systemic hypertension. They are also known to cause gastropathy and upper gastrointestinal bleeding, due to decreased levels of prostaglandin-induced mucus and bicarbonate production. Older patients, those with a history of gastritis or peptic ulcers, and those taking steroids or anticoagulants are at higher risk (Hawkey, 2000). NSAIDs inhibit platelet function and may exacerbate postoperative bleeding, especially when combined with other antiplatelet agents or anticoagulants.

COX-2-specific NSAIDs may have some theoretical advantages over agents that inhibit both COX-1 and COX-2. COX-2 inhibitors induce lower rates of bleeding and gastropathy, which would seem to make them advantageous for perioperative use. However, concerns have been raised about whether these medications predispose patients to adverse cardiovascular events. (Some similar concerns exist for nonspecific NSAIDs as well.) In a study evaluating the use of valdecoxib and parecoxib, two COX-2 inhibitors, after coronary artery bypass grafting (CABG), it was found that use of these drugs vs. placebo resulted in higher rates of adverse cardiovascular events, such as myocardial infarction, stroke, and pulmonary embolism (Nussmeier et al., 2005). Such concerns have resulted in valdecoxib being withdrawn from the market, although parecoxib, its prodrug, remains available in some countries. In the aggregate, it appears that NSAIDs have a useful role in perioperative pain control, but should be used carefully, with close monitoring for side effects.

Acetaminophen

Acetaminophen is one of the most widely used pain medications in the world due to its perceived efficacy and benign side effect profile. Acetaminophen is typically administered orally or rectally; an intravenous formation is also available for perioperative use. It produces analgesic and antipyretic effects. The mechanisms of acetaminophen's effects remain poorly understood. While it has some inhibitory effects on the COX enzymes and inhibits prostacyclin production, it does not seem to strongly affect thromboxane, and thus has minimal effect on platelets (Trettin et al., 2014). It has been proposed that acetaminophen

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may act via activation of central serotonergic pathways and stimulation of the endocannabinoid system, as its effect seems more dependent on CNS concentration than systemic levels (Anderson, 2008).

While acetaminophen itself is a relatively weak analgesic, it exhibits synergistic effects with opioids and other analgesics. Administration of acetaminophen after major surgery reduces morphine consumption in a similar fashion as that found with NSAIDs. However, NSAIDs seem to have more benefit in reducing gastrointestinal side effects and may result in slightly less sedation (McDaid et al., 2010).

While acetaminophen confers minimal risk of postoperative bleeding or gastropathy, it does have notable adverse effects, the most significant of which is hepatotoxicity. Acetaminophen is metabolized by cytochrome enzymes in the liver to a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine, which is usually bound and detoxified by glutathione. However, when large doses are given, glutathione is depleted and the metabolite binds to proteins. This results in oxidative stress, inflammation, and hepatic necrosis (Hinson, Roberts, & James, 2010). Certain patients, particularly those who habitually consume alcohol, are at higher risk, and total daily dosage may need to be limited. Theoretically, intravenous administration may be beneficial in these patients because it avoids first-pass metabolism in the liver.

REGIONAL ANALGESIA

While it requires some additional expertise, the use of regional techniques to provide analgesia after CT surgery has grown substantially. Use of regional analgesia can provide pain relief while avoiding some of the side effects of systemic medications.

Epidural Analgesia

Epidural analgesia involves the placement of a small flexible catheter in the space between the ligamentum flavum and the dura mater in the spinal canal. Medications, usually local anesthetics, opioids, or both in combination, are infused through the catheter. Pain is reduced via multiple mechanisms: local anesthetics block sodium channels on the spinal nerves in the paravertebral and intradural spaces, producing segmental analgesia that is proportional to the volume of medication administered (Bromage, 1975). Epidural opioids, in contrast, act on receptors both within the spinal cord and supraspinally. Supraspinal mechanisms seem to become more important when opioids are administered by infusion rather than bolus (George, 2006).

Thoracic epidural analgesia (TEA) has gained wide acceptance as the analgesic technique of choice for thoracotomy and, at some centers, video-assisted thoracic surgery (VATS). Typically, an epidural catheter is inserted in the mid-thoracic region, and a continuous infusion of medication is delivered, in some cases with additional bolus doses on demand via patient-controlled analgesia. When compared to systemic analgesics, TEA has been shown to reduce pain scores, time to extubation, ICU length of stay, consumption of rescue medication, and rates of postoperative arrhythmias, and seems to improve pulmonary measures such as functional residual capacity (FRC) (Guay, 2006). Other reviews have suggested that epidural analgesia reduces rates of pulmonary complications such as atelectasis and pneumonia (Ballantyne et al., 1998; Popping, Elia, Marret, Remy, & Tramer, 2008). On the other hand, these studies do not show an improvement in mortality with epidural analgesia. As an added benefit, some authors have concluded that use of TEA is associated with less chronic postthoracotomy pain, particularly if the epidural infusion is initiated preoperatively (Senturk et al., 2002). TEA can also be utilized in VATS, and

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has been shown to provide better analgesia than systemic analgesics (Yoshioka et al., 2006). However, it is not clear that this leads to fewer pulmonary complications after thoracoscopic surgery.

TEA has also been shown to have a role after cardiac surgery. Many of the same benefits posited in thoracic and abdominal surgery have been found in post-cardiac surgical patients. Several meta-analyses have found lower rates of arrhythmias and respiratory complications in cardiac patients who received epidural analgesia, as well as faster time to tracheal extubation (Liu, Block, & Wu, 2004; Svircevic et al., 2013). Notably, as with thoracic surgery, these investigators did not find any significant effect of TEA on rates of myocardial ischemia or mortality. The use of TEA in cardiac surgery has been limited by concerns over the risk of epidural hematoma, particularly when CPB requiring full heparinization is utilized. The risk of TEA-associated epidural hematoma after cardiac surgery has recently been estimated at roughly one in 5500, which is similar to the risk in the non-cardiac surgical population of one in 6600 (Hemmerling, Cyr, & Terrasini, 2013).

Besides the risk of hematoma formation, epidural analgesia has some other notable side effects. Epidural opioids can potentially cause any of the side effects of systemic opioids, although this is theoretically ameliorated by the lower total doses required. In particular, pruritus and urinary retention can be particularly severe (Ladak et al., 2009). Use of epidural local anesthetics substantially improves pain relief compared to epidural opioids alone and causes some degree of sympathectomy; while this may be beneficial in decreasing arrhythmias and myocardial oxygen demand, it can also lead to hypotension (Joshi, 2008). Hypotension may require reduction or temporary discontinuation of the epidural infusion.

Intrathecal Opioids

In some cases, intrathecal administration of opioids may be preferable to the epidural route. Because intrathecal injection is performed with a smaller needle than that used to place an epidural, it is theorized that the rate of spinal hematoma is lower with this technique. Additionally, because no catheter is left in place, there is no concern for how to accomplish catheter removal in patients who become coagulopathic or thrombocytopenic postoperatively or those who require postop anticoagulation. The rate of hematoma formation does seem to be somewhat lower with intrathecal injection, but such complications are extremely rare and thus rates vary widely across studies and between patient populations (Moen, Dahlgren, & Irestedt, 2004; Emazabel-Yunta et al., 2008).

Intrathecal opioids provide postop analgesia that is at least equivalent to that provided by intravenous narcotics. In cardiac surgery, they seem to reduce postoperative opioid requirements although they may also delay time to tracheal extubation (Chaney, Furry, Fluder, & Slogoff, 1997; Alhashemi, Sharpe, Harris, Sherman, & Boyd, 2000). Other data suggests an improvement in postoperative spirometric parameters and no change in time to extubation (Jacobsohn et al., 2005), but this has not been shown to translate into a decrease in complications, morbidity, or mortality. Studies examining patients undergoing thoracotomy also show decreased postop opioid requirements and improved respiratory function versus parenteral narcotics (Askar et al., 2007), although direct comparisons between epidural and intrathecal techniques are lacking.

One of the shortcomings of intrathecal injection is that because a catheter is generally not left in place, repeat doses of medication cannot be given, limiting the duration of analgesia. Intrathecal opioids can cause any of the side effects of systemic opioids, such as respiratory depression, nausea, vomiting, and pruritus. While this technique may be useful, it has not truly been shown to be superior to intravenous opioids, and since it is more expensive and effort-intensive, its appeal is likely to remain limited.

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Paravertebral Infusion

Another regional technique that can enhance postoperative analgesia is continuous paravertebral infusion (CPI). In this technique, a catheter is inserted into the paravertebral space on the surgical side and local anesthetic is infused, anesthetizing the spinal nerves as they exit the intervertebral foramina and producing unilateral pain relief (Richardson, Lonnqvist, & Naja, 2011). No opioids are included in the infusion, and the sympathectomy induced is unilateral, providing improved hemodynamic stability. Meta-analyses have shown that CPI seems to result in equivalent analgesia after thoracotomy when compared to TEA, and may induce less urinary retention, nausea, and hypotension (Ding et al., 2014). In an intriguing study in breast cancer patients, CPI was even found to reduce rates of cancer recurrence and distant metastasis when compared to systemic opioid analgesia (Exadaktylos, Buggy, Moriarty, Mascha, & Sessler, 2006). However, because the benefits in this study were suggested to be due to attenuation of the stress response and decreased use of opioids (which can be immunomodulatory), it is worth noting that TEA may result in similar benefits. This is still a question in the early stages of investigation.

While it appears to have a good side-effect profile compared to epidural analgesia, CPI does have its own risks. Because it is still an invasive procedure close to the spinal cord, its use is limited in coagulopathic patients. It also has limited utility in pain relief after sternotomy, when unilateral analgesia may be inadequate. However, this technique seems likely to grow in popularity in the future.

SEDATIVES

While analgesia forms the basis of patient comfort, additional sedatives are sometimes necessary to ensure anxiolysis and ventilator synchrony. Numerous agents are available which may act through different pathways and thus can have divergent effects. The choice of agent is largely dependent on a patient's individual medical condition and needs.

BENZODIAZEPINES

Benzodiazepines (BZDs) have a long history as ICU sedatives. They interact with the Gamma aminobutyric acid type A (GABA-A) receptor and enhance the action of GABA, the primary inhibitory neurotransmitter in the CNS. This produces numerous effects including sedation, anxiolysis, muscle relaxation, and anterograde amnesia. The BZD binding site on the receptor is distinct from the binding sites for other GABA-ergic sedatives, such as barbiturates and ethanol, and BZDs' effects can be synergistic with these agents (Olsen, 1981). BZDs are metabolized by CYP450 enzymes in the liver, then undergo glucuronidation, and are finally excreted renally. Some have active metabolites. Three of the most commonly used benzodiazepines in critical care practice are midazolam, lorazepam, and diazepam (See Table 6).

BZDs have several advantages: they provide reliable anxiolysis and have a high specificity for inducing amnesia, which can be very useful for procedural sedation. This anterograde amnesia appears to be strongly related to the lipid solubility of the drug (Fox, Liu, & Kaye, 2011). BZDs are also useful for treating alcohol withdrawal and delirium tremens.

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Table 6. Comparison of benzodiazepines most commonly used in the CTICU

Drug	Typical IV Dose	Onset	Duration of Action After Bolus	Elimination Half-Life	Active Metabolites?
Midazolam	1-5 mg	1-5 min	20-60 min	2-6 hrs	No
Lorazepam	0.5-4 mg	2-5 min	1-4 hrs	12-18 hrs	No
Diazepam	2-10 mg	15-20 min	1-6 hrs	20-70 hrs	Yes

Another advantage to BZDs in patients with cardiovascular disease is their relatively benign hemodynamic profile. When given at clinically relevant doses, BZDs may cause mild vasodilation but only a slight drop in arterial blood pressure (Stoelting, 1981). They seem to have minimal effects on coronary blood flow (Marty, Nitenberg, Blanchet, Zouioueche, & Desmonts, 1986) and are generally well-tolerated even in patients that are hemodynamically unstable.

As with other sedatives, BZDs have several drawbacks. Because they are hepatically metabolized and renally cleared, they may have prolonged effects in patients with end-organ dysfunction. While BZDs modestly decrease respiratory drive and response to hypercapnea and hypoxia (Forster, Gardaz, Suter, & Gemperle, 1980), clinical experience indicates that they rarely cause respiratory failure when given in isolation (Gaudreault, 1991). Flumazenil, a competitive BZD receptor antagonist, is readily available to reverse BZD effects. However, it should be reserved for severe cases because of the relatively benign nature of BZD overdose and the risk of seizures and agitation with flumazenil administration, particularly if a patient has been taking BZDs chronically. When indicated, flumazenil is given in increments of 0.2 mg IV, up to 1 mg total.

More concerning for intensivists, BZDs have been implicated in increasing the risk of ICU delirium. Lorazepam, in particular, has been associated with the transition to delirium in critically ill patients. This relationship seems to be dose-dependent, with an increasing risk of delirium at doses from 0 to 20 mg/day and a plateau in risk after 20 mg/day (Pandharipande et al., 2006). Additionally, total lorazepam dose has been correlated with risk of post-traumatic stress disorder (PTSD) after intensive care (Girard et al., 2007). However, these relationships are strongest in cohort and case-control studies and thus may be affected by unmeasured biases. One possible mechanism by which BZDs may increase risk of PTSD is the very amnesia that leads many intensivists to use them: at least one study has indicated that patients who have delusional memories of their ICU stay, with no true memories, are actually at higher risk for PTSD than those with true memories of the ICU (Jones, Griffiths, Humphris, & Skirrow, 2001).

Midazolam

Midazolam is a fast-acting, water-soluble BZD that is very lipophilic. This results in a speedy onset of action and a high level of anterograde amnesia. It is available in IV, oral, and intranasal forms, although it has low oral bioavailability. Midazolam's clinical action is typically terminated by redistribution out of the CNS, and, like fentanyl, its context-sensitive half-time increases after a prolonged infusion (see Figure 2). When given as an infusion, doses for midazolam range from 1-10 mg/hr.

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Lorazepam

Lorazepam has an onset of action that is similar to midazolam but has a longer duration of action. It also causes some anterograde amnesia. Because it undergoes direct glucuronidation without prior P450 metabolism and has no active metabolites, lorazepam's half-life is not substantially influenced by hepatic or renal dysfunction, making it an especially useful agent in critically ill patients (Olkkola & Ahonen, 2008). It can also be administered by continuous infusion at doses similar to midazolam. As previously mentioned, lorazepam has been associated with increased risk for delirium, but it is possible that this effect applies to all BZDs and lorazepam is simply the one most commonly used in the ICU setting.

Diazepam

Diazepam has a longer onset of action than midazolam or lorazepam. Because it has active metabolites (temazepam, oxazepam, and desmethyldiazepam), diazepam's apparent duration of action is much longer, and this effect is more pronounced in older patients or those with renal dysfunction. Indeed, it has been suggested that diazepam's half-life increases by 1 hour for every year of age after 40 (Griffin, Kaye, Bueno, & Kaye, 2013). Diazepam has prominent myorelaxant effects, especially at higher doses. Unlike midazolam and lorazepam, its IV formulation contains propylene glycol, which can cause injection pain and thrombophlebitis.

PROPOFOL

Propofol is a short-acting anesthetic that is chemically distinct from other sedatives. Because of its low solubility in aqueous solutions, it is prepared in a lipid emulsion containing soy and egg ingredients. Usual doses are 1-2.5 mg/kg for boluses and 1-5 mg/kg/hr for sedative infusions. Propofol has a very rapid onset (1-2 minutes) after bolus dosing and is cleared similarly quickly, with an initial redistribution half-life of 2-3 minutes and a longer elimination half-life of 34-56 minutes (Kanto & Gepts, 1989). Because the initial redistribution is what typically terminates the effect of propofol, its clinical effect usually dissipates in 5-10 minutes after a bolus. While propofol exhibits some increase in context-sensitive half-time with prolonged infusion, this effect is relatively mild compared to other sedatives (See Figure 2). In general, propofol induces a deeper state of unconsciousness than benzodiazepines or opioids, with more muscle relaxation (Dueck, Oberthuer, Wedekind, Paul, & Boerner, 2003). While this makes it useful for situations that demand heavy sedation, it also means that propofol must be given in a monitored environment and by clinicians experienced in its administration.

Propofol's metabolism is still a matter of some investigation. Very little propofol is excreted unchanged in the urine. While propofol undergoes hepatic metabolism, its rate of clearance seems to be greater than hepatic blood flow. The apparent explanation for this conundrum is that propofol is also metabolized in extrahepatic sites like the kidney and the lung. Experimental evidence suggests that 60% of propofol is metabolized in the liver and 33% in the kidney, with studies equivocal as to the lung's contribution (Hiraoka et al., 2005). Interestingly, propofol clearance does not seem to be affected significantly by cirrhosis or renal failure (Veroli et al., 1992; Morcos & Payne, 1985).

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Hemodynamically, propofol is known to cause hypotension after an IV bolus. This effect is more pronounced than that induced by BZDs or opioids. Much of this decrease appears to be related to decreased systemic vascular resistance (SVR), although propofol may also have some myocardial depressant effects, especially if PaCO2 is maintained at normal values rather than allowed to rise (Searle & Sahab, 1993). Propofol can depress the baroreceptor response to hypotension, which can exacerbate these effects. It is notable that much acute hypotension from propofol may be related to the fast rise and equally fast drop-off in blood levels that follows a propofol bolus. When an administration protocol was used that was specifically designed to avoid overshoot of desired blood levels, researchers found that hypotension was minimal (Roberts, Dixon, Lewis, Tackley, & Prys-Roberts, 1988). Propofol seems to cause some decrease in coronary blood flow (likely due to decreased blood pressure) but also decreases myocardial oxygen consumption by a similar magnitude; these changes seem to reverse with stimulation (Stephan, Sonntag, Schenk, Kettler, & Khambatta, 1986). Overall, propofol is likely safe to use in all but the most unstable cardiac patients, but should be administered with caution, especially when given as a rapid bolus.

As previously stated, propofol acts as a respiratory depressant. When used at anesthetic doses, propofol depresses minute ventilation and shifts the CO2 response curve to the right (Allsop, Taylor, Grounds, & Morgan, 1988). It can cause periods of apnea after bolus administration. However, lower doses of propofol used to induce conscious sedation have not been shown to have a significant effect on respiratory variables (Rosa et al., 1992). Higher doses of propofol can also cause a decrease in airway muscle tone, which may induce or exacerbate airway obstruction (Mathru et al., 1996).

One complication of propofol administration that has more recently come to light is propofol infusion syndrome (PRIS), a severe metabolic disorder. This syndrome is characterized by bradycardia, arrhythmias, rhabdomyolysis, lactic acidosis, fatty liver, renal failure, and cardiovascular collapse if unchecked. While this syndrome is rare enough that its prevalence is difficult to measure, its severity and high risk of mortality (estimated around 60%) make it significant (Kam & Cardone, 2007). Risk factors that have been proposed include higher doses of propofol (> 10 mg/kg/hr), prolonged infusion, impaired nutritional status, critical illness, and younger age. The most likely mechanism for PRIS is that propofol interferes with fatty-acid oxidation and may affect the electron transport chain (Wolf, Weir, Segar, Stone, & Shield, 2001). In those who are malnourished and dependent on fatty acids for energy, this may result in global metabolic failure.

DEXMEDETOMIDINE

One of the newest sedatives to gain popularity is dexmedetomidine, which acts as an α-2 adrenergic receptor agonist. Dexmedetomidine produces anxiolysis, mild analgesia, and a sedative state that has been termed "calm responsiveness." Patients receiving this agent will frequently remain asleep until stimulated, at which point they awaken and can follow commands, but will return to sleep when the level of stimulation drops. Dexmedetomidine has very little effect on respiratory drive and as such, is increasingly seen as an ideal choice for sedating patients who are extubated and breathing spontaneously or those who may benefit from early extubation (Venn, Hell, & Grounds, 2000). On the other hand, these characteristics may make dexmedetomidine unsuitable for situations requiring deep sedation. It is typically administered via a bolus of 1 mg/kg over 15 minutes, followed by an infusion of 0.2-1 mcg/kg/hr.

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Dexmedetomidine causes hemodynamic effects similar to other α-2 agonists such as clonidine: infusion of dexmedetomidine is associated with a decrease in heart rate and blood pressure, which may be quite profound in susceptible patients. Decreased blood pressure is primarily a result of reduced SVR, although cardiac output may decrease as well (Bloor, Ward, Belleville, & Maze, 1992). Bolus dosing can cause hypertension and bradycardia, which is thought to result from the increased α-1 agonist activity of the drug at higher concentrations, resulting in a temporary rise in SVR. In cardiac surgical patients, dexmedetomidine seems to decrease SVR and BP without significant effects on cardiac output, although mixed venous oxygen concentration has been noted to decrease (Ishikawa et al., 2006). It can blunt sympathetic responses to stimuli effectively but does not seem to have as significant an effect on baroreceptor response (Hogue et al, 2002).

Another advantage of dexmedetomidine is its fast onset and offset. Dexmedetomidine is metabolized both by the cytochrome P450 system and by direct glucuronidation and has no active metabolites. Like many other sedatives, it is redistributed extensively with an initial half-life of 6 minutes; its terminal elimination half-life is closer to 2 hours. Because dexmedetomidine has a high margin of cardiovascular and respiratory safety, it is usable in patients with hepatic impairment, although some dose adjustment may be necessary.

ANTIPSYCHOTICS

Antipsychotics are used both as a sedative and to treat delirium, a common problem in the CTICU. They produce varying degrees of sedation but have minimal effects on respiratory drive and thus can be advantageous in patients with compromised respiratory function (Tjia & Gitlin, 2010).

The two major categories of antipsychotics are typicals, such as haloperidol and thorazine, and atypicals, like olanzapine and quetiapine. Typical antipsychotics act primarily via dopaminergic (D2 receptor) antagonism and have a greater risk of associated extrapyramidal symptoms (EPS), such as Parkinsonism and tardive dyskinesia. Atypical antipsychotics modulate both dopaminergic and serotonergic systems and carry a lower risk of EPS, but have been associated with metabolic side effects such as weight gain and diabetes mellitus (Meltzer, 2012). Antipsychotics also have variable anticholinergic and α-adrenergic-blocking effects, which can potentially cause dry mouth, blurry vision, and hypotension (Guzman & Farinde, 2014). Lastly, but of substantial importance in the CTICU, is the risk of QTc interval prolongation with antipsychotics, which seems to be most prominent for haloperidol, droperidol, thioridazine, clozapine, and olanzapine, and can result in Torsades de Pointes in susceptible patients. Interestingly, low-dose quetiapine seems to have minimal effects on the QTc, while aripiprazole may actually shorten it (Goodnick, Jerry, & Parra, 2002). Careful ECG monitoring is essential in patients receiving these medications in the CTICU. In some studies of elderly dementia patients, antipsychotics (especially typicals) have been associated with increased overall mortality, but the relevance of this association with regard to shorter-term ICU therapy is unclear (Gill et al., 2007).

Antipsychotics, particularly haloperidol, have been shown to be efficacious in the treatment of acute delirium in the hospital setting (Lacasse, Perreault, & Williamson, 2006). However, this evidence has not always translated to the ICU, as other studies focusing specifically on critically ill patients have suggested no difference between groups receiving antipsychotics versus placebo in terms of days alive without delirium or coma (Girard et al., 2010). In contrast, Devlin et al. (2010) found that scheduled quetiapine combined with as-needed haloperidol produced faster resolution of delirium, reduced dura-

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tion of delirium, and less agitation than haloperidol alone, as well as possibly better functional status at discharge, although quetiapine had a higher incidence of somnolence. Studies evaluating whether scheduled haloperidol can prevent the onset of delirium in the ICU have similarly produced mixed results (Page et al., 2013; Wang et al., 2012). Overall, while it seems the efficacy of antipsychotics in treating ICU delirium is not well established, these drugs may be useful in patients with or without delirium whose conditions warrant a sedative with minimal respiratory effects. Further research is necessary to compare the efficacy of antipsychotics to other sedatives and better delineate their best role as part of an overall sedative regimen.

TRENDS IN ANALGESIA AND SEDATION

Before assessing what are the best agents and strategies to apply to CTICU patients, it is instructive to see what patterns are most common in current practice and what approaches are recommended by professional societies. A recent summary of international surveys by Mehta, McCullagh, and Burry (2009) showed that while there is much variation across countries, several trends are evident. Lighter sedation is becoming more common, and propofol is being used progressively more than benzodiazepines. Shorter-acting opioids are also gaining in popularity, with fentanyl and remifentanil taking the place of morphine. At the time of this review, notably, dexmedetomidine was not a commonly used agent.

The most recent guidelines from the Society of Critical Care Medicine suggest how to further refine practice to fit the best evidence (Barr et al., 2013). This set of recommendations emphasized pain control and frequent reassessment of pain as a primary objective. Other evidence-based suggestions were to titrate agents to achieve light rather than deep sedation, to monitor patients closely for delirium, and to use early mobilization as a tool to prevent delirium. Epidural analgesia was suggested for certain types of procedures, although some uncertainty about its benefits was noted. Few recommendations were made for the use of individual pharmacologic agents.

CHOICE OF MEDICATIONS: IS THERE A "BEST AGENT?"

Many factors need to be considered in choosing an analgesic and sedative strategy for a given patient. It is instructive to lay out goals for the patient on daily rounds: is deep sedation required, due to hemodynamic instability or severe hypoxia, or would the patient benefit from lighter sedation and a greater focus on weaning from the ventilator? And are there any measures that can be taken to reduce the need for sedation and analgesia, such as instituting physical therapy, environmental modifications, or an improved sleep regimen?

An additional question to consider is how to minimize the adverse effects of the sedative regimen on the patient. One population that warrants specific concern is patients at greater risk for delirium, such as those with advanced age, sensory impairment, alcohol abuse, or electrolyte abnormalities (Elie, Cole, Primeau, & Bellavance, 1998). This is of particular importance as delirium has been associated with increased mortality and length of stay in critically ill patients (Ely et al., 2004), as well as increased ICU and hospital costs (Milbrandt et al., 2004). In the cardiac surgery population, delirium may occur in more than 30% of patients. Risk factors include older age, medical comorbidities, decreased preoperative cognitive function, longer cardiopulmonary bypass time, and greater inflammatory response (Guenther

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et al., 2013). CT surgical patients who experience delirium have greater drops in postoperative cognition and are less likely to return to their preoperative baseline (Saczynski et al., 2012).

Other characteristics of the CT surgical population may impact choice of analgesics and sedatives. Hemodynamic instability may necessitate regimens that have less cardiovascular depressant effects. Organ failure may preclude the use of medications with long elimination half-lives or active metabolites. Patients with impaired pulmonary function may be unable to tolerate agents with prominent respiratory depressant effects. Considering these issues can help to tailor a patient's analgesic and sedative regimen to their needs.

A separate question is whether any one sedative regimen is better than another in terms of overall outcomes, and various studies have been undertaken to answer this question. As has been touched on before in this chapter, an important question is whether BZDs are associated with worse outcomes or more adverse events, particularly delirium. A meta-analysis examining BZD-based sedation vs. non-BZD-based sedation found that use of non-BZD regimens resulted in shorter ICU length of stay and shorter duration of mechanical ventilation but no significant difference in rates of delirium or mortality (Fraser et al., 2013). In a similar vein, another meta-analysis found that use of dexmedetomidine for ICU sedation was associated with a reduction in ICU length-of-stay and time to extubation, although this result was limited by high heterogeneity between studies (Pasin et al., 2013).

Several randomized studies have also been undertaken comparing specific sedatives in terms of efficacy and side effects. When midazolam and propofol have been compared, propofol seems to result in more time spent at target levels of sedation and earlier extubation than midazolam, especially after more prolonged infusions (Hall et al., 2001). This is intuitively sensible, since propofol has a substantially shorter context-sensitive half-time than midazolam. Even when the higher cost of propofol has been factored in, earlier weaning from mechanical ventilation seems to make propofol a more economically viable choice (Barrientos-Vega et al., 1997).

More recently, dexmedetomidine has grown in popularity and this is reflected in the literature. Pandharipande et al. (2007) conducted a randomized controlled trial (RCT) using lorazepam vs. dexmedetomidine for sedation in 106 mechanically ventilated medical and surgical patients. In this trial, patients receiving dexmedetomidine spent more time at their targeted level of sedation and also spent more days alive without delirium or coma. However, this latter composite outcome was driven primarily by more coma in the lorazepam group. There was a trend towards fewer days with delirium in the dexmedetomidine group but it did not reach significance. This study found no difference in mortality between groups.

A similar study was conducted in by Riker et al. (2009) comparing dexmedetomidine to midazolam for the sedation of critically ill, ventilated patients. In this instance, patients treated with dexmedetomidine spent less time on the ventilator, experienced less delirium, and were less likely to exhibit tachycardia and hypertension. Dexmedetomidine was, unsurprisingly, associated with more bradycardia, but the proportion of patients experiencing bradycardia that required treatment was much smaller. Notably, in this study, neither medication resulted in more time spent at the desired level of sedation.

Jakob et al. (2012) synthesized two multi-center Phase 3 RCTs, one comparing dexmedetomidine to midazolam and another comparing dexmedetomidine to propofol for sedation of mechanically ventilated ICU patients. This study found that all three agents were equivalent at maintaining desired levels of light to moderate sedation. Using dexmedetomidine seemed to lead to shorter duration of mechanical ventilation than midazolam and more ability to communicate with patients than either midazolam or propofol. However, dexmedetomidine caused more hypotension and bradycardia than either of the other sedatives used.

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Results of trials conducted specifically in CT surgical patients have found similar results. When comparing an analgesic regimen based on morphine vs. one based on dexmedetomidine, with open-label propofol administered as needed for additional sedation in both groups, Shehabi et al. (2009) found that the dexmedetomidine-based regimen resulted in shorter duration of delirium, earlier extubation, and less vasopressor use than the morphine-based regimen, although it also produced more episodes of bradycardia. In this study, there was also a trend towards a lower incidence of delirium in the dexmedetomidine group, but it did not reach significance. In an open-label study, Maldonado et al. (2009) found that dexmedetomidine administered after valvular cardiac surgery resulted in less opioid use and substantially lower rates of delirium than midazolam or propofol. Notably, this study had a number-needed-to-treat to prevent delirium of just over 2, implying a large clinical effect; however, it was limited by its smaller size, its open-label structure, and a high dropout rate. In the aggregate, while it seems dexmedetomidine may have some distinct advantages as a sedative in critically ill CT surgery patients, the choice of medication regimen still needs to be tailored to the individual patient.

CHOICE OF SEDATIVE STRATEGY

Several approaches towards analgesic/sedative administration seem to be useful regardless of the specific drugs chosen. One of these is the use of daily sedation interruption (DSI), sometimes referred to as a "sedation vacation." In this technique, a period each day is designated wherein sedatives are withheld and patients are allowed to awaken. This allows assessment of the depth of sedation, facilitates neurologic examination, and can be an ideal opportunity to conduct spontaneous breathing trials. The foundational study on interruption of sedation by Kress, Pohlman, O'Connor, and Hall (2000) revealed that this approach leads to shorter duration of ventilation and ICU length-of-stay, as well as fewer diagnostic studies, than usual care, with no increase in complications like self-extubation.

One concern with this strategy is that withholding sedation may lead to more stress for patients, greater impact of traumatic experiences, and even more PTSD. However, the evidence does not suggest this. In fact, examining retrospective data, it seems that DSI is associated with unchanged rates of adverse psychological outcomes, as well as fewer symptoms of PTSD, and possibly lower eventual rates of PTSD (Kress et al., 2003). Most of the studies examining DSI have been in mixed medical-surgical populations, with few CT surgical patients included, but there is no reason to think such an approach would not be equally effective in CT patients as long as severe hemodynamic instability is not present.

Not all studies on sedation interruption, however, have found the same results. A Cochrane review by Burry et al. (2014), which examined 9 trials of DSI, found that the intervention reduced rates of tracheostomy but did not change duration of mechanical ventilation or ICU stay. The studies analyzed had significant heterogeneity, which may have affected the results. In a randomized trial, Mehta et al. (2013) studied 430 critically ill, ventilated patients who were being treated using a protocol designed to target light sedation. In this case, again, DSI did not change length of stay or duration of ventilation. Notably, the DSI group in this study received higher daily doses of analgesics and sedatives than the group that was treated via sedation protocol alone. It seems that some of the benefit from DSI may be related to light sedation and not the daily interruption itself. This is consistent with the results of the original study by Kress et al. (2000), in which the DSI group received significantly less sedatives and analgesics than the control group.

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Another approach that has been evaluated is the use of analgesics with minimal or no sedation, even when patients are intubated. A trial by Strøm, Martinussen, and Toft (2010) randomized critically ill, mechanically ventilated patients to receive morphine boluses for analgesia with or without a concurrent propofol infusion. The group receiving propofol underwent daily interruption of sedation. Patients received haloperidol if they were felt to be delirious. Those in the morphine-only (or "no-sedation") group were assigned a dedicated caregiver to comfort and calm them if they were anxious or uncomfortable despite analgesics. If these measures failed repeatedly, patients in the "no-sedation" group could receive rescue sedatives by infusion. 18% of the patients in the "no-sedation" group eventually had to cross over and receive continuous sedatives. In the intent-to-treat analysis, this study found that the "no-sedation" group spent more time free from mechanical ventilation and had shorter LOS in the ICU and hospital. There was a trend towards lower ICU mortality in the "no-sedation" group but it was not significant. Conversely, the "no-sedation" group experienced more agitated delirium and received more haloperidol.

While this study creates interesting food for thought, it is not always feasible to forego sedation entirely, especially in patients who are unable to tolerate episodes of coughing, movement, or delirium. Again, while it seems prudent to limit any extraneous use of sedatives, a certain level may be necessary to avoid counterproductive stress responses. Intensivists must balance long-term outcome goals with the medical (and human) imperative to limit pain and anxiety when possible.

Another common strategy utilized in the CTICU is "fast-track" management. This term does not have a concrete definition, but involves various components of care in the intraoperative and postoperative periods that are designed to facilitate earlier ventilator weaning and extubation, shorter ICU stay, and improved recovery after CT surgery in appropriate patients. A meta-analysis of 25 trials by Zhu, Lee, and Chee (2012) evaluated two of the most common components of fast-track cardiac care, low-dose intraoperative opioids and early extubation protocols. They found that these 2 inverventions were associated with significantly shorter times until extubation and ICU length of stay; the fast-track patients had no increase in complications as a result. Other components not evaluated in this study include use of short-acting sedatives like remifentanil and dexmedetomidine and use of epidural analgesia. While fast-track recovery protocols do not clearly lead to shorter hospital stays or lower rates of complications, they appear safe and are consistent with the recognized goals of minimizing sedation and avoiding unnecessary interventions.

NON-PHARMACOLOGIC APPROACHES

While medications continue to be the mainstay of analgesia and sedation in the CTICU, other non-pharmacologic techniques may also prove useful. Physical therapy may facilitate early mobilization and can avoid painful stiffness and contractures, as well as potentially improve function and mood. Alternative therapies, such as acupuncture, massage, meditation, and visualization, may help to alleviate pain, although their use in the CTICU has not been well evaluated. Guided music therapy has been shown to decrease heart rate and myocardial oxygen demand and to improve sleep quality (Jacobi et al., 2002). As little of 20 minutes of music after cardiac surgery has been shown to significantly reduce anxiety and pain (Sendelbach, Halm, Doran, Miller, & Gaillard, 2006). Lastly, attention to sleep- frequently a rare commodity in the ICU- can potentially prevent delirium and help the healing process (Kamdar et al., 2013). Keeping the ICU quiet, turning down lights, and avoiding procedures overnight can all aid in this objective.

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CONCLUSION/DIRECTIONS FOR FURTHER RESEARCH

Patients in the CTICU are at risk for pain and anxiety from many sources, many of which are unavoidable in the perioperative period. Through judicious use of medications and non-pharmacologic interventions, intensivists and surgeons can alleviate much of their distress. However, it is of paramount importance to avoid adverse effects that can result from analgesics and sedatives, such as hypotension, bradycardia, delirium, and prolonged mechanical ventilation. There are many ways to achieve these goals, and more research is needed to better delineate whether certain agents may confer benefits in terms of mortality, neurocognitive dysfunction, or other long-term outcomes. It is also less clear whether certain populations may benefit from different agents more than others. While inferences can be made about this possibility, little empirical evidence exists. Dexmedetomidine seems to hold great promise both as a sedative and an analgesic adjuvant, and it may be informative to evaluate its efficacy when combined with other agents such as ketamine for postoperative analgesia. Unfortunately, dexmedetomidine's use is limited by its relatively high cost, although some studies imply that this is outweighed by the shorter duration of ventilation associated with dexmedetomidine (Lachaine, 2012). While antipsychotics are frequently used in the CTICU, there is little data as to their efficacy as sedatives and how best to use them as part of a sedative strategy.

Regardless of which agents are used, sedation interruption, sedation protocols, and other approaches that limit deep sedation seem to hold potential benefits for patients. More research may help to predict which patients are the best candidates for "fast-track" management and whether specific sedative and analgesic protocols may improve outcomes in subsets of the population.

The role of non-pharmacologic techniques in alleviating pain and agitation in the CT surgical population is a field that is ripe for study. These techniques are particularly attractive because they may help limit adverse drug effects, although their relative efficacy needs to be tested in a more formalized way. It may be useful in the future to combine multiple types of analgesics, sedatives, and alternative approaches to establish clinical pathways and protocols based on best evidence. This will help standardize care and make the perioperative process more efficient and less traumatic.

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KEY TERMS AND DEFINITIONS

Analgesic: An agent or technique that provides relief from painful sensations.

Cardiothoracic: Of or pertaining to the heart, lungs, or other intrathoracic organs.

Cardiothoracic Intensive Care Unit: An ICU providing specialized preoperative and postoperative care for patients undergoing cardiothoracic surgery.

Context-Sensitive Half-Time: The time required for blood levels of a drug to drop by 50%, which may be due to redistribution, metabolism, or elimination, and which may vary after different durations of drug infusion.

Delirium: A multifactorial syndrome characterized by acute onset, a fluctuating course, as well as possible attentional deficits and an altered level of consciousness.

Elimination Half-Life: The time required for blood levels of a drug to drop by 50% due to elimination from the body.

Intensive Care Unit: Specialized hospital unit wherein patients are cared for who require support of vital organ functions, including those suffering from respiratory, circulatory, and renal failure.

Opioid: One of a class of analgesics related to naturally-derived opium.

Regional Analgesia: A technique by which analgesia is provided through blockade or modulation of afferent pain signals, not via systemic medications.

Sedative: A medication that induces a depressed level of consciousness.

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Section 3
Functional Foods

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Chapter 20
Phytonutrients of Nutraceutical Importance: Exploring Antimicrobial, Antiproliferative, and Antioxidant Activities

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ABSTRACT

Phytotherapy re-emerged in the latest years as a healing system accepted and spread worldwide, and different molecules have been investigated due to their benefits to health. Nutraceutical formulations, which allow the intake of phytonutrients (generally in low levels in plant food) in concentrations that are enough to achieve the desired outcomes, represent feasible alternatives to improve general health and to prevent and treat varied diseases. Notwithstanding the incompleteness of an evidence-based clinical use of nutraceuticals, many questions remain unanswered regarding their global effects in humans and animals. Thus, the aim of this chapter is to provide recent evidence on chemical and pharmacological features of the main phytonutrients explored in nutraceutical formulations, focusing antimicrobial, antioxidant and antiproliferative potentials. Also, some insights on drug-phytonutrients interactions will be discussed.

INTRODUCTION

The rapidly increasing knowledge on nutrition, pharmacology and plant biotechnology has changed some concepts about food and health. Epidemiologic studies from the latest 50 years have provided evidence of an inverse association between the dietary intake of fruit and vegetables and occurrence of varied illness such as diabetes, osteoporosis, cancers, cardiovascular and infectious diseases. This protective effect obtained in food intake has been assigned to plant secondary metabolites; specifically, phytomol-

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ecules with nutritional properties, the phytonutrients. In a few words, phytonutrients can be defined as plant-derived compounds with nutritive properties that can have also curative or preventive effects over diseases (Michels et al., 2000). These phytomolecules are thought to be responsible for most of the health benefits observed in individuals that adopted long-time diets rich in fruits, vegetables, beans, cereals, and plant-based beverages such as tea and wine. The correlation between observed effects and the type or concentration of some phytomolecules has been described, such as Lycopene and Resveratrol, for instance, in cancer prevention. In plants, several phytomolecules of nutraceutical interest are part of defense mechanisms against environmental challenges such as damage due to ultraviolet light exposure and natural predators such as insects and other plagues (Hecht, 2000; Sharma et al., 2009).

The low bioavailability of phytomolecules is an important limitation on their use to prevent or treat diseases. The often low concentration found in plasma has led to some skepticism in this sense; however, in spite of their low bioavailability, varied studies in the latest years have provided evidence of their beneficial effects in humans and animals. To overcome the issue of low levels of phytonutrients in fruits and vegetables, pharmaceutical and nutritional companies have developed several pharmaceutical dosage forms of nutraceutical formulations and other dietary plant-based supplementary products. This becomes even more relevant considering that nutraceutical formulations provide adequate concentrations of phytomolecules and thus, different beneficial effects to health can be expected. Moreover, nutraceutical formulations make the use of phytonutrients feasible in a clinical perspective (McCullough et al., 2003; Key et al., 2004; Yang et al., 2010). Examples of Nutraceuticals are presented in Table 1.

The Dietary Supplement Health and Education Act, adopted by the American Association of Clinical Endocrinologists (Mechanick et al., 2003), provides adequate definitions of dietary supplements and nutraceuticals, which will be adopted and slightly adapted in this chapter for a better comprehension by non-specialist readers. According to Mechanick et al. (2003), dietary supplements comprise five main groups of ingredients/elements:

1. Vitamins or minerals;
2. General phytomolecules;
3. Amino acids;

Table 1. Examples of phytomolecules-based nutraceutical formulations

Formulation Brand	Phytomolecules	Manufacturer	Indications
Inneov Solar®	β-carotene, Lycopene, tocopherols and *Polypodium leucotomos* extract	Loreal and Nestlé	Oral photoprotection
Lauricidin®	Lauric acid	Med-Chem	Stimulation of the immune system
Extra-Strength Broccoli Extract with Glucosinolates®	Glucosinolates of broccoli extract	Green Foods	ROS/RNS scavenger, protection of the cardiovascular system
Flavonoid 1000®	Hisperidin, rutin, bromelin and citric bioflavonoids (200, 50, 50, 1000 mg, respectively)	DaVinci	Relief on varicose veins, reduction of inflammatory processes associated to pain, metabolism booster.

ROS: Reactive oxygen species; RNS: Reactive nitrogen species.

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4. Other substances that increase the total dietary intake such as enzymes;
5. Byproducts such as metabolites and constituents, or even extracts of any of the elements mentioned in topics 1 to 4.

Thus, dietary supplements are not foods in their natural form, but are not limited to common nutritional agents.

Nutraceuticals, on the other hand, are defined as sophisticated dietary supplements based in concentrated presentations of one or more pure bioactive substances, originally isolated from plant foods, although mineral and animal bioactive molecules are also included. These formulations are prepared in different pharmaceutical dosage forms and are used in dosages many times superior to those detectable in their original sources to improve health in varied aspects (Mechanick et al., 2003).

Most nutraceutical and dietary supplements formulations may be purchased without medical prescription in several countries, and in the latest years, pharmaceutical and health food companies have come up with new formulations claiming to have varied pharmacological effects of interest to prevent diseases (although some of them are still demanding more evidence from clinical studies, as will be discussed in this chapter). Different groups suggest that nutraceuticals present drug-like actions, and therefore, their formulations should be marketed, for instance, as over-the-counter medication. Considering this, a question arises: the rules of Lipinski et al. (1997), widely accepted to characterize a molecule or compound as not "drug-like", states that when a substance falls into one or more of the following properties:

1. More than five hydrogen-bond donors, considering OH and NH moieties;
2. More than ten hydrogen-bond acceptors, considering N and O atoms;
3. Molecular weight beyond 500 g/Mol;
4. Octanol/water partition coefficient log P higher than 5.

Therefore, as it is not reasonable to assume that the chemical properties of all nutraceuticals are within these reference levels, it becomes technically difficult to generalize that nutraceutical formulations could be regulated as over-the-counter drugs. Also, most of the phytomolecules used as nutraceuticals have not shown cytotoxic effects in several *in vivo* and *in vitro* models, being widely recognized as safe for human and animal use (Zhao et al., 2012).

Strong recommendations for consumption of nutraceuticals have become progressively popular to improve health, and to prevent and treat diseases. Nutraceuticals and dietary plant-based supplements, among other properties such as antioxidant and antiproliferative, can stimulate immune response and result in fewer infections or control of infectious diseases, particularly in the elderly and in malnourished, critically ill individuals (Pham-Huy et al., 2008; Lee et al., 2009).

Notwithstanding the incompleteness of an evidence-based use of nutraceuticals, many questions remain unanswered regarding their global effects in humans and animals. Therefore, the aim of this chapter is to provide recent evidence on chemical and pharmacological features of the main phytonutrients explored in nutraceutical formulations, focusing their antimicrobial, anti-oxidant and anti-inflammatory potentials. Also, some insights on drug-phytonutrients interactions will be discussed. With more direction on flavonoids, carotenoids, phytosterols, lauric acid and glucosinolates, we shall present recent evidence of the mentioned pharmacological activities and the possible benefits on the treatment of infectious diseases and cancers with important burden on human health worldwide. However, we do not intend here to exhaust the subject, but to propose discussions to open doors for more advances in this exciting research field.

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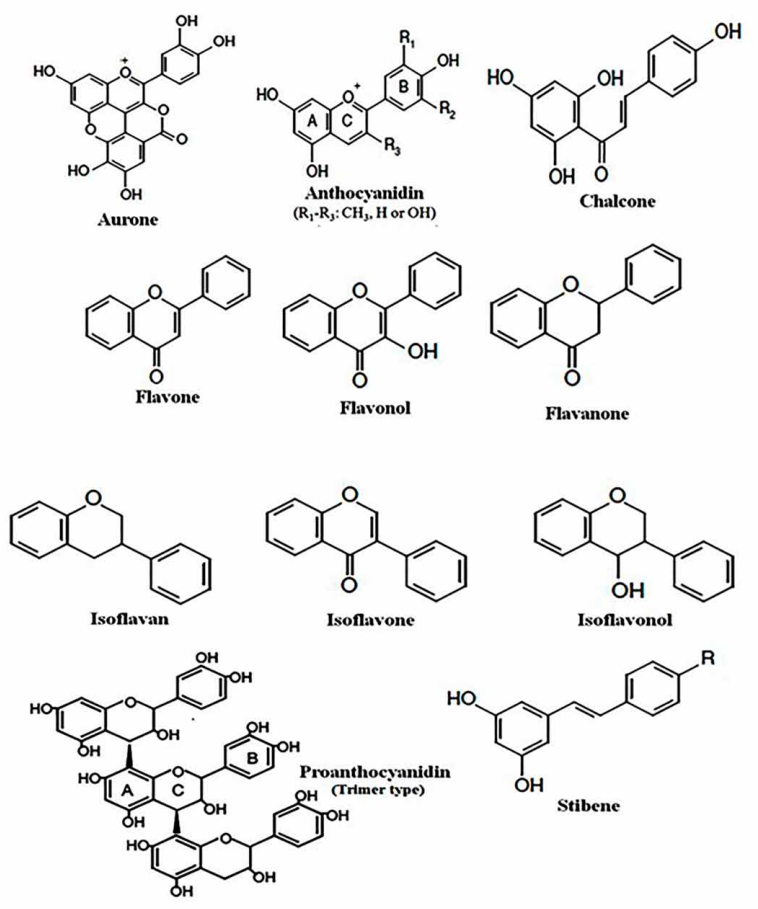
BACKGROUND

Flavonoids

Flavonoids are a diverse family of phytonutrients derived from phenylpropanoid and malonyl-coenzyme A pathways, characterized by two aromatic cycles (rings A and B) linked to a heterocycle (C ring) (Figure 1).

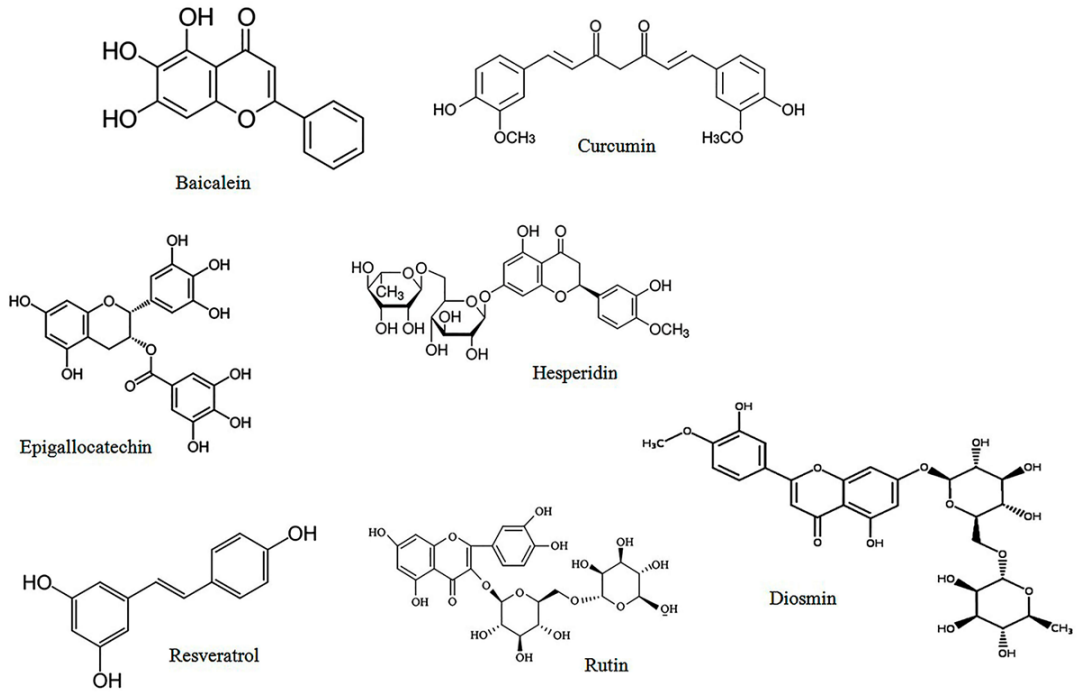
This phenylpropanoid core with 15 carbon atoms is usually modified by reactions such as alkylation, glycosylation, hydroxylation, methylation, methoxylation and oxidation, generating the major subgroups aurones, anthocyanins, chalcones, flavones, flavonols, flavanones, isoflavans, isoflavones, isoflavonols, stibenes, and proanthocyanidins. Currently, more than 10.000 flavonoid structures had been elucidated, and several pharmacological potentials have been assigned to them, such as antiproliferative, wound healing, anti-diabetic, cardioprotective, vasotonic, neuroprotective, antioxidant, antibacterial, antifungal, antiviral, and metabolism booster (Gutteridge, 1994; Haslam, 1996). Following, important flavonoids widely used as nutraceuticals will be discussed (Figure 2).

Figure 1. Structures of the major groups of flavonoids
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Figure 2. Structure of the flavonoids discussed in this chapter
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Baicalein

Baicalein ($C_{15}H_{10}O_5$) is found in citric fruits and mostly in *Scutellaria baicalensis*, a chinese herbal remedy known as skullcap or Huang Qin. Due to its good lipophilicity, baicalein can be transported into cells by passive diffusion. Most of the compound passes through glicuronization and and sulphation during absorption, and becomes too polar to pass through the lipid barriers. being transported in other ways inhibit CYP2C9 enzyme. Baicalein is able to (Stobiecki, 2000; Chen et al., 2006).

Chen et al. (2006) investigated the antimicrobial activity and the possible mechanism of action of baicalein on Methicillin-resistant *Staphylococcus aureus* (MRSA). Changes on membrane penetrability, protein synthesis, DNA topoisomerase activities and restriction map were assessed. The minimum inhibitory concentration reached 0.04 mg/mL, and after 16 h, the total soluble content of proteins decreased 44.62%. The flavonoid inhibited the activities of DNA topoisomerase I and II in a concentration of 0.025 mg/mL. Later, Moghaddam et al. (2014) examined the *in vitro* antiviral activity of baicalin, the main metabolite of baicalein, in different steps of dengue virus replication. Baicalin inhibited virus replication at IC50 = 13.5 ± 0.08 µg/mL with SI = 21.5 following virus internalization by Vero cells. Baicalin exhibited activity against DENV-2 extracellular particles and showed anti-adsorption effect.

Liu et al. (2015) assessed the protective effects of baicalein using an *in vivo* model of polymicrobial sepsis-induced liver injury. Polymicrobial sepsis was induced in C57BL/6 mice, which were treated with intraperitonial 100 mg/kg baicalein after 1, 6 and 12 h of the infection. Baicalein inhibited the mitogen-activated protein kinases (MAPKs) activation and suppressed the transcriptional activity of nuclear

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factor-kappa B (NF-κB). Resulting effects comprised improved survival of septic mice, ameliorated liver injury, and reduced both neutrophil infiltration and hepatic inflammatory cytokines expression and release.

Li et al. (2013) synthesized baicalein-8 benzyl amino acid derivatives and evaluated their cytotoxicity *in vitro* against HepG2 cells. Substitutions on positions C6 and C8 were considered, and amino acids were subjected to methylation and aminoacylation reactions. Through carboxyl group protection and aminoacylation of amino acid and benzyl protection of baicalein, derivatives of baicalein-6-O-amino acid esters were obtained. A total of 13 novel derivatives were synthesized and chemically characterized. Four derivatives showed a significant increase in cytotoxicity when compared to baicalein. Some baicalein-5-O-amino acid derivatives were also synthesized, but they lost cytotoxic activity. This suggested that 5-OH is an important structural feature for the cytotoxic activity of baicalein and its derivatives.

Luo et al. (2014) studied the *in vitro* antiproliferative effect of 30 novel synthesized baicalein derivatives. Three human cancer cell lines were used: HepG2 (human liver carcinoma), A549 (human alveolar basal epithelial adenocarcinoma) and BCG-823 (gastric carcinoma), and baicalein was used as a comparative control. The compounds were tested over a range of concentrations from 0.1 to 100 M for 48 h, and IC50 values were estimated. Apoptosis mechanism was identified for 8 compounds, which moiety of 7-benzyloxy group exhibited more potent anti-proliferative activity against the tumor cell lines when compared to baicalein. One of the molecules was found to be 14-fold, 16-fold, and 6-fold more potent than baicalein against HepG2, A549 and BCG-823 cancer cell lines, respectively.

Zeng et al. (2015) assessed the anti-allergic and anti-inflammatory activities of baicalein and chrysin, by analysing the interactions between hyaluronidase using experimental methods and molecular docking. With the increase of baicalein or chrysin concentration, the activity of the enzyme decreased significantly and the 50% relative activity (IC50) of baicalein and chrysin were estimated to be 52 ± 7and 267 ± 33 mol/L, respectively. Considering thermodynamic parameters and molecular modeling, authors suggested that each molecule could bind to hyaluronidase spontaneously through electrostatic forces, hydrophobic interactions and hydrogen bonding.

Curcumin

Curcumin ($C_{21}H_{20}O_6$) was firstly isolated from turmeric. The flavonoid is usually a bright orange to yellow powder, with melting point at around 183 ºC, sensible to light and of low bioavailability in humans, but is rapidly metabolized in the liver. Low levels of this substance can be detected in plasma and tissues, and due to its low water solubility and poor absorption, it is quickly excreted. Curcumin inhibits epigenetic enzymes such as histone deacetilases, and has been associated with protection against cancer and cardiovascular diseases. Although the route of biosynthesis of Curcumin is not fully understood, there are two proposed mechanisms that may help to explain its synthesis: the first mentions the extension reaction of cinnamic acid with five malonil-CoA molecules, and the second involves two cinnamate molecules joined by malonil-CoA (Kuzuyama et al., 2005; Rocks et al., 2012).

Rudrappa and Bais (2008) analyzed the effects of curcumin on the virulence of a *Pseudomonas aeruginosa* strain through *in vitro* assays for virulence factors production and tests in pathogenicity models using *Arabidopsis thaliana* and *Caenorhabditis elegans*. Curcumin inhibited the expression of virulence factors such as biofilm, pyocyanin, elastase, protease, and acyl homoserine lactone production, resulting in reduced pathogenicity of the strain. Transcriptomic analysis of the treated strain showed downregulation of quorum sensing genes. Kaur et al. (2010) also described a mechanism for the antimicrobial activity of curcumin in *Bacillus subtilis* and *Escherichia coli*, which relies on growth inhibition by blocking the

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assembly of the cytoskeletal FtsZ protein, involved in prokaryotic cell division and present in several bacterial species.

The possible synergism of mitomycin C and curcumin was studied by Zhou et al. (2011) in order to suppress human breast cancer *in vitro* and *in vivo.* Experiments were performed through assessing cell cycle phases of MCF-7 cells with flow cytometry. Tumor growth and cell cycle-associated protein expression were also monitored. It was seen that curcumin inhibited MCF-7 breast cancer cell viability in a concentration-dependent manner, and IC_{50} value was of 40 μmol/L.

Chhunchha et al. (2011) investigated the mechanisms involved in the antioxidant activity of curcumin, exploring peroxiredoxin 6 pathway, an anti-ROS pleiotropic oxidative stress-response protein, via specificity of protein 1 activity, against proapoptotic stimuli. Molecular analyses indicated that curcumin enhanced specificity protein 1 and peroxiredoxin 6 mRNA and protein expression in a concentration-dependent manner and downregulated ROS-mediated apoptosis by blunting ROS expression and lipid peroxidation. Curcumin could not activate peroxiredoxin 6 expression in the presence of specificity protein 1 inhibitors, suggesting that curcumin increased expression of peroxiredoxin 6 in a dependent way of specificity protein 1 activity.

Rocks et al. (2012) studied the effects of curcumin complexes with cyclodextrin alone or in combination with gemcitabine on the growth of orthotopically implanted lung tumors *in vivo*. Tumors were monitored on 7, 10, 14, 18 and 21 days after cell injection. Tumors development was almost completely abolished when mice were treated by the association with gemcitabine. The association of curcumin–cyclodextrin-gemcitabine resulted in a two-fold reduction of proliferation index in mouse lung tumours when compared to mice treated with the vehicle alone or non-soluble curcumin.

Diosmin

Diosmin ($C_{28}H_{32}O_{15}$) is a flavone originally isolated from *Teucrium gnaphalodes,* and is quite abundant in citric fruits, with concentrations ranging from 17.8 to 245 mg/100 g. This flavonoid is hydrophobic and has a melting point of around 274 ºC, being obtained via dehydrogenation of hesperidin,. Its half life is of around 11 hours, being mostly eliminated (80%) in stool. Diosmin has anti-inflammatory, venotonic vasoprotective effects, being the latest ones associated to the extension of norepinephrin activity in veins, increasing the venous tonus and the intensity and frequency of contractions in the lymphatic vessels (Bae et al., 2000).

Studies of antimicrobial activity of diosmin are, curiously, rare. Bae et al. (1999) described the anti-Helicobacter pylori activity of varied flavonoids, and in their study, the minimum inhibitory concentration of diosmin was higher than 100 μg/mL. Later, Bae et al (2000) assessed the antiviral activity of several flavonoids, and the inhibitory effects of diosmin in cell lines infected wih rotavirus was reached with 10 μg/mL.

Regarding its antioxidant activity, Akroum et al. (2010) described effective free radical scavenging activity of diosmin in the DPPH assay, reaching 50.35% activity at 10 μg/mL. Also, the effect of diosmin in oxidative stress in streptozotocin-nicotinamide induced diabetic rats was studied by Srinivasan and Pari (2012) through assessing lipid peroxidation and ameliorative properties. Diabetic animals had increased plasma glucose concentration with significant decrease in plasma insulin levels. The activity of superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase were reduced. The levels of vitamins C, E and reduced glutathione decreased while lipid peroxidation markers increased in

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liver and kidney tissues of diabetic control rats, when compared to healthy control rats. The oral treatment with diosmin showed ameliorative effects on the analyzed biochemical parameters.

Barreca et al. (2013) demonstrated that diosmin binding to human serum albumine can contribute to protect it against degradation. Fluorescence experiments indicated that the binding site of the flavonoid involved modifications around Trp214 at the subdomain IIA. A decrease in α-helix structure following flavonoid binding was also detected. *In silico* analyzes suggested that the primary binding site of diosmin was located in a microenvironment able to stabilize the binding of the ligand in non-planar conformations, known as Sudlow's site I.

Using animal model of Wistar rats, Tahir et al. (2013) described the effect of diosmin on attenuation of hepatic toxicity induced by 2-acetylaminofluorene and, the chemopreventive efficacy of the flavonoid against hepatocarcinogenesis induced and increased by diethylnitrosamine and 2-acetylaminofluorene, respectively. The protective effect of diosmin was demonstrated by reductions of visible hepatic nodules, restoration of early tumor markers such as Ki67 and ODC, and reductions of serum cytotoxicity enzyme markers AST and ALT, oxidative stress biomarkers, cell necrosis markers like TNF-α and inflammatory enzymatic markers COX-2 and iNOS.

Epigallocatechin

This polyphenol of molecular formula $C_{22}H_{18}O_{11}$, is available at higher levels in the leaves of *Camelia sinensis* (around 7400mg/100 g as green tea, and around 4300 mg/100 g as white tea). It can also be found in lower concentrations in the skin of apples, plums, onions, walnuts and hazelnuts. This flavonoid is characterized as a white to pink powder, with melting point around 218 ºC. Different from other flavonoids, it is soluble in water, and also in ethanol and DMSO (Assis et al., 2015).

Lipson et al. (2015) recently used epigallocatechin against rotavirus. After 3 days in culture, pure epigallocatechin concentrations of 80, 160, and 320 μg/mL reduced the virus infectivity titer levels to 50, 20, and 15% of the control, respectively. No cytotoxicity was detected in the concentrations used.

To increase the antimicrobial activity and stability of epigallocatechin, Sun et al. (2011) complexed the flavonoid with Cu^{+2} into polyvinyl alcohol nanofibers. MICs of the complex against different bacteria and yeasts ranged from 8 to 600 ug/mL, and high values of MIC are related to *S. cerevisiae* and *C. albicans*. The bactericidal activity of the complex against *E. coli* and *P. nitroreducens* was not significantly weaker than that against Bacillus cereus and *B. subtilis.* The activity against *S. aureus* was lower than that against the other bacteria (including Gram-negative).

Nevertheless, epigallocatechin was not effective in eliminating *Streptococcus mutans* from dentin caries-like lesions. Assis et al. (2015) evaluated the efficacy of epigallocatechin at 0.5%, 1%, and 2% concentrations in an in vitro model of dentin caries-like lesions. When compared, chlorhexidine digluconate, an antimicrobial molecule widely used against caries, the antimicrobial efficacy of epigallocatechin was significantly lower against the tested *S. mutans* strain.

Harpole et al. (2015) investigated the effects of epigallocatechin against SK-OV-3 ovarian cancer cell line model, aiming to explore antioxidant exposure to slow the progression of the disease. A statistically significant increase in nitric oxide following the administration of epigallocatechin alone and in combination with thymoquinone was detected at 24, 48 and 72 hours, being approximately three times higher than the control.

Considering that high doses of epigallocatechin can induce toxicity in the liver, kidneys, and intestine, Cai et al. (2015) investigated the proliferation and apoptosis of H9C2 cardiomyocytes treated with

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high doses of epigallocatechin. Using the CCK8 and TUNEL assays, and western blotting detection of alterations of Sirtuin 1 (SIRT1), it was seen that different from other organs, epigallocatechin inhibited H9C2 cardiomyocytes proliferation and induced apoptosis in a time-dependent and dose-dependent way. The downregulation of SIRT1 protein expression is possibly involved in this mechanism.

Hesperidin

The name "hesperidin" is derived from the term "hesperidium", which means "fruits produced in citric trees". Hesperidin ($C_{28}H_{34}O_{15}$) is able to inhibit LDL oxidation and thus, prevent atherosclerotic plaque growth. It is also able to inhibit the intestinal absorption of cholesterol and increase its hepatic catabolism. It is usually the most abundant molecule among the flavonoids present in lemon or orange. Hesperidin is hydrophobic, of pale yellow color and with melting point ranging from 262 to 258 ºC (Wilcox et al., 2001).

Abuelsaad et al. (2013) performed susceptibility tests through agar-diffusion against *Aeromonas hydrophila.* Hesperidin was tested in the concentrations of 100, 50, 25, and 12.5 mg/ml. The concentration of 100 mg/ml was the most effective in reducing bacterial growth. Using borth microdilution, authors investigated minimal inhibitory concentrations (MIC) and minimal bactericidal concentration (MBC). Hesperidin MBC was of 12.500 μg/mL and MIC was of 3.125 μg/mL. Similarly, Abass et al. (2014) assessed the antimicrobial activity of crude extracts of orange peels, rich in hesperidin, against *Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli* and *Salmonella typhi* by agar diffusion. The extract was effective mostly against *B. subtilis* (45 μg/mL), and E. coli (75 μg/mL), and less effective against *S. typhy* and *S. aureus* (175 μg/mL)

In one of the first studies with commercial formulations of phytomolecules of nutraceutical importance, using animal (rats) model, Damon et al. (1987) administered hesperidine tablets (Daflon® 500 mg) by gastric intubation for a period of 26 weeks. There were no deaths, no alteration of weight and standard functional tests had no modifications.

Bigoniya & Singh (2014) investigated the anti-ulcer potential of hesperidin through evidences of histological analyzes of its antioxidant activity in mucosal tissue of the stomach. Ulcers were induced with indomethacin (oral doses of 150, 300 and 450 mg/kg) and hypothermic restrain stress. At the doses of 300 and 450 mg/kg, hesperidin induced pH increase, with histological evidences of cytoprotection. Glutathione, super oxide dismutase and catalase, important antioxidant enzyme systems, and mucin levels (for tissue protection) increased significantly at 450 mg/kg. Thus, the antioxidant, mucoprotective and cytoprotective effects of hesperidin are strongly associated to its anti-ulcer properties.

Using a murine model, Man et al. (2015) demonstrated that topical applications of hesperidin improved epidermal functions in aged skin. Hesperidin did not stimulate epidermal proliferation, but decreased the pH in aged skin. Slight increase in transepidermal water loss rates (however ranging within normal loss rates of a healthy skin) was detected in treated skin, and accelerated barrier recovery was detected at 2 and 4 hours after the treatment. The expression of filaggrin, involucrin, and loricrin genes, all trivial for skin integrity, was increased in adult keratinocytes cultures treated with hesperidin. Topical hesperidin increased the levels gene expression of HMG-CoA and FAS in aged skin.

Resveratrol

Resveratrol ($C_{14}H_{12}O_3$) is a stilbene (3,5,4′-trihydroxystilbene) produced by several unrelated plant species like blackberry, ginger, blueberries and grapes, in response to injury, ultraviolet irradiation, micro-

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bial infections and other stressing conditions. Takaoka (1939) isolated this flavonoid for the first time from the roots of *Veratrum grandiflorum*. This flavonoid is also present in processed products based on grape and wine, appearing in other foods at lower concentrations. It is poorly soluble in water and is described as a yellowish-white powder, with melting point ranging from 261 to 263 ºC. The molecule exists as cis- (Z) and trans- (E) diastereomers. The (E) diastereomer is the form in which resveratrol is found in nature. When exposed to ultraviolet irradiation, resveratrol can undergo isomerisation to the (Z) form.When administered orally, less than 5% of the flavonoid can be found in blood plasma. Among its biological activities, important anti-inflammatory mechanisms comprise the modulation of NF-κB and inhibition of action and expression of cyclooxygenases (COX). It has been described that resveratrol can also induce apoptosis, indicating its antiproliferative potential (Chen et al., 2012; Kumar & Pandey, 2013; Fremont, 2014).

Hwang & Lim (2015) investigated the mechanisms of the antimicrobial potential of resveratrol over E. coli species. It was seen that the production of reactive oxygen species (ROS) increased in the strains, although cell growth was not recovered by ROS quenching. Thus, ROS formation and cell death were not directly correlated to resveratrol treatment. DNA fragmentation increased in cells, and cell elongation was also observed. FtsZ, an essential cell-division protein involved in cell elongation in prokaryotes, had its gene expression and its formation strongly inhibited in *E. coli* strains.

A recent mechanism of the antifungal activity of resveratrol against *C. albicans* yeasts was proposed by Lee & Lee (2015). Interestingly, resveratrol can induce apoptosis in clinical isolates of *C. albicans*, as shown by the loss of mitochondrial membrane potential, an important event in resveratrol-induced apoptosis. Resveratrol also activated metacaspase and promoted cytochrome c release, confirming the apoptosis-induced mechanism.

Resveratrol is effective in scavenging free radicals to prevent LDL oxidation (Chang et al., 2011), and as an effective *in vivo* inhibitor of cyclooxygenase activity, resveratrol can significantly reduce oedema, osteoarthritis, airway inflammation, and can also prevent allograft rejection (Elmali et al., 2005Wu et al., 2005 a and b). In this context, the elegant study of Park et al. (2012) provided data on a new pathway in which resveratrol directly inactivates phosphodiesterase enzymes, indicating that the metabolic effects of this flavonoid are result of competitive inhibition of cAMP-degrading phosphodiesterases, leading to elevated cAMP levels and to a signaling cascade that activates situin 1.

The antiproliferative activity of resveratrol is also widely recognized. Reis-Sobreiro et al. (2009) analyzed the antimyeloma and antileukemia potentials of resveratrol. The flavonoid induced apoptosis in multiple myeloma and leukemia T-cell through coclustering of Fas/ CD95 death receptor and lipid rafts. Resveratrol-induced disruption of mitochondrial transmembrane potential was inhibited by a FADD dominant negative mutant by downstream signaling of Fas/CD95 blockage. This mechanism also inhibited apoptosis induced by resveratrol. Cells deficient in Fas/CD95 did not undergo resveratrol-induced apoptosis.

Frampton et al. (2010) assessed the antiproliferative effects of resveratrol on cholangiocarcinoma cells, in combination to chemotherapeutic drugs. Cholangiocarcinoma cell lines were treated with resveratrol and following, with 5-fluorouracil, gemcitabine, or mitomycin C. The combined treatment with resveratrol decreased cell proliferation and increased apoptosis in all in vitro assays, and more extensively than any of the chemotherapeutic drugs alone.

Scherzberg et al. (2015) studied the antiproliferative effect of the a methylated resveratrol analog Z-TMS [(Z)-3,5,4'-Trimethoxystilbene] over varied cancer cell lines, and compared the analog molecule results to pure resveratrol. Flow cytometric analysis revealed a G2/M cell cycle phases arrest after Z-TMS

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treatment, whereas resveratrol caused S phase arrest. Furthermore, Z-TMS impaired tubulin and microtubule polymerization, and no beneficial effects on lipid accumulation were observed for this molecule. The lacking beneficial metabolic effects of Z-TMS were assigned to a possible loss of activity on genes that may have the expression regulated by resveratrol.

Rutin

Rutin ($C_{27}H_{30}O_{16}$) has been described to be an effective angiogenesis inhibitor that is also able to block vascular endothelial growth factor (VEGF), what can potentially contribute to the control of some types of cancer. This flavonoid is present in fig, apples and wheat, and is usually presented as a solid yellow to white powder of melting point around 242 °C, being more soluble in methanol than in water. Other pharmacological properties described for rutin comprise anti-inflammatory activity and platelet aggregation inhibition, decreasing capillary permeability and improving microcirculation. These potentials contribute to prevent clots formation and thus, prevention of infarcts and strokes (Haslan, 1996; Ugusman et al., 2014).

Interestingly, the antimicrobial activity of rutin is still controversial somehow. Singh et al. (2008) identified rutin as the major flavonoid in *Pteris vittata* (Brake Fern). Hydromethanolic extracts of *P. vittata* (70, 80, and 90%) were tested against Gram-negative bacteria, and the 70% extract was the most effective against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Orhan et al. (2010) reported antimicrobial activity of rutin against MRSA, *E. coli, P. mirabilis, K. pneumoniae, P. aeruginosa* and *A. baumannii*. However, no activity was observed for rutin against Gram-negative bacteria in the study of Alves et al. (2013).

Ananth et al. (2015) developed thioglycolic acid-capped cadmium telluride quantum dots (TGA-CdTe QDs) and conjugated them to rutin, aiming to enahnce its antimicrobial activity. These complexes were tested against *E. coli*. Adsorption of conjugated QDs on cell wall and its penetration through porins were described as the main possible mechanisms of action of the antimicrobial activity of the complex. QDs also induced stable oxidative stress by ROS production, what contributes to increase the membrane permeability.

Rutin is mostly recognized for its potent antioxidant and anti-inflamatory activities, control of high blood pressure, protection of UV–radiation and mainly, its ability to decrease capillary fragility (Ugusman et al., 2014). Also, effects on bone resorption have been studied. Using a cell culture of bone marrow cells isolated from 4-5-week-old C57BL/6J mice, Kyung et al. (2008) described the effect of rutin on osteoclast formation. Rutin inhibited osteoclast formation induced by receptor activator of NF-κ B ligand (RANKL) at an early stage by reducing levels of ROS and TNF-α, which are elevated via RANKL. Rutin also lowers NF-κ B activation in response to RANKL.

Rutin contains disaccharide moieties and is generally better distributted on the body than other flavonoids. Choi et al. (2013) investigated the mechanisms of rutin and its direct targets involved in reduction of COX-2 expression in JB6 P+ cell lines by benzo[a]pyrene-7,8-diol-9,10-epoxide (B[a] PDE), its active metabolite. AP-1 and NF-kB activities (at DNA level) were mediated by the activation of MAPK-signaling pathways when induced by B[a]PDE, involving ERK, JNK, and p38 proteins. Thus, the transactivation of AP-1 and NF-kB was inhibited, an important mechanism of downregulation that affects the immune system. Rutin suppressed phosphorylation of ERK and upstream kinases such as MEK and Raf induced by B[a]PDE. Akt phosphorylation induced by B[a]PDE was suppressed in JB6

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P^+ cells as well. Although rutin reduced B[a]PDE-induced Raf/MEK/ERK and Akt activation, it had no effect on the phosphorylation of the epidermal growth factor receptor (EGFR). Even $EGFR^{-/-}$ mouse embryonic fibroblast cells had COX-2 expression diminished after exposed to B[a]PDE. EGFR appeared to be directly targeted by rutin, and the suppressive effect on COX-2 expression is likely to be mediated by Raf/MEK/ERK and Akt signaling.

Xu et al. (2014) studied the effects of rutin in the clinical evolution of Alzheimer's disease (AD) in a murine model, and proposed the use of this molecule in AD treatments. Orally administered rutin attenuated memory deficits in AD-induced mice, and decreased the levels of oligomeric extracellular β-amyloid (Aβ) plaques. Super oxide dismutase (SOD) activity and glutathione (GSH)/glutathione disulfide (GSSG) ratio were increased, demonstrating its antioxidant activity in the central nervous system. Rutin also reduced GSSG and malondialdehyde (MDA) levels, downregulated microgliosis and astrocytosis, and decreased interleukin (IL)-1β and IL-6 levels in the brain, indicating its anti-inflammatory neuroactivity. The pretreatment with rutin reduced the activity of hepatic transaminases and improved the histological signs of acute liver damage in intoxicated mice. Rutin was effective in the suppression of nitrotyrosine formation, but had weak effects in attenuation of the inflammation in the liver, as indicated by measurements of nuclear factor-kappa B (NF-κB), tumor necrosis factor-α (TNF-α) and cyclooxygenase (COX-2). The expression of inducible nitric oxide synthase (iNOS) was potently suppressed by rutin. Thus, protection against nitrosative stress and hepatocellular damage can be effectively achieved by rutin, in spite of the weak antioxidant and anti-inflammatory activities.

Carotenoids: Lycopene and β-Carotene

Carotenoids are widespread natural pigments (mostly yellow, orange, red, salmon and other variants), and almost all are from the family of terpenoids (tetraterpenes). They can be found in plants, bacteria, fungi, yeasts, algae, and non-mammalian animals. More than 700 different carotenoids have been isolated from natural sources, including some molecules with hydrophilic profile, and more than 600 molecules are fully characterized.

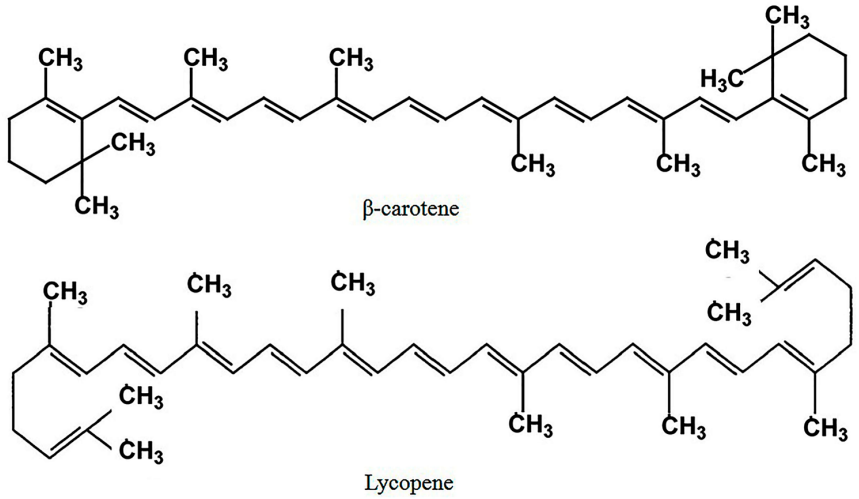
Carotenoids photoprotection and the antioxidant potential for preventing cancer, scavenging of reactive species and diseases of the eye remain important active fields of investigation. Also, carotenoids may boost the immune system by increasing the number of white blood cells. On the other hand, rare studies have demonstrated antimicrobial potentials for this chemical group. Until the time of preparation of this chapter, the exact mechanisms of these biological potentials of carotenoids remain unknown.

In humans, the role that carotenoids play in protecting those tissues that are most heavily exposed to light is the most explored. Oral photoprotection formulations, for instance, are already commercially available. β-carotene, α-carotene, lutein, zeaxanthin and β-cryptoxanthin are major carotenoids detected in human blood and tissues; nevertheless, β-carotene and lycopene are the most studied carotenoids, and the most used for nutraceutical purposes. Thus, certain aspects of these molecules will be more explored here (Figure 3).

Lycopene is a red pigment found in plant food like tomatoes, guava and watermelon. This molecule consists in a long acyclic chain of chemical formula $C_{40}H_{56}$. It is usually presented as a dark red hydrophobic solid with melting point ranging between 172 and 173 ºC. Lycopene can be found in foods such as tomatoes in an average of around 3.31 mg/100 g, and papaya, in an average of around 3.39 mg/100 g. (Hark & Deen, 2005).

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Figure 3. Structure of β-carotene and lycopene
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Lycopene levels in vegetables are usually analyzed through spectrophotometric techniques, however, it can be measured indirectly through chromaticity values obtained via colorimetric analysis. This last technique, nevertheless, is recommended for plants with high production of Lycopene.

The synthesis of lycopene and its decomposition are quite pronounced during the ripening of fruit, and at temperatures above 30 °C, the synthesis is inhibited, but it can be reversed as the vegetable return of to lower temperatures. External factors such as pH, temperature and oxidation, can influence the stability of carotenoids.

β-carotene is commonly found in many vegetables, fruits and plants such as carrots. The molecule also has molecular formula $C_{40}H_{56}$. When the cyclisation of lycopene occurs at both ends of the molecule, β-carotene is then formed. It is presented as an orange or red-to-purple solid with a density ranging from 0.935 to 0.947 g/cm^3 and has melting point ranging from 180 to 182 ºC. The molecule is hydrophobic and very slightly soluble in methanol.

β-carotene is a precursor of liposoluble retinol. A significant part of the formation of retinol metabolism occurs in the gastrointestinal mucosa, being its absorption pH sensitive. β-carotene is also able to enhance the induction of CYP2E1, important in the metabolism of several drugs. Its average concentration in carrots usually reaches around 7 mg/100 g.

Because the bioavailability of β-carotene is very variable among people, Borel et al. (2015) investigated the possible effects of variations in genes involved in β-carotene absorption and postprandial metabolism on this variability on the bioavailability of the carotenoid. Single-nucleotide polymorphisms (SNPs) were genotyped with the use of whole-genome microarrays. A total of 33 healthy and nonobese adult men were enrolled. After an overnight fast, patients consumed a test meal containing 100 g tomato paste with 0.4 mg β-carotene. Postprandial plasma chylomicron β-carotene concentration was then measured at 8 h intervals, and 54 genes representing 2172 SNPs were investigated. The postprandial chylomicron β-carotene response was highly variable and positively correlated to the fasting plasma β-carotene concentration. A total of 25 SNPs in 12 genes affected the long-term blood β-carotene status and thus, explained (69%) of β-carotene bioavailability variance.

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Horváth et al. (2015) proposed that carotenoids might modulate transient receptor potential ankyrin 1 and vanilloid 1 (TRPA1 and TRPV1) activation predominantly on peptidergic sensory nerves, considering that carotenoids are incorporated into membranes. β-carotene, lutein and lycopene were used in a murine model of cutaneous neurogenic inflammation, induced by mustard oil and capsaicin. Administration of 100 mg/kg subcutane lutein or β-carotene (three times during 24 h) resulted in significant swelling reduction, and Lutein decreased neutrophil accumulation induced by TRPA1 activation, although mustard oil-evoked intestinal contraction was not influenced. Curiuously, lycopene had no effect in none of the tested parameters. None of the three carotenoids could overcome capsaicin-evoked inflammation.

Woodside et al. (2015) investigated the possible benefits of anti-inflammatory and antioxidant properties on the risk of diseases that predominate in older people. Authors suggested that carotenoid intake and status are associated with reduced risks of cardiovascular diseases, although β-carotene supplementation increases the risk of lung cancer and does not reduce the risk of cardiovascular diseases. Increased lycopene intake may reduce prostate cancer progression.

Photoprotective effects of these carotenoids are still in debate. Stahl & Sies (2012) reviewed the topic and provided evidence that a carotenoid-rich diet or carotenoid supplementation might have photoprotective benefits through scavenging of ROS. Considering that carotenoids are part of a complex blend nutrients in food, other components can contribute to this effect. As expected, photoprotection through dietary intake of β-carotene or lycopene is lower than the achieved by topical sunscreens. the combination of both strategies, however, increases skin protection against UV irradiation and contributes to preserve skin health and appearance.

Ascenso et al. (2014) proposed that lycopene modulation of gap-junctional communications, involved in cell growth and molecular signalling of proliferation and apoptosis through proteic channels such as connexin 43, are important in cell cycle dynamics and for carotenoids behaviour as cellular anti-oxidants *in vivo*. Fernández-García (2014) assessed the antioxidant effect of lycopene alone or combined to α-tocopherol or naringenin on human dermal fibroblasts (hdf) exposed to ultraviolet-A light. UVA irradiation led to reduced cell viability in a dose dependent manner, and similar effects were observed when cells were exposed to lycopene. However, this reduction was suppressed in the presence of naringenin. α-tocopherol had no effect in this parameter. Lycopene combined to naringenin was able to inhibit ROS formation and increased the stability of the carotenoid. Thus, combinations of dietary antioxidants might be more effective than the sole use of lycopene.

Nevertheless, the work of Sokoloski et al. (2015) reported that lycopene regular intake in pills or tomato paste, although safe and with no side effects, did not provide systemic photoprotection against ultraviolet-B light. A total of 20 subjects using lycopene pills or tomato paste received only ultraviolet B irradiation and the variation of color was compared to normal skin after 24 h. Evaluations were made at baseline and after 4, 8, and 10 weeks. Conversely, no difference was detected in the mean of minimal erythematous dose at baseline between groups.

Phytosterols

In the latest years, several pharmacological potentials of phytosterols have been described, including cardiovascular protection, antiproliferative and antimicrobial effects and lipid metabolism improvement. However, they still lack of evidence generated in clinical studies. Currently, most of their biological activities have been demonstrated *in vitro*, one of the reasons that explains the controversial status of most of the existing investigations. Also, nutraceutical formulations developed with these molecules are

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scarce, but several types of food are enriched with these compounds, such as dairy products. Phytosterols are of abundant occurrence in plant and marine species, and are hydrophobic white crystalline solids. Phytosterols present from twenty-seven to twenty nine carbon atoms resembling the cholesterol molecule. These can be distinguished by the arrangement of the nucleus, the structure and composition of the side chain and by polar moieties of the molecule (Robbers et al., 1997; Brafau et al., 2008).

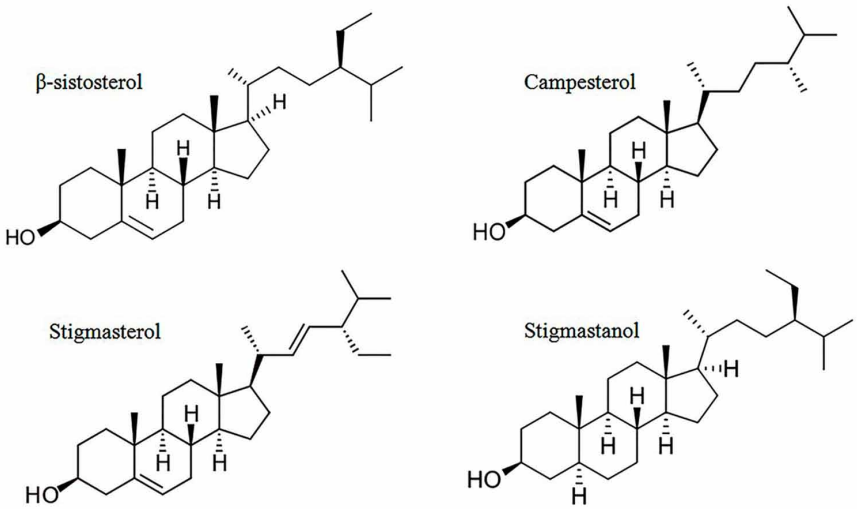
The biosynthesis of these compounds usually begins with Coenzyme A generating mevalonate. A set of similar reactions pathways is observed in phytosterols when compared to the synthesis of terpenes. Due to structural characteristics very similar to those of cholesterol, phytosterols are able to perform common cellular functions of cholesterol by interfering in membrane fluidity and water permeability. They can also act as phytormones precursors (Robbers et al., 1997; Thomas et al., 2006).

Phytosterols may be found in foods with high levels of lipids such as sesame seeds, walnuts and peanuts, as well as being present in some vegetables, grains and fruits. These compounds make up part of the unsaponifiable fraction of vegetable oils. In soy and corn, phytosterols might be detected in up to 330 and 970 mg/100g of total oils, respectively (Thomas et al., 2006) (Figure 4).

Monu et al. (2008) investigated the potential antimicrobial activity of a commercial formulation of phytosterols (ss-sitosterol, campesterol, sitostanol, and campestanol) in milk and their effect on yogurt starter cultures. No antimicrobial effect was detected on plate count or on psychrotroph population in pasteurized milk stored at 4 °C. A microbial challenge test of the formulation using *Pseudomonas spp*., *Saccaromyces cerevisiae* and *Aspergillus ochraceous*, in cold milk, confirmed that the blend of phytosterols had no antimicrobial activity. The formulation also had no effect on the growth of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* during yogurt production. When combined to dispersing agents, the formulation presented some antimicrobial effects.

Mahmood et al. (2013) studied the effects antimicrobial activities of β-sitosterol complexed to Cu (II), Co (II), and Fe (III), important trace elements in the human organism. All the complexes presented antimicrobial potential against highly pathogenic bacterial and fungal species. More recently, a group headed by the same author described the complexation of β-sitosterol with Palladium (Pd) (Mahmood

Figure 4. Structures of major phytosterols
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et al., 2015). This complex showed significant antibacterial and antifungal activity, being superior to the previous ones.

Ramprasath & Awad (2015) recently reviewed the biological potentials of phytosterols. Although the mechanisms of action remain to be elucidated, improvements in blood lipid profile have been described as the main pathway to its cardioprotective effects. However, the levels of phytosterols in natural diets might range from less than 60 mg to more than 500 mg/2000 Kcal, and in an optmistic perspective, they might reduce only 3-14% LDL levels, what suggests the urgent need of phytosterols-based nutraceutical formulations (Racette et al., 2015). Reductions in growth of various cancer cells have been demonstrated, and mechanisms such as inbihition of cell growth and multiplication, reduction of angiogenesis and impairment of tissues invasion and metastasis, have been proposed to explain its antiproliferative activity (Ramprasath & Awad, 2015).

Ravikumar et al. (2009) isolated both α-spinasterol and spinasterol from a petroleum ether extract fraction of *Polyalthia cerasoides* seeds, and tested them against Caco-2 cell line. These molecules exhibited antiproliferative potential at different concentrations, and IC50 was of 57.7±6.81 nM/mL for spinasterol and 60.0±7.10 nM/mL for α-spinasterol. Carange et al. (2011) analyzed the protective potential of 24-epibrassinolide, a natural brassinosteroid (a type of phytosterol with potential of inducing stress tolerance of vegetables) in an *in vitro* model of Parkinson's disease. Neuronal PC12 cells were treated with 1-methyl-4-phenylpyridinium, which induced oxidative stress and consequent apoptosis in dopaminergic neurons. The treatment with the phytosterol reduced the levels of intracellular ROS and modulated the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. 24-epibrassinolide also inhibited apoptosis by reducing DNA fragmentation as well as the Bax/Bcl-2 protein ratio.

On the other hand, Cilla et al. (2015) tested the antiproliferative effect of β-cryptoxanthin and phytosterols (β-sitosterol, campesterol and stigmasterol) against human colon cancer Caco-2 cells. The molecules showed no antiproliferative activity against Caco-2 cells, nor alone nor in combination. Caco-2 cells were treated with β-cryptoxanthin and/or phytosterols alone or in combination, at concentrations commonly detectable in human serum levels. Decreased cell viability was detected due to apoptosis, which was followed by dephosphorylation of BAD enzyme, mitochondrial depolarization and caspase 3-dependent PARP receptor cleavage. Also, intracellular Ca^{+2} influx and increased ROS/RNS levels were detected.

Lauric Acid

Lauric acid, also known as dodecanoic acid, is a fatty acid of structural formula $CH_3(CH_2)_{10}CO_2H$. Lauric acid is available mainly in coconut oil (from 43 to 53% of total fatty acids), palm kernel oil (40 to 52%) and in smaller fractions in other food, such as 5.8% of the total milk fat, 2.2% of total cow's milk and 4.5% of the fat present in goat's milk. The biosynthesis of this acid occurs through a reverse β-oxidation. Generally, it is presented as a hydrophobic white solid, with slight soap-like odor and lacking uniformity. It has an approximate density of 0.88 g/cm^3, and melting point ranging between 44 and 46 ºC.

Lauric acid can activate the release of interleukin 2 and stimulate the bone marrow to produce white blood cells. The anti-inflammatory potential of lauric acid is belived to be associated to local inhibition of prostaglandin E_2 (PGE_2) and Interleukin 6 synthesis, pro-inflammatory mediators in arthritis, rheumatism and muscle inflammation. Upon arriving in the intestine, it is metabolized by lipases to monolaurin, which is absorbed and has the ability to solubilize virus capsules, causing its destruction, what indicates

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its antiviral properties. Lauric acid induces the opening of cell membranes allowing glucose and insulin levels to normalize. It is also able to balance HDL levels and to reduce LDL oxidation (Figure 5).

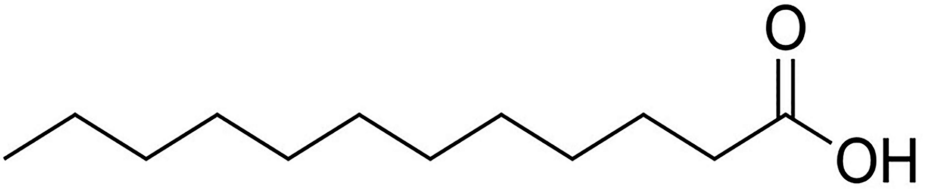
Rouse et al. (2005) combined lauric acid monoesters to lactic, mandelic, malic, or benzoic acid, and evaluated them against MRSA in a murine model of nasopharyngeal colonization. Administration of the molecules 128774-49E or 128774-53A was associated to reduction of MRSA carriage in 71% and 83% of animals, respectively. Administration of the compound 128774-53A resulted in 50% more reduction of MRSA carriage than mupirocin. Also, in vitro experiments were performed to assess the antimicrobial activity of 13 lauric acid monoester formulations and mupirocin against 30 methicillin-susceptible *S. aureus* (MSSA) isolates and 30 (MRSA) isolates. MSSA and MRSA MIC90 values were 0.25 µg/mL for mupirocin and ≤4 µl/ml for all LAM formulations tested.

Skrivanová et al. (2005) determined the susceptibility of *Clostridium perfringens* strains to C(2)-C(18) fatty acids. Among the tested molecules, lauric acid showed the highest antimicrobial activity, followed by myristic, capric, oleic and caprylic acid. Cultures of *C. perfringens* strains treated with capric and lauric acid at pH 5.0-5.3, the number of viable cells was very reduced; however, only lauric acid reduced the number of viable cells of the strains below 10(2) ml(-1) at pH > 6. The proposed mechanism of action for lauric acid was separation of inner and outer membranes, and cytoplasma disorganization, suggesting its use for controling of clostridial infections.

Nakatsuji et al. (2009) demonstrated that lauric acid can be used to treat acne. This compound showed antimicrobial activity against clinical isolates *of Propionibacterium acnes, Staphylococcus aureus* and *Staphylococcus epidermidis*, both *in vitro* and *in vivo* (using a murine model). Lauric acid yielded minimal inhibitory concentration (MIC) values against more than 15 times lower than those of benzoyl peroxide (a molecule highly used in topical formulations) in the tested species. *P. acnes* was the most sensitive bacteria to lauric acid among the tested species. No cytotoxicity was detected in human sebocytes exposed to lauric acid. *In vivo*, intradermal and epicutaneous applications of lauric acid decreased the count of *P. acnes* isolated from mouse ears.

The antinflammatory potential of lauric acid has been explored in the latest years. The classical study of Henry et al. (2002) described high inhibitory activity of lauric acid over cyclooxygenase enzymes COX-I and COX-II at 100 µg/mL. Further, Fauser et al. (2013) compared lauric acid (C12:0) and butyrate (C4:0) regarding their potentials to induce apoptosis, modify glutathione levels, generate ROS and modify phases of the cell cycle in Caco-2 and IEC-6 cancer cell lines. Lauric acid was more potent to induce apoptosis in Caco-2 and IEC-6 cells when compared to butyrate. In Caco-2 cells, lauric acid was more efficient to reduce GSH availability and in generation of ROS when compared to butyrate. Also, butyrate protected IEC-6 cells from ROS-induced damage, but lauric acid induced high levels of ROS when compared to butyrate.

Figure 5. Lauric acid molecule
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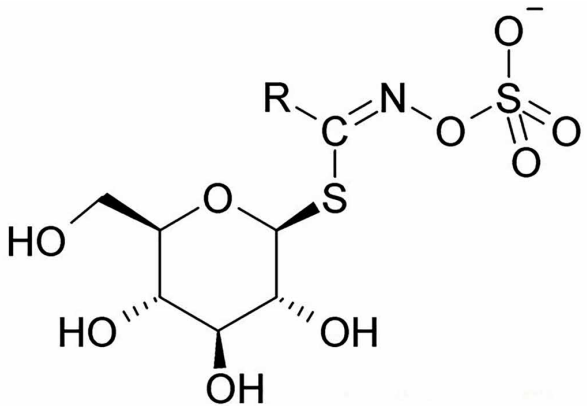
Glucosinolates

Glucosinolates are generally found in plants with spicy flavor such as leaves and roots of the turnip and mustard seeds, and in vegetables like broccoli and cauliflower. This effect is mostly perceived when these plants are chewed. Most of glucosinlates are water soluble, and when plants suffer mechanical damage, 60 to 80% of total glucosinolates are converted into sulfurano-nitrile compounds. When cooking, the enzyme miriosinase is denatured and glucosinolates can be absorbed with no chemical alteration, being converted into sulforaphane only in the intestines after exposed to bacterial tioglucosidases. Most of the glucosinolates are excreted in the form of N-acetyl-cysteine. among others (Nastruzzi et al., 2000) (Figure 6).

Leoni et al. (1997) investigated the antiproliferative effects of glucosinolates and their isothiocyanate-derived products on K562 (erythroleukemic human) cells. Strong inhibitory activity on K562 cells growth was detected for sinigrin, progoitrin, epi-progoitrin, glucotropaeolin and glucocheirolin. Later, researches conducted by this group (Nastruzzi et al., 2000) described the effects of isothiocyanates and several glucosinolates-derived nitriles (epi-progoitrin, glucocheirolin, glucoraphenin, glucotropaeolin and sinalbin) in an *in vitro* model of colorectal cancer using K562 cells. Isothiocyanates were more potent to inhibit cancer cell growth than the glucosinolates-derived nitriles epi-progoitrin, glucotropaeolin, glucoraphenin, and glucocheirolin.

Lampe and Peterson (2002) reviewed the effects of genetic polymorphisms on the chemoprotective properties of cruciferous vegetables against different types of cancer. This pharmacological activity of cruciferous vegetables is generally assigned to the high content of glucosinolates and its metabolites detectable in them. They can modulate important biotransformation enzymatic systems such as the monooxygenases from cytochrome P450, influencing, for instance, scavenging and immune regulation. Beyond the (somehow) expected failure of chemoprotective activity due to genetic and biochemical alterations that influence receptors and transcription factors that interact with these compounds, functional variations due to genetic and biochemical instabilities in enzymes like glutathione S-transferase (mainly M1 and T1 isotypes), which metabolize isothiocyanates, can increase cancer risk due to the intake of cruciferous vegetables.

Figure 6. General structure of glucosinolates
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More recently, Calmes et al. (2015) investigated the mechanism by which glucosinolate-derived isothiocyanates could cause fungal cell death using *Alternaria brassicicola*, a *Brassica* species pathogens. Exposure of the strain to isothiocyanates led to a decreased rate of oxygen consumption, intracellular accumulation of ROS and depolarization of the mitochondrial-membrane, impairing its function in fungal cells. The expression of different oxidative-response genes was increased in the fungus when exposed to isothiocyanates-derived ROS, what may explain the protection of the fungus against isothiocyanates, as mutant strains deficient in the specific transcription factors were hypersensitive to the tested isothiocyanates.

Interactions of Phytomolecules of Nutraceutical Interest and Antimicrobial Drugs

As mentioned, most of the phytonutrients discussed present varied spectrum of antimicrobial activity. Varied practices of traditional medicine in countries such as, Brazil, China, India and several African countries include the use of plant extracts for several illnesses, including infectious diseases. In many cases, patients and health professionals employ a drug-therapy regimen in which the patient used natural products rich in phytonutrients and other phytomolecules of (generally) recognized biological activity combined to medication. However, these interactions are poorly investigated and represent a serious problem in drug therapy due to poorly predictable interactions (Saavedra et al., 2010; Dias-Souza et al., 2013). We will present here in brief some discussion in this topic.

When these combinations include antimicrobial drugs, the risk of favoring microbial resistance is high, given that the efficacy of synthetic antimicrobial drugs can be severely impaired, due to antagonistic interactions. However, it can also be enhanced, as a result of synergic interactions. Saavedra et al. (2010) evaluated the antimicrobial activity of several phytonutrients against *E. coli, P. aeruginosa, L. monocytogenes* and *S. aureus*, and the possible synergism of combinations of streptomycin and these molecules. Phenolic compounds (caffeic acid, chlorogenic acid, ferulic acid, gallic acid, tyrosol, chalcone - phloridzin; flavan-3-ol - (-) epicatechin; seco-iridoid -oleuropein glucoside) and glucosinolate hydrolysis products (allylisothiocyanate, benzylisothiocyanate and 2-phenylethylisothiocyanate) were tested in combination to ciprofloxacin, gentamicin and streptomycin in a disc diffusion assay. All of the tested isothiocyanates had superior antimicrobial activity when compared to phenolic molecules, being *P. aeruginosa* the most sensitive microorganism. Synergy was demonstrated between streptomycin and gallic acid, ferulic acid, chlorogenic acid, allylisothiocyanate and 2-phenylethylisothiocyanate against Gram-negative bacteria.

Sato et al. (2006) provided evidence of the synergic effect of the combination of vancomycin and isoflavones isolated from *Erythrina poeppigiana* against (MRSA). Isolupalbigenin and erythrinin B exhibited the highest anti-MRSA activity. Later, Gould et al. (2009) described the synergic effect of the combination of pomegranate rind extract and cupric sulphate against methicillin-sensitive and resistant *S. aureus,* and Panton-Valentine Leukocidin positive community acquired MSSA. The authors observed reduction in bacterial growth for 80% of the isolates.

Because of the strong resistance of bacteria to the currently available antimicrobial drugs, natural products and specific molecules have been investigated as alternatives to synthetic drugs, and our group has been exploring chemical, microbiological and pharmacological aspects of some natural products that might be interesting candidates for using in antimicrobial therapy (Dias-Souza et al., 2013; Marçal

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et al., 2014; Figueiredo et al., 2015; Pereira et al., 2015). Concerning drug interactions with natural products, our group is currently investigating possible synergic or antagonist effects of the combination of antimicrobial and antioxidant natural products with synthetic antimicrobial drugs (Dias-Souza et al., 2013; Santos et al., 2015; Santos et al., 2016).

Annacardium occidentale (cashew) is a plant used in Brazil and India for treating infectious diseases and for wound healing, and the fruit is widely explored for juice pulp commercialization. The antimicrobial activity of *A. occidentale* stem bark hydroethanolic extract was demonstrated by our group by the first time against *S. aureus* and *S. epidermidis* by the first time (Dias-Souza et al., 2013), and using an adapted disc diffusion method, strong antagonism was detected when the extract was combined to antimicrobial drugs against *S. aureus* strains (Dias-Souza et al., 2013).

When analyzing specific phytomolecules, we demonstrated that some flavonoids and carotenoids can impair the antimicrobial activity of some drugs, but can also enhance this pharmacological activity. We developed an adaptation to the method used with *A. occidentale* extract and tested phytonutrients against clinical isolates of *S. aureus, E. coli* and *P. aeruginosa*. Interestingly, none of the phytonutrients presented antimicrobial activity against any of *S. aureus* and *E. coli* strains. Nevertheless, most of the tested combinations generated antagonistic effects for these species, being more pronounced for *S. aureus* strains. In investigating *E. coli* strains, the antimicrobial activity of cefalexin and amoxicillin was strongly reduced, whereas most of the drugs tested for *S. aureus* lost their pharmacological activity completely. Conversely, the antimicrobial activity of the drugs tested against *P. aeruginosa* strains was strongly favored, and synergism was evidenced (Santos et al., 2015; Santos et al., 2016).

Like most of the scarce studies on antimicrobials-phytomolecules interactions studies, the mechanisms by which these interactions work remain to be elucidated. We hypothesized that some sort of membrane injury might be involved, considering that, until the preparation of this chapter, the tested phytonutrients does not present any evidence that they may overcome the resistance mechanisms for the tested drugs. An important aspect of investigating such interactions is the necessity of exploration of isolated molecules with pharmacological activity, after the interference of a crude extract on specific drugs is well described.

The research performed by our group and others are being directed for, in a near future, begin investigations with animal models, such that the *in vitro* findings can be confirmed. With *in vivo* data from animal models, it will be possible to plan the use of some associations for clinical studies, in order to reach a combined formulation to be used as a medication.

CONCLUSION

The phytonutrients discussed have many chemical and pharmacological aspects concerning their antimicrobial, antiproliferative and antioxidant activities described, but several points remain to be elucidated, especially the ones related to mechanisms of action. The safety of these molecules for human consumption is largely recognized; however, the combined use with medications may induce unpredictable effects. The evidences of the wide spectrum of antimicrobial and antiproliferative activities of the discussed molecules open doors for more investigations in order to reach the point in which the development of pharmaceutical dosage forms will extrapolate nutraceutical purposes and will be no more a promising alternative to clinical treatments, but effective tools of large scale production against several diseases.

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Chapter 21
Prediction of Structural and Functional Aspects of Protein: In-Silico Approach

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ABSTRACT

To predict the structure of protein from a primary amino acid sequence is computationally difficult. An investigation of the methods and algorithms used to predict protein structure and a thorough knowledge of the function and structure of proteins are critical for the advancement of biology and the life sciences as well as the development of better drugs, higher-yield crops, and even synthetic bio-fuels. To that end, this chapter sheds light on the methods used for protein structure prediction. This chapter covers the applications of modeled protein structures and unravels the relationship between pure sequence information and three-dimensional structure, which continues to be one of the greatest challenges in molecular biology. With this resource, it presents an all-encompassing examination of the problems, methods, tools, servers, databases, and applications of protein structure prediction, giving unique insight into the future applications of the modeled protein structures. In this chapter, current protein structure prediction methods are reviewed for a milieu on structure prediction, the prediction of structural fundamentals, tertiary structure prediction, and functional imminent. The basic ideas and advances of these directions are discussed in detail.

INTRODUCTION

A protein is a sequence produced by combination of 20 amino acids. Amino acids are characterized by polar (hydrophilic) and non polar (hydrophobic) based on its residue. These amino acids are associated to each other with peptide bond to form a protein sequence (Laskowski*et al.,* 2003). The fabrication of a protein sequence is much effortless than the determination of a protein structure. However, the structure of a protein gives much more impending in the function of the protein than its sequence. Consequently, a number of methods for the computational prediction of protein structure from its sequence have been

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developed. Protein structure prediction is the prediction of the three-dimensional structure of a protein from its amino acid sequence that is, the prediction of its secondary, tertiary, and quaternary structure from its primary structure. The native structure is the 3D structure of the protein sequence. It necessitates knowing the native protein structure so that we can find out the function of that protein structure. There are numerous algorithms used to predict the native confirmation of a protein. Protein sequences are folded on lattice with non overlapping amino acid chain. These self circumventing conformations produced native structures which have smallest energy configuration. The protein sequence-structure gap is broadening speedily. The number of identified protein sequences (*Bairoch and Apweiler, 2000)* is blasting as a outcome of genome and other sequencing projects. The rising number of protein sequences is much bigger than the escalating number of known protein structures (Berman *et al*., 2000). Hence, computational predictive tools for protein structures are badly needed to narrow the widening gap. Prediction of 3D protein structure from the amino acid sequence is one of the most exigent tribulations in theoretical structural biology (Montelione *et al*., 1999; Skolnick *et al*., 2000).Three-dimensional protein structures still cannot be correctly predicted straight from sequences. A transitional but helpful step is to predict the protein secondary structure, which is a way to simplify the prediction problem by projecting the very complicated 3D structure onto one dimension, i.e. onto a string of secondary structural coursework for each residue.

Fortitude of the exclusive tertiary (three-dimensional) structure of a protein from its amino acid sequence alone is one of the most significant and exigent problems in modern biology. The information on the tertiary structure of a protein is pretty decisive in accepting the function and biological responsibility of the protein. At present, genome-sequencing projects are producing an extraordinary amount of linear amino-acid sequences. An exponential growth of protein sequence database in current years by far outpaces the experimental determination of protein tertiary structures. Structure prediction is vitally dissimilar from the inverse problem of protein design. Protein structure prediction is one of the most key targets accomplished by bioinformatics and theoretical chemistry; it is extremely important in medicine and biotechnology for designing of the drug and novel enzymes.

This chapter begins with a thorough introduction to the protein structure prediction problem and is divided into four themes: a background on structure prediction, the prediction of structural elements, tertiary structure prediction, and functional insights.

The Protein Structure

Proteins are building blocks of life. It reveals more sequence and chemical complexity than DNA or RNA. It is a polymeric macromolecule fictitious of amino acid building blocks set in a linear chain and coupled jointly by peptide bonds. A protein sequence is a linear hetero polymer made up of one of the 20 different amino acids. Protein structures range in size from tens to several thousand residues (Brocchieri and Karlin, 1987). Protein structure is the bimolecular structure of a protein molecule that is a sequence formed from various residues. To be able to perform their biological function, proteins fold into one or more specific spatial conformations, driven by a number of non-covalent interactions such as hydrogen bonding, ionic interaction, Van der waals forces, and hydrophobic packing. The primary structure is usually symbolized by a sequence of letters above a 20-letter alphabet linked with the 20 naturally arising amino acids. Proteins are the foremost building blocks and useful molecules of the cell, pleasing up roughly twenty percent of a eukaryotic cells weight, the biggest involvement after water. They execute ample range of functions in the living organism, playing diverse catalytic, structural, regulatory and sig-

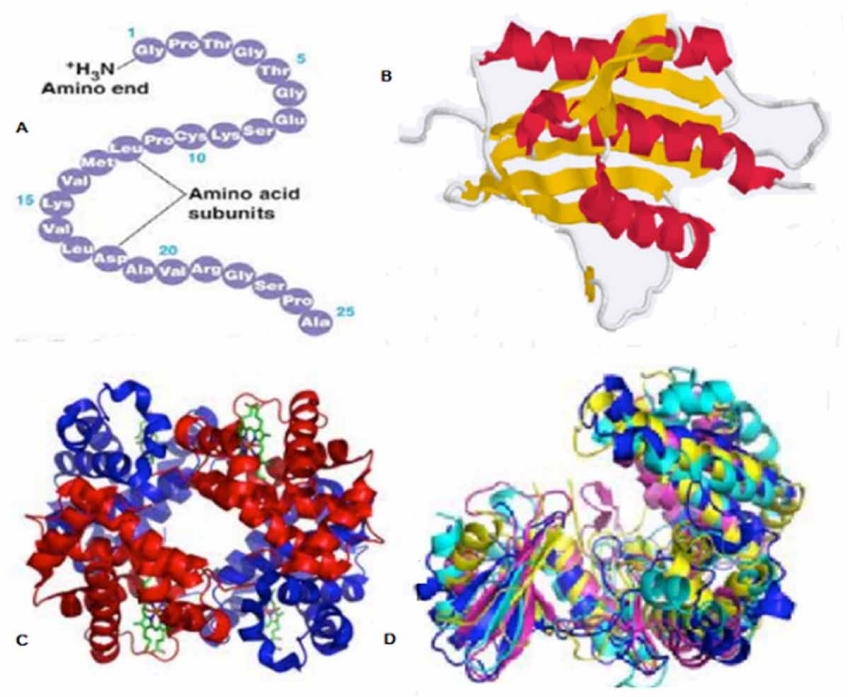
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naling roles essential for the cellular development, differentiation, replication and survival. The secondary structure is precised by a sequence classifying each amino acid into the consequent secondary structure constituents (e.g., alpha, beta, or gamma). The 3D structure of proteins can be solved by 1) Experimental methods, or 2) Structure prediction. It is incredibly inflexible to solve the structures experimentally. Solving through X-ray crystallography produces awfully excellent results but we require to have a very pure protein sample which should form crystals that are fairly perfect. Solving through NMR is restricted to tiny soluble proteins. The acquaintance of the 3D structure is helpful for rational drug design, protein engineering, exhaustive study of protein bimolecular interactions, study of evolutionary relationship between proteins or protein families etc. Structural features of proteins are frequently described at four levels of complexity. The primary structure of a protein can voluntarily be deduced from the nucleotide sequence of the analogous messenger RNA. Based on primary structure, various features of secondary structure can be predicted with the assist of computer programs. Conversely, predicting protein tertiary structure relics a very rough problem, although some progress has been made in this important area.

Primary Structure

The primary structure refers to linear arrangement of amino acids in a protein and the location of covalent linkages such as disulfide bonds between amino acids. The primary structure of a protein is determined by the gene corresponding to the protein. This structure is held together by covalent or peptide bonds, which are made during the process of protein biosynthesis or translation. The sequence of a protein can be determined by methods such as Edman degradation or tandem mass spectroscopy. Figure 1 A. Depicted a linear chain of amino acids.

Figure 1. A) Primary Structure of protein, B) Secondary Structure C) Tertiary structure and D) Quaternary structure of Protein



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Secondary Structure

Secondary structure is a confined frequently occurring structure in proteins and is mainly formed through hydrogen bonds between backbone atoms called random coils, loops or turns. There are two types of stable secondary structures: Alpha helices and beta-sheets (Figure.1B). Alpha-helices and beta-sheets are preferably located at the core of the protein, whereas loops prefer to reside in outer regions. Secondary structure prediction is a set of techniques in bioinformatics that aim to predict the local secondary structures of proteins. For proteins, a prediction consists of assigning regions of the amino acid sequence as likely alpha helices, beta strands, or turns. These secondary structures are defined by patterns of hydrogen bond between the main-chain peptide groups. They have a regular geometry, being constrained to specific values of the dihedral angles ψ and φ on the ramchandran plot (Chiang *et al*., 2007). Specialized algorithms have been developed for the detection of specific well-defined patterns such as transmembrane helices and coiled coils in proteins, or canonical microRNA structures in RNA(Mount, 2004)

Tertiary Structure and Quaternary Structure

The three-dimensional structure of a protein thatresults from a large number of non-covalent interactions between amino acids (figure.1C). Quaternary structure is the 3D structure of a multi-subunit protein and how the subunits robust together. In this perspective, the quaternary structure is stabilized by the matching non-covalent connections and disulfide bonds as the tertiary structure. Complexes of two or more polypeptides are called multimers. Distinctively it would be called a dimer if it contains two subunits, a trimmer if it contains three subunits and a tetramer if it contains four subunits. The subunits are recurrently associated to one another by symmetry maneuver, such as a 2-fold axis in a dimer. The best example of quaternary structure is hemoglobin (Figure.1D).

Protein Structure Prediction and Modeling

Protein structure prediction is one of the largely imperative problems in modern computational biology. It is consequently becoming gradually more significant to predict protein structure from its amino acid sequence, using impending obtained from already known structures. Amino acid sequence analysis offers essential insight into the structure of proteins, which in turn deeply assisted the perceptive of its biochemical and cellular function. Endeavors to exploit computational techniques in predicting protein structure based only on sequence information started 40 years ago (Chou and Fasman, 1974). The tertiary structure of proteins involves the folding of the secondary structural elements. The physical properties that determine fold are the backbone rigidity, interaction between the amino acids which include the electrostatic interaction, the van der waals interaction, hydrogen and disulphide bonds and interaction with water. (Dunbrack *et al*., 2000). The structure of an organized protein is indispensable for the understanding of its function. Even though the number of experimental accessible proteins is exponentially rising over the last several years, there are a large number of proteins with unidentified fold and devoid of a noticeable homology with any protein, which has been determined. Experimentally, protein structures possibly will be gritty *via* the X-ray crystallography or protein NMR (Chance *et al.*, 2002). Other experimental methods are currently of a lesser practical consequence. In practice, *abinitio* and molecular dynamics (MD) techniques are too sluggish or too imprecise. Thus, the best *de novo* prediction methods use mainly statistical information from known structures (Rohl et al., 2004). The most

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truthful prediction method so far is the template, or homology modeling approach, which predicts the structure by a comparison to a similar sequence. Understanding of a protein three-dimensional structure is aninput to understanding the protein biological function, to the rational drug design, protein engineering, etc. (Simons*et al.*, 2001).

Types of Protein Structure Predictions

There are assorted approaches and algorithms to predict the structure may be recapitulate as follows, 1) Prediction in 1D engross, Prediction of secondary structure, Prediction of solvent accessibility, Prediction of trans-membrane, helices. 2) Prediction in 2D engross, Prediction of the inter-residue and strand contacts. 3) Prediction in 3D engross, searching the database to locate a appropriate template for modeling. There are three methods for protein structure prediction namely, 1) homology modeling, 2) Fold recognition or threading, and 3) Ab-initio method. All these methods engage searching the database for a homologue to the target protein.

Homology Modeling

Homology modeling is sustained on the actuality that if two sequences have a high sequence similarity then they have similar 3D structure. But this is forever not the case. The free modeling technique has predicted some structures with very elevated precision; sometimes even exceeding the correctness of template/homology based approaches. The step in conventional homology modeling consists following possessions. Database search: Sequence similarity searching is a crucial step in analyzing newly determined protein sequences. A template for modeling desires to be recognized and sequence alignment between the template and target sequences has to be generated. Recurrently, template identification is executed by firm standard tools, such as PSI-BLAST, and the resulting alignment is consequently rectified by other tools and ultimately by physical professional corrections. The aligned fragments of templates are used to construct the corresponding fragments of the target structure. Multiple sequence alignment (MSA), main chain modeling, loop modeling, side chain modeling, energy refinement are the important parameters. The final, step of modeling is structure refinement which rivets repacking the side chains and energy minimization of the entire structure. The model obtained may be evaluated using PROVE, PROCHECK and what-if checks. There is some useful software routinely used for homology modeling like SwissPDB. TASSER, newer software also using the fragment approach, is very attractive because it is capable to predict the targets with similar precision while needing far less computing time (Zhou et al., 2007). Rosetta is also lucratively used for the homology modeling target as a refinement step.

Fold Recognition or Threading

Protein three-dimensional structure resolves protein function. But what resolves 3D structure is the question. The hypothesis that structure also referred to as ‘the fold’, is exclusively determined by the specificity of the sequence, has been confirm for many proteins (Kolinski and skolnick, 2004). Although it is now acknowledged that fussy proteins like chaperones frequently play a role in the folding pathway, and in correcting misfolds, it is still commonly implicit that the final structure is at the free-energy minimum (Sali, 1993,1995). The threading and sequence-structure alignment looms are stands on the observation that many protein structures in the PDB are incredibly analogous. The mainly exact prediction method

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so far is the template, or homology modeling approach, which predicts the structure by a contrast to a similar sequence. In favor of two thirds of sequences, a similar sequence can be establish and consequently the structure can be predicted by homology modeling with good exactitude for those with fewer than 300 residues. Nevertheless, it is expected that most of the remaining third also has identified folds. Homology modeling does not work for sequence disparity larger than 20% and thus *de novo* methods are desirable for those (Zhou and Zhou, 2002). A cluster of methods, called "threading", were developed which used structural information from several less intimately related structures. Conversely, they are less truthful than modern fragment methods and are thus only used as a module in some software (Moult et al., 2005).The accomplishment of the prediction of protein three-dimensional structure from its amino acid sequence is inadequate by paucity in the conformational search procedures aimed at finding the global energy minimum and in the efficient impending used to evaluate the free energies of possible structures (Cymerman et al., 2004). The protein fold-recognition approach to structure prediction aims to recognize the known structural scaffold that accommodates the target protein sequence in the finest way. Classically, a fold-recognition plan encompasses four mechanism: (1) the illustration of the template structures (2) the evaluation of the compatibility between the target sequence and a template fold (3) the algorithm to compute the optimal alignment between the target sequence and the template structure, and (4) the way the ranking is computed and the statistical significance is estimated (Fischer and eisenberg, 1996). Homology modeling and fold recognition are the most steadfast structure-prediction practice is to identify its matching structural folds from offered known structures with or without significant sequence similarity. This approach is also referred as template based modeling. Template-based modeling becomes progressively more dominant because most popular structure folds are known (Daiet al., 2011). On the other hand, recognizing structurally similar folds in the absence of sequence similarity or fold recognition is exigent. New methods ORFeus (Observatories and Research Facilities for European Seismology) uses sequence profiles and ignore the experimental structural information from the template, and attempts to predict the structure de novo both for the target and the template families (Ginalskiand Rrychlewski, 2003). Protein fold recognition scheme endeavor to recognize the suitable template from a structure template library for a query protein and create an alignment between the query and the recognized template protein, from which the structure of query protein can be predicted. Protein fold recognition using the protein threading procedure has established enormous success (Moult et al., 2003).

Unusual techniques can be used in order to face the computational difficulty of protein fold recognition problem such as Molecular Dynamics, Monte Carlo, Genetic Algorithms and Neural Network. The GenTHREADER method uses a conventional sequence alignment algorithm to produce alignments which are then appraised by a method derived from threading techniques. All threaded model is evaluated by a neural network in order to produce a single gauge of assurance in the projected prediction (Jones, 1999).

Lin *et al(*2002) have developed a new program TUNE (Threading Using Neural network based on an artificial neural network model to predict compatibility of amino acid sequences with structural environment. Jiang *et al (*2005) anticipated a new fold recognition model with mixed environment-specific substitution mapping called MESSM.A support vector machine is engaged to measure the significance of the sequence-structure alignment. MESSM shows good performance on protein fold recognition. Support Vector Machine (SVM) regression approach to directly predict the alignment accuracy of a sequence template alignment has been implemented by (Xu, 2005). Threading or remote homologue

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design is a protein structure prediction technique carried when there is not enough sequence similarity between the target and template. The recognition of the template is a problem by itself and hence it is also called fold recognition.

AB-Initio Prediction

Proteins are among the foremost significant groups of biomolecules, with their biological functions ranging from structural elements to signal transducers between cells. Independently from their biological role, incident related to protein behavior in solutions and at solid interfaces can find an extensive range of engineering applications such as in biomedical implants, scaffolds for artificial tissues, bioseparations, biomineralization and biosensors. The functionality of a protein is directly related to its three-dimensional structure (i.e. conformation) for both biological and engineering applications. Process such as homology and threading that depend on a large database of accessible experimental data are the most popular means of predicting the conformation of proteins in their native environment. Ab-initio prediction is conceded out when there is no appropriate homologue found in the database. Prediction is done completely from the sequence that may be based on An finsen's hypothesis that the native state of the protein represents the global free energy minimum. Ab-initio method tries to find these global minima of the protein. *Ab initio* protein folding has regularly been an area of entirely academic interest characterized by relatively slow progress.

The purpose of ab initio structure prediction is effortless: Given a protein's amino acid sequence predict the structure of its native state. It is normally understood that a protein sequence folds to a native conformation or ensemble of conformations that is at or near the global free-energy minimum. Thus, the problem of finding native like conformations for a given sequence can be decomposed into two sub problems: (a) developing an accurate potential and (b) developing an efficient protocol for searching the resultant energy landscape. There are two mechanisms to *ab initio* prediction: devising a scoring (i.e, energy) function that can distinguish between correct (native or native-like) structures from incorrect ones, and a search method to investigate the conformational space. In various techniques, the two apparatus are coupled together such that a search function drives, and is driven by, the scoring function to find native-like structures. Unlike computational approaches to the problem fluctuate as to which assumptions are made. A promising approach, based on the discretization of the conformational space, is that of deriving a protein-centric lattice, by allowing the backbone torsion angles, phi, psi, and omega, to take only a distinct set of values for each different residue type. Under biological circumstances, the bond lengths and bond angles are fairly rigid. Consequently, the internal torsion angles along the protein backbone determine the main features of the final geometric shape of the folded protein. To predict protein structure *de novo* for larger proteins will necessitate superior algorithms and larger computational resources like those afforded by either influential super computers e.g. Blue Gene or MDGRAPE-3) or distributed computing. Even though these computational barriers are vast, the potential benefits of structural genomics make *ab initio* structure prediction an active research field. *Ab initio* prediction methods use just the sequence of the protein. Threading and Homology Modeling methods can build a 3D model for a protein of unknown structure from experimental structures of evolutionary related proteins (Zhang,2008).There are two kinds of model.

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1. **Lattice Model:** This represents peptide chain as lattices. But fails to represent subtle geometric consideration like strand twist and its backbone prediction is not all that accurate.
2. **Discrete State off-Lattice Model:** It improves upon the lattice modelby applying restraints like allowing only certain side chain structure and limiting the peptide bond rotation. In order to reduce the complexity, local structure biases are used. But the strength and multiplicity of the local structure prediction is highly sequence dependent. There are two type of scoring functions namely knowledge based scoring function and force field based function which are used. Currently there does not exist a reliable scoring function or search method. There is a new fully automated ab-intio prediction method in which the Monte Carlo fragment insertion method (ROSETTA) of Baker and others has been merged with the Isite (library of sequences structure motifs) and the HMMSTR model for local structure in proteins.

Comparative Modeling

Comparative modeling predicts the 3D structure of a specified target protein sequence based principally on its alignment to one or more proteins of known structure i.e. templates. The prediction method consists of fold assignment, target–template alignment, model building, and model evaluation. The number of protein sequences that can be modeled and the accuracy of the predictions are growing progressively because of the growth in the number of known protein structures and because of the improvements in the modeling software. The aspire of comparative or homology protein structure modeling is to build a three-dimensional (3D) model for a protein of unknown structure on the basis of sequence similarity to proteins of known structure. Two situations must be met to build a useful model. First, the likeness between the target sequence and the template structure must be obvious. Second, a substantially correct alignment between the target sequence and the template structures must be calculated (Marti-Renom et al., 2002). Comparative modeling methods are useful for predicting the tertiary structure of the target protein if there is a template for a homologue with a comparatively high sequence similarity. During comparative modeling, the target sequence is aligned to templates, and then all atom structures of the target protein are formed ensuing to fulfilling in alignment gaps and correctly orienting side chains (Baker and Sali, 2001). Comparative modeling is presently the most habitually used approach to the theoretical prediction of protein structure (Holm and Sander, 1996).

Whereas a protein structure and function is evolutionary too much conserved than the protein sequence, similarity of sequence of a new protein to a protein of known structure entails their structural similarity (Clark, 1999).

A template protein probably will be identified by a sequence comparison method (Altschul*et al.*, 1997) or by a threading algorithm (Kiharaand Skolnick, 2004).

The almost all conservative approach to the comparative modeling occasionally called homology modeling, although meticulously speaking homology is not required for the sequence and structure similarity. Comparative modeling ruins the most dependable and routinely used method for protein structure prediction (Eswar*et al.*, 2008, Ginalski, 2006). The unusual term of homology modeling is regularly used. That is because the identification of a structural template is classically based on the homology relation between the target protein and the templates, which is usually reflected by a certain level of sequence similarity. All existing comparative modeling methods consist of four sequential steps: fold

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assignment and template selection, template–target alignment, model building, and model evaluation. If the model is not satisfactory, template selection, alignment, and model building can be repeated until a satisfactory model is obtained.

Applications

Comparative modeling is an increasingly efficient way to obtain useful information about the proteins of interest. Comparative models can be helpful in designing mutants to test hypotheses about a protein's function

- Identifying active and binding sites,
- Identifying, designing and improving ligands for a given binding site,
- Modeling substrate specificity,
- Predicting antigenic epitopes,
- Simulating protein–protein docking,
- Inferring function from a calculated electrostatic potential around the protein,
- Facilitating molecular replacement in X-ray structure determination,
- Refining models based on NMR constraints,
- Testing and improving a sequence–structure alignment,
- Confirming a remote structural relationship and rationalizing known experimental observations.

Transmembrane Topology

Transmembrane protein topology prediction methods play significant roles in structural biology, since the structure determination of these types of proteins is enormously tricky by the common biophysical, biochemical and molecular biological methods. The need for accurate prediction methods is high, as the amount of known membrane protein structures plunge extreme behind the estimated number of these proteins in various genomes. The precision of these prediction methods emerges to be higher than most prediction methods applied on globular proteins, however it reduces vaguely with the increasing number of structures. A transmembrane protein is a protein that spans an entire biological membrane. These proteins go from one side of a membrane through to the other side of the membrane. Transmembrane proteins are aggregate and precipitate in water. They need nonpolar solvents for extraction, while some of beta-barrels can be also extracted using denaturing agents. Transmembrane proteins control the flow of information and substances into and out of the cell and are involved in a broad range of biological processes. All transmembrane proteins are integral membrane proteins, but not all integral membrane proteins are transmembrane proteins.

There are two fundamental types of transmembrane proteins: 1) Alpha-helix- This is the major category of transmembrane proteins present in the inner membranes of bacterial cells or the plasma membrane of eukaryotes. In humans, 27% of all proteins encompasses predictable to be alpha-helical membrane proteins. 2) Beta-plated sheets-These proteins are found only in outer membranes. Membrane proteins are crucial for survival. They constitute key components for cell–cell signaling, mediate the transport of ions and solutes across the membrane, and are crucial for recognition of self (Chen *et al.*, 2002). Most

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TM proteins are helical (TMH) proteins. Helices in membrane spanning regions are more tightly packed than the helices in soluble proteins. The topology of TMH proteins, includes, the membrane spanning regions and the amino-terminal orientations. The determination of the topology of a TMH protein is useful for the annotation of its biological function (Zhou, 2003). Many different methods have been developed to predict the topology of transmembrane protein TMH proteins. Transmembrane helices can be predicted based on the distinctive patterns of hydrophobic and polar regions within the sequence. Kyte and Doolittle developed one of the first methods that evaluated the hydrophilicity and hydrophobicity of a protein along the amino acid sequence (Kyte and Doolittle, 1982). The first HMM-based transmembrane protein topology predictors were introduced ten years ago: TMHMM and HMMTOP (Tusnady, 1998). TMpred is one of the methods using such statistical preferences to predict membrane helices taken from an expert-compiled data set of membrane proteins. The HMM Phobius was designed to combine the signal peptide model of Signal P-HMM [Nielsen and krogh, 1998] with the transmembrane topology model of TMHMM (Sonnhammer et al., 1998). Reynolds et al, 2008 described Philius, a DBN-based approach to transmembrane protein topology prediction. Hidden Markov models (HMMs) have been successfully applied to the tasks of transmembrane protein topology prediction and signal peptide prediction. Consensus methods based on multiple prediction algorithms were also developed (Drew et al., 2002).

Ligand-Binding Residue Prediction

In this section, we explore means for predicting protein residues that interact with small molecules. The accurate prediction of ligand binding residues from amino acid sequences is imperative for the programmed functional annotation of new proteins. The rationale of the ligand binding site residues in a protein is also vital, because substrate specificity of an enzyme is determined by the well information of the binding site residues, such as side chain orientation and physiochemical properties (Schwede, et al. 2009).

The conquering identification of ligand-binding sites on protein surfaces is usually the opening point for the annotation of protein function and drug discovery. The identification of ligand-binding sites is frequently the starting point for protein function annotation and structure based drug design. Many computational methods for the prediction of ligand-binding sites have been developed in latest decades. The protein surface can form pockets that are binding sites of small molecule ligands. Therefore, the identification of pocket sites on the protein surface is often the starting point for protein function annotation and structure-based drug design. The prediction of protein-ligand binding sites has great significance for protein function annotation and computer-aided drug design. Besides the binding site identification, the binding sites' ranking according to their likeliness to accept a molecule is also an important and challenging issue (Gao et al., 2012**).** Identifying ligand-binding residues reliably aids the overall understanding of the role and function of a protein by using them to subsequently predict the types of ligands to which they bind and, in the case of enzymes, the types of reactions that are catalyzed. Moreover, knowledge of the residues involved in protein-ligand interactions has broad applications in drug discovery and chemical genetics, as it may be used to better virtually screen large chemical compound libraries and to aid the process of lead optimization. In addition, the ligand-binding residues of a protein can be used to influence target-template sequence alignment in comparative protein modeling which has been shown to improve the quality of the 3D models produced for the target's binding site. These quality improvements in the binding site's 3D model are critical to docking-based approaches for virtual screening (Kauffman and Karypis, 2009).

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The number of predictors that submitted new methods in this category was highly encouraging and suggests that current technology is at the level that experimental biochemists and structural biologists could benefit from what is clearly a growing field. Several different protein ligand binding site prediction methods have been developed, which mostly fall into two major categories: sequence-based methods and structure based methods. The sequence-based methods rely on identifying conserved residues that may be structurally or functionally important and include methods such as firestar, WSsas, FRcons, ConFunc, ConSurf, FPSD, Pand INTERPID. The structure based methods can be further subdivided into the geometric methods utilized by FINDSITE and Site Hunter; energetic methods utilized by SITEHOUND and Q-Site Finder. The FunFOLD software implements a competitive method for the prediction of protein binding site residues, which can also be used to determine the putative ligands interacting with a protein. The FunFOLD software is freely available as both a standalone package and a prediction server, providing competitive ligand binding site residue predictions for expert and non-expert users alike. The software provides a new fully automated approach for structure based function prediction using 3D models of proteins. Nevertheless, for the 3D models the LigandSite and Phyre server are frequently used. Similarly MAMMOTH used for structural superposition of similar templates with bound ligands (Roche et al., 2011).

To predict the potential binding site, several computational methods have been developed. In brief, these algorithms can be divided into three categories (1) purely geometry-based methods, which follow the assumption that the protein-ligand binding sites are generally located at crevices on the protein surface or cavities in the protein. Methods falling in this category include POCKET, LIGSITE, PASS, SURFNET, and Pocket Picker. (2) energetic-based methods, which coat the protein surface with a layer of probes to calculate van der Waals interaction energies between the protein and probes. As an example, Q-Site Finder is a classical tool falling in this category (3) knowledge based methods, which includes various statistical methods, machine learning methods and similarity comparison methods. Besides, a part of them predict protein-ligand binding sites by searching for clusters or patterns of the conserved residues.These methods include POCKET, LIGSITE, LIGSITEcs, SURFNET,CAST, PASS and Pocket Picker all of which use pure geometric characteristics and do not require any knowledge of the ligands (Roche et al.,2010). Identification of binding sites is of fundamental importance in SBDD and virtual ligand screening. Identifying sequence and structural similarity with proteins with known functional sites is an emerging area for identifying ligand binding sites. Energy- based methods of pocket detection include those of Meta Pocket and Q-Site Finder is online.

Loop Modeling

Loop modeling is conceivably the hardest step in comparative modeling process along with alignment. When target and template share above 35% sequence identity, errors in loops are the dominant problem in comparative modeling. *Loop modeling* is a crisis in protein structure predictions necessitating the prediction of the conformations of loop regions in protein with or without the use of a structural template. Computer programs that resolve these troubles have been used to research a extensive sort of scientific issues from ADP to breast cancer (Baloria et al., 2012). Since protein function is gritty by its shape and the physiochemical properties of its exposed surface, it is essential to build an accurate model for protein or ligand interaction studies (Fiser and Sali, 2003). The process for modeling a loop in a given situation is illustrated here by specifying its three main mechanisms: The depiction of a protein; the manacles that define the objective or energy function; and the scheme for optimizing the energy function. The

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modeling method is completely automated and is implemented in the program MODELLER-5. Whilst the mostly recurrent application of the technique is to predict single loops, it is also technically suitable for modeling any set of contiguous or noncontiguous residues or atoms e.g., several loops, a loop with a ligand, a huddle of side chains in the preset environment created by the respite of the protein. Consequently, ingenuous modeling of loops is indispensable for structure-based prediction of function from sequence. For example, a comparative model can occasionally be used with computational ligand docking to locate a putative ligand or determine preferences within a limited set of ligands (Xu et al., 1996). A somber complication is that the ligand may persuade conformational changes in loops with which it interacts. Loop modeling methods can be classified in two approaches: *Ab initio* methods and database searching or knowledge-based methods.

Ab Initio Methods

Ab initio loop prediction is based on a conformational search guided by a scoring or energy function -the later describing the physico-chemical properties of a protein and its environment. It is also called as non-template based technique. The non-template based looms employ a statistical model to seal in the gaps created by the unknown loop structure known as an *ab initio* method. Several of these programs include MODELLER, Loopy, and RAPPER; but each of these programs approaches the problem in a dissimilar way. Further programs exploit a de novo approach that samples sterically feasible loop conformations and selects the best one. The determining a best model means that a scoring method must be fashioned to compare the diverse conformations (Adhikariet al., 2012)

Knowledge-Based Methods

Knowledge-based approaches, which are the most widely used also called as template based technique. It utilizes libraries of regular rotamers extracted from high resolution X-ray structures. This approach is executed in most automatic homology modeling events. The CORALL module of WHATIF and the SCWRL program important software using such approach. Sections of a structural model that are predicted by non-template-based loop modeling tend to be greatly less perfect than regions that are predicted using template-based techniques. Conversely, loops are commonly too short to present sufficient information about their local fold. Fragment of up to nine residues occasionally have totally unrelated conformations in different proteins (Cohen and Pressnell, 1993).It is furthermore referred as database searching approach. This is more precise and resourceful if it is precedent by a structural classification of the loops here in the database. This approach to loop prediction initiated by finding segments of main chain that fit the two stems of a loop. The stems are defined as the main chain atoms that herald and follow the loop but are not part of it. The search is carrying out through a database of many known protein structures, not only homologues of the modeled protein. The preferred segments are then superposed and annealed onto the stem regions. These initial crude models are often refined by optimization of some energy function. The database search ensue to loop modeling is truthful and proficient when a specific set of loops is created to tackle the modeling of that group of loops like b-hairpins and the hyper variable regions in immunoglobulins (Sibanda et al.,1989). There are many such methods, exploiting different protein representations, energy function terms, and optimization or enumeration algorithms. ModLoop is a web server for automated modeling of loops in protein structures. The server relies on the loop modeling usual in MODELLER that predicts the loop conformations by satisfaction of spatial restraints, without relying

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on a database of known protein structures (Fisher, 2009). FREAD is a database search loop modeling algorithm. Its principal use is to fill in the gaps in imperfect 3D models of protein structures. The input is a 3D model of a protein structure as well as the location and amino acid sequence of the loop region to be modeled (Choi et al.,2009). FALC-Loop is an internet server for protein loop modeling. The FALC-Loop server uses the protein loop modeling method that utilizes fragment assembly and analytical loop end developed in the Reference. The method shows a brilliant presentation when compared to earlier loop sampling methods (Lee et al., 2011).YASARA Structure features a loop modeler, that can swiftly retrieve loop conformations that overpass two sets of anchor residues from the Protein Data Bank. This knowledge-based approach profits extremely from the increasingly growing PDB, and has been shown to be a strong competition for ab-initio loop prediction (Michalsky et al., 2003).

CONCLUSION

The 3D structures of proteins are preset by a linear sequence of amino acid residues. To predict structure from sequence is an intricate job enough to have occupied a generation of researchers. Remarkable looms to predict the structure obtain into account unlike chemical and physical properties. Nonetheless these are not appreciably perfect or trust worthy adequate to predict all kinds of proteins. Ab-initio prediction methods even with their very low accuracy can give a reliable functional annotation.

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KEY TERMS AND DEFINITIONS

Ab Initio Prediction: The ab initio structure prediction of proteins is to correctly characterize the 3D structure of a protein using only the amino acid sequence as input.

Insilico Analysis: The term 'in silico' is a modern word usually used to mean experimentation performed by computer and is related to the more commonly known biological terms in vivo and in vitro. These in silico methods include databases, quantitative structure-activity relationships, pharmacophores, homology models and other molecular modeling approaches, machine learning, data mining, network analysis tools and data analysis tools that use a computer.

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Molecular Modeling: Molecular modeling is an application of computers to generate, manipulate, calculate and predict realistic molecular structures and associated properties. The techniques are mainly used in the fields of computational chemistry, drug design, computational biology and materials science for studying molecular systems.

Protein Structure: Proteins are single, unbranched chains of amino acid monomers. Protein structure is biological polymers composed of amino acids. Amino acids, linked together by peptide bonds, form a polypeptide chain. One or more polypeptide chains twisted into a 3-D shape form a protein. Proteins have complex shapes that include various folds, loops, and curves. Folding in proteins happens spontaneously.

Visualization Software: Visualization software is required to view the structure that is encoded by atomic coordinate PDB files and to be able to manipulate the images to view the molecule from various perspectives. Visualization typically takes the form of two dimensional displays, where interactive controls allow the user to explore and experience the three-dimensional structure of the protein under study.

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Chapter 22
Flavonoids: Prospective Strategy for the Management of Diabetes and Its Associated Complications

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ABSTRACT

Diabetes Mellitus is one of the major healthcare problems faced by the society today and has become alarmingly epidemic in many parts of the world. Despite enormous knowledge and technology advancement, available diabetes therapeutics only provide symptomatic relief by reducing blood glucose level, thereby, just slows down development and progression of diabetes and its associated complications. Thus, the need of the day is to develop alternate strategies that can not only prevent the progression but also reverse already "set-in" diabetic complications. Many flavonoids are reported, traditionally as well as experimentally, to be beneficial in averting diabetes and lowering risk of its accompanying complications. In the present chapter we have convened different flavonoids beneficial in diabetes and comorbid complications and discussed their mechanisms of action. Further, we conclude that coupling current therapeutics with flavonoids might provide exceptional advantage in the management of diabetes and its complications.

INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder arising from variety of factors, genetic or environmental, resulting in hyperglycemia. Hyperglycemia leads to acute and chronic metabolic abnormalities, amongst which neuropathy, nephropathy, retinopathy, learning and memory impairment and cardiovascular complications are the major causes of morbidity (Goodman, 2011). Hyperglycemia is a consequence of either pancreatic β-cells destruction, thereby decreasing the level of insulin secretion

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(Type-1 Diabetes Mellitus; T1DM) or due to decreased responsiveness of target cells towards insulin with or without insulin secretory defects (Type-2 Diabetes Mellitus; T2DM) (American Diabetes Association, 2013). In 2013, 382 million people across the globe were reported to be diabetic amongst which 5.1 million lost their lives and therapeutic management of diabetes costs $548 billion. By the year 2035, diabetic population of the world is projected to increase by 55% and its management cost is expected to reach $627 billion. Major concern is that 46% cases of DM remain undiagnosed for longtime (International Diabetes Federation, 2013) and by the time clinical symptoms appear, damage has already been done. Majority of current diabetic research is focused on identifying various therapeutic targets with protective as well as restorative ability to reverse the diabetic damage. Currently available antidiabetic medications controls hyperglycemia by reducing dietary glucose absorption, disposing blood glucose into muscles, liver or adipose tissue and by increasing its excretion form the body thereby providing only symptomatic relief. None of the available treatments are capable of reversing the damage inflicted by diabetes and ceasing its progression (Tripathi, 2013; Verma, Itankar, & Arora, 2013). Hyperglycemia in diabetes generates excessive reactive oxygen/nitrogen species (ROS/RNS) by oxidation of glucose. These highly reactive ROS/RNS binds and disturb normal functioning of biomolecules such as proteins, ribonucleic acid (RNA), deoxy-ribonucleic acid (DNA), etc. and thus, directly or indirectly aids in the development of various diabetic complications (Rochette, Zeller, Cottin, & Vergely, 2014).Thus the use of flavonoids, which are natural antioxidants, may provide additional advantage over current antidiabetic therapy. Present chapter discusses various targets that are currently used for the management of DM and potential of various flavonoids to exploit them. It also provides the list of flavonoids that were reported to be effective in managing diabetes or diabetic complications with their possible mechanisms, which needs further exploration to fully understand their antidiabetic potential.

All the therapeutic interventions currently used clinically for the management of DM exploit primarily following biochemical processes of human body.

1. **Glucose Absorption:** Dietary polysaccharides are converted to glucose prior to their absorption in gastrointestinal tract by α-glucosidase and α-amylase. Therefore, inhibition of these enzymes reduces glucose absorption and ultimately lowers blood glucose level (Etxeberria, de la Garza, Campión, Martínez, & Milagro, 2012; Kim, Kwon, & Son, 2000; Kim, Nguyen, Kurihara, & Kim, 2010; Sales, Souza, Simeoni, Magalhães, & Silveira, 2012). Reduction in blood glucose level results in enhanced glycogenolysis and lipolysis for generating glucose to fulfill the energy requirements of body and thus provide beneficial outcomes in obesity and associated insulin resistance (InR). Both of these enzymes are important targets for antidiabetic therapy and compounds such as acarbose andglucsidase inhibitors are clinically used for the management of diabetes (Goodman, 2011).
2. **Glucose Disposal from Blood:** Insulin is a peptide hormone responsible for maintaining glucose homeostasis by disposing blood glucose into skeletal muscles (for energy), adipose tissue (lipogenesis) and liver (glycogenesis). Mechanistically, this is achieved through interaction of insulin with heterotetrameric insulin receptor (IR) which ultimately leads to the transcription and translation of glucose transporter-4 (GLUT4), responsible for glucose disposal. Insulin potentiators (sulfonylureas etc.), insulin analogues (beef and pork insulin preparations etc.) and insulin signaling modulators (peroxisome proliferator activated receptors-γ (PPAR-γ)) are extensively used clinically for the management of diabetes (Goodman, 2011; Tripathi, 2013). Further, glucagon like peptide-1 (GLP1) is responsible for disposing glucose from the blood by lowering glucagon and increasing insulin blood levels. GLP1 is degraded by enzyme dipeptidyl peptidase-4 (DPP4), inhibition of DPP4

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results in GLP1 mediated lowering of blood glucose. DPP4 inhibition is a newly discovered and promising antidiabetic target for the clinical management of hyperglycemia (Janardhan & Sastry, 2014).

3. **Glucose Metabolism:** Glucose is the primary energy source of our body and is oxidized by a series of enzymatic reactions to produce energy in the form of adenosine triphosphate (ATP). In one such reaction, aldose reductase (AR) converts glucose into sorbitol by utilizing nicotinamide adenine dinucleotide (NAD) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) during the process. During hyperglycemic state, excessive sorbitol generation inflicts osmotic stress and thereby damages microvasculature, especially in the eyes causing retinopathy. Enormous depletion of NADPH (antioxidant) further augments oxidative stress within the body which is responsible for the initiation and constant progression of diabetic complications. Inhibition of AR and other glucose metabolizing enzymes is very effective in reducing the progression of diabetic complications by lowering osmotic and oxidative stress. But AR inhibitors do not have any significant effect on blood glucose level and hence are not able to halt the progression of DM (Sharma & Sharma, 2008).
4. **Glucose Excretion:** Kidneys filter about 180g of glucose per day which is entirely reabsorbed from proximal tubule by transporters such as sodium glucose co-transporter-2 (SGLT2). Drugs which inhibit SGLT2, such as canagliflozin, results in the excretion of glucose in urine and thereby reduces blood glucose level. This transporter has recently gained attention as a potential target for lowering blood glucose level (Tahrani, Barnett, & Bailey, 2013). Although it slows down the progression of diabetes associated complications, but like other targets, it is also ineffective in halting or reversing these complications.
5. **Oxidative Stress Induced Damage:** High blood glucose level inflicts excessive oxidative stress by generating advanced glycation end-products (AGE), ROS, RNS and depleting natural antioxidant defense of the body (viz. catalase, superoxide dismutase (SOD), glutathione reductase (GSH), NADPH etc.) through activation of stress sensitive signaling, hexoseamine, polyol and AGE pathways. All these factors sum up to induce neuropathy, nephropathy, vascular and other diabetic complications (Fisher, Zhang, Geoffrey, & Steven, 2006; Johansen, Harris, Rychly, & Ergul, 2005). Compounds that lower oxidative stress may not be able to reduce blood glucose levels but can be very effective in reducing diabetic complications. These agents can be given along with hypoglycemic agents for the effective management of diabetes and its complications.

FLAVONOIDS AND DIABETES

From the above discussion, role of oxidative stress in development and progression of diabetic complications becomes evident. Thus, the use of flavonoids, which possesses natural antioxidant properties, may provide additional advantage over traditional therapeutic strategies. Flavonoids are the 15 carbon polyphenolic compounds in which two aromatic rings are linked by a 3 carbon bridge i.e. C6-C3-C6 linkage (Jaganath & Crozier, 2010). Todate, more than 6000 different flavonoid molecules have been characterized, which are produced by plants as secondary metabolites in fruits, vegetables, nut, seeds, flowers, stem etc. (Harborne & Williams, 2000; Jaganath & Crozier, 2010). Rapid rise in the metabolic and neurodegenerative diseases worldwide has necessitated to speed-up the search of effective alternatives to the available therapeutics. According to a World Health Organization (WHO) report, about 90%

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of T2DM, 80% of cardio vascular disorders and 33% of cancers can be avoided just by adopting necessary lifestyle and dietary changes (World Health Organization, 2003). A number of studies conducted in recent past have established the inverse relationship between enhanced intake of dietary flavonoid and epidemiology of neurodegenerative disorders. These studies demonstrated that occurrence of DM and neurodegenerative disorders can be reduced by consuming diets rich in polyphenols and flavonoids (Anderson, Anthony, Cline, Washburn, & Garner, 1999; Law & Morris, 1998; Steinmetz & Potter, 1996). Meta-analysis study involving 2,84,806 subjects revealed that the risk of T2DM is reduced significantly by enhanced consumption of dietary flavonoids (Liu et al., 2014). Further, evidence suggests that flavonoids can control DM and its associated complications by reducing hyperglycemia, hyperlipidemia (Bansal et al., 2012; Chen et al., 2013; Nisha & Mini, 2013; Zheng et al., 2011), inflammation stress (Lin, Wu, Fang, & Yen, 2012; Nisha & Mini, 2013; Zheng et al., 2011), oxidative stress (Bansal et al., 2012; Chen et al., 2013; Nisha & Mini, 2013), improving insulin release and its signaling(Jin, Shi, Tang, Qiao, & Duan, 2013; Zheng et al., 2011), glucose tolerance (Bansal et al., 2012), diabetic profile of pancreas and liver (Bansal et al., 2012; Bansal et al., 2011; Dias, Bronze, Houghton, Mota-Filipe, & Paulo, 2010) and by regulating PPAR-γ activity (Zheng et al., 2011). Apart from these, few other studies depicts that flavonoids control diabetes by inhibiting α-amylase and α-glucosidase(Yao, Zhu, Chen, Tian, & Wang, 2013), AMP-activated protein kinase (AMPK) signaling (Jin, Shi, Tang, Qiao, & Duan, 2013;Kim, Kim, & Kim, 2011)andprotein-tyrosine phosphatase 1B(PTP1B)(Li et al., 2013). All these reports provide us with the strong justification regarding the antidiabetic potential of the dietary flavonoids. However, only few flavonoid molecules have been studied extensively for their antidiabetic activity, but the mechanisms through which they act remains unclear to large extent till date. In this chapter flavonoids that had been reported to possess antidiabetic activity are categorizes according to their possible mechanism of action.

TARGETS OF FLAVONOIDS FOR ANTI-DIABETIC POTENTIAL

1. Aldose Reductase (AR)

AR is a monomeric reduced NAD/NADPH-dependent enzyme which belongs to aldo-keto reductase superfamily of enzymes. In 1956, Hers reported that AR inhibition results in lowering of blood glucose level (Hers, 1956) and later on, in 1959, its elevated level during diabetes was confirmed (Van Heyningen, 1959). Counting on this information, Kinoshita *et al.* and Varma and Kinoshita demonstrated that AR inhibitors prevented cataractogenesis in diabetic rats and rabbits (Kinoshita, Dvornik, Kramil, & Gabbay, 1968; Varma & Kinoshita, 1976). Since then it has been looked upon as a potential target for the management of diabetic complications, especially retinopathy.

AR acts on a variety of substrates in the body and is responsible for glucose metabolism through polyol pathway, catecholamine metabolism, steroid metabolism and detoxification of aldehydes(Yabe-Nishimura, 1998).Under normoglycemic condition, majority of body's glucose is converted to glucose-6-phosphate (G6P) by hexokinase and about 3% of total glucose enters polyol pathway, where it is metabolized by AR. However during hyperglycemia, AR work load shoots up by 10 fold and more than 30% of the total glucose enters polyolpathway (González, Barnett, Aguayo, Cheng, & Chylack, 1984; Morrison, Clements Jr, Travis, Oski, & Winegrad, 1970). AR initially catalyzes theNADPH-dependent reduction of glucose into sorbitol, which is then converted to fructose by sorbitol dehydrogenase through NAD utilization (Hers, 1956). Sorbitol accumulates in the body, especially in ocular lens, and inflicts osmotic stress

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which disrupts cell membranes, leading to various diabetic complications amongst which retinopathy is the best known (Nagata et al., 1989). Further during hyperglycemia, NADPH is depleted and reduced NAD is accumulated which consequently give rise to abnormalities in body's antioxidant enzymes, GSH and nitric oxide synthase (NOS), thereby inflicting oxidative stress. Endothelial cells cultured in high glucose media showed lower level of GSH and were more liable to the damage inflicted by hydrogen peroxide (H_2O_2)(Kashiwagi et al., 1994). Likewise, NOS production from L-arginine was reduced due to non-availability of NADPH cofactor and thus elicits microvasculature damage coupled with sluggish nerve conduction (Cameron, Cotter, Dines, & Maxfield, 1993; Stevens et al., 1994).

Elevated NADH/NAD^+ ratio favor the synthesis of diacylglycerol (DAG) by increasing dihydroxyacetone phosphate availability and enhancing the reduction of *sn*-glycerol-3-phosphate, which is intermediate in DAG synthesis (Pugliese, Tilton, & Williamson, 1991). DAG in turn activates protein kinase C (PKC) which increases sodium-proton antiport activity, responsible for regulating intracellular pH, cellular growth and differentiation (Williams, 1995). All these biochemical changes associated with high glucose induced vascular complications were demonstrated experimentally in various tissues including retina, aorta, and renal glomeruli and were ameliorated by using β isoform of PKC (Ishii et al., 1996).

AR metabolizes toxic aldehydes, such as 4-hydroxynonenal (HNE) and glutathione-HNE (GS-HNE), generated by lipid peroxidation during hyperglycemia. AR efficiently reduces HNE and GS-HNE to non-toxic 1,4-dihydroxy-2-nonene (DHN) and GS-DHN. However in doing so, AR, phospholipase-C (PL-C) and PKC activates mitogen activated protein kinase (MAPK) and nuclear factor kappa B (NFκB) which enters nucleus and initiates transcription of their target genes (Pladzyk, Ramana, Ansari, & Srivastava, 2006; Zeng et al., 2013). Tumor necrosis factor-α (TNF-α), inter leukin-6 (IL-6), IL-1 and other cytokines and cytokines like substances, thus generated, induces InR and adds up in the development of diabetic complications (Goldberg, 2009).

To date numerous flavonoids have been shown repeatedly in literature to havethe potential to inhibit AR enzymatic activity *in-vitro* (Table 1; Figure 1) (Matsuda, Morikawa, Toguchida, &Yoshikawa, 2002; Shimizu et al., 1984; Varma and Kinoshita, 1976). Mok and Lee (2013) isolated quercetin, kaempferol, afzelin, myricitrin, quercitrin and myricetin from the flowers of *Rhododendron mucronulatum*(Ericaceae) and reported them to be a potent inhibitor of AR activity, quercitrin being most potent showing IC_{50} value 0.13 μg/ml (Mok & Lee, 2013). Similarly, 2'.4-dihydroxy-4'-methoxydihydro chalcone and davidigenin(Govorko et al., 2007; Logendra et al., 2006), erigeroflavanone(Yoo et al., 2008), butein, sulfuretin(Lee et al., 2008), engeletin and astilbin(Wirasathien et al., 2007) are also known to inhibit AR activity. From these, apigenin, guaijaverin, desmanthin-1 and luteolin inhibited AR with IC_{50} value in the range of 0.082-7.67 μM (Yoo et al., 2008; Matsuda, Morikawa, Toguchida, & Yoshikawa, 2002). In a separate study, cosmosiin and lonicerin were reported to be potent AR inhibitors with 99 and 91.5% inhibition of enzyme activity respectively (Shimizu et al., 1984).All these reports are based on *in-vitro* screening of these compounds against AR enzyme and further animal experimentation is needed to gain better understanding of their antidiabetic potential.

2. α-Glucosidase and α-Amylase

α-glucosidase (EC 3.2.1.20) is an exo-type α-glucosidic*O*-linkage-hydrolase enzyme located in the surface of intestinal villi and micro-villi. It catalyzes the final step in digestion of carbohydrates where it breaks oligosaccharides and α-glucans through hydrolytic reaction, which splits α-1,4 glycosidic bond between glucosyl residue and glucosidic oxygen followed by proton exchange reaction between water

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and glucosyl residue, thus giving absorbable D-glucose as the final product (Kim et al., 2010). α-amylase (EC 3.2.1.1.) is a calcium metalloenzyme present in saliva and pancreatic juice. It is responsible for the hydrolysis of α-D-(1,4)-glycosidic bond of starch, amylose, amylopectin, maltodextrins and glycogen, converting them to shorter oligosaccharides so that they can be absorbed into the body (Etxeberria, de la Garza, Campión, Martínez, & Milagro, 2012; Sales, Souza, Simeoni, Magalhães, & Silveira, 2012). Inhibition of these enzymes aids diabetes management by suppressing the absorption of glucose from the ingested meals and thus keeping the blood glucose level low.

Many flavonoids are capable of inhibiting α-glucosidase and α-amylase *in-vitro* which makes them an appropriate moiety for antidiabetic research (Table 2; Figure 2). Daidzein (Park, Ju, Park, & Han, 2013), trilobatin (Dong, Li, Zhu, Liu, & Huang, 2012), baohuoside I (Phan, Wang, Tang, Lee, & Ng, 2013) and luteolin (Kim, Kwon, & Son, 2000), inhibited both enzymes whereas saponarin (Sengupta, Mukherjee, Goswami, & Basu, 2009), pelargonidin-3-α-O-rhamnoside and cyanidin-3-α-O-rhamnoside (Cherian, Kumar, Augusti, & Kidwai, 1992; Hanamura, Hagiwara, & Kawagishi, 2005) are effective in inhibiting α-glucosidase only. Antidiabetic potential of majority of these findings needs to be further justified through *in-vivo* experimentation.

3. Antioxidant Pathway

Oxidative stress is characterized by excessive amount of ROS/RNS in the body either because of excessive production or insufficient removal from the body. ROS and RNS are highly reactive molecules which react with biomolecules such as proteins, lipids and DNA and consequently inflict enormous oxidative damage. Diabetes associated complications are attributed mainly to the involvement of superoxide ($^{\bullet}O_2^-$), nitric oxide ($^{\bullet}NO$), and peroxynitrite ($ONOO^-$) free radicals which are produced either through nitric oxide synthase pathway ($^{\bullet}NO$ and $ONOO^-$) or by NADPH oxidases and mitochondrial electron transport chain (Johansen, Harris, Rychly, & Ergul, 2005). Human body has its own defense mechanism against these free radicals in the form of SOD, GSH, glutathione peroxidase (GSH-Px) and catalase enzymes which convert them to non-toxic forms. SOD is present nearly in all cells and is the first line of defense against $^{\bullet}O_2^-$ converting it to H_2O_2, which is further, degraded by GSH-Px or catalase to non-reactive forms. GSH-Px and catalase are located in mitochondria and peroxisomes respectively and are responsible for detoxifying H_2O_2 (Fisher et al., 2006; Johansen, Harris, Rychly, & Ergul, 2005). During hyperglycemia ROS/RNS are produced excessively, thereby, antioxidant capacity of these defense enzymes gets saturated. This renders biological system open to the damaging effect of these free radicles and activates oxidative stress pathway. Generally five oxidative stress pathways are involved in diabetes and its complications (Figure 1).

3.1 The Polyol Pathway

In polyol pathway, NADPH is a co-factor which is responsible for regenerating antioxidant molecule, GSH. GSH is an important scavenger of ROS. During hyperglycemia, NADPH level is reduced markedly through polyol pathway because of which reduced glutathione fails to regenerate. This in turn exacerbate intracellular oxidative stress (Giacco & Brownlee, 2010). Further sorbitol dehydrogenase oxidizes sorbitol to fructose which activates PKC through elevated $NADH/NAD^+$ ratio causing redox imbalance mediated oxidative stress (Brownlee, 2001).

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3.2 Hexosamine Pathway

Fructose-6-phosphate (F6P) is the substrate for hexosamine oxidative stress pathway. Under normoglycemic condition almost entire F6P is involved in glycolysis and very little amount is available for hexoseamine pathway. However, hyperglycemic condition produces excessive F6P which ultimately becomes available for hexosamine pathway where glutamine-fructose-6-phosphate amidotransferase (GFAT) converts it to UDP-N-acetylglucosamine, an essential metabolite for producing glycosyl chains in proteins and lipids. O-glucosamine-N-acetyl transferases enzyme utilizes UDP-N-acetylglucosamine for making post-translational changes at serine and threonine residues of cytoplasmic and nuclear proteins involved in diabetes such as insulin receptor substrate(IRS), pancreatic duodenal homeobox 1 (PDX1), endothelial nitric oxide synthase (eNOS) and sp1, rendering them non-functional, leading to InR and other related complications (Buse, 2006; Dias & Hart, 2007; Giacco & Brownlee, 2010).

3.3 Diacylglycerol (DAG) and Protein Kinase C (PKC)

During diabetes, dihydroxyacetone phosphate level is elevated in body via glycolysis. It is then reduced to glycerol-3-phosphate which conjugates with fatty acids to chronically enhance the de novo synthesis of DAG. DAG activates the signaling cascade mediated through the activation of PKC, which is a serine/threonine kinase (Geraldes & King, 2010).PKC is also activated as a consequence of inhibition of glycolytic enzyme, glyceraldehide-3-phosphate dehydrogenase, (Brownlee, 2001; Giacco & Brownlee, 2010) and through interaction between AGEs and their extracellular receptors (Scivittaro, Ganz, & Weiss, 2000). PKC subsequently activates MAPK, NADPH oxidase and NF-κB, which upsurges oxidative and inflammatory stress induced diabetic complications (Ceriello, 2006).

3.4 Advanced Glycation End-Products (AGEs)

AGEs are associated with both T1DM (Chiarelli et al., 1999; Hwang, Shin, & Yang, 2005) and T2DM (Aso, Inukai, Tayama, & Takemura, 2000; Tan et al., 2002; Tan, Chow, Tam, Bucala, & Betteridge, 2004). Degree of association is related to the severity of diabetic complication which includes microvascular complications (Aso, Inukai, Tayama, & Takemura, 2000;Chiarelli et al., 1999; Wautier et al., 2003), macrovascular complications (Aso, Inukai, Tayama, & Takemura, 2000; Kilhovd et al., 2007; Kilhovd, Berg, Birkeland, Thorsby, & Hanssen, 1999), insulin resistance (Tan, Shiu, Wong, & Tam, 2011) and enhanced marker of inflammation and endothelial dysfunctioning (Tan, Chow, Tam, Bucala, & Betteridge,2004). AGE production is initiated by nucleophilic addition reaction between amino group of protein and carbonyl group of a reducing sugar, forming a Schiff base, which rearranges itself to more stable ketoamine or amadori product. Initial part of reaction is reversible as the concentration of reactants is low and thus under normoglycemic condition Schiff base breaks into its reactants in reversible manner. However during hyperglycemia, excessive reactant concentration leads to the formation of more stable amadori product, which is non-reversible reaction. Further, glycated proteins undergo a series of dehydration, oxidation-reduction and other rearrangements reactions to produce AGEs. Interaction of AGE and its receptor depends upon the load of the AGE available. Under normoglycemic conditions, when AGEs are low, the expression of receptor for advanced glycationendproducts (RAGE) is downregulated and expression of advanced glycationendproduct receptor-1 (AGER1) is upregulated. AGER1 activation has inhibitory effect on the RAGE mediated activation of transcription factors nuclear NFκB, activator

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protein 1 (AP-1) and forkhead box protein-O-subclass (FOXO) and consequently transcription of the genes initiated by them. Besides inhibiting RAGE activity, AGER1 also initiate degradation process of AGE itself. The entire scenario changes during hyperglycemic state when AGE are produced excessively, causing upregulation of RAGE and downregulation of AGER1, leading to the activation of inhibited transcription factors and subsequent transcription of genes related to oxidative and inflammatory stress (Bansal et al., 2013; Morita, Yano, Yamaguchi, & Sugimoto, 2013; Nass & Simm, 2009).

3.5 Stress-Sensitive Signaling Pathways

Apart from inflicting damage on biomolecules, oxidative stress is known to be involved in the activation of various stress sensitive signaling cascade which ultimately result in the enhanced cytokine release, inflammation and apoptosis. These signaling mechanisms mainly involve MAPK, stress-activated protein kinase (SAPK) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway during diabetes. p. 38 MAPK is a regulatory protein activated through chemokines, neurotransmitters, radiation, physical, and chemical stresses including oxidative stress. Hyperglycemia enhance the activity of p38 MAPK in vascular smooth muscle cells, retinal pericytes (Igarashi et al., 1999), and mesangial cells where it regulates inflammation, differentiation and cellular proliferation through transcription of its specific genes. Further, inhibition of p38 MAPK reduces apoptosis (Liu et al., 2003),arachidonic acid release (Igarashi et al., 1999) and improves sensory nerve conduction (Agthong & Tomlinson, 2002) in diabetes along with enhancing translocation of the insulin-responsive GLUT4 to the cell membrane (Carlson, Koterski, Sciotti, Poccard, & Rondinone, 2003). Furthermore, oxidative stress activates SAPK and JAK/STAT signaling cascade which inflicts inflammatory stress induced damage by enhancing cellular proliferation, apoptosis and inflammatory response along with sensitizing cellular response to interferons and interleukins (Gomperts, Kramer, & Tatham, 2009).

Flavonoids possess antioxidant property and many of them are also known to possess significant anti-diabetic potential (Table 3; Figure 3). Quercetin improved blood glucose profile, body weight, macrophage count and biochemical profile in alloxan induced diabetic rats. Though its exact antidiabetic mechanism still remains unclear but prevention of DNA damage, antioxidant and anti-inflammatory property suggest that it probably act by reducing oxidative stress (Bhutada et al., 2010; Maciel et al., 2013; Oršolić et al., 2011). Bucolo et al., (2012) evaluated effect of eriodictyol on streptozotocin (STZ) induced diabetic rats and reported it to attenuate inflammation, lipid peroxidation and reduce production of inflammatory mediator viz. TNF-α, intercellular adhesion molecule-1 (ICAM-1), vascular endothelial growth factor (VEGF), and eNOS level in retina (Bucolo, Leggio, Drago, & Salomone, 2012). Diosmin normalized oxidative status by elevating plasma level of SOD, catalase, GSH-Px, glutathione-S-transferase (GST) and non-enzymatic antioxidants such as vitamin C and vitamin E. Further, it was reported to reduce plasma glucose and glycosylated haemoglobin (Hb1Ac) levels and elevate insulin and hemoglobin level (Pari & Srinivasan, 2010; Srinivasan & Pari, 2012). Epigallocatechin-3-O-gallate eliminated oxidative stress by enhancing SOD and GSH-Px enzyme activity and by reducing malondialdehyde (MDA) and 8-isoprostaglandin plasma levels in Wistar rats. It also reduced free fatty acid (FFA) level thereby abolishing InR, probably through inhibiting lipolysis (Li et al., 2011). Fisetin and luteolin reduced the levels of blood glucose and HbA1c and increased plasma insulin level. They further reduced the circulating levels of lipid peroxides and hydroperoxides in hepatic tissues and normalizing altered alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), SOD, catalase, ROS, GSH-Px, GST and GSH activity. Thus, they seems to alleviate diabetes induced oxidative stress to

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counteract its complications (Kim, Kwon, & Son, 2000; Liu et al., 2013; Prasath & Subramanian, 2013; Qian et al., 2010; Wang, Lu, Li, Zhao, & Zhang, 2011). Treating STZ-diabetic rats with hesperidin, naringin, epicatechin and rutin showed reduction in blood glucose, HbA1c, thiobarbituric acid reactive substances (TBARS), MDA, nitric oxide (NO), TNF-α, IL-6 level and lipid peroxides in serum and hepatic tissue with enhanced GSH, catalase, GSH-Px, and SOD enzymatic activity. Though not clear, but their antidiabetic potential also seems to be mediated by eliminating oxidative stress (Kamalakkannan & Prince, 2006; Mahmoud, Ashour, Abdel-Moneim, & Ahmed, 2012; Quine & Raghu, 2005). *In-vitro* studies conducted on puerariafuran, coumestrol, sulfuretin, astilbin, pelargonidin-3-α-O-rhamnoside and cyanidin-3-α-O-rhamnoside reports significant inhibition of AGE, which is a key mediator of diabetic complications (Hanamura, Hagiwara, & Kawagishi, 2005; Jang et al., 2006; Lee et al., 2008; Wirasathien et al., 2007). 4,2',4'-trihydroxy-3'-[6-hydroxy-3,7-dimethyl-2(E),7-octadienyl] chalcone, 3,4,2',4'-tetrahydroxy-3'-geranyldihydrochalcone and 4,2',4'-trihydroxy-5'-geranyldihydrochalcone inhibited mRNA expression of RAGE, IL-6, TNF-α and cyclooxygenase-2 (COX-2) and reduced ROS generation along with improving mitochondrial morphology and functioning in diabetic animals (Lin, Wu, Fang, & Yen, 2012). Pelargonidin and glabridin facilitated oxidative defense of body by increasing SOD activity and lowering MDA levels in different tissues of diabetic animals (Mirshekar, Roghani, Khalili, Baluchnejadmojarad, & Moazzen, 2010; Wu, Jin, & Jin, 2013). Similar elevation of antioxidant status in diabetic animals after apigenin and puerain treatments had been reported previously (Rauter et al., 2010; Yoo et al., 2008; Zhu et al., 2010).

4. Insulin Signaling

As discussed earlier, Insulin is responsible for maintaining normal glucose homeostasis in the body, which is achieved through binding of insulin to α-subunit of IR and subsequent auto-phosphorylation of its tyrosine residue on β-subunit. This triggers phosphorylation and activation of insulin receptor substrates proteins 1-4 (IRS1-4), Shc, Gab-1, Cbl, and APS (Saltiel & Pessin, 2002), and switches "on" downstream signaling cascade by activating numerous intermediate effectors, which ultimately leads to the activation of protein kinase B (PKB)/Akt.Akt, a serine-threonine kinase, enters nucleus to induce transcription and translocation of GLUT4 to the membrane. Akt independent pathway are also known to be involved in the translocation of GLUT4 via phosphorylation of proto-oncogene Cbl, thereby aiding in the disposal of glucose (Cole, Astell, Green, & Sutherland, 2007; Plum, Schubert, & Brüning, 2005; Saltiel & Kahn, 2001). PKB is known to promote fatty acid synthesis, protein synthesis, glycogen synthesis, PIP3-dependent kinase (PDK) mediated inhibition of lipolysis through cyclic adenosine monophosphate(Camp)/protein kinase A(PKA) pathway and thus aids in establishing glucose homeostasis (Proud, 2006).

Since insulin/insulin signaling is directly involved in regulating blood glucose level, it is thus the most important target for the management of diabetes. Many flavonoids have been reported to effectively counter diabetes by their direct action on insulin receptor and by modulating insulin signaling (Table 4; Figure 4). Apigenin-6-C-β-fucopyranoside enhances insulin signaling and produces subsequent dose dependent disposal of blood glucose into liver and muscles of diabetic animals (Cazarolli et al., 2012). Karanjin enhances GLUT4 translocation and AMP-activated protein activity to produce antidiabetic effect inL6-GLUT4 mycmyotubes (Jaiswal, Yadav, Maurya, Srivastava, & Tamrakar, 2011). Myricetin was reported to enhance phosphorylation of IRS1 and Akt. It further elevated protein level of IRS1, GLUT4 and phosphatidylinositol-3 kinase (PI3K) with subsequent reduction in blood glucose and

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HbA1c levels (Liu, Tzeng, Liou, & Lan, 2007). Puerarin upregulated IRS-1 and insulin-like growth factor-1 (IGF-1) expression and thus reduce circulating blood glucose level in STZ challenged rats (Wu et al., 2013). Baicalein was reported to enhance phosphorylation and activation of the IRS1/PI3K/Akt pathway thereby improving insulin signaling. Further, it abolished InR and improved glucose intolerance (Pu et al., 2012). Morin activate and potentiate insulin signaling on HepG2 cells (Paoli et al., 2013) and thus can be a molecule of interest for future anti-diabetic research. Two flavonol glycosides isolated from *Cinnamomumosmophloeum* leaves showed promising result in an *in-vitro* model for mimicking insulin action. They enhanced GLUT4 translocation and phosphorylation of IRβ-subunit (Lee, Rao, Chen, Lee, & Tzeng, 2009). Rutin showed antidiabetic potential by increasing glucose uptake into soleus muscle through activation of PI3K and MAPK pathway (Kappel, Zanatta, Postal, & Silva, 2013). Epigallocatechin-3-O-gallate improved insulin signaling and InR by enhancing AMPK level, Akt level and GLUT4 translocation in skeletal muscle and adipose tissue in diabetic animals (Li et al., 2011). *In-vitro* and *in-vivo* studies conducted on tangeretin reported its ability to improve insulin signaling and translocation of GLUT4 to cellular membrane. This action may be a result of enhanced phosphorylation of AMPK, which itself is a key component of insulin signaling cascade (Kim, Hur, Kwon, & Hwang, 2012). Antidiabetic reports of all these molecules are just preliminary and needs to be explored further so as to understand the detailed mechanism through which they control diabetes.

5. Peroxisome Proliferator-Activated Receptors (PPAR) Receptor

PPARs belong to the nuclear receptor superfamily of transcription factors which have been extensively exploited as therapeutic targets for T2DM and dyslipidemia in resent past. PPARs consist of three different subtype of receptors i.e. PPAR-α, PPAR-γ and PPAR-α/γ amongst which PPAR-γ plays an important role in T2DM because of its influential effect on fatty acid flux from adipose tissue which alleviates InR and reduces blood glucose levels. PPAR-α on the other hand has lipid modifying properties. Upon activation, PPAR-γ conjugates with retinoid-X receptors (RXRs) forming a heterodimer (PPAR-RXR). This heterodimer migrates to nucleus and binds to specific DNA sequence, peroxisome proliferator–response elements, and initiate the transcription of GLUT4 genes. PPAR-α has regulatory effect on genes controlling the fatty acid uptake, oxidation process, inflammation, and vascular function whereas PPAR-γ regulates genes for fatty acid uptake and storage, inflammation and glucose homeostasis in the body (Mello, Polvani, & Galli, 2009; Plutzky, 2011).

Non-responsiveness of fat cells to insulin results in chronic elevation in plasma level of FFA, which in turn induces resistance towards insulin in liver and skeletal muscles. This further induces hyperglycemic state by increasing gluconeogenesis and reducing glucose disposal into these organs. Resistant fat cells produces excessive amount of cytokines (TNF-α, IL-6 and restin) which further adds up to InR and inflammatory stress. It has been observed that about 70% T2DM patients have some degree of dyslipidemia (Fagot-Campagna et al., 2000) which is characterized by increased triglycerides (TGs) and reduced high density lipoprotein (HDL) cholesterol levels (Malmberg et al., 2000). PPAR-γ has direct effect on lipid and glucose metabolism in adipose tissue, liver and skeletal muscles. PPAR-γ promotes differentiation of adipocyte, FFA uptake and facilitates its storage in subcutaneous adipose tissue rather than visceral adipose tissue. This lowers FFA level in blood and restores insulin sensitivity. It further enhances glucose disposal into liver and skeletal muscle by increasing expression and translocation of glucose transporters (GLUT1/GLUT4) thereby reducing the level of blood glucose (Kramer, Shapiro, Adler, Bush, & Rondinone, 2001).

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In recent past, few flavonoids have shown good potential to upregulate PPAR-γ receptor and thus they alleviated InR and reduced hyperglycemia and associated complications (Table 5; Figure 5). Isoginkgetin(Liu et al., 2007), luteolin(Ding, Jin, & Chen, 2010; Puhl et al., 2012) and other flavonoids isolated from *G. glabra*(Kuroda et al., 2010) are reported to upregulate PPAR-γ receptor in various *in-vitro* and *in-vivo* studies, developed for the management of InR in T2DM and its associated complications.

6. Glucose Metabolic Pathway

Glucose is the primary energy source of our body. After entering cells, it is oxidized to generate ATPs through glycolysis. Glucose is first converted to G6P by the reaction catalyzed by hexokinase which is further converted to F6P by phosphoglucoisomerase enzyme. F6P, thus formed, either enters pentose phosphate pathway leading to the generation of NADPH or is converted into glyceraldehyde-3-phosphate by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to yield pyruvate as an end product of glycolysis through series of reactions. Pyruvate now enters Kreb's cycle and mitochondrial metabolism to further enhance energy production in the form of ATP (Nelson, Lehninger, & Cox, 2008).

During hyperglycemia, ROS generated by mitochondria inhibits GAPDH and activates poly-ADP-ribose polymerase-1 (PARP-1) enzyme which is involved in DNA repair and apoptotic pathways (Brownlee, 2001; Brownlee, 2005; Giacco & Brownlee, 2010). DNA damage inflicted by ROS activates PARP-1 which consumes NADPH excessively thereby elevating oxidative stress as discussed above. GAPDH inhibition (Brownlee, 2005) results in accumulation of glyceraldehyde-3-phosphate which in turn activates two major pathways of diabetic complications, AGE and DAG pathway. In AGE pathway, excess of glyceraldehyde-3-phosphate is converted to methylglyoxal through non enzymatic process. DAG activates PKC pathway and inflicts subsequent damage as discussed previously. GAPDH inhibition further leads to the accumulation of F6P upstream which enter hexosamine pathway and is converted to UDP–N-Acetylglucosamine by GFAT resulting in various diabetic complications. Lastly, GAPDH inhibition results in accumulation of glucose which becomes available to polyol pathway in excess and initiate diabetes induced damage (Giacco & Brownlee, 2010).

Interfering with the enzymes of the glucose metabolic cycles so as to reduce the blood glucose level is another mechanism through which antidiabetic activity of many flavonoids has been observed (Table 6; Figure 3). Diosmin (Pari & Srinivasan, 2010), eupatilin (Kang et al., 2008)and biochanin A (Harini, Ezhumalai, & Pugalendi, 2012) reduced the activity of glucose-6-phosphatase, fructose 1,6-bisphosphatase, phosphoenol-pyruvate carboxykinase and increased activity of glucokinase, glucose-6-phosphate dehydrogenase in diabetic animals, and thereby controlling hyperglycemia.

7. Other Targets

7.1 Protein-Tyrosine Phosphatase 1B (PTP1B)

PTP1B is a member of protein tyrosine phosphatases family of enzymes and is reported to be linked to InR and T2DM by several experimental studies. These studies demonstrated that PTP1B dephosphorylates the IR and IRS-1 and thus acts as a negative regulator of insulin signaling (Bandyopadhyay et al., 1997; Goldstein, Bittner-Kowalczyk, White, & Harbeck, 2000). Overexpression of PTP1B in cell cultures lowered insulin mediated phosphorylation of IR and IRS-1whereas reduction in PTP1B level enhanced insulin signaling (Byon, Kusari, & Kusari, 1998; Kenner, Anyanwu, Olefsky, & Kusari, 1996;

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Sreejayan, Lin, & Hassid, 2002; Tagami et al., 2002). Recently, studies conducted on PTP1B knockout mice clarified its involvement in insulin signaling, T2DM, inflammation and obesity (Grant et al., 2014) thus making it as a promising target for antidiabetic therapy. Secondly, PTP1B is known to down regulate leptin, which is an adipocyte-secreted hormone responsible for reducing food intake and enhancing energy expenditure through its action on hypothalamus. PTP1B binds and dephosphorylates JAK2, which is required for downstream signaling of leptin pathway (Lund, Hansen, Andersen, Møller, & Billestrup, 2005; Zabolotny et al., 2002). Further, neuronal PTP1B regulates leptin production in adipocytes and leads to the development of leptin resistance (Bence et al., 2006). Thus, impairment of leptin signaling by PTP1B over activity also adds on to the progression of diabetes and its associated complications.

Flavonoids capable of inhibiting PTP1B are listed in Table 7. 2',4-dihydroxy-4'-methoxydihydro chalcone and 2',4'-dihydroxy-4'-methoxydihydro chalcone, isolated from *A. dracunculus*, lowered PTB1B activity by 36% and 26% respectively with previous one having IC_{50} value of 61 μM (Wang et al., 2006). Morin was also reported to possess PTP1B inhibitory activity through *in-vitro* assay (Paoli et al., 2013). PTP1B signaling, as indicated, plays effective role in progression of diabetes and thus these compounds can be looked upon as potential agents to explore this target for controlling diabetic complications.

7.2 Sodium Glucose Co-Transporter 2 (SGLT2)

In healthy human being, kidney filters approximately 180g glucose/day which is completely reabsorbed by the highly selective sodium coupled glucose transporters, SGLT2 and SGLT1, located in the proximal tubule of the kidney. Out of these two, 90% of the glucose is reabsorbed by SGLT2 and remaining by SGLT1 (Gerich & Bastien, 2011). Therefore, inhibition of SGLT2 increases urinary glucose excretion and reduces blood glucose level in hyperglycemic condition in insulin independent manner. Recent clinical studies conducted on canagliflozin, a SGLT2 inhibitor, demonstrated its exceptional therapeutic efficacy and safety in diabetic patients in comparison to placebo and other established antidiabetic drugs (Cefalu et al., 2013; Lavalle-Gonzalez et al., 2013; Wilding et al., 2013).

SGLT2 inhibiting potential of flavonoids is yet to be demonstrated experimentally, either *in-vivo* or *iv-vitro*, though luteolin had shown potential to inhibit SGLT2 through *in-silico* docking study using 2QX2 PDB (Table 7) (Annapurna et al., 2013). However, *in-silico* studies cannot be considered conclusive and experimental justification needs to be established.

7.3 Nuclear Factor Kappa B (NFκB)

As discussed earlier, NF-κB is a cytoplasmic transcription factor which on activation migrates to nucleus and regulates the expression of genes for TNF-α, tissue factor, cell adhesion molecules and chemotactic proteins. Along with PARP, it activates inducible-NOS (iNOS), intercellular Adhesion Molecule-1 (ICAM-1) and histocompatibility complex class II, and thereby enhancing inflammatory damage. Activation of NFκB in endothelial cells induces apoptosis (Du, Stocklauser-Farber, & Rosen, 1999; Gomperts, Kramer, & Tatham, 2009). A study conducted by Kim et al. (2007) demonstrated that inhibition of NFκB signaling through activation of PPAR-γ protects pancreatic β-cells from cytokines induced cytotoxicity/cell death. In animal experiments, NFκB inhibition is known to provide protection against chemical induced diabetes (Ho, Chen, & Bray, 2000; Park & Park, 2001). Recently, NFκB was reported to play crucial

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role in diabetic cardio-vascular complication and its suppression by various agents provided beneficial outcomes in these complications (Parra et al., 2014; Xiang, Wang, Zhang, Ling, & Xu, 2012). Further, inhibition of NFκB protected apoptosis of cardiomyocytes via reduction in ROS generation (Kuo et al., 2013). These reports thus make NFκB an auspicious target for management of diabetes.

Two flavonoids (Table 7), baicalein (Pu et al., 2012)and scutellarin (Luo et al., 2008), were reported to counteract hyperglycemia elicited metabolic abnormalities and vascular inflammation directly by interfering with the expression and signaling mechanism of NFκB. Thereby, they can be useful in impeding the progression of diabetic complications, especially cardio-vascular.

7.4 Jun N-Terminal Kinase (JNK) Signaling

JNK signaling is activated by cytokines, endoplasmatic reticulum stress and hyperlipidemia, all of which are elevated in DM (Hotamisligil, 2010). Insulin gene expression is markedly reduced by the activation of JNK signaling pathway through oxidative stress and JNK inhibition protected pancreatic cells from oxidative damage (Kaneto et al., 2002; Kim et al., 2010). Recently, Norseen et al. (2012) demonstrated that retinol binding protein-4 play an important role in insulin signaling and development of InR. This action was attributed to the involvement of JNK signaling pathway via its effect on pro-inflammatory cytokines Evidence suggest that, elevated JNK level reduces insulin sensitivity (Ye, Xue, Sha, Li, & Zhen, 2009) whereas lower levels enhance the utilization of fatty acids thereby improving insulin sensitivity. Further, it diverts glucose from oxidative pathway towards glycogenesis, and thus, lowered blood glucose level and subsequent oxidative stress (Vijayvargia, Mann, Weiss, Pownall, & Ruan, 2010).

Pu et al., (2012) reported that baicalein alleviated diabetes and counteracted its complications through insulin and NFκB signaling. Same study also depicted that along with insulin and NFκB signaling, JNK signaling cascade may also play crucial role in the development of diabetic complications (Table 7) (Pu et al., 2012). Thus inhibition of JNK signaling may also prove useful in countering diabetic complications.

8. Unknown Mechanisms

Many flavonoids have been screened for their antidiabetic activity through preliminary *in-vitro* and *in-vivo* studies but the exact mechanism through which they benefit diabetic complications is still not clear (Table 8). Chrysoeriol reduced circulating glucose level, improved glucose tolerance, ALT and AST level of kidney and liver (Rauter et al., 2010). Naringenin prevented DNA damage in the alloxan treated animals through unknown mechanism (Oršolić et al., 2011). Quercetin-3'-methoxy-3-O-(4''-acetylrhamnoside)-7-O-α-rhamnoside, kaempferol-4'-methoxy-3.7-dirhamnoside and isorhamnetin-3-O-β-D-glucoside enhanced *in-vitro* glucose disposal into myotubes and thus can be assumed to show same effectiveness in animal models as well (Motaal, Ezzat, & Haddad, 2011). Silymarin improved body weight and hepatic glycogen level along with reducing daily urinary output volume in STZ treated rats. It further normalized MDA, NO, total thiol, AST and ALT level, improved histopathological profile of liver, downregulated cytochrome P450-3A2 (CYP 3A2) and upregulated GSH-Px expression. Though their effect can be attributed to the alleviation of oxidative stress but still no clear-cut observation is there (Malekinejad, Rezabakhsh, Rahmani, & Hobbenaghi, 2012). Kaempferol 3-*O*-α-(2''-galloyl) rhamnoside, quercetin-3-*O*-α-rhamnoside, kaempferol 3-*O*-α-rhamnoside, quercetin 3-*O*-α-arabinoside

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and quercetin 3-*O*-α-(2″-galloyl) rhamnoside were reported to inhibit rat liver microsomal glucose-6-phosphatase enzyme. Glucose-6-phosphatase is a key enzyme involved in the glucose metabolic cycle and its regulation affects the accumulation or disposal of glucose from the circulation. Thus these molecules can be further subjected to *in-vivo* antidiabetic study to test their activity in living system (Estrada et al., 2005). 4-hydroxyderricin and xanthoangelol were reported to enhance differentiation of 3T3-L1 cells to adipocytes and increase glucose disposal into these cells. They also reduced blood glucose level and alleviated polydipsia in diabetic KK-A^y mice through unknown mechanism (Enoki et al., 2007). Likewise delphinidin had shown effectiveness in controlling hemoglobin and albumin glycosylation in *in-vitro* and *in-vivo* study and may be considered as a good candidate for future diabetic research (Gharib, Faezizadeh, & Godarzee, 2013). Genistein improved glucose tolerance, reduced circulating glucose and modified the levels of ALT, AST, SOD and MDA in STZ induced diabetic rats (Baluchnejadmojarad & Roghani, 2008; Rauter et al., 2010). Kaempferitrin reduced blood glucose level in alloxan induced diabetic rats by enhancing glucose disposal into soleus muscle (Jorge, Horst, de Sousa, Pizzolatti, & Silva, 2004). Only preliminary studies have been conducted on these molecules and majority of them are reported to normalize altered biochemistry and hematological parameters, which are generally seen in diabetes. These results cannot be considered conclusive and cannot be attributed to any single mechanism and require further investigation.

CONCLUSION

Current chapter discusses the impact of diabetes on global health and current strategies that are being used to counter diabetes and its associated complications. It is well known that the currently available diabetic therapeutics is not capable of preventing or reversing the complications associated with DM. In this regard, current focus of diabetic research is to identify and counter mechanisms responsible for the development of these complications. Oxidative stress, in form of ROS/RNS that are generated excessively during hyperglycemia, is one of the major reasons for development and progression of diabetic complications. Evidence suggests that antioxidants are capable of preventing and reversing these secondary complications in early stages in diabetic animals (Abiko et al., 2003, Koya, Lee, Ishii, Kanoh, & King, 1997) and also prevent the hyperglycemia induced biological changes within the body (Greene, Nelson, Robinson,& Buse, 2001;Montero et al., 2000;Studer, Craven,&DeRubertis, 1997).

Flavonoids are natural antioxidants and are well known for their antioxidant potentials. Many of them, such as quercetin, rutin, etc., had been reported to counter diabetes and its complications through enhancing oxidative defense and other mechanisms. Flavonoids may benefit in diabetes by abolishing oxidative stress induced damage by eliminating free radicals and by potentiating oxidative defense of the body. Exceptional beneficial effect of flavonoids become evident from findings that suggests that enhanced intake of diets rich in polyphenols lowers risk of diabetes and development of its complications by 80% (Steinmetz & Potter, 1996; Law & Morris, 1998; Anderson, Anthony, Cline, Washburn, & Garner, 1999; WHO, 2003). Thus, it can be concluded that effective and efficient diabetic therapeutics can be achieved by coupling traditional diabetic treatment strategies together with flavonoids. Flavonoids can be used as dietary supplements and may prove to be efficient in eliminating development and progression of diabetes and its associated complications.

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KEY TERMS AND DEFINITIONS

Aldose Reductase: Aldose reductase is an oxidoreductase enzyme responsible for catalyzing biochemical reaction that leads to detoxification of aldehydes and carbohydrates, especially glucose.

Antioxidant: Substance that inhibits oxidation is referred to as antioxidant. In biological system, any substance which either directly inhibits oxidative stress or indirectly facilitates regeneration or formation of antioxidant enzymes is referred to as antioxidant.

Diabetic Complications: Complications in the biological system that arise secondary to long standing diabetes are known as diabetic complications. They include neuropathy, nephropathy, retinopathy, etc.

Flavonoids: Flavonoids are 15 carbon polyphenolic secondary metabolites of plants having two aromatic rings linked by a 3 carbon bridge i.e. C6-C3-C6 linkage. Flavonoids are mainly responsible for antioxidant property of the plants.

Herbal Therapy: Whole plant or its any part or extract or other such preparations or isolated compound or mixture of these when used for preventing or curing any disease or disease condition, such therapy is referred to as herbal therapy.

Insulin: Insulin is a 51 amino acid peptide hormone produced in the β-cells of pancreas. It is responsible for maintaining normal blood glucose level in the body.

Type 2 Diabetes Mellitus: The most common type of diabetes, characterized by insulin resistance leading to hyperglycemia. Thus it is also known as non-insulin-dependent diabetes.

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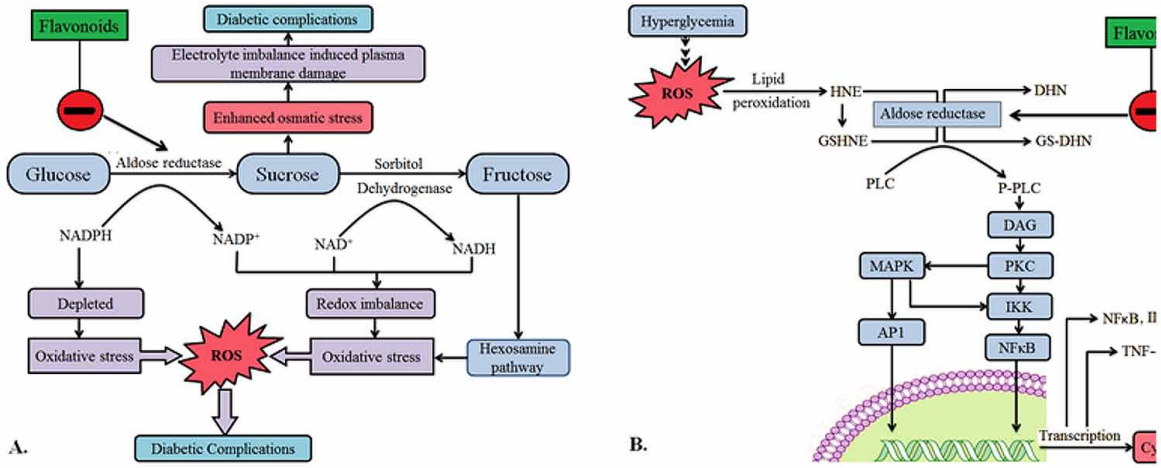
APPENDIX: FIGURES AND TABLES

In Figure 1, (A) AR converts glucose into sorbitol which directly leads to retinopathy by inflicting osmotic stress. This process consumes and depletes antioxidant NADPH, leading to excessive oxidative stress induced damage at cellular and molecular levels. (B) AR is also responsible for the detoxification of aldehydes from the body. This process leads to the transcription of excessive inflammatory stress markers, causing cytotoxicity through DAG-PKC-NFκB-MAPK pathway. Flavonoids are known to inhibit AR activity thereby prevents the development of subsequent damage inflicted by hyperglycemia.

Dietary polysaccharides are broken down into absorbable monomers (D-glucose) by α-glucosidase and α-amylase in small intestine. Flavonoids inhibit these enzymes and thus polysaccharides remain in non-absorbable form which automatically reduces the dietary glucose uptake and hyperglycemia as shown in Figure 2.

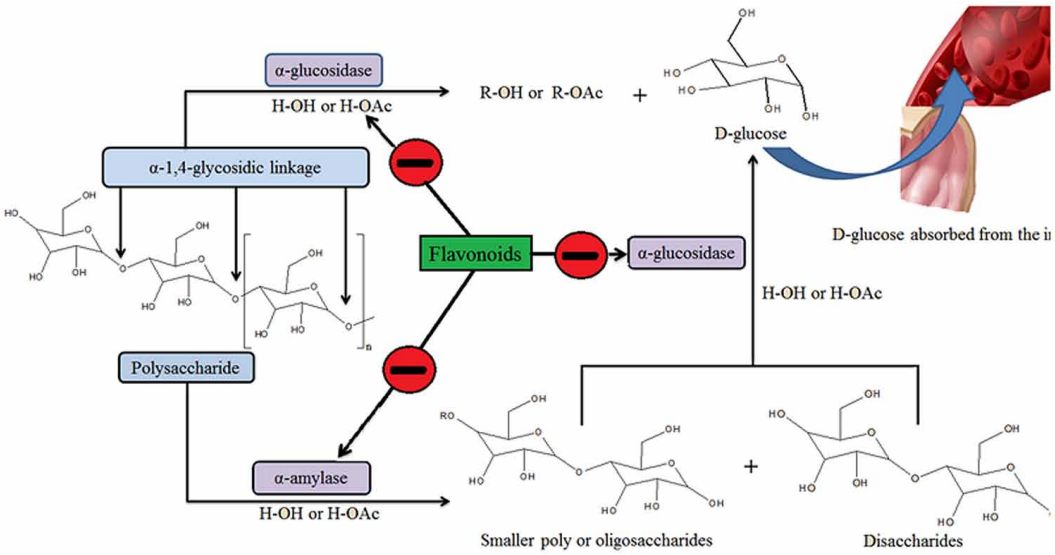
Glucose is the primary energy source for every cell of our body. Glucose is oxidized to produce ATP, through various biochemical processes (glycolysis, Kreb's cycle and polyol pathway). During hyperglycemia, oxidation of excess glucose generates enormous ROS and AGE through various metabolic pathways. These highly reactive molecules reacts with biomolecules (proteins, RNA, DNA etc.) thereby compromising their biological functions leading to various diabetic complications. Flavonoids are natural antioxidants and help to reduce oxidative stress by direct free radical scavenging ability, directing excess glucose to glycolysis and Kreb's cycle for energy production and by blocking various pathways that leads to generation of ROS and AGE (Figure 3).

Figure 1. Role of AR and its inhibition by flavonoids in the development of diabetic complications



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Figure 2. Role of α-glucosidase and α-amylase and their inhibition by flavonoids in diabetes



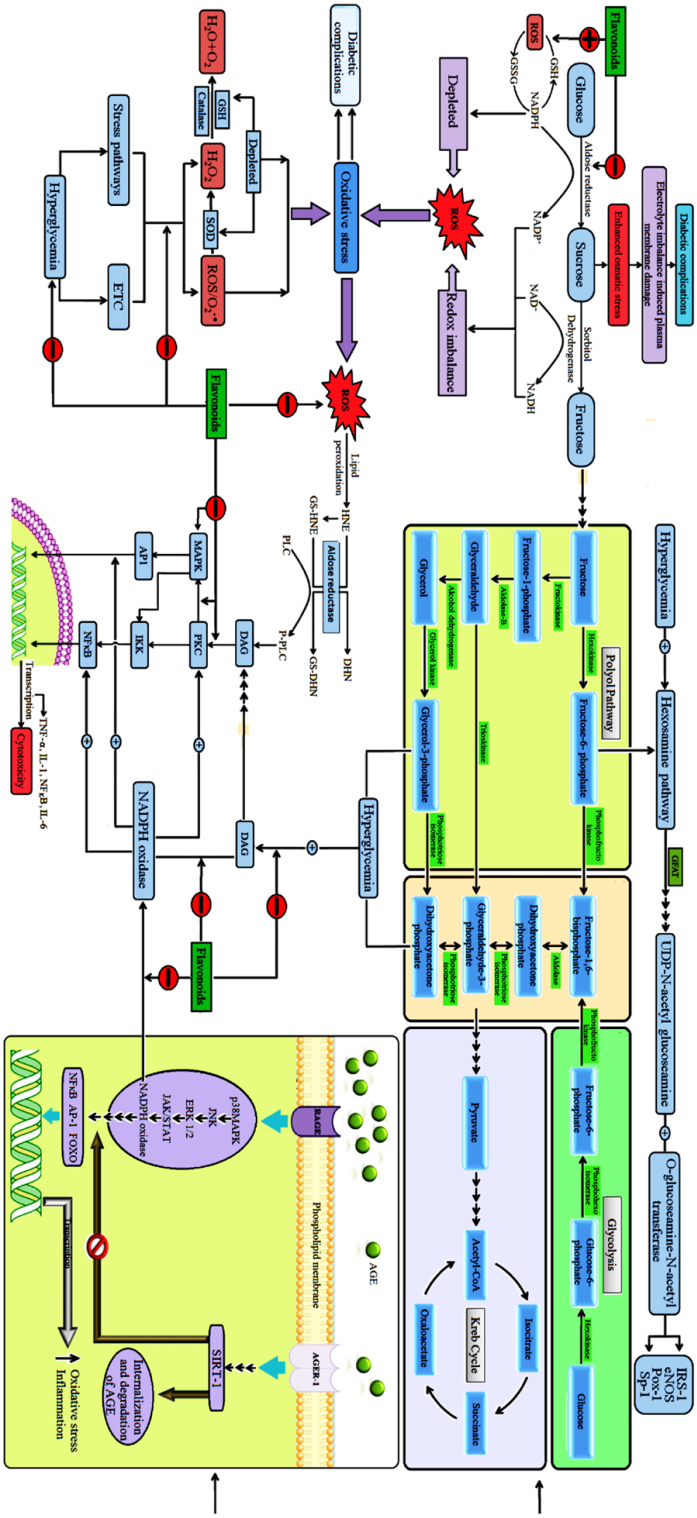
Insulin is responsible for maintaining glucose homeostasis by potentiating transcription and translocation of GLUT4 to cell membrane via multistep signaling cascade and thereby disposing blood glucose into skeletal muscle (energy production), adipose tissue (stored as fat) and liver (converted to glycogen). Flavonoids potentiate insulin signaling and GLUT4 translocation thereby enables disposal of glucose from the blood and reduces hyperglycemia (Figure 4).

PPAR-γ on activation binds to RXR forming an activated dimer which binds to specific target genes and initiates transcription of insulin receptor protein. IR is then translocated to cell membrane where it binds to insulin and result in insulin mediated disposal of glucose into skeletal muscle, adipose tissue and liver. Flavonoids binds to PPAR-γ and initiates dimerization with RXR and initiate further downstream signaling to ultimately dispose excess glucose from the blood (Figure 5).

Tables 1-8.

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Figure 3. Schematic representation of the involvement of oxidative stress (ROS/AGE) and glucose metabolic pathways in diabetes and role of flavonoids in protecting against oxidative stress



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Figure 4. Schematic representation of insulin signaling and its potentiation by flavonoids for the management of diabetes

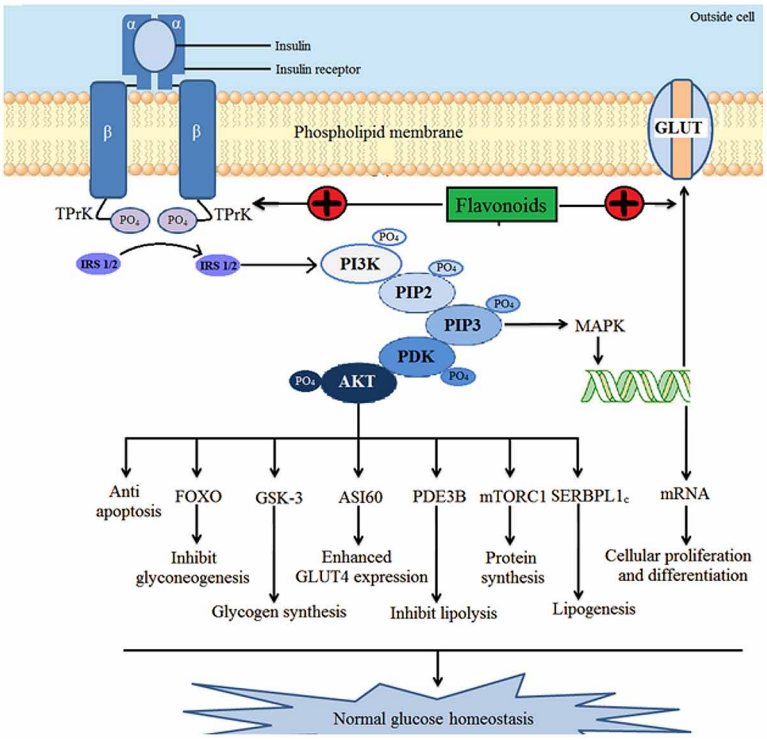
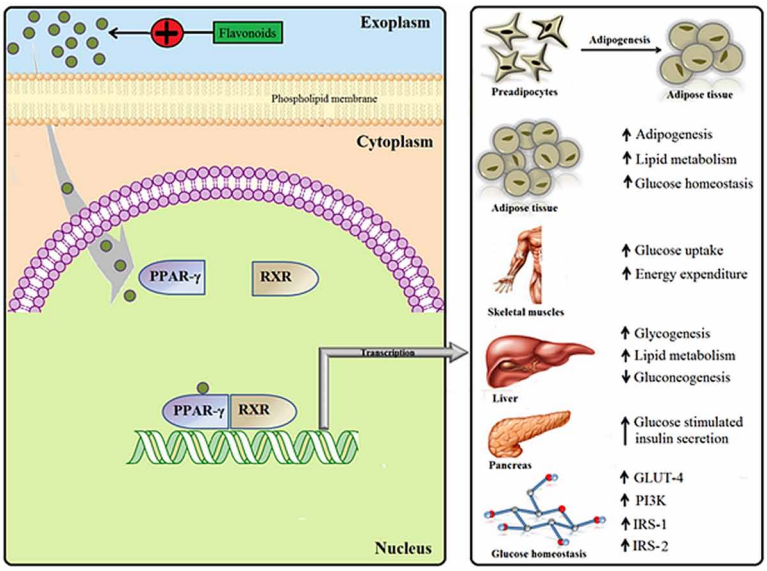


Figure 5. Schematic representation of the role of PPAR-γ and its potentiation by flavonoids in the management of diabetes



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Table 1. List of flavonoids having AR inhibitory activity

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
1.	Quercetin; Fruits, vegetables etc.	*In-vitro* 01, 0.5 and 1 µg/mL	Inhibition of AGE formation and rat lens AR assay	2. IC_{50} = 0.48 µg/mL and 7.21 µM)	Mok & Lee, 2013; Yoo et al., 2008
2.	Kaempferol; *Rhododendron mucronulatum* (Ericaceae)*; Erigeron annuus* (Asteraceae)	*In-vitro* 0.5, 1 and 5 µg/mL	Inhibition of rat lens AR assay	2. IC_{50} = 0.79 µg/ml and 1.33 µM	Mok & Lee, 2013; Yoo et al., 2008
3.	Afzelin; *R. mucronulatum* (Ericaceae)	*In-vitro* 01, 0.5 and 1 µg/mL	Inhibition ofrat lens AR assay	2. IC_{50} = 0.31 µg/mL	Mok & Lee, 2013
4.	Myricitrin; *R. mucronulatum* (Ericaceae)	*In-vitro* 0.5, 1 and 5 µg/mL	Inhibition of rat lens AR assay	2. IC_{50} = 2.67 µg/mL	Mok & Lee, 2013
5.	Quercitrin; *R. mucronulatum* (Ericaceae)	*In-vitro* 0.05, 0.01 and 1 µg/ mL	Inhibition of rat lens AR assay	2. IC_{50} = 0.13 µg/mL, 0.16 µM and 50.15 µM	Mok & Lee, 2013; Yoo et al., 2008; Matsuda et al. 2002
6.	Myricetin; *R. mucronulatum* (Ericaceae)	*In-vitro* 1, 5 and 10 µg/mL	Inhibition of rat lens AR assay	2. IC_{50} = 11.92 µg/mL	Mok & Lee, 2013
7.	2',4-dihydroxy-4'-methoxydihydro chalcone; *Artemisiadracunculus* (Asteraceae)	*In-vitro* 3.75 µg/ml	Human recombinant AR2 enzyme inhibition assay	2. Inhibited AR activity (58.2% inhibition)	Logendra et al., 2006
8.	Davidigenin; *A. dracunculus* (Asteraceae)	*In-vitro* 3.75 µg/ml	In-vitro human recombinant AR2 enzyme assay	2. IC_{50} = 10 - 12.70 µM	Logendra et al., 2006
9.	Erigeroflavanone; *E. annuus* (Asteraceae)	*In-vitro* Multiple concentrations	Rat lens AR inhibition assay	2. IC_{50} = 6.67 µM	Yoo et al., 2008
10.	Butein; *Rhusverniciflua*(Anacardiaceae)	*In-vitro* 0.5, 1 and 2 µM	Recombinant human AR inhibition assay	2. IC_{50} = 0.7 µM	Lee et al., 2008
11.	Sulfuretin; *R. verniciflua* (Anacardiaceae)	*In-vitro* 0.5, 1 and 2 µM	Recombinant human AR inhibition assay	2. IC_{50} = 1.3 µM	Lee et al., 2008
12.	Engeletin; *Stelechocarpuscauliflorus* (Annonaceae)	*In-vitro* 2.5 and 5 µg/ml	Recombinant human AR inhibition assay	2. IC_{50} = 1.16 µM	Wirasathien et al., 2007
13.	Astilbin; *S. cauliflorus* (Annonaceae)	*In-vitro* 2.5 and 5 µg/ml	Recombinant human ARinhibition assay	2. IC_{50} = 26.7 µM	Wirasathien et al., 2007
14.	Apigenin; *Genistatenera* (*Fabaceae*)	*In-vitro* multiple concentrations	Rat lens AR inhibition assay	2. IC_{50} = 6.67 µM	Yoo et al., 2008
15.	Guaijaverin; *Myrciamultiflora* (Myrtacea)	*In-vitro* 25 µL sample solution in 0.5 ml volume	Rat lens AR inhibition assay	2. IC_{50} = 0.18 µM	Matsuda et al. 2002

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Table 1. Continued

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
16.	Desmanthin-1; *M. multiflora* (Myrtacea)	*In-vitro* 25 µL sample solution in 0.5 ml volume	Rat lens AR inhibition assay	2. IC_{50} = 0.082 µM	Matsuda et al. 2002
17.	Cosmosiin; *Various plants* (Myrtacea)	*In-vitro* 25 µL sample solution in 1.5 ml volume	Rat lens AR inhibition assay	2. 99% inhibition at 10^{-5} M	Shimizu et al., 1984
18.	Lonicerin; *Various plants* (Myrtaceae)	*In-vitro* 25 µL sample solution in 1.5 ml volume	Rat lens AR inhibition assay	2. 91.5% inhibition at 10^{-5} M	Shimizu et al., 1984
19.	Luteolin; *E. annuus* (Asteraceae)	*In-vitro* Multiple concentrations	Rat lens AR inhibition assay	2. IC_{50} = 1.54 µM	Yoo et al., 2008
20.	Apigenin-7-O-β-D-glucuronide; *E. annuus*(Asteraceae)	*In-vitro* Multiple concentrations	Rat lens AR inhibition assay	2. IC_{50} = 1.28 µM	Yoo et al., 2008

Table 2. List of flavonoids having α-glucosidase and α-amylase inhibitory activity

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
1.	Daidzein; fruits, nuts, soybeans, legumes etc.	*In-vitro* 0.04-0.4 mM	α-glucosidase and α-amylase inhibition assay	2. Inhibited α-glucosidase (45.64-88.37%) and α-amylase (22.53-60.87%) enzymatic activity	Park et al., 2013
2.	Trilobatin; *Lithocarpuspolystachyus* (Fagaceae)	*In-vitro* 0.2. 0.4, 0.8, 1.2, 1.6 and 2 mg/ml	α-glucosidase and α- amylase inhibitory assay	3. α-glucosidase IC_{50} = 0.37 mg/ml 4. Week inhibitor of α-amylase activity (21.2 ± 3.2%)	Dong et al., 2012
3.	Baohuoside I; *Epimediumbrevicornum* (Berberidaceae)	*In-vitro* 1, 10 and 100 µM	α-glucosidase and α-amylase inhibitory assay	3. α-glucosidase IC_{50} = 28.9 µM/L 4. Week inhibitor of α-amylase activity	Phan et al., 2013
4.	Luteolin; *Lonicerajaponicum* (Caprifoliaceae)	*In-vitro* various concentrations	α-glucosidase and α- amylase inhibitory assay	3. α-glucosidase IC_{50} = 0.5-1 mg/ml 4. α-amylase IC_{50} =50-500 µg/ml	Kim et al., 2000
5.	Saponarin; *Tinosporacordifolia* (Menispermacea)	*In-vitro* multiple doses; *In-vivo* 20-80 mg/kg/ day/p.o. (maltose fed rats)	α-glucosidase inhibition assay	2. Competitive inhibited α-glucosidase IC_{50} = 48 µM (intestinal maltase) and 35µM (intestinal sucrose)	Sengupta et al., 2009
6.	Pelargonidin-3-α-O-rhamnoside; *Malpighiaemarginata*(Malpighiaceae)	*In-vitro* 4.79 mM	α-glucosidase inhibition assay	2. Inhibited α-glucosidase enzymatic activity (approx. 9-23%)	Hanamura et al., 2005
7.	Cyanidin-3-α-O-rhamnoside; *M. emarginata* (Malpighiaceae)	*In-vitro* 4.62 mM	α-glucosidase inhibition assay	2. Inhibited α-glucosidase enzymatic activity (approx. 12-20%)	Hanamura et al., 2005

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Table 3. List of flavonoids having oxidative stress protective effect during hyperglycemia

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
1.	Quercetin; Fruits, vegetables etc.	*In-vitro* Various concentrations *In-vivo* 5, 25 and 50 mg/kg/p.o. weekly; (male wistar rats)	Inhibition of AGE formation assay; STZ induced diabetes in rats	4. Normalized TBARS level in serum 5. Improved catalase and SOD activity in liver and kidney 6. Inhibited formation of AGE (IC_{50} = 23.9 μM)	Maciel et al., 2013; Yoo et al., 2008
2.	Sulfuretin; *R. verniciflua* (Anacardiaceae)	*In-vitro* 0.5, 1 and 2 μM	AGE inhibition assay	2. IC_{50} = 124 μM	Lee et al, 2008
3.	Astilbin; *S. cauliflorus* (Annonaceae)	*In-vitro* 2.5 μg/ml μ	AGE inhibition assay	3. 64.9% inhibition of AGE formation 4. IC_{50}= 0.76 μM	Wirasathie-n et al., 2007
4.	Luteolin; (commercial)	*In-vivo* 50, 100, 200 mg/kg/day/p.o(male SD rats)	STZ induced diabetes	6. Reduced MDA levels in cerebral cortex 7. Elevated GSH, SOD and catalase levels in diabetic brain 8. Inhibited ROS levels in aorta 9. Elevated NO level, NOS and SOD activity in aorta 10. AGE formation IC_{50} = 22.7 μM,	Liu et al., 2013; Qian et al., 2010; Wang et al., 2011; Yoo et al., 2008
5.	Pelargonidin-3-α-O-rhamnoside; *M. emarginata* (Malpighiaceae)	*In-vitro* 0.125, 0.25, 0.3, 0.5 and 2 mg/ml	Inhibition of AGE formation	2. Strongly inhibited AGE formation (approx. 78-82%; 0.3 mg/ml)	Hanamura et al., 2005
6.	Cyanidin-3-α-O-rhamnoside; *M. emarginata* (Malpighiaceae)	*In-vitro* 0.125, 0.25, 0.3, 0.5 and 2 mg/ml	Inhibition of AGE formation	4. Strongly inhibited AGE formation (approx. 70-79%; 0.3 mg/ml)	Hanamura et al., 2005
7.	Eriodictyol; *Eriodictyoncalifornicum*(Hydrophyllaceae)	*In-vivo* 0.1, 1, 10 mg/kg/day/p.o(male SD rats)	STZ induced diabetes	4. Reduced blood glucose, retinal TNF-α, ICAM-1 and eNOS levels 5. Preserved blood-retinal barrier by attenuating retinal inflammation and lipid peroxidation 6. Prevented early retinal abnormalities linked to diabetes	Bucolo et al., 2012
8.	Diosmin; Citrus fruits	*In-vivo* 100 mg/kg/day/p.o(male wistar rats)	STZ-nicotinamide induced diabetes	4. Potentiated SOD, catalase, GPx, GSH and GST antioxidant activity in liver and kidney 5. Elevated vitamin C and vitamin E level in liver and kidney homogenate 6. Lowered lipid peroxidation in tissue homogenate	Srinivasan & Pari, 2012

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Table 3. Continued

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
9.	Epigallocatechin-3-O-gallate; *Camellia sinensis* (Theaceae)	*In-vivo* 5 and 10 mg/kg/i.p. every 8 h; (male wistar rats)	Free fatty acid induced InR	3. Reduced MDA and 8-isoprostaglandin plasma level 4. Enhanced antioxidative enzymes level (SOD and GPx) in serum	Li et al., 2011
10.	Fisetin; strawberries, onion, persimmon etc	*In-vivo* 10 mg/kg/day/p.o (male wistar rats)	STZ-induced diabetes	3. Reduced TBARS, lipid peroxides and hydroperoxides levels in hepatic tissue 4. Normalized SOD, catalase, GPx, GST and GR activity in liver	Prasath & Subramani-an, 2013
11.	Hesperidin; Citrus sp. (Rutaceae)	*In-vivo* 50 mg/kg/day/p.o (male albino rats)	High fat fed/ STZ-induced diabetes	4. Reduced lipid peroxidation, MDA and NO levels in serum 5. Elevated serum GSH, vitamin C and E levels 6. Elevated GSH, catalase, GPx, GR and SOD activity in hepatic tissue	Mahmoud et al., 2012
12.	Naringin; Citrus sp. (Rutaceae)	*In-vivo* 50 mg/kg/day/p.o (male albino rats)	High fat fed/ STZ-induced diabetes	4. Reduced lipid peroxidation, MDA and NO levels in serum 5. Elevated serum GSH, vitamin C and E levels 6. Elevated GSH, catalase, GPx, GR and SOD activity in hepatic tissue	Mahmoud et al., 2012
13.	Epicatechin; tea, grapes, red wine, coca etc.	*In-vivo* 15 and 30 mg/kg/day/i.p. (male wistar rats)	STZ-induced diabetes	3. Reduced TBARS and hydroperoxides level in liver, kidney and heart 4. Elevated GSH, catalase, SOD and GSH-Px activity in liver, kidney and heart tissues	Quine &Raghu, 2005
14.	Rutin; Fruits and vegetables	*Iv-vivo* 100 mg/kg/day/p.o(male wistar rats)	STZ-induced diabetes	3. Reduced TBARS and hydroperoxides level in brain, kidney and liver 4. Improved GSH, GSH-Px, SOD, catalase, vitamin C and vitamin E antioxidant activity in liver, kidney and brain	Kamalakkannan & Prince, 2006
15.	Puerariafuran; *Puerarialobata* (Fabaceae)	*In-vitro* Multiple dose	AGE inhibition assay	2. Inhibited AGE formation (IC_{50}= 0.53 μM)	Jang et al., 2006
16.	Coumestrol; *P. lobata* (Fabaceae)	*In-vitro* Multiple dose	AGE inhibition assay	2. Inhibited AGE formation (IC_{50}= 0.19 μM)	Jang et al., 2006

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Table 3. Continued

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
17.	4,2',4'-trihydroxy-3'-[6-hydroxy-3,7-dimethyl-2(E),7-octadienyl] chalcone; *Artocarpuscommunis*(Moraceae)	*In-vitro* 2.5 μM	Human THP-1 monocytes ROS production assay	4. Reduced generation of ROS 5. Improved functioning and morphology of mitochondria 6. Inhibited mRNA expression of RAGE	Lin et al., 2012
18.	3,4,2',4'-tetrahydroxy-3'-geranyldihydrochalcone; *A. communis*(Moraceae)	*In-vitro* 2.5 μM	Human THP-1 monocytes ROS production assay	4. Reduced generation of ROS 5. Improved functioning and morphology of mitochondria 6. Inhibited mRNA expression of RAGE	Lin et al., 2012
19.	4,2',4'-trihydroxy-5'-geranyldihydrochalcone; *A. communis*(Moraceae)	*In-vitro* 2.5 μM	Human THP-1 monocytes ROS production assay	4. Reduced generation of ROS 5. Improved functioning and morphology of mitochondria 6. Inhibited mRNA expression of RAGE	Lin et al., 2012
20.	Pelargonidin; (Marketed)	*In-vivo* 10 mg/kg/day and/ or 2 days/ p.o. (male wistar rats)	STZ induced diabetes	3. Reduced MDA and nitrite levels in whole brain homeogenate 4. Elevated SOD activity in brain	Mirshekar et al., 2010
21.	Glabridin; *Glycyrrhizaglabra*(Fabaceae)	*In-vivo* 10, 20, 40 mg/kg/ day/p.o (male kunming mice)	STZ induced diabetes	3. Increased SOD activity in the liver, kidney and pancreas along with improvement in body weight and glucose tolerance 4. Lowered fasting blood glucose level and MDA level in the liver, kidney and pancreas	Wu et al., 2013
22.	Apigenin; *Genistatenera* (Fabaceae)	*In-vitro* Various concentrations *In-vivo* 4 mg/kg/day/i.p. (wistar rats)	Inhibition of AGE formation assay; STZ-induced diabetes	3. Inhibited AGE formation (IC_{50} = 89.9 μM) 4. Protected liver and kidney from diabetic damage, probably through antioxidant effect	Rauter et al., 2010; Yoo et al., 2008
23.	Puerarin; *Radix pueraria* (Fabaceae)	*In-vitro* 10, 25, 50 and 100 μM *In-vivo* 100 mg/kg/ day/p.o (male lean and obese zucker rats)	*In-vitro* assayson vascular smooth muscle cells; Neointimal formation in carotid arterial balloon injury model (obese Zucker rats)	3. Inhibited ROS formation and NADPH oxidase activity in vesicular smooth muscle 4. Inhibited phosphorylation and translocation of PKCβ2, Rac1, p47phox, and p67phox subunits responsible for activation of NADPH oxidase in vesicular smooth muscle and in rats	Zhu et al., 2010

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Table 4. List of flavonoids having insulin mimetic and insulin signaling modifying potential

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
1.	Myricetin; *Abelmoschus moschatus*(Malvaceae)	*In-vitro* 20 µmol/L; *In-vivo* 1 mg/kg/i.p. thrice daily (insulin resistant obese zucker rats)	InR in skeletal muscle cell line (C2C12 myoblasts); Obese Zucker rats	5. Reduced InR 6. Enhanced expression of GLUT4 in soleus muscle 7. Elevated phosphorylated IRS-1 protein, p85 PI3K expression and Akt serine phosphorylation level in soleus muscle 8. Attenuated InR in C2C12 myotubes by activating Akt and AMPK	Ding, Dai, Zhang, & Li, 2012; Liu et al., 2007
2.	Epigallocatechin-3-O-gallate; *C. sinensis* (Theaceae)	*In-vivo* 5 and 10 mg/kg/i.p. every 8 h; (male wistar rats)	Free fatty acid induced InR	4. Enhanced phosphorylation of AMPK, Akt and translocation of GLUT4 in skeletal muscle and adipose tissue 5. Alleviatedfree fatty acid induced peripheral InR 6. Improved insulin signaling	Li et al., 2011
3.	Rutin; Fruits and vegetables	*Iv-vitro* 500 µM and 10^{-18} -10^{-7} M; *Iv-vivo* 100 mg/kg/day/p.o. (male wistar and SD rats)	STZ-induced diabetes and isolated soleus muscles	4. Reduced glucose and elevated insulin levels in plasma 5. Stimulated glucose uptake into soleus muscle through PI3K, MAPK and PKA pathways 6. Activated calcium-calmodulin-dependent protein kinase II (CaMKII) mediated translocation of GLUT4 to plasma membrane and glucose uptake	Hao et al., 2012; Kamalakkannan & Prince, 2006; Kappel et al., 2013; Virginia Demarchi Kappel et al., 2013
4.	Apigenin-6-C-β-fucopyranoside; *Averrhoacarambola* (Oxalidaceae)	*In-vitro* 1, 10, 100 µM. *In-vivo* 20, 50 mg/kg/day/p.o. (male wistar rats)	Glucose uptake assay on soleus muscle; Glucose challenged rats	3. Dose dependently reduced serum glucose level 4. Improved insulin signaling via PI3K, PKC and MAPK and enhanced glucose disposal into skeletal muscle	Cazarolli et al., 2012
5.	Karanjin; *Pongamiapinnata* (Fabaceae)	*In-vitro* 2.5, 5, 10, 25 µM	Assayed on L6-GLUT4 mycmyotubes	4. Dose dependent glucose uptake and translocation of GLUT4 5. Unaltered GLUT4 protein, GLUT4 mRNA and other key molecule in insulin signaling 6. Increased activity of AMPK	Jaiswal et al., 2011
6.	Puerarin; *P. lobata* (Fabaceae)	*In-vivo* 20, 40 and 80 mg/kg/day/i.p. (male BALB/c mice)	STZ-induced diabetes	8. Elevated insulin level and reduced body weight, TG, total cholesterol (TC) and blood glucose level 9. Upregulated protein levels of IRS-1 and IGF-1 in pancreas 10. Elevated mRNA level of IR and PPAR-α in skeletal muscle	Wu et al., 2013
7.	Tangeretin; *Poncirus trifoliate* (Rutaceae)	*In*-vitro 25, 50, 100, 200 µM *In-vivo* 200 mg/kg/day/p.o. (male C57BL/6 J mice)	Various *in-vitro* assays on C2C12 myotubescells. High-fat diet induced obese mice	3. Improved insulin signaling, GLUT4 translocation, glucose tolerance and phosphorylation of AMPK and AS160 4. Reduced high fat diet-induced fat accumulation and TC/TG levels	Kim et al., 2012

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Table 4. Continued

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
8.	Baicalein; *Scutellaria baicalensis* (Lamiaceae)	*In-vivo* 400 mg/kg/day/p.o. (C57BL/6J male mice)	High fat diet induced diabetes	4. Phosphorylated and activated IRS1/PI3K/Akt pathway 5. Lowered abdominal obesity and abolished InR 6. Improved glucose intolerance and lipid profile	Pu et al., 2012
9.	Morin; Fruits, vegetables etc.	*In-vitro* 50 µM	Assayed on HepG2, NIH-3T3, and PC3 cells	3. Activated and phosphorylated IR and Akt 4. Inhibited gluconeogenesis and enhanced glycogenesis	Paoli et al., 2013
10.	Kaempferol 3-O-β-D-glucopyranosyl-(1→4)-α-l-rhamnopyranosyl-7-O-α-L-rhamnopyranoside(CO-1); *Cinnamomumosmophloeum*(Lauraceae)	*In-vitro* 5 and 20 µM	Assayed on 3T3-L1 adipocytes	4. Insulin mimetic effect with 12.2 fold elevation in adiponectin secretion 5. Increased phosphorylation of IRβ and activated insulin signaling pathway 6. Enhanced GLUT4 translocation	Lee et al., 2009
11.	Kaempferol 3-O-β-D-apiofuranosyl-(1→2)-α-larabinofuranosyl-7-O-α-l-rhamnopyranoside(CO-1); *C. osmophloeum*(Lauraceae)	*In-vitro* 5 and 20 µM	Assayed on 3T3-L1 adipocytes	4. Insulin mimetic effect with 12.2 fold elevation in adiponectin secretion 5. Increased phosphorylation of IRβ 6. Enhanced GLUT4 translocation	Lee et al., 2009

Table 5. List of flavonoids having PPAR-γ receptor modulating activity

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
1.	Isoginkgetin; *Gingko biloba* (Ginkgoaceae)	*In-vitro* 0.1, 0.5 and 1 µM	Assayed on LiSa cells and mouse adipocyte 3T3-L1 mouse fibroblasts	3. Week agonistic activity on PPAR-γ receptor 4. Enhanced AMPK signaling and subsequent adiponectin production in LiSa and 3T3-L1 cells	Liu et al., 2007
2.	Luteolin; *Cirsiumjaponicum* (Asteraceae) and *Cynodondactylon* (Poaceae)	*In-vitro* various concentrations *In-vivo* 50, 100 and 200 mg/kg/day/p.o. (male SD rats)	3T3-L1 adipocytes and primary adipose cell lines;. STZ induced diabetes	4. Elevated PPAR-γ mediated transcription and insulin sensitization in 3T3-L1 adipocytes 5. Possessed PPAR-γ partial agonistic activity 6. Enhanced phosphorylation of Akt2 and lowered mRNA levels of inflammatory mediators along with increased adiponectin and leptin gene expression in adipocytes and primary mouse adipose cells	Ding et al., 2010; Puhl et al., 2012
3.	Licoflavone A, echinatin, kanzonol X, kanzonol W, shinpterocarpin, glabrol, shinflavanone, gancaonin L, glabrone and 5'-formyl glabridin; *G. glabra*(*Fabaceae*)	*In-vitro* 10 µg/ml	GAL-4-PPAR-c chimera assay(CV-1 monkey kidney cells)	2. Significant PPAR-γ ligand binding activity	Kuroda et al., 2010

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Table 6. List of flavonoids capable of modulating enzymes involved in glucose metabolism

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
1.	Diosmin; Citrus fruits	*In-vivo* 25, 50, 100 mg/kg/day/p.o. (male wistar rats)	STZ induced diabetes	3. Increased hexokinase and glucose-6-phosphate dehydrogenase activity in liver 4. Reduced glucose-6-phosphatase and fructose-1,6-bisphosphatase enzymatic activity in liver	Pari & Srinivasan, 2010
2.	Eupatilin; *A.princeps*(Asteraceae)	*In-vivo* 0.005 g/100 g or 0.02 g/100 g diet (male C57BL/KsJ-db/db mice)	db/db type 2 diabetic mice	4. Improved hepatic glucose metabolism 5. Lowered hepatic glucose-6-phosphatase and phosphoenol-pyruvate carboxykinase activity 6. Increased liver glucokinase activity	Kang et al., 2008
3.	Biochanin A; *Cicerarietinum* and other legumes (Fabaceae)	*In-vivo* 10 mg/kg/day/p.o. (male wistar rats)	STZ induced diabetes	4. Increased glucokinase, glucose 6-phosphate dehydrogenase activity in liver 5. Suppressed glucose-6-phosphatase and fructose 1,6-bisphosphatase activity in diabetic liver 6. Lowered hepatic glycogen content	Harini et al., 2012

Table 7. List of flavonoids having PTP1B, SGLT2, NFκB and JNK modulating activities

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
1.	2',4-dihydroxy-4'-methoxydihydro chalcone; *A. dracunculus* (Asteraceae)	*In-vitro* 2.5, 5, 10, 25 µg/ml and 3.75 µg/ml	PTP1B activity in primary human skeletal muscle culture	2. Inhibited PTP1B activity (IC_{50} = 61 µM) Reduced PTP1B activity by 36% Reduced level of PTP1B mRNA by 29%	Wang et al., 2006
2.	2',4'-dihydroxy-4'-methoxydihydro chalcone; *A. dracunculus* (Asteraceae)	*In-vitro* 2.5, 5, 10, 25 µg/ml and 3.75 µg/ml	PTP1B activity in primary human skeletal muscle culture	2. Reduced PTP1B activity by 26%	Wang et al., 2006
3.	Morin; Fruits, vegetables etc.	*In-vitro* 50 µM	Assayed on HepG2, NIH-3T3, and PC3 cells	*3. In-vitro* and *in-silico* inhibition of PTP1B 4. Non competently inhibited PTP1B activity (IC_{50} = 15.0 ± 0.9)	Paoli et al., 2013
4.	Luteolin; *C.dactylon* (Poaceae)	*In-silico* docking	*In-silico* docking study on 2XQ2 PDB of SGLT2	*2. In-silico* docking analysis revealed inhibition of SGLT2	Annapurn--a et al., 2013
5.	Scutellarin; *E.multiradiatus*(Asteraceae)	*In-vitro* 0.1 and 1 µM	Assayed on human endothelial ECV304 cells	2. Inhibited high glucose induced elevation of NF-κB expression in ECV304 cell	Luo et al., 2008
6.	Baicalein; *S. baicalensis* (Lamiaceae)	*In-vitro* 400 mg/kg/day/p.o. (C57BL/6J male mice)	High fat diet induced diabetes	2. Inhibited MAPK, NF-κB and AMPKα2 signaling	Pu et al., 2012

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Table 8. List of flavonoids which controls diabetes and its associated complications through unknown mechanism

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
1.	Chrysoeriol; *G. tenera* (*Fabaceae*)	*In-vitro* 4 mg/kg/day/i.p. (wistar rats)	STZ-induced diabetes	3. Improved glucose tolerance and reduced circulating glucose level 4. Improved urea, ALT and AST profile in liver and kidney	Rauter et al., 2010
2.	Naringenin; Fruits and vegetables	*In-vivo* 50 mg/kg/ day/i.p. (CBA inbred mice)	Alloxan-induced diabetic	4. Improved body weight and reduced mortality 5. Reduced RBCs and Hb level and elevated leukocyte level in blood 6. Reduced DNA damage, % apoptosis in kidney and liver	Oršolić et al., 2011
3.	Quercetin-3'-methoxy-3-O-(4''-acetylrhamnoside)-7-O-α-rhamnoside *Cleome droserifolia* (Cleomaceae)	*In-vitro* 50 µM	Glucose uptake assay in differentiated C2C12 cells	2. Insulin equivalent value = 2.3 ± 0.05	Motaal et al., 2011
4.	Kaempferol-4'-methoxy-3,7-dirhamnoside *C. droserifolia* (Cleomaceae)	*In-vitro* 50 µM	Glucose uptake assay in differentiated C2C12 cells	2. Insulin equivalent value = 2.0 ± 0.04	Motaal et al., 2011
5.	Isorhamnetin-3-O-β-D-glucoside *C. droserifolia* (Cleomaceae)	*In-vitro* 50 µM	Glucose uptake assay in differentiated C2C12 cells	2. Insulin equivalent value = 1.3 ± 0.05	Motaal et al., 2011
6.	Silymarin; *Silybummarianum* (Asteraceae)	*In-vivo* 50 mg/kg/ day/p.o. (male wistar rats)	STZ-induced diabetes	6. Normalized MDA, NO and total thiol molecules level 7. Improved body weight and hepatic glycogen level 8. Reduced blood glucose, urine output and serum ALT and ALP 9. Downregulated CYP 3A2 and upregulated GSH-Px expression in liver 10. Histopathological improvement in diabetic liver profile	Malekinej-ad et al., 2012
7.	Kaempferol 3-*O*-α-(2''-galloyl) rhamnoside; *Bauhinia megalandra*(Fabaceae)	*In-vitro* 50 µM	Rat liver microsomal glucose-6-phosphatase bioassay	3. Inhibited liver microsomal glucose-6-phosphatase (IC_{50} = 31 ± 2) 4. Inhibited the neoglucogenic capacity of rat liver slices (IC_{50} = 30 µM)	Estrada et al., 2005
8.	Quercetin 3-*O*-α-rhamnoside; *B. megalandra*(Fabaceae)	*In-vitro* 50 µM	Rat liver microsomal glucose-6-phosphatase bioassay	2. Inhibited liver microsomal glucose-6-phosphatase (IC_{50} = 246 ± 5)	Estrada et al., 2005

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Table 8. Continued

S. No.	Flavonoid (Source)	Dose	Model/Assay	Major Findings	Reference
9.	Kaempferol 3-*O*-α-rhamnoside; *B. megalandra*(Fabaceae)	*In-vitro* 50 µM	Rat liver microsomal glucose-6-phosphatase bioassay	2. Inhibited liver microsomal glucose-6-phosphatase ($IC_{50} = 186 \pm 4$)	Estrada et al., 2005
10.	Quercetin-3-*O*-α-arabinoside; *B. megalandra*(Fabaceae)	*In-vitro* 50 µM	Rat liver microsomal glucose-6-phosphatase bioassay	2. Inhibited liver microsomal glucose-6-phosphatase ($IC_{50} = 1069 \pm 31$)	Estrada et al., 2005
11.	Quercetin 3-*O*-α-(2″-galloyl) rhamnoside; *B. megalandra*(Fabaceae)	*In-vitro* *50* µM	Rat liver microsomal glucose-6-phosphatase bioassay	2. Inhibited liver microsomal glucose-6-phosphatase ($IC_{50} = 27 \pm 1$)	Estrada et al., 2005
12.	4-hydroxyderricin; *Angelica keiskei* (Apiaceae)	*In*-vitro 1.25-20 µM *In-vivo* 0.15% test drug in diet (diabetic KK-A^y mice)	3T3-L1 cell culture and differentiation assay and diabetic KK-A^y mice	3. Enhanced differentiation and glucose uptake into 3T3-L1 cells 4. Reduced blood glucose level and alleviated polydipsia	Enoki et al., 2007
13.	Xanthoangelol; *A. keiskei* (Apiaceae)	*In-vitro* 1.25-20 µM *In-vivo* 0.15% test drug in diet (diabetic KK-A^y mice)	3T3-L1 cell culture and differentiation assay and diabetic KK-A^y mice	3. Enhanced differentiation and glucose uptake into 3T3-L1 cells 4. Reduced blood glucose level and alleviated polydipsia	Enoki et al., 2007
14.	Delphinidin; *Vitisvinifera* (Vitaceae)	*In-vitro* 100 mg/ml *In-vivo* 100 mg/kg/day	Hemoglobin and albumin anti-glysation activity in diabetic animals	*3. In-vitro* reduction in albumin glysation *4. In-vivo* reduction in albumin and hemoglobin glycosylation	Gharib et al., 2013
15.	Genistein; *G. tenera* (*Fabaceae*)	*In-vivo* 1 and 4 mg/kg/day/i.p. (male wistar rats)	STZ-induced diabetes	3. Improved glucose tolerance, body weight, ALT, AST and SOD activity in liver and kidney 4. Reduced blood glucose and MDA level	Baluchnejadmojarad &Roghani, 2008; Rauter et al., 2010
16.	Kaempferitrin; *B. forficata* (Caesalpiniaceae)	*In-vivo* 100 mg/kg/p.o. (male wistar rats)	Alloxan induced diabetes	3. Reduced blood glucose 4. Enhanced dose dependent glucose uptake into soleus muscle of rat	Jorge et al., 2004

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Chapter 23
Food Allergy and Food Poisoning:
Toxicology on Culinary Sciences

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ABSTRACT

Taken into account data from which is considered a product not safe, estimate the safe level of a contaminant on food, for example, always have many unavoidable uncertainties. It cannot be overemphasized enough, that this also happens as in any other human activity. In most cases, we hope, to define as clearly as possible the eventual risk associated with particular conditions of exposure to a given substance in food. There are numerous toxic compounds that reside naturally in certain foods that unable these to be consumed above certain limits or even are fully prohibited in some other countries. Chapter starts with a clear explanation of differences and relationships between food allergy and food poisoning, continued with main allergens in food and main toxics. Finally, authors summarize different origins of toxins and allergens (natural from foods, from additives, pollutants and food processing).

INTRODUCTION

Nowadays food allergy is a public health problem and a matter of concern for consumers. This is firstly, due to the way that affects the quality of life of individuals who suffer them, because is often difficult to choose the right foods for diet. Keep in mind that sometimes symptoms can be serious, or even lead to death. Secondly, the importance of food allergies prevalence that has been observed in the recent years, despite the lack of concrete data to estimate the prevalence precisely is because, among other factors,

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the lack of uniformity of diagnostic methods and confusion regarding other adverse reactions to foods, it is estimated that between 1 and 3% of adults and between 4 and 6% of children have food allergies (CODEX, 2006).

This chapter reviews the general characteristics of food allergies and their current impact on society, based on a literature review of published literature.

It is known as food allergy a set of adverse reactions to food, due to their ingestion, or inhalation, proven immune pathogenesis. This only occurs in some individuals, maybe after taking small amounts of food not relating them with any physiological or pathophysiological effect. The term "food allergy" is being abused, improperly applied to any adverse reaction to a food or food additive, which is in fact a toxic effect.

Cooking related toxicology has achieved a leading state in recent years, as shown a considerable amount of information on social media (TV and news) and in different scientific journals and texts (Tareke et al, 2000; Berjia et al, 2014; Kima, 2015). Regarding the origin of toxins in the culinary, we can consider four main sources: natural, accidental / allergens, industrial and process generated. Although in some cases, toxins may belong to more than one of these categories simultaneously. Natural toxins can occasionally cause problems, because they can be found unexpectedly in foods with a higher concentration than usual, or toxic species can be confused with harmless ones as often happens with some herbs and plants. Accidental intoxication generally represents the greatest risk to health, unlike the previous ones, not the quantity, frequency, type of associated food is known, or how it came to food. Sometimes it is a little known toxic or popular use abroad as Tonka beans (Dolan et al, 2010) and difficulty to diagnose poisoning. Moreover, the presence of allergens in food is extremely difficult to control, despite the most advanced legislation (European) ((UE) Nº 1169/2011) is always possible the presence of uncontrolled allergens (Alcalá AESAN 2012). Industrial poisons are foreign substances to food, added in amounts known to achieve a particular purpose, such as additives or uncontrolled such as pollutants. These compounds are not safe, even some of them are potentially toxic to most consumers; who may have symptoms with varying intensity. However if additives were not used would be very difficult to have a wide variety and quantity of food in urban areas, which has concentrated the highest percentage of the population in recent years, demanding food for subsistence. Toxic generated by the process, are the result of the transformation of food through different stages of development; from cooking, stabilization, formulation, mixing, sterilization, transportation, etc. These toxins can result from such simple processes such as roast meats, doing different polycyclic aromatic hydrocarbons, many with mutagenic and carcinogenic molecules finally generated.

BACKGROUND

Some eats are inherently poisonous while others can lead to health conditions that sometimes prove fatal. From pre-hominids time to now every time that we eat something, we are suffering a (more or less) controlled risk to death. This risk will come, from the toxicity of the food eaten or from and adverse reaction of our organism to normal food composition. Obviously the age, sex, size and previous exposition, are factor that modulate the negative action of eaten food. Modern industrial processes, as well as culinary ones, introduce new molecules in our food-stock, creating new sceneries for food poisoning.

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METABOLIC DIFFERENCES BETWEEN ALLERGY AND POISONING

In fact body reactions due to food intake may create a wide range of results. Adverse food reactions (food hypersensitivities,) include any abnormal reaction resulting from the ingestion of a food and might be the result of food intolerances (non-allergic food hypersensitivities) or food hypersensitivity/allergy (food allergy).

In many cases, differences between intolerance and a clear allergy are imperceptibles. Food intolerances (non-allergic food hypersensitivities) are adverse responses caused by some unique physiologic characteristic of the host, such as metabolic disorders. Food hypersensitivities/allergies are adverse immunologic. Toxic reactions might mimic food hypersensitivities and typically are due to factors inherent in a food, such as toxic contaminants (e.g., histamine in scombroid fish poisoning) or pharmacologic substances within the food, which can affect most healthy individuals when given in appropriate doses.

Food aversions also might mimic adverse food reactions but are not mounting a brisk protective response to dangerous pathogens (Sampson 2004).

The gastrointestinal immune response thus plays a pivotal role in maintaining protective immunity in health and a critical role in the pathogenesis of a wide variety of clinical disorders associated with food allergy (MacDonal & DiSabatino, 2009). Within both (food allergy and poisoning), food allergy reactions mediated by IgE immune pathogenesis (immediate hypersensitivity or allergic type) such as those produced by other immune mechanism known (non-IgE mediated reactions) are included. It is characteristic of the early presence of high titers of antibodies against IgE in food. (Bellanti et al, 2004) Also it can be found increased specific antibodies from other classes of immunoglobulins, immune complexes and most probably mediated immunity responses cells. The existence of a joint involvement, i.e. IgE mediated reactions partially and cells, in varying degrees are also possible. In any case, plus evidence of dependency between ingestion of a food and the appearance of symptoms, it is necessary that these altered immune parameters are directly involved in the pathophysiology responsible for the symptoms.

Some enteropathy such as celiac disease and enteropathy and other food protein enterocolitis (cow's milk, rice, chicken, soy, etc.) may be included within the concept of food allergy, because in them is proven or probably the existence of an underlying immune mechanism. Allergic reactions to foods must be distinguished from food intolerance reactions and toxic reactions to foods.

Food intolerance is a form of adverse reaction to a food or food additive in the pathogenesis does not exist or has not been demonstrated, unlike food allergy, immune-based mechanism. Senses, food intolerance and food allergy have been used and, sometimes, are still used indiscriminately and even with a similar meaning, which has favored the emergence of confusion when analyzing the pathogenic, clinical, diagnostic and therapeutic processes. Despite all, more than 20 years ago, for the first time was defined with a pathogenic base, the concepts used to describe adverse reactions to foods (Metcalfe, 1984), whose use, has allowed better communication in the scientific literature regarding the different reactions to food components.

Food intolerance reactions can occur by the following mechanisms:

- Enzymatic or metabolic, defects related to enzymes involved in the metabolism of food (Table 1).
- Pharmacological foods or intolerance due to the pharmacological action of chemical compounds naturally or added to food, such as caffeine, theobromine that may cause heart rhythm disturbances, tremor or migraine in certain individuals, even when ingested in moderate doses (Table 2).

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- Finally, there is food intolerance called indeterminate, to denote any intolerance reaction whose mechanism is not clarified production. At last, most adverse reactions include food additives.

Toxic reactions caused by food (food poisoning), include those for foodborne diseases and produced by chemical or biological agents contained therein.

Its main feature is that anyone can be susceptible to poisoning from these products, although the vast majority of them are not dangerous, because the amount that can be ingested in a normal diet is insufficient to cause any harm. Table 3 shows a classification of the agents capable of causing food poisoning. Some, such as additives or histamine may act by different mechanisms as poisoning agents.

Intolerance reactions and toxic reactions are characterized both by being dose-dependent. The clinical manifestations that occur are the more intense the higher amount of ingested food or food additive. This dose-response relationship cannot exist in allergic reactions, so that, in some of them, very small doses can lead to an exaggerated clinical response. Another peculiar feature is that toxic reactions can occur in any individual, whenever you eat food in sufficient quantity. Instead, both toxic and allergic reactions in intolerance, the onset of clinical manifestations before the ingestion of a particular product at a given dose is only in relation to individual characteristics, so that the same dose is perfectly tolerated by any other individual, otherwise healthy (Table 4). Probably small quantities of poisoning products (histamine-like), during the early development of immune system, are responsible of immunoglobulin's alterations in childhood and in correspondence early food allergy development (Taylor et al, 1984).

Issues, Controversies, Problems

Despite these differences, sometimes toxic reactions, intolerance or allergy can lead to very similar clinical pictures. This situation will occur when the agent responsible, in addition to a general pharmacological action, is acting on an individual who, for certain characteristics, has a response threshold decreased. It is also possible that the responsible agent, ex, histamine, also is a mediator of anaphylactic reactions, which may result in similar clinical pictures to them ("anaphylactoid reactions", "food pseudoallergy").

In contrast to the ingrained idea among laymen and even among some medical doctors, it is likely that food allergy constitutes only a quantitatively very small part of all the adverse reactions to foods. Data on prevalence of food allergy in the population are very limited in both children and adults, because the conceptual gap and the different diagnostic criteria used. If a method of controlled provocation is used to assess a possible adverse reaction to a food. No more than one third of the supposedly allergic children respond positively (Bock 1987) and less in the adult population (Jansen et al., 1994) (Young & Stoneham, 1994). Also, within the pediatric population, it appears that its incidence is higher in younger children.

Food Allergy

All foods can be potentially sensitizing. Its prevalence is related, among other factors, to feeding habits of the population studied. However, when excluding sensitizations with no clinical relevance, most patients respond only to few foods. Also, its prevalence may vary with age, associates regarding exposure to allergens and mainly to immunological and physiological processes of maturation factors. Approximately half of the cases of childhood food allergies are associated with animal allergens. In our environment, the child, the egg (protein clear) is the most common food allergen, followed by cow's milk and fish (Crespo et al., 1995). After that become dominated by sensitization to vegetables, such as

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nuts, fresh fruits, legumes and vegetables; frequently found in these patients, associated sensitization to pollens (Kany et al., 2001). Food allergens are water-soluble proteins or glycoproteins with a size between 10 and 70 kDal. and consequently more stable to heat, acid and proteases. In foods are often one or several main, major or larger and other secondary or minor allergens allergen and which are regarded as respectively allergens to which respond most immunologically sensitized patients or relevant food allergens to which respond only a few individuals. Some are characteristic of a particular food can be shared with other species of the same or close family (eg, fish, legumes) and others may be present even in phylogenetically distant families.

This usually correspond to proteins with similar function (profilin, Trophomyosins, serum albumins, etc.). Some are widespread, being known as "panallergens". These panallergens may be involved in the poly-sensibilities detected in many patients,

1. **Food Allergens of Animal Origin:**
    a. **Cow Milk:** Although, it has been demonstrated, various mammalian milks cause allergic reactions. Cow milk, is most common and best studied. Milk human species, cow's milk, or a derivative formula are usually the first food that non-homologous individual receives in significant quantities. This means, that it is also the first food antigen which humans come into contact with in a usual way. Therefore, is not surprising that is, in the early childhood, the food that causes the greatest number of adverse reactions. In Spain, the incidence data of immediate allergy to cow's milk proteins (PLV) in infants varies between 0.4 and 1.9 per 100 (Sanz et al., 2001) (García et al., 2003).

Major allergen components include serum proteins in cow's milk (particularly, beta-lactoglobulin, alpha-lactalbumin and, to a less extent bovine serum albumin) and casein (alpha, beta and kappa). Comparing with purified proteins from cow's milk, it was observed that beta-lactoglobulin is the protein that more often induces clinical responses.

    b. **Egg:** The egg is a widely consumed food worldwide and the leading cause of allergic reactions in children (Crespo et al.,1995). The light seems to be more allergenic than the yolk. Egg the major allergen present in the clear, is the ovomucoid (Gal d 1) (Bernhisel-Broadbent et al., 1994). Is a trypsin inhibitor and represents 10% of the protein content of the egg white. The main feature of ovomucoid is its extreme resistance to degradation by proteases and thermostability, so allergen resulting affected by cooking in minimal amounts. Another allergenic protein is egg white or ovotransferrin, conalbumin (Gal d 3) having homology with other iron binding proteins, such as serum transferrin and more labile than previously commented. Other allergen is white egg white lysozyme (Gal d 4), 14.3 kDa protein, composed of a single polypeptide chain linked by four disulfide bridges.

Also the yolk can have a major allergen paper, since the involvement of yolk livetins. In syndrome called "feather-egg" (Mandallaz et al., 1988), based on clinical observation of patients showed that starts with respiratory allergy awareness feathers and subsequently develops egg allergy (in adults), with some special features like its beginning at a later age, presence of asthma due to sensitization to feathers and a recognition profile egg allergens different. In children with this clinical framework, feathers sensitization, usually occur, previous to egg sensitization (Añibaro et al., 1997). These patients exhibit

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IgE antibodies to a protein of 65-70 kDa yolk, alpha-livetin, identified as chicken serum albumin and responsible for cross-reactivity.

c. **Fish:** Fish allergy is relatively common in countries where consumption is high, like Japan, Scandinavian countries and Spain. However, this distribution tends to change due to market entry of new food products derived from fish such as surimi, or by the use of fish oil rich in polyunsaturated fatty acids, given their beneficial effects on the cardiovascular system.

The fish species most frequently involved in allergic reactions are gadiformes (cod, hake) and flatfish (flounder, rooster); less salmon, clupeiforms (sardine, anchovy) and tuna (Pascual et al., 1992).

The first purified and well-characterized allergen fish was Gad c 1 (previously designated as Allergen M), found in cod (*Gadus callarias*) (Aas 1966). This allergen belongs to a group of proteins known as parvalbumins muscle, which control the flow of calcium through the cell membrane and are only found in the muscle of fish and amphibians. Gad c 1 is very heat-stable and resistant to proteolysis. Allergenicity is considerably reduced in canned fish.

The structural similarity of different fish parvalbumins makes in sensitized patients very often to arise cross reactions (Pascual et al., 1992). However, the differences make its importance not alike in all species of fish. A third of those allergies to fish, tuna patients tolerate as albacore (*Thunnus alalunga*).

d. **Crustaceans and Mollusks:** Crustaceans have been recognized as a common cause of food hypersensitivity reactions. Their allergens are thermostable. In recent years described the existence of cross-reactivity between different species of crustaceans and less, but also relevant to other allergens present in insects and mites (Dermatophagoides spp.).

Shellfish can be gastropods such as snails, bivalves such as clams, mussels, oysters and cephalopods such as octopus and squid. The major allergen seems to be also tropomyosin and is often is associated with sensitization to shellfish (Leung et al., 1996).

2. **Food allergens of plant origin:**

a. **Vegetables:** Legumes are dicotyledonous plants botanical order of *Fabaces* comprising three families: *Mimosaceae, Papilionaceae* and *Cesalpiniaceae.* Major species responsible for allergic reactions (lentil, pea, pea, peanut, soybean, lupine) belonging to the *Papilionaceae* family, commonly known as legumes. The vegetables are among the food allergens that most often cause immediate allergy in children (Pereira et al., 2002). In Mediterranean countries the introduction of nuts, including peanuts, not done before 2 or 3 years, although in recent times the use of peanut, soybean and lupine as additives makes its consume earlier and also hidden-form, which could be contributing to the incidence of allergy to legumes.

Peanut (*Arachis hypogaea*) is responsible for many serious allergic reactions, whose frequency has not only increased in recent years, but also its appearance is earlier, partly due to increased consumption, possessing proteins Very versatile, easily digested and various presentations; aperitif, fresh, grilled, butter or an oil.

The peanut is the major food allergen in US and the leading cause of anaphylaxis. It is estimated that allergy in the general US population may be about 1%. According to some authors would be the major

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food allergen in children over three years (Bock and Atkins, 1989) but it is also rising at younger ages. In Europe, more specifically in Anglo-Saxon countries, their allergenic importance is increasing in recent years by the similarity of dietary patterns with the US, due to both changes in dietary habits (increased consumption of fast food, immigration and different dietary habits), and its role as hidden allergen. It has been found that roasting increases allergenicity, possibly due to changes in the molecular structure or enzyme function (Beyer et al., 2001).

In peanuts have been identified several major allergens in sensitized patients. The Ara h 1 allergen is a 63.5 kDa glycoprotein that has high homology with vicilins (storage proteins present in the seeds of many plants). The identification of this protein family may partly explain the wide immunological cross-reactivity between peanut and other legumes.

Soybean (*Glycine max*) is the basis of tofu, soybean paste and soy milk, also consumed fresh. It is common cause of adverse reactions, possibly helped by its wide use in the West as an additive and as a food supplement to enrich the protein content of other foods. Soy can be found in bread, cakes, ice cream, breakfast cereals, packet soups, canned tuna, hot dogs, sauces, Chinese food, etc. Also, their use in dietary formulas has been considered as a possible cause of sensitization in infants, sometimes with the presence of specific IgE to soy, but generally no demonstrable clinic for soybean (Cordle, 2004) (Martin, 2006).

Among the various allergens identified in soybeans, fractions with molecular weight 13-14 kDa and 25 kDa 22-, are the most powerful in three different groups except;whole soybean powder and shell, and have proven their allergenicity both inhalation and through the digestive tract (González et al., 1995).

The lentil (*Lens sculenta*) is the most consumed vegetable in Spain. In this country with a traditionally Mediterranean diet, lentils are legumes that most often cause IgE-mediated hypersensitivity, followed by chickpea. They are the fourth or fifth leading cause of food allergy in the child population. In addition, half the cases begin between the first and third year of life, predominantly in the second year (Pereira et al., 2002).

In lentil described a protein band between 12 and 16 kDa, which would be called a gamma-Len c 1 vicilin subunit, and a weight of approximately 66 kDa, Len c 2, corresponding to a biotinylated seed protein.

Has been identified Len c 1 as a major allergen and Len c 2 as a minor allergen lentil (López-Torrejón et al., 2003). Len c 1 shows a 45% similarity with Ara h 1 Peanut allergen greater.

b. **Other Vegetables:** The chickpea (*Cicer arietinum*) is similarly important cause of allergic reactions in the East and in our environment, due to their high consumption. The bean (*Phaseolus vulgaris*) is native to Central and South America. There are many edible species and can be divided broadly into two types: those that are eaten with pods whose seeds are not developed (green beans) and those which only the seeds (beans, beans, pinto bean) are eaten. The lupine (*Lupinus sativus*) is used as a protein rich in pastries and other food for its high content of nitrogenous substances, fats and minerals. It has been identified as a hidden allergen responsible for anaphylactic reactions (Rojas-Hijazo et al., 2006). The pea (*Pisum sativum*) is an herbaceous legume from Asia. It is rich in proteins, lipids, lysine, vitamins A, B and E, phosphorus and calcium. In children ingestion is rare before 2 or 3 years. There have been rare allergic reactions to this legume.

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c. **Cereals:** Described the major allergens in wheat flour, barley and rye are water soluble (albumins and globulins) and related symptoms are derived from no exposure inhalation and ingestion with (Houba et al., 1998).

The higher in baker's asthma allergen is a group of inhibitors of alpha-amylase. A 15-kDa allergen in wheat flour and other flour 14.5 kDa barley inhibitors trypsin / alpha-amylase have been identified. Also, homodimeric 25 kDa proteins of rye and barley have been isolated. Swallowed allergy to wheat and other cereals is rare in the West (Crespo et al., 1995a). Regarding intake, has been identified, frequently involved in sensitization to wheat flour in children with atopic dermatitis and more recently been shown that the alpha-amylase inhibitor is a wheat allergen relevant allergenic protein of 50 kDa Patients who experience hypersensitivity reactions with wheat protein intake (Palosou, 2003).

The major allergens of rice (Oryza sativa) have been identified as albumins 14 to 16 kDa molecular weight, belonging to the family of inhibitors of trypsin / alpha-amylase (Alvarez et al., 1995). They have also identified a lipid transfer protein as a major allergen in individuals with corn clinical signs after ingestion (Pastorello et al, 2000).

d. **Gluten:** The term "gluten"as a fraction of proteins from wheat, rye, barley, and oats, or crossed varieties and derivatives thereof and which is insoluble in water and in 0.5 M NaCl Prolamines form the gluten fraction which can be extracted with a 40-70 percent ethanol. The prolamin from wheat is gliadin, from rye is secalin, barley hordein and oats avenin. Oats currently appears separated from the three cereals referred to above and it may not be disease-inducing like other cereals (rice, corn), although this phenomenon is correlated with possibly fewer oats contains prolamine proportionally to the others mentioned cereals.

According to their amino acid sequences, the gliadins are subdivided into alpha, gamma and omega-gliadins and are rich in proline and glutamine. These prolamins are harmful to certain individuals and their intake triggers them celiac disease.It is often said that these patients have "gluten sensitivity". Prolamin content of gluten is generally reduced by 50 percent (Codex 2006).

e. **Fruits and Vegetables:** In the Mediterranean area fruits are a common cause of food allergy, particularly of oral allergy syndrome. They have been identified only in a few clinical studies species, causing IgE mediated allergy. The family *Rosaceae* (apple, pear, peach, albarcoque, plum, cherry) and *Cucurbitaceae* (melon, watermelon, cucumber, pumpkin, zucchini) have received special attention in clinical studies as well as bananas (family *Musaceae*) and kiwi (family *Actinidiaceae*).

Other families of fruits are abundant immunological and molecular data, but very little information on its possible clinical significance. Among vegetables, celery allergy is of particular interest for their ability to produce anaphylactic reactions (Ballmer-Weber et al., 2000).

The tomato, both raw and cooked is a regular feature in the diet. The awareness of this vegetable is particularly prevalent in the Mediterranean area, especially in children and adolescents, within is the most common clinical oral allergy syndrome. However, when there is an awareness associated latex, the most common manifestations are urticarial and anaphylaxis (Reche et al., 2001).

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There are very few communications allergy to other vegetables. It is very rare lettuce allergy can cause allergic contact dermatitis, but not enough of allergic reactions upon ingestion data (Franck et al., 2000).

The allergens in fruits and vegetables are thermolabile, which facilitates tolerance sensitized individuals after specific treatments that inactivate their antigens.

f. **Nuts ("Nuts in Shell"):** Under the name of nuts, tree nuts, walnuts ("nuts") various seeds from different botanical families are included. It is a heterogeneous group of foods without Botanical related, but with great allergenic similarities and consumption patterns. Oleaginous fruits are consumed in dried form among which are: almonds, hazelnuts, cashews, walnuts, Brazil nuts, Pecan nut, pistachio, pine nuts, and sunflower seeds. Its stability against heat and digestive processes favor its high allergenicity, so often the reactions are intense and immediate. Allergy to nuts is one of the most common food allergies, although its prevalence varies according to age and geographic area.

They occur most often in children than in adults (Ewan, 1996) and, sometimes, may appear before the first known contact with the nut. It is likely that exposure to small amounts unnoticed or sensitization by other means explain these intense reactions. They can lead to severe clinical manifestations, even in small quantities, due to its presence as food ingredients or flavorings in various food products.

Allergy to nuts is often associated with allergies to various pollens. Most individuals with allergies to hazelnut are sensitized to pollens of birch and hazel (Ortolani et al., 2000) and confirmed that the major allergen in hazelnut is a 18 kDa protein homologous to Bet v 1 birch (Pastorello et al., 2002).

The major allergen of Brazil nuts (*Bertholletia excelsa*), Ber e 1 is a methionine-rich 2S protein composed of two subunits of 9 and 3 kDa. This protein has been used in transgenic studies to correct the deficit in methionine levels having soybeans. However, the commercial development of this GM soy has been abandoned, and that gene transfer of allergen (Ber e 1), of the Brazil nut, also transfers its allergenicity of soybean (Nordlee et al, 1996).

g. **Sesame:** The *Sesamum indicum* is native to tropical Africa, whose cultivation is widespread, for the use of plant seeds. Allergenic importance is great in those places where consumption is common. It is an ingredient that should be clearly identified and separated from nuts and peanuts, which can also contaminate. The prevalence and severity of allergy seed sesame has increased in recent years in Europe (Gangur et al., 2005) due to its introduction from Asian countries in the diet as food ingredient or oil, especially in bakery products, fast food, new foods and vegetarian products. Given its characteristics, sesame must be identified when used as ingredient in packaged foods, as indicated by Directive 2003/89 of the European Union (EU, 2003). However, many products and ready meals containing sesame are sold at fast food places, bakeries, restaurants, etc., whose ingredients are not easily identifiable and where cross contamination is more likely.

h. **Mustard:** Mustard is one of the most allergenic of spices on record to date, therefore its prevalence as allergenic potency. As for other food allergens, sensitization depends on the eating habits of the population. Allergens have been isolated from the seed of yellow mustard (*Sinapis alba*) and Eastern (*Brassica juncea*) and their primary structures have been studied in great detail (González de la Pena et al., 1993) (Monsalve et al., 1993). No all allergens of yellow mustard seed is a 15 kDa protein. Structural homology of an occupational allergen,

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Ric c 1, *Ricinus communis*, is known and is probably related to other 2S albumin present in soybean, cottonseed and pea.

3. **Other:** Latex, Anisakis, Mites, etc.: They are sometimes cause of serious clinical pictures, plus unexpected. Besides food used as additives, other conveyed by foods that can act as allergens. As most important, the following can be considered:
   a. **Latex:** Or natural rubber latex is a processed plant product obtained from the milky sap of many plants, though the only one who has been commercially exploited is from *Hevea brasiliensis* tree. Due to its elastic properties is used to make multiple everyday items such as gloves, condoms, pacifiers, baby bottle nipples, balloons, stickers, etc..; and more specific as medical supplies, catheters, tourniquets, gloves, masks, etc. It is estimated consumption worldwide of 5.5 million tons per year. Latex allergy occurs by type I or immediate hypersensitivity mediated by IgE antibodies to latex proteins.

The relationship between latex allergy and food allergy has two aspects to consider. On the one hand, it acts as a contaminant, in their contact with food proteins, handled with latex gloves. In this situation, there may be apparent signs of food allergy in patients allergic to latex. Furthermore, it is often an association of latex allergy and various fruits and vegetables allergens such as kiwi, chestnut, avocado, banana, papaya, figs, potatoes, peach, pineapple, grapes, celery, plum, tomato, etc…, which is known as "latex-fruit syndrome" (Wakelin & White, 1999), which is related to the existence in latex similar to those found in these plant antigens.

   b. **Anisakis:** The anisakiosis is a parasitic disease that is caused by parasitized fish larvae of Anisakis spp., consumed rare or undercooked. It is particularly important as health problem, in countries with high fish consumption. The adult parasite is found in the digestive tract of a variety of marine mammals, which act as definitive hosts (wales, dolphins, etc.) The larvae mature in the water and are eaten by small crustaceans that feed on various species of fish and cephalopods, which reached the stage of L3. The cycle is completed when the definitive hosts acquire carriers L3 intermediaries. Humans are an occasional host a result of eating contaminated fish larvae and he cannot complete its life cycle.

Clinical signs and symptoms develop as a result of inflammatory reaction caused by the penetration of larvae in the mucosa of the gastric wall. It can also cause manifestations of allergic reaction (hypersensitivity) immediate type ranging from urticarial or angioedema to anaphylactic shock and mixed frameworks with gastrointestinal and allergic clinic symptoms.

In patients IgE antibodies primarily directed against antigens of the various structures of Anisakis larvae are detected. To the consumer, it should not consume rare fish that has not been previously frozen under 24 h, or fresh fish cooked to no more than 60 ° C for at least 5-10 minutes. The cooked on the grill or microwave are less secure than cooking or frying (AESAN, 2005) procedures.

   c. **Mites (Dermatophagoides):** There are several publications of asthmatic patients sensitized to mites, *Dermatophagoides farinae* mainly no history of food allergy, anaphylactic reactions have presented different intensity by ingesting contaminated products made from flour mite. This contamination is common in places with warm, moist environment that favors growth

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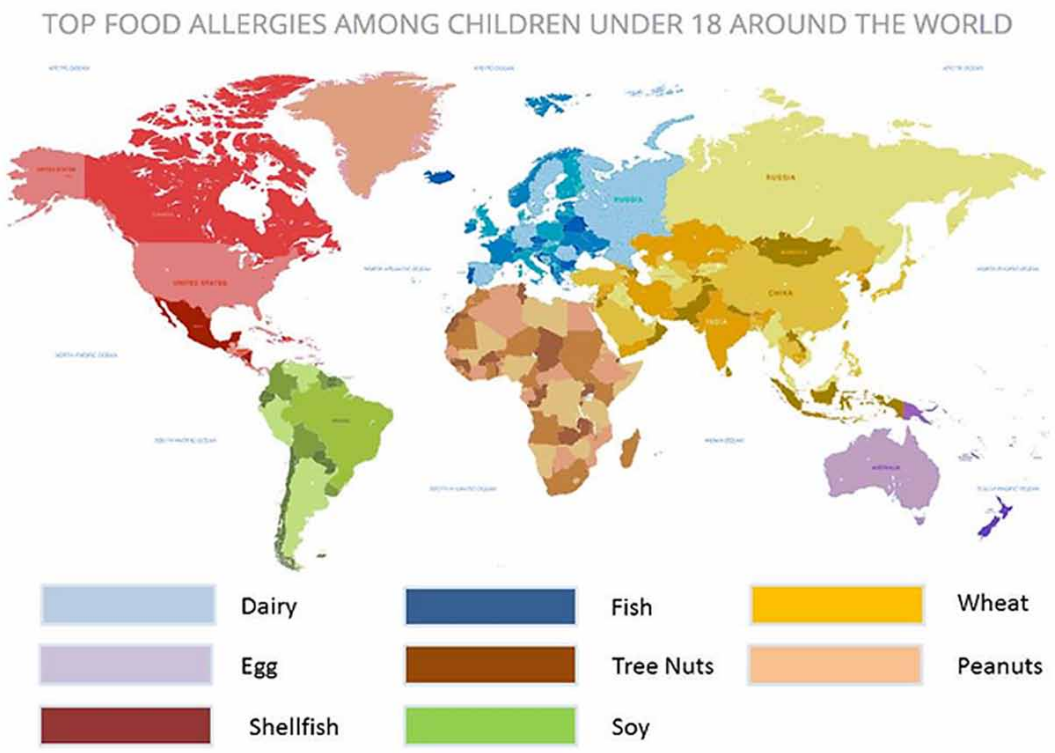
of these mites. In Spain is described on inhabitants of the Canary Islands (Garcia-Robaina et al., 1997).

d. **Residues of Veterinary Drugs:** Sometimes animal foods may contain residues from veterinary treatment. Depending on the nature and risk of the same to human health, mandating absence or maximum limits in foodstuffs is regulated. Specifically, allergic reactions have been reported after ingestion of foods with residues of antibiotics such as ß-lactams and macrolides for which there are maximum permitted residues (Dewdney et al., 1991). Long such allergic reactions have been associated with the presence of penicillin in milk (Wicher and Reisman, 1980). Surveillance studies show a low incidence of detectable residues in most products (Figure 1).

Toxics in Foods

Although an agent causes food poisoning may be chemical or physical, in food toxicology it refers exclusively to chemicals. A term widely used in the drug area to define any foreign substance to the body in question is that of xenobiotic agent. However as Paracelsus said: "dosis sola facit venenum" even oxygen, that is essential to maintain the life of any aerobic organism, an atmosphere of pure oxygen is

Figure 1. Top eight allergies distribution, in children around the world
Extracted from Prevalence of allergies around the world: the big eight (ten, thirteen?). M. Fernandez-Rivas, Symposium on Sensitizing Properties of Proteins, Prague, 11-13 April 2012 (http:www.hesiglobal.org/files/public/Committee%20Presentations/PATC/Fernandez%20Rivasfor%20websiteAPPROVED.pdf)



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harmful to any mammal, as it is quickly consumed aminobutyric acid, moderator cerebral and nerve transmission, decrease and consequently serious nervous disorders occurs that end to convulsions and death (Repetto, 1981) occur. One could mention many examples which show the dictum of Paracelsus, indicating that the beneficial and harmful effect, of a substance depends on the dose. However, for each chemical there is a certain degree of toxicity.

Currently it is managing the concept of "potential toxicity," which has been proposed by Luckey and Venugopal, in an attempt to have a parameter to assess the toxicity of substances more accurately. This potential toxicity (pT) depends among other factors, the experienced animal species and route of administration. By definition, pT is the intake of base 10 logarithm of the inverse of the dose of a substance expressed in mol / kg, given produces a toxic effect. (Luckey & Venugopal, 1977; Repetto, 1981).

$pT = -\log T$

where,

T = toxic dose molar base.

Knowing the molecular weight (MW) of xenobiotic compound or agent is easy to calculate the pT, as shown in Table 5.

Fungal toxins are probably the main cause of chronic toxicity in foods. Since the discovery of protein synthesis inhibitory fungal toxins (Fando et al, 1985), many fungi species has shown dangerous toxins able to appear in foods (Varga et al, 2003, Abarca et al, 2000). These fungi and their spores are sprayed around human environment in building (Cooley et al, 1998, Shelton B.G. et al, 2002), air (Zukiewicz-Sobczak 2013), water (Gonçalves et al, 2006) and food making places (Simeray et al, 1995).

Aspergillus species are probably the most extended fungi and play a key role in fungal toxicity. *Aspergillus oryzae* is used, in much classical Asian fermentations like, soy sauce,

sake and miso. Unfortunately is very difficult to distinguish at lance, between *Aspergillus oryzae,* and *A.flavus, A.restrictus, A.fumigatus* etc… species producers of fungal toxins.(EPA 1997).

Biological Factors that Influence Toxicity

The biological system which acts on the toxic agent is critical, because the effect will vary considerably according to the agency. This factor should be taken into account, since it is well known that among the different species of animals and humans there is great variation in sensitivity to toxic agents. The knowledge of the origin, development and course of poisoning in a particular animal should be established to scientifically based, extrapolate to humans. Precisely in toxicology, a specific branch known as Comparative Toxicology, which indicates on the basis of substantiated studies, animal model can be used to extrapolate experimental results to man. About the above, are some examples of animal models (Melby & Altman, 1976; Hodgson & Gutherie, 1980).

The effect of a toxic agent on a biological system results in an alteration of physiological equilibrium (homeostasis), so that poisoning is an illness and as such should be considered under pathos- chronic criterion, or must observe the developments in line time and we can classify it as acute poisoning, chronic or subacute.

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Acute poisoning is defined as exposure to a xenobiotic agent that produces an almost immediate manifestation (sometimes in minutes), with a single administration of the poison, which can lead to poisoned to death, or total or partial recovery, which they may be or not sequels or nagging injuries.

In many cases of acute poisoning phenomenon, it happen reversibility except death. Overall subacute poisoning does not imply a lesser degree of severity of acute intoxication, but an evolution of subclinical disorders poisoning will appear.

Although this type of poisoning requires repetitive administration of toxic, not visible at the start conditions are present; however, the short term can present evidence of intoxication and some toxicologists believe that it is present, be exposed to the toxic biological system over a period that can range from one month to three months. Chronic poisoning occurs due to repeated exposure to the toxic agent.

This absorption occurs with relatively small toxic, which by itself does not produce visible amounts disorders initially, but the accumulation of xenobiotic agents in the body (typically in a specific organ or tissue) and over time are presented pathological conditions, and in most cases are irreversible. In this type of poisoning, initial conditions remain latent (subclinical) and are usually reversible; until for some reason, as it could be a decrease in normal physiological conditions (illness), you can highlight the poisoning. A classic chronic intoxication example, is the effect of carcinogenic substances, since in sub lethal doses, these compounds are not evident in the short term.

Poisoning requires three months to one or more years and when it manifests itself in clinical form, is very difficult to reverse the damage produced (Fabre & Truhaut, 1976; Repetto, 1981; Klaassen et al, 1986).

The concept of acceptable daily intake (ADI) or also called Acceptable Daily Intake (ADI), refers to the reduced expression of all available toxicological data for a given xenobiotic agent. DDA itself corresponds to the amount of a substance that can be ingested daily by an individual throughout his life, without as to cause damage to health. This level or dose is generally set by animal experimentation on acute and chronic toxicity (currently investigating mutagenic, teratogenic and carcinogenic) and preferably taking the value of the dose without observable effect (NOEL) for the most sensitive species. The maximum amount of xenobiotic that the most sensitive animal can ingest daily without adverse effect sought. However, it can be extrapolated to the human you have to take the safety factor (FS), which should generally consider interspecies or intra-species variation and the value of 100 (Pennington., 1998; Larry et al, 1990; Derache, 1990; Kotsonis et al, 1994).

Therefore the DDA is generally hundredth of NOEL for the most sensitive animal and expressed in mg / kg body weight. This DDA allows a high degree of probability, ensure a low risk of xenobiotic in question, but you will never say with absolute certainty safety.

Getting the DDA from NOEL: Acceptable Daily Intake or ADI

DDA = NOEL / FS

where,

NOEL = no-observed-effect dose (mg / kg pc- day)
SF = Safety factor (10 when the NOEL is of the same species; 100 for another)

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Naturally Toxic Agents in Foods

Natural toxins found inherently in plant or animal foods, can be harmful when consumed in sufficient quantities. Natural toxins serve specific functions in ecosystems as chemical signals or as chemical defenses against predators, parasites or infectious microorganism. Obviously the molecules used have a wide range of chemical structures and toxicity levels.

The most common intoxications are those produced by toxic fungi. Every year inexpert people around the world die by toxic fungi ingestion. Although only a few of the 70-80 species of poisonous mushrooms are actually fatal when ingested, many of these deadly fungi bear an unfortunate resemblance to edible species and are thus especially dangerous. (Karlson-Stiber., Perssons, 2003)

They are near 2.000 species of poisonous plants in the world. Many times more than those considered as food (few hundred). Even many of this "nutritious" plants can become toxic if they are taken in excess or if they are not cooked in the right way. In addition, the toxicity of poisonous plants will change in dependence of sub-specie, as well as the geographical and climate conditions.

In culinary sciences are usual some plants that are very dangerous if they are ingested untreated: Potatoes (Solanine and glycoalkaloids), manioc, bamboo (Cyanide generating compounds and saponines), green beans (enzyme inhibitors). (Wink et al, 2008).

In animals, toxins may come from their proper metabolism or a chemical passed along the food chain. Is more frequent, that poisoning happens, eating marine animals than terrestrial ones. Marine toxins generated by toxic microalgae (Dinoflagellates) are accumulated in shellfish, fish and crustaceans and at end consumed by humans. Also microorganism and coral will produce neurotoxins (Tetrodotoxin) that may cause lethality after ingested (Chun-Fai Yu et al, 2004).

Culinary technics are very important in decrease or annulation of heat-labile toxins. One of most relevant examples is squid ink, used in Spanish and Italian gastronomy. The squid ink, contains toxic microorganism, melanin, melanin related products and L-Dopa. A single thermic treatment of 20 min. at 80º C is enough to neutralize toxicity of squid ink, and let it ready for food use (Fiore et al, 2004).

Food Additives and Toxicity

Food additives are natural or artificial substances, which are added to food in order to preserve or enhance its qualities, either preserving them from microorganisms, enhancing its taste, color or improving its texture. Safety or toxicity of additives it's depends on the substance itself and the amount used in final food. Both natural and synthetic products can be potentially mutagenic or cause allergic reactions (flour improvers, etc.) Another danger is the possibility to be used as cover, tampering or alterations of food, causing poisoning. (FAO/OMS, expert committee, 1965).

Pollutants Toxicity

During the past two hundred years humanity, has been discharging industrial waste in natural environment. Many of this industrial waste are chemically organic and may introduce itself inside food chain.

Persistent organic pollutants (POPs), are chemicals of global concern due to their potential for long-range transport, persistence in the environment, ability to bio-magnify and bio-accumulate in ecosystems, as well as their significant negative effects on human health and the environment. Humans are exposed to these chemicals in a variety of ways: mainly through the food we eat, but also through the air we breathe,

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in the outdoors, indoors and at the workplace. Many products used in our daily lives may contain POPs, which have been added to improve product characteristics, such as flame retardants or surfactants. As a result, POPs can be found virtually everywhere on our planet in measurable concentrations.

The most commonly encountered POPs are organic-chlorine pesticides, such as DDT, industrial chemicals, most notably polychlorinated biphenyls (PCB), as well as unintentional by-products of many industrial processes, especially polychlorinated di-benzo-p-dioxins (PCDD) and dibenzofurans (PCDF), commonly known as 'dioxins'.

However, an overall dietary risk assessment would require inclusion of other dietary sources of dioxins and dioxin-like PCBs. In many countries, an inadequate use of newspapers as starting of fire in home grills will produce the excess of POP needed, to appear a disease.

In order to address these risks, several steps have been taken to reduce or eliminate emissions of dioxins, dioxin-like PCBs and other related persistent organic pollutants (often referred to as POPs). Many countries have now implemented the Stockholm Convention on POPs (2001), which suggests ending commercial use of 12 POPs and reducing or eliminating their emission into the environment. To monitor reduction in human exposure WHO and UNEP run a monitoring program for POPs in breast-milk. A steady decline in levels has been observed since 1980 for most countries. (ONU, UNEP/POPS/POPRC.10/10, 2014)

In regard to food contamination, the FAO/WHO Codex Alimentarius Commission, which is a risk management body comprised of 169 member countries, is developing a draft code of practice for dioxins and dioxin-like PCBs in food, which identifies source-directed measures to reduce their presence in food, including fish, as well as a position paper, which provides an evaluation of the need for possible regulatory measures, such as limits in food and feed. (WHO, Food Safety Program updates, 2015)

Toxic Agents Generated in Food Processing

1. **Ethil-Carbamate/Urethane:** Ethyl carbamate (EC, urethane), is a naturally occurring component of all fermented foods and beverages. Because EC has shown a potential for carcinogenetic when administered in high doses in animal tests, production of EC is tried to be reduced during food production. The winemaker industry is interested in reducing EC levels in their products (Butzke & Bisson 1997).
2. **Allyl-Isothiocyanate:** Onion derived substances (Allyl-isocianates) has been used as bio-protective gas additives, to increase self-live of packed foods. Their use is not extended inside industry and exist some controversial opinions, about the advantages of their use. In a hand, there are natural biocides in the other hand may change the natural flavor of the product by adsorption.
3. **Pyrrolizidine Alkaloids:** Thirteen plant families are well known to contain pyrrolizidine alkaloid (PA) (IPCS, 1988). PA's are hepatotoxic, carcinogenic, genotoxic, teratogenic and sometimes pneumo-toxic. World-wide many episodes of PA intoxications have been reported involving ruminants as well as humans. Ruminants may eat the PA-plants directly as part of the forager, humans in an unconscious way taking contaminated grain or through ruminant's products (milk, meat, etc...). Also the microflora of the rumen and the origin specie of plant PA may vary the intensity of poisoning (Wiedenfeld & Edgar 2011).
4. **Alkenyl Benzenes and Derivatives:** Eugenol and methyleugenol, are aromatic compounds present in many natural herbs and plants used in culinary (Miele et al, 2001). Also may be presents in packaging of foods (Aurela et al, 2001), as pollutants of non-vegetable foods. In both cases the

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presence of these molecules, at high concentratios may increase the risk of cellular mutagenicity and finally cancer (Dean 1978).

5. **Tannins:** Tannins are in all plants as part of the metabolism of cellulosic structures and have potential antioxidant activity (Hagerman, et al, 2015) and biocidal and antibiotic activity (Scalbert, 1991). These biological activities give to tannins relative toxic activities at high concentrations in animal feeds (Makkar 2003)
6. **Ethanol and Coffee:** Both may be qualified as "social drugs" and need to be ingested in baste quantities to become deadly toxic for humans. Many foods and food additives that are safe for human consumption can be extremely toxic to pets (Gugler et al, 2013). Even chronic ingestion of ethanol causes digestive mucosa irritation and changes in pets' intestine adsorption patterns (Bell et al, 1985).
7. **Diacetyl from Adverse Fermentations:** Diacetyl, is an important component in the flavor of food and it is known to be the chief component of the aroma of butter. It has been found in adverse fermentations of *Sacharomices Cerevisae* and has long been known. (Suomalainen & Ronkainen, 1968). Recently some strain of *Enterobacter Aerogenes*, are found to be able produce high levels of diacetyl during fermentation. (Zhao Ling et al, 2007). The absorption of diacetyl by air in mice shows relationship with obliterative bronchiolitis (OB) disease. This relation it has been observed also by Centers for Disease Control and Prevention (USA), in flavor-manufacturing industry workers. (CDC, 2007).
8. **Flavonoids:** Flavonoids are phenolic compounds that represent substantial constituents of the non-energetic part of the human diet. A group of metabolites of increasing interest are the phytoestrogens and phytosteroids, which are agonist or antagonist to the action of human endogen steroids. There are many chemical characterization and several biological studies of these compounds and many of them indicate the various possible medical applications, for example, in the decrease of symptomatology of menopause and the prevention and treatment of related diseases, in the prevention and treatment of cardiovascular diseases and in some types of cancer (Leyva et al, 2011). The studies about the toxicity of this natural compounds show a protective effect more than toxic one. (Fotsis et al, 1998) (Mishra et al, 2008).
9. **Maillard Reactions:** The Maillard reaction, is fundamental in culinary sciences for the production of aroma, taste, and color during the cooking of many foods (meat, bread, etc..). Recently it has been shown that these reactions may be accompanied by a reduction in nutritive value and the formation of toxic compounds (Ledl, & Schleicher, 2003). The increase of Strecker degradation reactions (Van Boekel M.A.J.S, 2006) larger the amount of toxic by-products found. Mutagenic compounds like acrylamide are the final result of this decomposition reaction (Mottram et al, 2002).
10. **Lipid Thermodegradation:** When we heat food in contact with oil, a lot off physicochemical reactions happen at same time. To heat the frying oil to cooking, we have to raise the temperature above 100 ° C, trying not rise above the smoke point (which depends on the type of oil we use), normally in our houses, 120ºC. At this temperature, begins to realize the Maillard reaction. The sugars and amino acids from food react to give candy type products and finally decompose it into aldehyde compounds and pyrazines (Strecker degradation reaction) that are unhealthy enough.

Simultaneously, fatty acids (all oils are fatty acids, more or less purified), are degraded in a real chain of chemical reactions. Fatty acid of our oil, bind with oxygen from air to give hydro-peroxide molecules.

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Food fat decomposition give more (generally saturated, climbing of cholesterol) free fatty acids and semi-decomposed fats (mono-glycerides, di-glycerides and glycerin). Finally, with increasing temperature, we obtain dimers, trimers, epoxides, alcohols, simple acids, hydrocarbons and ketones. That is, all a wide variety of organic compounds, more or less toxic.

Regarding the type of oil oxidation by heat, always increases the acidity, so if we start heating an oil of lower acidity, it will take longer to upload this to levels that makes it disgusting sensory. Special oils, which sell for fryers are almost pure fatty acids (high oleic, etc....), this means they can be used longer without degradation, but its organoleptic characteristics are very low. In the market are also sold anti-oxidant compounds to add to the frying oil that make it longer shelf-life, but usually is cheaper to optimize the use of standard oil in the kitchen.

11. **Sucrose and Tooth Decay:** Relationship between sucrose, bacterial adhesion and tooth decay are well stablishing. Glycoprotein and plaque formation is a fast process that fixes anaerobic bacteria like *Streptococcus mutans*, in teeth surface. Lactic acid generated by bacterial metabolism causes teeth cavity.

In modern cuisine, use of polysaccharides based, food additives (thickening and gel-forming agents), modulate the effect of bacterial adhesion to tooth (Steinberg et al, 2005).

FUTURE RESEARCH DIRECTIONS

The main matter is the difficulty to obtain data about ADIS and NOEL, fine referenced, that may be used in food or culinary process. In the other hand, to understand this data is mandatory a minimum level of toxicology knowledge. Basic food toxicology and culinary sciences should be a basic knowledge to be study in food technology, food production and culinary sciences careers. Day by day, allergen and toxin detection thresholds are increasing in the same ratio than technology, but some answer remains unresolved (Taylor S.L. et al, 2004). Protective measures are running, but their real effect on population is unknown at time (Moneret-Vautrin, & Kanny 2004). Many review on food allergens thresholds, have

Table 1. Main food intolerances by enzymatic effect

Situation	Enzyme Deficiency
Disaccharidase deficiency	Lactose, sucrose
Galactosemia	Galactose-*1*-phosphate uridyl transferase
Hyperbilirubinemia	uridine diphosphate -epimerase
phenylketonuria	Fenilalanin-hidroxilasa
Alcohol intolerance	Aldehyde-dehydrogenase
Favism	*Glucose-6*-phosphate dehydrogenase

Table 2. Endogenous agents responsible for the pharmacological action of food

Vasoactive Amines	Diamines	Histamine
	Monoamines	Tyramine *Phenylalanine* Serotonin Dopamine
Methylxanthines		Caffeine Theobromine Theophylline
Others		Capsaicin Ethanol Myristin

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Table 3. Toxic food reactions

Due to Added Chemicals	
Agricultural chemicals Chemicals packaging Industrial chemicals	Insecticides, herbicides, fungicides, fertilizers Lead, tin, copper, cinc Polychlorides and biphenyl polibromuros, mercury
Due to Natural Contaminants	
Microorganisms Toxins	Campylobacter, Salmonella, Sighella Clostridium botulinum (botulism) Estaphylococcus aureus (staphylococcal intoxication) Neurotoxin dinoflagellate (ciguatera) Mycotoxins (ergotism, aflatoxins)
Due to Persistent Toxic Chemicals	
Dangerous at any dose	Poisonous animals Poisonous plants Poisonous mushrooms
Dangerous if ingested in Large Quantities	Cyanogenic Glycosides (plumlike) Latirógenos (almortas) Bociógenos (berros, nabos) Atropina (estramonio) Solanina (patata y tomate verdes)
By Generated Natural Toxicants	
	Histamine poisoning (scromboid)

Table 4. Differences and similarities between allergic reactions, intolerance reactions and toxic reactions to food

	Allergic Reaction	**Intolerance Reaction**	**Toxic Reaction**
Depending on the individual factors	(+)	(+)	
Depending on food factors	(-)	(-)	
Pathogeny	Inmunological	Diverse, non inmunological	Diverse, non inmunological
Examples	Urticaria	Lactose intolerance	Escombroid
	Anaphylaxis	Favism	Lathyrism
	Enteropathies	Galactosemia	Botulism
	Colitis	Phenylketonuria	Toxic Oil syndrome

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Table 5. Acute toxicity of some representative substances

Substance	DL 50 (mg/K)
Ethylic Alcohol	10.000
Sodic Phenobarbital	150
Strychnine	2
Nicotine	1
Tetrodotoxine	0,1
Dioxin	0,001
Botox	0,00001

From Klaassen et al., 1986.

try to clarify this questions, but the relationships between allergen presence and toxic/immune-reaction remain unclear (Kirsch et al, 2009) (Green et al, 2002) (Crevel et al, 2008) (Morisset, et al, 2003) (Nowak-Wegrzyn et al, 2009) (Fernandez 2012)

CONCLUSION

Adverse reactions to foods produced by allergies, food intolerances and toxins, are an emerging health problem. The incidence of these diseases presents a continued increase in recent years. The most effective way to avoid the adverse food reactions is eliminated from the diet of susceptible people components that trigger the adverse effect, making an exclusion diet. For consumers with allergies, exposure to a small amount of food that is sensitive can provoke severe reactions, including anaphylactic shock which can compromise their life. There is great variation in sensitivity of different food allergens among the population, and there is currently no consensus on allergen levels, with the exception of gluten, which may lead to an allergic reaction in susceptible people. That is why they have to have all the necessary information about the composition of foods to make a right choice according to the needs and becomes an essential element of food safety.

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Chapter 24
Food and Cardiac Health:
Protective Effects of Food on Cardiovascular System

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ABSTRACT

Emerging influence of Cardiovascular Diseases (CVDs) and its impact on the society has raised much awareness for its prevention. Healthy food habits and physical exercise has drawn a lot of attention of the people from scientific as well as common world. The role of food-based bioactive compounds in reducing risk of CVDs has been established with various health benefits apart from the basic nutrition have been reported. The present chapter provides an overview of the role of different foods on cardiovascular health of humans. Biological effects of plant derived food products and their bioactive compounds in the context of relevance to cardiovascular health promotion are discussed in detail. The chapter also covers the effects of the consumption of functional food on the intermediate clinical markers of CVDs including cholesterolemia, hypertension, endothelial function and inflammation. The chapter will enable the better understanding of the current knowledge on the potential health benefits of different functional foods and bioactive compounds on cardiovascular health.

INTRODUCTION

Cardiovascular Diseases (CVDs) have a substantial influence on public health from past several decades and it still remain the major cause of mortality and morbidity throughout the globe. CVDs comprise group of different cardiac and vascular complications including hypertension, coronary heart, atherosclerosis, cerebrovascular disease (stroke), peripheral artery disease, rheumatic heart disease, heart failure etc. Major lifestyle causes of CVDs include tobacco intake, physical inactivity and unhealthy diet (World health organization, 2009). Major CVD events arise due to atherosclerosis, a pathophysi-

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ological complication of innermost layer of arterial wall and such events can be prevented by nutritional supplementation (O'Toole et al. 2008). Different parameters have been studied in this regard and life-style changes have been suggested as the most helpful practices including physical activity and healthy food habits. Healthy diet and food habits show an inverse relationship with onset of vascular diseases as well as affect the longevity. Food provides both the essential nutrients required for basic life processes as well as bioactive compounds that help in disease prevention and health enhancement. Balanced diet including fruits, vegetables, whole grains and other plant foods helps in acquiring the required amounts of nutrients, antioxidants, bioactive compounds and phytochemicals thereby prevents various health related complications. Potential health promoting benefits of the natural bioactive compounds have been studied over time. There is keen interest in assessing the role of food-based bioactive compounds in reducing risk of chronic diseases including Cancer, CVD and diabetes mellitus.

For the reduction of coronary heart diseases, much emphasis is given on reducing the saturated fat, trans fat and cholesterol to lower the low-density lipoprotein-cholesterol (LDL-C) levels. Other pathways involved in the protective effects mediated by different food products with respect to CVDs onset has also been studied extensively. Oxidative stress results in cellular damage by affecting proteins, DNA and lipids, thereby increasing the risk CVDs. Oxidized LDL-C are a major factor that contributes to the cardiac diseases. Antioxidant potential of fruits and vegetables accounts for the most of the health promoting benefits and CVD prevention (Pandey & Rizvi, 2009). Major oxidative stress mediated events that lead to various cardiovascular complications are summarized in the Figure 1. Other factors include preventing vascular inflammation, reducing platelet hyper activity, vasodilation, cardiac hypertrophy etc. Inflammation is a critical factor in CVDs and its systemic marker, C-reactive protein, plays an important role in disease progression. Inflammation promotes atherosclerosis initiation and progression resulting in severe thrombotic complications of atherosclerosis (Shrivastava et al, 2015). Platelet activation and aggregation play a key role in the pathogenesis of myocardial infarction and ischaemic cardiac complications. Cardiac hypertrophy is a compensatory patholphysiological response of the heart to chronic pressure, and is a critical risk factor for ischemic heart disease, arrhytmia and sudden death. Dietary supplementation can promote the proper platelet function by reducing the hyper-reactivity of platelets and maintain cardiovascular health.

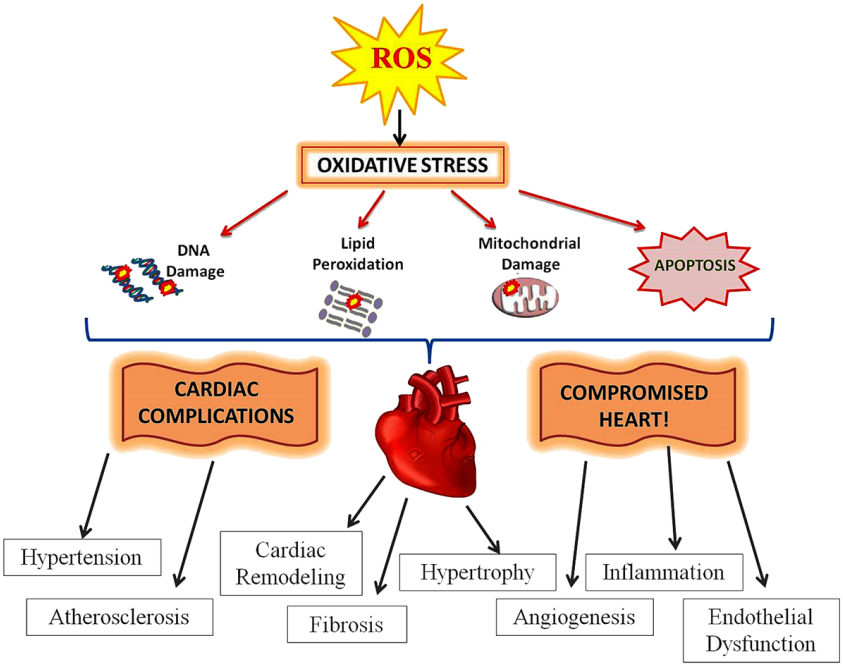
Based on the extensive studies done in the previous decades, a throughout general observation has been made to support the hypothesis that the precise intake of foods and beverages with high amount of flavonoids and polyphenols play an important role in reducing CVD risk by improving vascular function and modulating inflammation (Habauzit & Morand, 2012). There are ample epidemiologic evidences from numerous studies that suggest a strong relationship between the consumption of diets high in fruits and vegetables, legumes, whole grains, fish and cardiovascular disease protection. Phenolic compounds including flavonoids, tannins etc. are present in almost all the plants and vegetables. These compounds have been studied in detail for their protective and beneficial roles in human health.

NATURAL FOODS WITH CARDIOPROTECTIVE BENEFITS

There are increasing facts suggesting the uptake of natural food products as they are enriched in micro-nutrients including minerals, vitamins and essential fatty acids that are required to sustain the cellular antioxidant levels and other stress response mechanisms (Visioli & Hagen, 2007). It has also been demonstrated in various studies that intake of a healthy balanced diet including whole food is evidently

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Figure 1. Oxidative stress induced cardiac complications



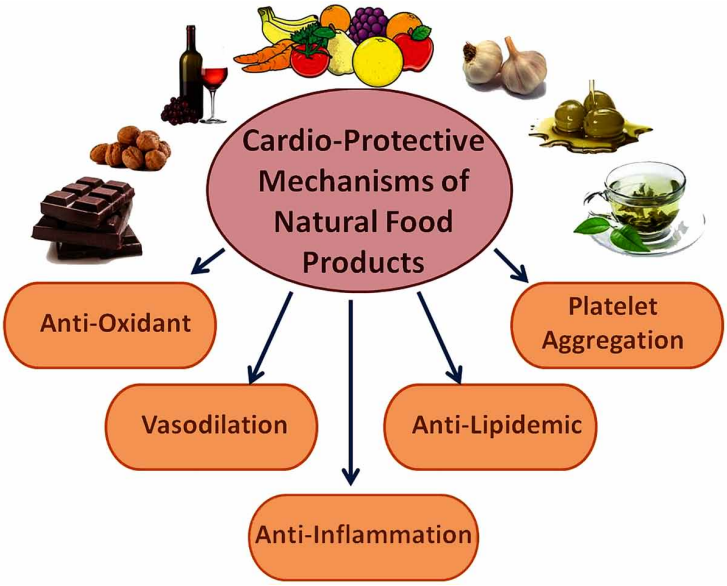
more beneficial as compared to the consumption of processed food. It may be contributed to the fact that isolated compounds either loses their biological activity or may not behave in the similar way as in the whole foods. Hence, the usually present combination of phytochemicals of natural food products is responsible for the various potential preventive effects. Number of food item has been reported to play a significant role in prevention and cure of CVDs. These are discussed in detail in the following section and their different mechanisms are depicted in Figure 2.

Fruits, vegetables, legumes, whole grains and fish are high in fiber and omega 3 (or *n*-3) fatty acids as well as low in saturated fat, trans fat and dietary cholesterol. Whole grains, nuts and green leafy vegetables are the rich source of vitamin E, which is well known for reducing the risk for atherosclerosis and coronary heart diseases. Fruit and vegetables have sufficient amount of antioxidants including phytochemicals, phenolics and carotenoids that protect cellular systems and reduces the risk of various diseases including CVDs. In a very recent UK Women's Cohort Study, it has been eastablished that total fruit intake lowers the risk of CVD mortality, with a 6–7% reduction in risk for each 80 g/day portion consumed. Also, women in the highest intake group of grapes and citrus experienced a significant reduction in risk of CVD and stroke as compared with the non-consumers (Lai et al., 2015).

Garlic, or *Allium sativum* has been used in daily food practices across the worlds from centuries and it has been studied for various medically important effects including hypocholesterolaemic, anti-atherosclerotic, antioxidant and cardioprotective (Bayan et al, 2013). Garlic comprises specific allyl sulphides and other organosulpfur compounds that express the characteristic protective properties of garlic in wide variety of patho-physiological conditions. These sulphide compounds restrict the oxidized LDL mediated cellular eNOS degradation as well as prevents the vasculae endothelium from oxidative stress mediated effects (Seki & Hosono, 2015). Garlic is well known for reducing total cholesterol and TAG thereby preventing cardiac complications (Tapsell et al, 2006). Garlic consumption decreases mortality and morbidity rates

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Figure 2. Cardio protective mechanisms of different natural food products



of CVDs through its antithrombotic, hypoglycemic and lipid-lowering properties. Garlic oil is shown to decrease hypercholesterol mediated cardiac hypertrophy *in vivo* by modulating different signaling pathways and molecules including IL-6, p-extracellular signal-regulated kinase-5, p-MAPK-5, calcineurin, p-GATA binding protein 4 thereby improving hypertrophy-associated cardiovascular complications (Hseieh et al., 2014). It is also known for maintaining the homeostasis of the immune system by stimulating specific cell types and other mechanisms important for the development and advancement of various diseases including CVDs. Garlic modulates cytokine secretion thereby contributing to its action for many of the therapeutic effects (Arreola et al., 2015). Garlic supplements have also shown to reduce the high blood pressure. In a meta-analysis conducted by Wang et al., it was observed that garlic consumption directly modulates blood pressure and it is significantly effective in the hypertensive patients (Wang et al., 2015). Preventive effects of garlic powder on CVDs and risk factors were studied in another meta-analysis where it was found that garlic significantly reduces blood total cholesterol and LDL-C levels. Garlic was also found to decrease systolic and diastolic blood pressure (Kwak et al, 2014).

Green Tea, derived from *Camellia sinensis*, is well studied for its role in cardiac protection and CVD prevention (Cooper & Morre, 2005; Kuriyama et al., 2006; Sumpio et al., 2006). Green tea consumption has shown to inhibits cardiomyocyte apoptosis, a critical factor in the transition from hypertrophy to heart failure, prevents cellular damage caused by oxidative stress, p53 modulation and decrease in Bcl-2 expression (Sheng at al., 2007). It also reduces LDL-C levels thereby directly reducing different cardiac complications.

Syzygium cumini, also known as black plum or jamun, is a well-known for its medicinal properties including anti-inflammatory, hypoglycemic, anti-oxidant, antibacterial and cardioprotective (Chagas et al., 2015). Various *in vitro* and *in vivo* studies have demonstrated the cardioprotective potential of *S. cumini.* It has been shown to reduce isoproterenol-induced myocardial infarction in rats by inducing biochemical alterations (Mastan et al., 2009). It also exerts cardioprotective effects against glucose induced oxidative stress on cardiac myocytes by exerting free radical scavenging activity (Atale et al., 2013).

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In studies, nuts consumption as a part of diet has shown different potential health benefits despite of their high fat content (Coates & Howe, 2007). Nuts intake markedly decrease the risk of sudden cardiac arrest and other associated heart problems like high cholesterol levels. Walnuts, almonds and hazelnuts have drawn ample attention in the recent years for their cardio protective and other health benefits.

Oil derived from olives has been shown to decrease CVD risk by reducing LDL oxidizability and downregulate CD40-ligand expression and their downstream products (Castaner et al., 2012). Canola oil can reduce plasma cholesterol levels and influences various biological functions and biomarkers of cardiac disease risks. Canola oil consumption also increases tocopherol levels and effects insulin sensitivity, lipid peroxidation, inflammation, energy metabolism, and cancer cell growth (Lin et al., 2013).

In a recent study, it has been shown that the consumption of beans have hypercholesterolaemic with resulting in reducing the LDL-C levels without disturbing the HDL-c levels (Winham & Hutchins, 2007).

Xuezhikang, Chinese red-yeast rice, comprises statins that are used to supplement the diet for the prevention of cardio vascular diseases. Studies have shown that this rice can prevent the occurrence of cardiac complications in diabetic patients by 50% (Zhao et al., 2004). It has no adverse effects associated with the regular consumption and its commercial production should be encouraged in the other parts of the world as well. Buttermilk consumption has shown to be associated with reduced cholesterol and triglycerides concentrations in men and women by inhibiting intestinal absorption of cholesterol. Therefore, it should be recommended as a natural and low cost dietary product for improving lipid profiles in low risk patients (Conway et al., 2013).

These remarkable effects of natural food products thus reinforce the recommendation to raise the intake for the cardiovascular protection.

FUNCTIONAL FOOD AND CARDIAC HEALTH

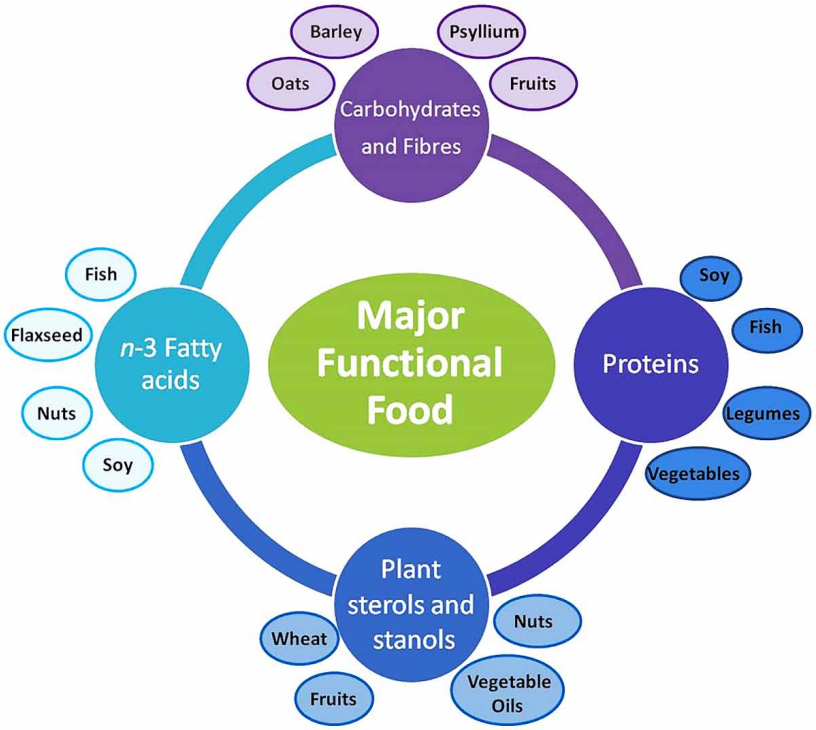
Functional food displays advantageous effect on different body functions by improving the state of health and reducing the disease risk apart from its nutritional importance. Functional food comprises the food that we consume as a part of our diet with beneficial effects. It has been observed in various clinical and epidemiological studies that protective nutritional practices can reduce the risk of chronic heart disease upto 60% (Everitt et al., 2006; Kris-Etherton et al., 2002; Habauzit & Morand, 2012). These protective ingredients in our food reduce the major risk factors of cardiac abnormalities like reduction in blood cholesterol levels, hypertension and diabetes. Functional foods majorly comprise soluble fibres, phytostenols, sterols, long-chain *n*-3 fatty acids, soya proteins etc. These components majorly act upon the serum lipids and reduce the risk of heart diseases. The major classes of functional foods along with their main sources are illustrated in the Figure 3.

Dietary fibres help in reducing the cholesterol levels and considered as a safe approach for the prevention as well as cure of high cholesterol levels (Erkkila & Lichtenstein, 2006). Soluble fibres decrease the LDL-C levels thereby exerting hypocholestrolaemic effects and hence, recommended harmless. They are majorly present in fruits, oat bran, barley, flaxseed and psyllium.

β-glucan from soluble oats and barley is also well known for the reduction of LDL-C levels. It is primarily associated with the reduction in the bile acid absorption and cholesterol levels. Its consumption in the form of a fruit drink has shown significant reduction in cholesterol levels (Naumann et al, 2006; Nwachukwu et al., 2015).

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Figure 3. Major categories of different functional foods and their sources



Long-chain fatty acids of the *n*-3 series are essential dietary compounds as they cannot be synthesized in mammals. They play an important role in modulation of membrane properties and lipid mediated signaling pathways by incorporating into the structural lipids (Sirtori et al, 2009). Intake of *n*-3 fatty acids, like eicosapentaenoic and docosahexaenoic acids result in decreased incidence of coronary heart disease and unexpected cardiac death (von Schacky C, 2004). They are generally present in animal diets, especially fish, with few exceptions of certain seeds like nuts, flaxseeds, linseed, rapeseed and soy. The role of fish consumption in reducing the risk of cardiovascular diseases was established several years back and has become the basis of using fish oils and other fish derived products in purified forms for supplementing the diet for the disease prevention (Elvevoll et al, 2006). It has been observed that *n*-3 fatty acids from fish oil are involved in a number of nuclear receptor-regulated mechanisms thereby regulating tissue lipid metabolism and other associated metabolic and lipoprotein disorders (Dubey & Cheema, 2006). The *n*-3 fatty acids also help in improving atherosclerosis risk factors like hypertension apart from triacylglycerolaemia and HDL-cholesterolaemia (Borghi & Cicero, 2006). Because of these beneficial effects, different forms of *n*-3 fatty acids have been incorporated into variety of easily available food items, for example, cow's milk and bread spreads.

Sterol compounds produced by plants can decrease the intestinal absorption of dietary and biliary cholesterol by interfering with their transporters thereby altering the cholesterol metabolism. Stanols are formed by saturating β-sitosterol and campesterol and incorporated in the different varieties of functional foods (Winter, 2005). Phytosterols and phytostanols have hypocholesterolaemic efficacy as the esterification of the fatty acids with plant sterols, improve the LDL-C:HDL-C ratio (Chan et al., 2007).

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Soya proteins have shown various cardio protective effects in various animal and human studies mainly by reducing the total cholesterol, LDL-C and ischaemic events. Consumption of low carbohydrate diet including either soya or vegetable proteins have shown to reduce the cardiovascular risk (Halton et al, 2006). In 1999, US Food and Drug Administration approved the health claim for the role of soya protein intake in cardiovascular disease risk reduction based on the cholesterol lowering effect of soya proteins. Similarly to soya protein, fish protein also reduces cholestrolaemia and blood pressure thereby preventing cardiovascular risk. Protein derived from lupin seeds has also shown remarkable cholesterol-lowering effects in various studies (Martins et al, 2005). Lupin has an advantage of having the whole protective ingredients for the cardiac protection including fibres, phtosterols and protein. Lupin protein isolates have also shown to decrease the development of hypertension (Pilvi et al, 2006). Red wine and purple-grape juice has shown to exert platelet-inhibitory properties. The antioxidant potential of red wine provides cardioprotection by reducing oxidative stress in different pathological conditions of cardiac system (Das et al., 2007).

BIOACTIVE COMPOUNDS FROM FOOD AS CARDIOPROTECTANTS

Bioactive compounds are defined as the extranutritional components that are present in food in small quantities. Phytochemicals are the bioactive nonnutrient plant compounds present in fruits, vegetables and other plant based foods. They are known to reduce the risk of various chronic diseases directly or indirectly. Phenolic compounds and flavonoids are the extensively studied bioactive compounds as they are present in all the plants. They have been studied widely in cereals, nuts, olive oil, legumes, vegetables, fruits, tea etc. Studies have reported protective relationship among flavonoids and CVD. Their antioxidant potential reflects favorable effects on CVD risk factors. Different classes of bioactive compounds along with their natural sources and protective roles have been summarized in Table 1.

Various plant-derived foods and drinks, comprising chocolate, wine, berry juices, tea, coffee etc., have been extensively studied *in vitro* and *in vivo* for their potential benefits on cardiovascular health. They hold a wide range of phenolic compounds, including flavonols, flavanols, procyanidins and anthocyanins (Pascual-Teresa et al, 2010).

Flavonoids are the most common polyphenolic compounds present in plant derived food products. The reduction in CVD risk as observed after flavonoid intake is clinically significant (Hooper et al, 2008; Mursu et al, 2008). Based on an hypothesis for understanding the polyphenols portective effect, acidic nature of flavonoids may act as chemical uncouplers in the mitochondria leading to an increased resting energy expenses and results in the increased cell longevity (Modriansky & Gabrielova, 2009). Cocoa is rich in flavonoid content that has been well studied for its protective role in cardio vascular disorders (Ding et al., 2006). Other than flavonoids, Cocoa also contains catechins and procyanidins, which can reduce the oxidant-induced peroxynitrite production in vitro, enhance the NO synthase expression and NO-dependent vasodilation in the aorta and prevent hypertension (Grassi et al, 2005; Kurosawa et al, 2005). Cocoa polyphenols exhibit antioxidant, anti-inflammatory and anti-atherogenic activity and targets NF-κB and iNOS (Rimbach et al, 2009). These benefits make dark chocolate consumption, either in powder, as chocolate or from different cocoa flavanol-rich drinks, a very important measure for reducing cardiovascular risks (Corti et al., 2009).

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Table 1. Bioactive compounds, their natural sources, and protective roles in human health

Bioactive Molecules	Major Natural Sources	Protective Roles
Anthocyanins	Red, blue and purple berries	Anti-oxidant, cardio protective, DNA damage protection
Proanthocyanins and Procyanidins	Cocoa, grapes, apple, cinnamon	Anti-oxidant, anti-carcinogenic
Phytoestrogen	Soy, flaxeed oil, whole grains, fruits and vegetables	Anti-oxidant, cardio protective
Hydroxytyrosol	Olive oil	Anti-oxidant, cardio protective
Reserveratol	Nuts, red wine	Anti-oxidant, anti-thrombonic, anti-inflamatory, anti-carcinogenic
Lycopene	Carotenoid fruits including tomatoes	Anti-cancerous
Organosulphur	Onion, garlic	Anti-carcinogenic, cardio protective, anti-oxidant
Isothiocyanates	Cruciferous vegetable	Anti-carcinogenic, cardio protective
Monoterpenes	Citrus fruits, cherries, herbs	Anti-carcinogenic, cardio protective
Quercetin	Onions, fruits, broccoli	Anti- inflammatory, cardio protective, anti-hypertensive, anti diabetic, anti cancerous
Myricetin	Berries, fruits, vegetables, herbs, tea and wine	Anti-oxidant, anti diabetic, anti cancerous
Kaempferol	Aloe vera, fruits	Anti-inflammatory, anti-apoptotic, anti oxidant, anti cancerous, anti bacterial
Luteolin	Fruits, vegetables, medicinal herbs	Anti cancerous, anti oxidant, anti- inflammatory, anti-hypertensive
Fisetin	Strawberry, onion, grapes, wine	Bone protective, neuro protective
Catechin and Epicatechin	Tea, chocolate, apples, pears, grapes and red wine	Anti oxidant, anti-obese, cardio protective
Tannins or Proanthocyanidins	Fruits and vegetables	Anti oxidant, cardio protective, anti-inflammatory, anti-carcinogenic and anti-mutagenic

Phenolic compounds and polyphenols have shown to regulate various signaling pathways involved in cell survival, growth and differentiation. Polyphenols represents the major contender to explain the protective effects of plant-derived food products. The anti-inflammatory action of phytochemicals present in wide variety of plants may play an important role in the prevention of CVDs. Phytochemicals have also been shown to have roles in the reducing platelet aggregation, cholesterol synthesis modulation and blood pressure maintenance.

Catechin and epicatechin are the most common flavonoids. Catechins are also called monomeric flavonols and they are the biosynthetic precursor of proanthocyanidins and well known for their anti-oxidant potential, but their protective effect on cardiovascular health is also accounted by other effects including mediating important mechanisms like hypertension, inflammation, cellular proliferation, hyperglycaemia and hypercholesterolemia. Epigallocatechin gallate is a major catechin of green tea and it has been shown to decrease vascular inflammation by synthesizing more of nitric oxide thereby blocking endothelial vascular inflammation (Yamakuchi et al, 2008). Nitric oxide production is also restricted by PPAR receptors in response to catechins (Danesi et al, 2009). Catechin consumption has also shown to decrease decreased atherosclerotic factors including TNF-α, sICAM-1, MCP-1 and CRP suggesting the effective role in reducing atherosclerosis and proinflammation (Hsu et al, 2007).

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Quercetin is another flavonoid present in fruits, vegetables, nuts, seeds and flowers and has epidemiologic evidence for a protective effect against CVDs. It inhibits platelet aggregation and reduces thromboxane synthesis and may be helpful in reducing CVD risks (Egert et al., 2009).

Oligomeric procyanidins or flavan-3-ols have shown to activate of eNOS and mediating an endothelium dependent relaxant effect that modulates the NO/cGMP pathway (DalBo et al., 2008). Proanthocyanidins present in grape seeds have demonstrated to reduce the levels of proinflammatory markers such as CRP, IL-6, TNF-α and an increase in the production of antiinflammatory cytokines thereby suggesting the obesity-related adipokine dysregulation to manage cardiovascular and metabolic risk factors (Terra et al., 2009). In an *in vitro* study, catechin and proanthocyanidins have shown the cardioprotective effects on myocardial cells against the toxicity originated by the administration of anticancer drugs like doxorubicin (Du et al., 2008).

Anthocyanins are the bioactive compounds with dual benefits, firstly for their impact on the sensorial characteristics of food products and secondly for their health related properties with proposition on cardiovascular disease risk protection. Anthocyanins are the pigments responsible for the bright colors of many fruits and vegetables. (de Pascual-Teresa & Sanchez-Ballesta, 2008). They can act upon different cells involved in the pathogenesis of atherosclerosis, in addition to the effects on cholesterol distribution thereby protecting endothelial cells from CD40- induced proinflammatory signaling (Wallace, 2011; Xia et al., 2007). Anthocyanins have also shown decrease in NF-kB–related proinflammatory chemokines, cytokines, and inflammatory mediator responses in the plasma suggesting inhibition of NFkB activation by anthocyanins (Karlsen et al, 2007).

Curcumin or diferuoylmethane is an active ingredient of and well known for its different protective properties including anti-inflammatory, antioxidative and cardiovascular effects (Miriyala et al., 2007; Ahuja et al., 2011). Curcumin treatment reduces free radical generation, monocyte adhesion to activated endothelial cells and phosphorylation of c-Jun N-terminal kinase, p38 mitogen activated protein kinases. These effects results in the prevention of myocardial function following ischemic or biochemical stress to the heart. Curcumin can also reduce cardiac remodeling and suppresses cardiac hypertrophy (Kohli et al., 2013; Srivastava & Mehta, 2009).

Various phytoestrogens are present in soy, but also in flaxseed oil, whole grains, fruits, and vegetables. Many phenolic compounds have antioxidant properties Hydroxytyrosol is a phenolic compound present in olives and olive oil and well known as a potent antioxidant. Resveratrol is found in nuts and red wine and exhibits antioxidant, antithrombotic and anti-inflammatory properties. Lycopene is also an effective antioxidant present in tomatoes and other fruits. Organosulfur compounds present in garlic, isothiocyanates in cruciferous vegetables and monoterpenes in citrus fruits, cherries, and herbs are few other examples of cardioprotective effects mediating bioactive compounds.

CONCLUSION

Prevention is better than treatment and the same applies to the onset of CVDs. Novel dietary advances for cardiovascular prevention are of major significance at present as there is an increasing despise in primary prevention of CVDs by chronic drug usage. A correlation between CVD prevention and healthy diet has been demonstrated from the various studies. There are sufficient evidences recommending the consumption of food sources rich in bioactive compounds including flavonoids, fibres, plant proteins and phytosterols, polyphenols etc. Integrating plant foods with high levels of flavanols in the diet of healthy

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individuals can decrease the risk of CVD significantly. The active compounds present in the plants have huge potential to reduce serum total cholesterol and LDL-C levels, thus directly improving cardiac health. Phenolic compunds are potent effectors of biological processes and influence CVD risk via modulating cellular pathways like MAPK signaling pathway and important mediators like NF-κB, AP-1 and Nrf.

There are sufficient evidences for recommending the consumption of food sources rich in bioactive compounds, that is, recommending a diet including variety of fruits, vegetables, whole grains, legumes, oils, and nuts. Various mechanisms of action of most the bioactive compounds have been conducted but more scientific research is need to be done to embark on science based dietary recommendations. Well-designed future investigations with the isolated bioactive compounds are needed to be done to derive the valuable information regarding the mechanism of action and doses to be taken. This will lead to commence recommendations for both the nature of the compounds and the optimal dose and better targets can be achieved in the area of cardiovascular health protection.

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Section 4

Molecular Modeling and Drug Design

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Chapter 25
Molecular-Docking-Based Drug Design and Discovery:
Rational Drug Design for the Subtype Selective GPCR Ligands

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ABSTRACT

Currently 30-50% of drug targets are G Protein-Coupled Receptors (GPCRs). However, the clinical useful drugs for targeting GPCR have been limited by the lack of subtype selectivity or efficacy, leading to undesirable side effects. To develop subtype-selective GPCR ligands with desired molecular properties, better understanding is needed of the pharmacophore elements and of the binding mechanism required for subtype selectivity. To illustrate these issues, we describe here three successful applications to understand the binding mechanism associated with subtype selectivity: 5-HT2B (5-Hydroxytryptamine, 5-HT) serotonin receptor ($HT_{2B}R$), H_3 histamine receptor (H_3HR) and A_3 adenosine receptor (A_3AR). The understanding of structure-function relationships among individual types and subtypes of GPCRs gained from such computational predictions combined with experimental validation and testing is expected the development of new highly selective and effective ligands to address such diseases while minimizing side-effects.

INTRODUCTION

G protein-coupled receptors (GPCRs) with >800 including 340 non-olfactory receptors are the largest superfamily in the human genome. These are customarily partitioned into 5 families: glutamate, rhodopsin, adhesion, frizzled, and secretin (Fredriksson, Lagerstrom, Lundin, & Schioth, 2003). GPCRs regulate essential physiological processes (e.g. cellular metabolism, cell growth, secretion, immune

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defense, neurotransmission, and differentiation) through various endogenous ligands which include biogenic amines, peptides, lipids, nucleotides, and proteins, modulate (Lefkowitz, Pierce, & Luttrell, 2002). GPCRs also involves in various important cell recognition and communication processes (Ellis, 2004). Thus, GPCRs are important drug targets for all major disease areas, including neurodegenerative, psychiatric, metabolic, cardiovascular, cancer, and infectious diseases (Tang & Insel, 2005). Indeed, currently 30-50% of drug targets are GPCRs (Hopkins & Groom, 2002). The currently marked GPCR-targeting drugs with ~80 account for ~$50 billion in annual sales, and many have annual sales > $2 billion (Goddard III & Abrol, 2007). Identifying GPCR subtypes with specific cell and tissue has been accelerated for target evaluation, lead identification, and optimization of GPCRs. However, the clinical use as drugs for targeting GPCR have been limited by the lack of subtype selectivity or efficacy, leading to undesirable side effects.

BACKGROUND

Endogenous ligands regulate multiple GPCR subtypes. Serotonin activates 15 serotonin (5-HT) receptors. With the exception of the 5-HT_3 receptor, a ligand-gated ion channel, all other 14 serotonin receptors are GPCRs. The 5-HT_1 (1A- 1F) and 5-HT_5 (5A, 5B) receptors decrease cellular level of cAMP through coupling with Gi/ Go protein, while $5HT_4$, 5-HT_6, and 5-HT_7 receptors increase cellular level of cAMP through coupling with Gs protein. 5-HT_2 (2A– 2C) receptors increase cellular level of inositol triphosphate (IP_3) and diacylglycerol (DAG) through coupling with G_q/ G_{11} protein (Nichols & Nichols, 2008). Various biological and neurological processes were regulated through the serotonin receptors such as aggression, anxiety, appetite, cognition, learning, memory, mood, nausea, sleep, and thermoregulation. Thus, the serotonin receptors are the target of a variety of pharmaceutical drugs, including many antidepressants, antipsychotics, anorectics, antiemetics, gastroprokinetic agents, antimigraine agents, hallucinogens, and entactogens (Nichols & Nichols, 2008).

Histamine acts via four histamine receptors (HRs); H_1, H_2, H_3 and H_4. Histamine has a critical role in immumomodulation and allergic diseases. Other biological activities include cell proliferation, differentiation, hematopoiesis, embryonic development, regeneration, wound healing, aminergic neurotransmission, secretion of pituitary hormones and regulation of gastrointestinal and circulatory functions (Jutel, Blaser, & Akdis, 2005). The H_3 and H_4 HRs inhibit the cellular level of cAMP through coupling with Gi/ Go protein, while the H_2HR activates the cellular level of cAMP through coupling with Gs protein. The H_1HR increases the cellular level of IP_3 and DAG through coupling with G_q/ G_{11} protein.

Adenosine binds all 4 subtypes of adenosine receptors (ARs), denoted A_1, A_{2A}, A_{2B}, and A_3, which coupled to G proteins. Activation of the A_1 and A_3 ARs inhibit the cellular level of cAMP via Gi/ Go protein, while the A_{2A} and A_{2B} ARs activate the cellular level of cAMP via Gs protein (Olah & Stiles, 1995). ARs are involved in many of the body's cytoprotective functions. Thus, ARs are important pharmacological targets in the treatment of a variety of diseases because of their key roles in controlling numerous physiological processes. For example, many therapeutic agents under development for treatment of central nervous system disorders, inflammatory diseases, asthma, kidney failure and ischemic injuries exert their effects via interactions with ARs.

To achieve high selectivity for specific GPCR subtype, an alternative approach is the development of selective allosteric modulators of the specific receptor subtypes. There are two marketed GPCR allosteric modulators, a positive allosteric modulator of the calcium sensing receptor used to treat hyperparathy-

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roidism and Maraviroc a negative allosteric modulator of chemokine receptor 5 that inhibits HIV entry into cells and is used to treat HIV infections (Conn, Christopoulos, & Lindsley, 2009). However, in this review we will focus on the subtype selective orthosteric ligands. Several groups studied the subtype selectivity. The 3D models of all four subtypes using the crystal structure of the $hAA_{2A}R$ as a template were generated by homology modeling and applied the methodology of ligand-guided receptor optimization for ređnement (Katritch, Kufareva, & Abagyan, 2011). The crystal structures of 5-HT_{1B} and 5-HT_{2B} receptors were compared (Wang, Jiang, Ma, & Xu, 2013). The 5-HT_{2A} selectivity was discussed based on the steered molecular dynamics (MD) approach of the 5-HT_{2A} model (Isberg, Balle, Sander, Jørgensen, & Gloriam, 2011). The development of subtype selective nicotinic acetylcholine receptor ligands was discussed (Astles et al., 2002). Fine-tune ligand selectivity for the angiotensin 2 receptors AT2R subtypes through electronic control of ligand aromatic-prolyl interactions results in an AT2R high affinity (Ki = 3 nM) agonist analogue with 18,000-fold higher selectivity over AT1R(Magnani et al., 2014). More recently, the binding pocket and subtype specificity in the X-ray structures of opioid receptors (OR) were discussed (Manglik et al., 2012b). In the binding pocket of the δ-OR within 4 Å of the ligand, 11 out of the 14 residues, are identical between μ-OR and δ -OR. The three differences are at μ-OR positions $E229^{EC2}$, $K303^{6.58}$ and $W318^{7.35}$, which are Asp, Trp and Leu in the δ-OR, respectively. In particular, the point mutation $W318L^{7.35}$ markedly increases the affinity of the δ-OR selective ligands at the μ-OR. But the qualitative analysis is not done for which residues are involved in ligand subtype selectivity according to energy. The interaction energy includes the non-bonding van der Waals (vdW) and electrostatic Coulombic energy between the ligand and protein to see how much or which interaction the subtype selective residues contribute in the binding site.

MAIN FOCUS OF THE CHAPTER

Issues, Controversies, Problems

To develop subtype-selective GPCR ligands with desired molecular properties, we need much better understanding of the binding sites for ligands to GPCRs and of how the mechanism for subsequent activation is affected by the structural parameters. Particularly difficulty here is that there are often many subtypes for the same endogenous ligand (4 for histamine and adenosine, 13 for serotonin), which we can expect might have someone similar binding sites. Moreover, there is clear evidence that the GPCR structure suitable for recognizing antagonists can be quite different than those for agonists. Thus even with a crystal structure of a GPCR bound to a specific antagonist or agonist, the best GPCR conformation for binding a different agonist or antagonist might be very different. Thus to predict the proper binding site to a particular GPCR, we need to first predict the ensemble of stable apo-protein structures which might be suitable for the various ligands.

To predict the ensemble of low energy GPCR conformations, we developed the GEnSeMBLE (GPCR Ensemble of Structures in Membrane BiLayer Environment) Monte Carlo Method. GEnSeMBLE compares the sequences of any one target to those of all other GPCRs to predict the hydrophobic regions likely to span the membrane and then predicts the optimum packing of the 7 transmembrane (TM) domains into 7-helix bundles. To ensure sampling of all possible packings, GEnSeMBLE starts with templates for GPCRs obtained previously from experiment (or theory) and samples up to ~12 trillion tilts and rotations of these helices, which it reduces down to the best ~10 to 20.

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Then the DarwinDock Monte Carlo Technique was performed to predict the best binding pose for each ligand to each of the 7-helix bundles in this 10-20 bundle ensemble.

These procedures, which have been validated against several X-ray crystal structures, are applied here to three systems which were compared with the X-ray studies and validated by other experiments. We consider here three successful applications of computations to predict the binding mechanism for subtype selectivity:

1. Human 5-HT_{2B} (5-Hydroxytryptamine, 5-HT) serotonin receptor ($hHT_{2B}R$), (S.-K. Kim, Li, Abrol, Heo, & Goddard III, 2011)
2. Human H_3 Histamine receptor (hH_3HR) (S. K. Kim, Fristrup, Abrol, & Goddard III, 2011) and
3. Human A_3 adenosine receptor (hA_3AR) (S. K. Kim, Riley, Abrol, Jacobson, & Goddard III, 2011)

These calculations improved understanding of how these subtype selective ligands interact with each subtype at the molecular level and would be useful to diminish undesired side effects through the cross-selectivity with other subtypes and to design novel classes of subtype selective drugs.

1. METHODS

1.1. GEnSeMBLE Monte Carlo Protocol (Abrol, Bray, & Goddard III, 2011b)

The MembEnsemb (later version of the MembStruck) techniques which were used to predict the 3D structure for the hHT2BR and hHT2CR is the earlier version of the GEnSeMBLE Monte Carlo method. For each of the 7-helix conformations selected from the BiHelix analysis we built full 7-helix bundle, scream all 7 helixes simultaneously and evaluated the energy, ScreamTot. In addition, we evaluated the membrane solvation free energy (MembSolE, Es) using a multi-dielectric (80|7|2|7|80) implicit membrane model of the total solvation free energy change associated with transfer of protein from implicit bulk water. In the GEnSemBLE method, the scoring energies are ranked 4 different ways the charged total energy, the neutralized total energy, the charged interhelical energy, and the neutralized interhelical energy. Although we did not apply the same methods for the three successful applications, we validated our previous result using our new methods.

The GEnSeMBLE method (Abrol, et al., 2011b) was used to generate the 3D structures for the ensemble conformations of GPCRs, as summarized in Figure 1. GEnSeMBLE generates a complete sampling (millions to trillions) of rotations and tilts of the helices for the ensemble of low lying structures expected to be energetically accessible for binding of ligands. This method replaced our earlier MembStruk method which sampled helix rotations helix by helix, rather than combinatorial (Vaidehi et al., 2002).

The structure prediction methodology described previously (Abrol, Bray, & Goddard III, 2011a) is summarized as follows:

1. **PredicTM:** Uses multiple sequence alignments of over 2,000 GPCR sequences using the MAFFT (Katoh, Kuma, Toh, & Miyata, 2005) program combined with the hydrophobicity scales from White and von Heijne(Wimley, Creamer, & White, 1996) to predict the TM domains for membrane protein.

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These TM domains were extended by capping rules and the secondary structure prediction using PORTER(Pollastri & McLysaght, 2005) and SSPro(Pollastri, Przybylski, Rost, & Baldi, 2002).

2. **OptHelix:** OptHelix uses molecular dynamics to predict the structure for each TM domain, which is usually helical except for kinks that may be caused by Proline residues.
3. **Homologize Helices:** When closely related X-ray structures with higher than 30% of sequence identity are available, we also use homology models to determine the helix shape. We consider the currently available X-ray structures; human nociceptin/ orphanin FQ receptor,(Thompson et al., 2012) mOPRD mouse delta opioid receptor,(Granier et al., 2012) mouse mu opioid receptor,(Manglik et al., 2012a) kappa opioid receptor,(H. Wu et al., 2012) human D_3 dopamine receptor,(Chien et al., 2010) turkey β_1 adrenergic receptor,(T. Warne, Serrano-Vega, M.J., Baker, J.G., Moukhametzianov, R., Edwards, P.C., Henderson, R., Leslie, A.G.W., Tate, C.G., Schertler, G.F.X., 2008) human adenosine A_{2A} receptor,(Jaakola et al., 2008) human β_2 adrenergic receptor,(V. Cherezov, Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G.F., Thian, F.S., Kobilka, T.S., Choi, H-J., Kuhn, P., Weis, W.I., Kobilka, B.K., Stevens, R.C., 2007) human sphingosine 1-phosphate,(Hanson et al., 2012) human Chemokine CXCR4,(B. Wu et al., 2010) human H_1 histamine receptor,(Shimamura et al., 2011) rat M_3 muscarinic receptor,(Kruse et al., 2012) human M_2 muscarinic receptor,(Haga et al., 2012) and bovine rhodopsin (Li, 2004; T. Okada, Fujiyoshi, Y., Silow, M., Navarro, J., Landau, E.M., Shichida, Y., 2002; T. Okada, Sugihara, M., Bondar, A.N., Elstner, M., Entel, P., Buss, V., 2004; Palczewski, 2000; Teller, 2001) Based on the sequence identities of the TM regions for these systems and the diversity of structures, we chose a starting template.
4. **BiHelix:** To cover all possible rotations of all 7 helices, consider 30° increments in the rotation about each TM axis (eta η angle), leading to $(12)^7 \sim 35$ million packings. The energies for all 35 million packing are estimated in by evaluating the energies of all $(12)^2=144$ interactions for each of the 12 pairs of helices in direct contact. For each of the 1,728 pairwise interactions, SCREAM(V.W.T. Kam & W.A. Goddard III, 2008) was used to optimize the side-chains. The BiHelix mean field energies for all 35,000,000 packings are used to select the best 2000. We then build the full 7-helix bundle for each of these 2,000 structures, optimize the side-chains for each using SCREAM, and neutralize the charged residues for more accurate energy scoring. The Dreiding D3 force field (D3FF)(Mayo, Olafson, & Goddard III, **1990**) was used throughout wherever energies were evaluated.
5. **SuperBiHelix(Bray, Abrol, Goddard III, Trzaskowski, & Scott, 2014):** Starting with the X-ray tilt angles we validated(Abrol, et al., 2011a) that the BiHelix analysis always identifies the experimental set of rotation angles correctly (this validates the procedure and the scoring function). However we have shown that even for closely related GPCRs, we must optimize the tilt angles: theta θ (tilting away from the z-axis) and phi φ (the azimuthal angle of the tilting from the xy plane) angle in order to get an accurate structure for a structure based on a different template (Abrol, Griffith, Bray, & Goddard III, 2012) Here we have found that starting with the best angles from the BiHelix optimization, it is sufficient to sample $\pm 10°$ for θ tilt angle while simultaneously sampling by $\pm 30°$ for both φ and η angles, leading to a total of $(3x5x5)^7 \sim 10$ trillion combinations. Generally the hydrophobic center (HPC) for each TM domain from the PredicTM analysis is placed at z=0, but we can use SuperBiHelix to optimize translation along the TM axis simultaneously with optimizing rotations.

In SuperBiHelix, The BiHelix mean field energies for all 10 trillion combinations packings are used to select the best 2000, which are then examined much more accurately by building the 7 TM bundles

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and optimizing side chains. Our experience is that the mean field energies are sufficiently accurate that the best 7-helix bundle energies are all in the first 700 of the 2,000, indicating that analyzing the best 2,000 is sufficient.

6. **Loop Generation:** The loops and the N/C terminus including helix 8 for a new structure are modeled through homology with the template structure, and then relaxed through 10 cycles of simulated annealing between 50K and 600K each for 0.1 ps, followed by minimization. The disulfide bridge between C3.25 and Cys in the second extracellular loop (EC2), which is conserved among Class A GPCRs, is constructed. Then keeping the 7 TM bundle fixed, we first optimize the loop structures (up to1000 steps or down to 0.5 RMS force) followed by quench annealing between 50 K and 600K for 10 cycles.

The best examples of using our procedures to successfully predict mechanisms relating to activation or inactive character of GPCRs are our studies on various mutants of CB1 that were subsequently confirmed by experiment. We were able to show that mutations that strengthening the coupling of TM6 to TM2 and TM3 make the CB1 receptor totally inactive, while mutations that break this interaction are essentially totally active (Ahn, Scott, Abrol, Goddard III, & Kendall, 2013) Moreover we predicted mutations that would switch either from totally inactive to constitutively active or from totally active to constitutively active that were confirmed by subsequent experiments.

1.2. DarwinDock

The lowest energy predicted structures from BiHelix/ SuperBiHelix were used for docking several ligands, using DarwinDock. The starting structure and charges of the ligands are calculated using quantum mechanics (B3LYP with the 6-311G** basis set). Then we develop a set of conformations by LigCluster and select 10 to 20 for docking.

1. **Scanning the receptor for potential binding regions:** Using the predicted 7-helix bundle structure, we predicted putative ligands binding regions as follows. The 6 hydrophobic residues, I, L, V, F, Y, and W, were alanized for the whole protein and scanned for potential binding regions. The overall molecular surface of the protein structure was mapped with spheres representing the empty volume of the protein (currently using the Sphgen procedure in DOCK4.0 suite). The entire set of protein spheres is partitioned into ~30 to 50 overlapping cubes of 10 to 14 Å sides. For each of these 30 - 50 regions, 1,000 poses were generated.
2. **DarwinDock:** The GenMSCDock is the later version of the GenMSCDock techniques to predict the binding site for HT2B/ HT2CR agonists. The GenMSCDock procedures sampled a complete set of configurations for each conformation of the ligand. This leads to a hierarchy of families with a specified range of diversities, allowing the best to be selected at a coarse level by evaluating energies of family heads. This also leads to an ensemble of the best diversity families that are used for higher-level calculations. The GenMSCDock method does two rounds, one to ensure completeness and the other to enrich the better families. To achieve an average of 6 children in each family, we resort to obtain 0.6 Å families in the enrichment step. The steps in GenMSCDock include bulky residue alanination, diversity finder, Voronoi reclustering, side-chain refinement and neutralization. The DarwinDock method which is the later version of GenMSCDock increases the sampling

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number for all binding configurations from 2500 to 5000 with a larger diversity parameter of 1.2 – 1.4 Å in the completeness step. In addition, we include the scoring energies of ligand strain and solvation.

DarwinDock has replaced our earlier methods, the HierDock and GenMSCDock. For each ligand conformation, DarwinDock iteratively produces a total of ~50,000 poses into the putative binding regions of the alanized protein. The poses partitions into families based on CRMS and the energy scoring of family heads and select the top 10% ordered by total energy. The 100 conformations are scored for the children of these top families for further optimization. For 100 conformations, we dealanize the protein side-chains to optimize the side chains using SCREAM. Then we neutralize the protein and ligand by transferring protons within salt bridges and protonating or deprotonating exterior ligands, followed by further full geometry minimization.

We validated DarwinDock using 3 crystal structures of ligand/ GPCR complexes: human β_2-adrenergic receptor (0.4 Å RMSD),(V. Cherezov et al., 2007) human $AA_{2A}R$ (0.8 Å RMSD),(Jaakola, et al., 2008) and turkey β_1-adrenergic receptor (0.1 Å RMSD) (T. Warne et al., 2008) This supports that we can accurately identify ligand binding sites in proteins and optimize the ligands with desirable properties.

3. **Neutralization for scoring energy:** Quantum mechanics (QM) calculations show that if an effective dielectric constant is < 8Å, the extra proton on a Lys or Arg transfers back to the negative carboxylate of an Asp or Glu.[22] Thus buried salt bridges is expected to have neutral residues. We find that neutralizing these exposed residues removes the sensitivity to details of the distances of charged residues (and counter ions) remote from the binding cavity. This neutralization leads to differential binding energies that are dominated by the local cavity interactions and leads to much smaller solvation energies.

We used the unified cavity energy as the scoring function for docking. It was calculated by the total non-bonding energy in 4 Å of ligand which is the sum of van der Waals (vdW), Coulomb, and H-bond energy (kcal/mol) in the unified cavity.

An excellent example validating our DarwinDock is the predicted binding site for the Maraviroc anti HIV drug from a selection of 8 diverse packings for the chemokine CCR5 GPCR (Abrol et al., 2014) Then we predicted 12 mutations designed to increase or decrease binding significantly. These mutations, carried out after the predictions, led to a mean error of <1 kcal/mol in the predicted correlation of relative binding energies. Interestingly, the optimum protein packing for binding Maraviroc was different than for the original wild type binding site for each of the 12 mutations.

2. APPLICATIONS

2.1. Subtype Selectivity of Antagonists for $hHT_{2B}R$ (Kim et al., 2011)

Three 3 subtypes of 5-HT_2 GPCRs are important drug targets for schizophrenia, depression, migraines, anxiety, hallucinogens, feeding disorders, perception, hypertension, and gastrointestinal dysfunctions. In particular, $hHT_{2B}R$ antagonists have a possible therapeutic treatment of pulmonary hypertension (Fredriksson, et al., 2003).

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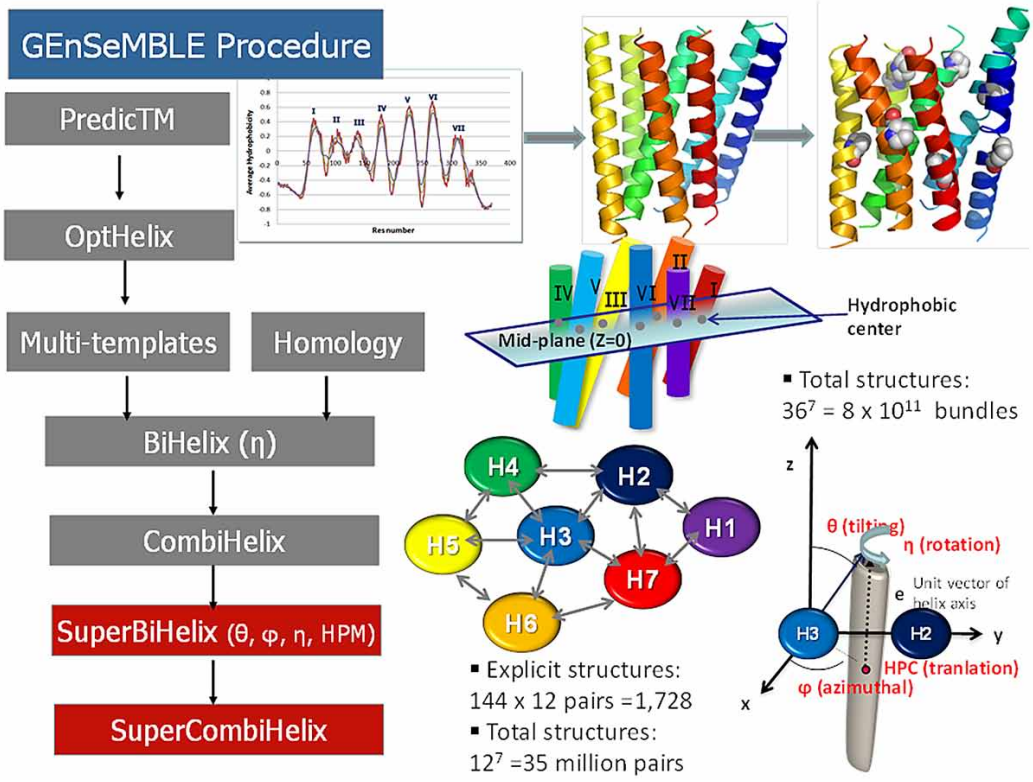
The sequence identities of overall and TM region between $hHT_{2B}R$ and $hHT_{2C}R$ were 57.38 and 72.51%, respectively. The sequence identities of each TM region have 65.22% for TM1, 75.00% for TM2, 87.50% for TM3, 55.56% for TM4, 62.50% for TM5, 81.82% for TM6, and 80.80% for TM7. The predicted structure of $hHT_{2B}R$ has an average RMSD of 0.39 Å relative to the $hHT_{2c}R$ TM bundle (RMSD of TM1: 0.63 Å, TM2: 0.39 Å, TM3: 0.26 Å, TM4: 0.52 Å, TM5: 0.45 Å, TM6: 0.21 Å, TM7: 0.30 Å).

We compare here the binding sites and energies for two cases

- A non-selective antagonist (SB-206533 **1**:5 -methyl-1-3,5-dihydro-2H-pyrrolo[2,3-f]indole-1-carboxylic acid pyridine-3-ylamide) bound to hHT_{2B} and $hHT_{2C}R$ (serotonin) GPCR
- A selective antagonist **2** (1-methyl-1-1,6,7,8-tetrahydro-pyrrolo[2,3-g]quinoline-5-carboxylic acid pyridine-3-ylamide) bound to hHT_{2B} (serotonin) GPCR

Figure 1 shows the predicted structures(S.-K. Kim, et al., 2011) for non-selective SB-206533 **1** in cyan, to both of the $hHT_{2B}R$ and $hHT_{2C}R$. For residue numbering, we used the Ballesteros-Weinstein number; the most conserved residue in each of the 7 TM domains is taken as the reference and numbered as 50. This residue is designated x.50 where x is the number of the TM helix (Ballesteros & Weinstein, 1995) From the cavity analysis, we find similar interaction energies for this antagonist to both $hHT_{2B}R$ and the $hHT_{2C}R$. For example the hydrogen bonding (HB) interactions between the ureido-CO group and the

Figure 1. Flowchart of GEnSeMBLE (GPCR Ensemble of Structures in Membrane BiLayer Environment) Monte Carlo method



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Ser-OH side chain at S139$^{3.36}$ is conserved among 5-HT2 family receptors, 2A, 2B, and 2C. The complex is stabilized through additional hydrophobic interactions at conserved hydrophobic residues, V136$^{3.33}$, F274$^{6.51}$ (2B)/ F272$^{6.51}$ (2C), and F299$^{7.38}$/ F298$^{7.38}$. The N-methyl group in the cyclopenta-indole ring forms the hydrophobic interactions with the subtype variable residues, Non-bond energies of L132$^{3.29}$, I186$^{4.56}$, and V190$^{4.60}$ in the $hHT_{2B}R$ are -2.48, -0.10, and -0.76 kcal/mol, respectively. The corresponding amino acids, I132$^{3.29}$, V186$^{4.56}$, and I190$^{4.60}$, at the $hHT_{2C}R$ also have favorable non-bond energies. Thus, similar total cavity energies of SB-206533 **1** at the $hHT_{2B}R$ and $hHT_{2C}R$ are -42.48 and -42.28 kcal/mol, respectively. In addition, subtype-selective residues also reveal similar interactions (Table 1).

Then we studied the conformational restricted analogue **2** (1-methyl-1-1,6,7,8-tetrahydro-pyrrolo[2,3-g] quinoline-5-carboxylic acid pyridine-3-ylamide) known to bind much more strongly to 5-$HT_{2B}R$ affinity (pA2, 5-HT2B: 7.27 vs. pKi, 5-HT2C: 5.39) (Forbes et al., 1995)

Table 1. Cavity analysis of SB-206533 **1** *and* **2** *at human 5-HT2B and 2C receptors. The residues are ordered by the non-bonding energy difference (2C -2B)*

Ballesteros-Weinstein #	2 (pKi, 2B: 7.3)			2 (pKi, 2C: 5.4)			Diff.	1 (2B: 7.6)	1 (2C: 7.9)
	Res	#	NonBond	Res	#	NonBond	2C-2B	NonBond	NonBond
3.29	**LEU**	**132**	**-2.35**	**ILE**	**131**	**6.12**	**8.46**	**-2.48**	**-0.84**
6.55	ASN	344	-2.40	ASN	331	-1.54	0.86	-2.19	-0.47
5.47	PHE	226	-2.05	PHE	223	-1.38	0.68	-1.02	-2.39
6.54	THR	343	-1.33	THR	330	-0.66	0.67	-1.17	0.00
3.33	VAL	136	-5.34	VAL	135	-4.91	0.43	-3.78	-5.44
5.46	ALA	225	-1.09	ALA	222	-0.68	0.41	-0.48	-1.12
6.47	MET	336	-0.40	MET	323	0.00	0.40	-0.06	0.00
6.52	PHE	341	-0.38	PHE	328	0.00	0.38	0.00	0.00
3.34	LEU	137	-0.38	LEU	136	0.00	0.38	0.00	0.00
6.58	**LEU**	**347**	**-0.56**	**SER**	**334**	**-0.23**	**0.33**	**-0.67**	**0.00**
5.39	**MET**	**218**	**-0.32**	**VAL**	**215**	0.00	**0.32**	**-0.35**	**-0.28**
5.40	LEU	219	-0.28	LEU	216	0.00	0.28	-0.27	0.00
5.44	**LEU**	**223**	**-0.26**	**PHE**	**220**	0.00	**0.26**	**-0.20**	**-0.30**
3.40	ILE	143	-0.51	ILE	142	-0.26	0.25	-0.28	-0.31
5.43	SER	222	-1.70	SER	219	-1.49	0.21	-2.03	-2.92
6.44	PHE	333	-0.59	PHE	320	-0.42	0.17	-0.52	-0.09
6.53	ILE	342	-0.16	ILE	327	0.00	0.16	0.00	-0.10
7.45	SER	372	-0.15	CYS	360	0.00	0.15	0.00	0.00
7.41	ILE	368	-0.12	ILE	356	0.00	0.12	-0.10	0.00
5.41	**PHE**	**220**	**-0.07**	**ILE**	**217**	**0.00**	**0.07**	**0.00**	**-0.08**
3.36	*SER*	*139*	*-6.34*	*SER*	*138*	-6.28	*0.06*	*-5.67*	*-6.95*
5.45	**ALA**	**224**	**-0.05**	**VAL**	**221**	0.00	**0.05**	**0.00**	**-0.14**
6.49	CYS	338	-0.03	CYS	325	0.00	0.03	-0.03	0.00
6.46	**LEU**	**335**	**-0.03**	**ILE**	**321**	**0.00**	**0.03**	**-0.03**	**0.00**

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Table 1. Continued

Ballesteros-Weinstein #	2 (pKi, 2B: 7.3)			2 (pKi, 2C: 5.4)			Diff. 2C-2B	1 (2B: 7.6)	1 (2C: 7.9)
	Res	#	NonBond	Res	#	NonBond		NonBond	NonBond
6.51	PHE	340	-5.95	PHE	327	-5.93	0.02	-4.43	-5.80
5.32	**LYS**	**211**	**-0.01**	**VAL**	**208**	0.00	**0.01**	**-0.06**	0.00
6.45	**ILE**	**334**	**0.00**	**LEU**	**321**	0.00	**0.00**	**-0.02**	0.00
3.28	TRP	131	-0.32	TRP	130	-0.32	**0.00**	-0.37	-0.44
4.56	**ILE**	**186**	**0.00**	**VAL**	**185**	**0.00**	**0.00**	**-0.10**	**-1.27**
4.57	**ALA**	**187**	**0.00**	**SER**	**186**	**0.00**	**0.00**	**-0.12**	**-0.58**
5.37	ASP	216	0.00	ASP	213	0.00	0.00	-0.38	0.00
7.46	SER	373	-0.85	SER	361	-0.86	0.00	-0.77	0.00
7.33	**MET**	**360**	**0.01**	**LYS**	**348**	**0.00**	**-0.01**	0.00	0.00
2.57	VAL	107	-0.08	VAL	106	-0.10	-0.02	-0.10	0.00
7.34	LEU	361	-0.23	LEU	349	-0.26	-0.03	-0.26	0.00
6.42	PHE	331	0.05	PHE	318	0.00	-0.05	0.00	0.00
3.35	PHE	138	-0.70	PHE	137	-0.76	-0.06	-0.63	0.00
7.43	TYR	370	-1.59	TYR	358	-1.65	-0.06	-1.35	-0.53
3.37	THR	140	-1.16	THR	139	-1.32	-0.17	-0.96	-2.12
4.60	**VAL**	**190**	**-0.51**	**ILE**	**189**	-0.72	**-0.20**	**-0.76**	**-1.24**
7.42	GLY	369	-0.87	GLY	357	-1.10	-0.23	-0.73	-0.31
6.48	TRP	337	-0.84	TRP	269	-1.14	-0.30	-1.42	-1.77
7.39	VAL	366	-1.72	VAL	354	-2.19	-0.48	-1.83	-0.09
7.35	LEU	362	-1.05	LEU	350	-1.59	-0.54	-1.21	-0.69
3.32	ASP	135	-2.87	ASP	134	-3.63	-0.75	-3.81	-3.50
7.38	PHE	365	-1.95	PHE	353	-2.76	-0.82	-1.87	-1.54
	SUM		**-47.53**	**SUM**		**-36.06**	**11.47**	**-42.48**	**-42.28**

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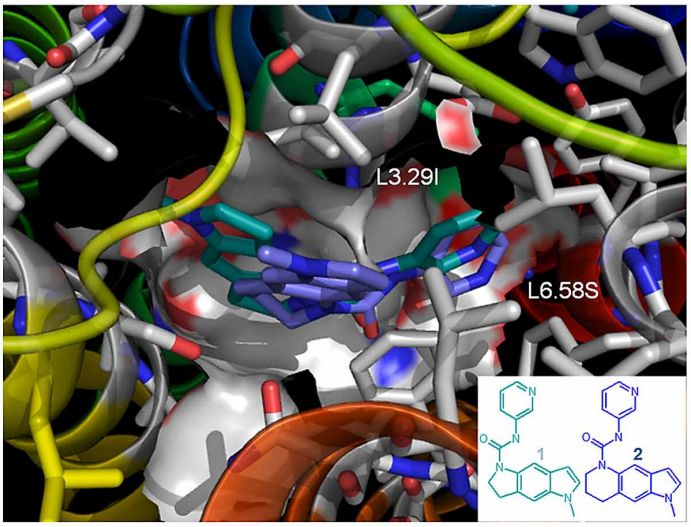
Indeed we find that arises because the introduction of the six membered ring is clearly detrimental for $hHT_{2C}R$. The major hydrogen bond interaction is at the ureido-CO group with Ser-OH at conserved $S139^{3.36}$. Conserved hydrophobic residues, $V136^{3.33}$, $F274^{6.51}$ / $F272^{6.51}$ (2C), and $F299^{7.38}$/ $F298^{7.38}$ (2C) also stabilize the complex through hydrophobic interactions. We observed that bad contacts at $I132^{3.29}$ with unfavorable vdW interaction (+6.24 kcal/mol) result in reduced interaction at the $hHT_{2C}R$, compared with the favorable vdW interaction at the $hHT_{2B}R$ (-2.32 kcal/mol).

In addition, similar binding energies were shown at other variable residues, $V190^{4.60}$ / $I190^{4.60}$ in 2C, $L281^{6.58}$/ $S279^{6.58}$ in 2C. We find favorable interaction at $hHT_{2B}R$ (-47.53 kcal/mol) but unfavorable interaction at the $hHT_{2C}R$ (+11.47 kcal/mol), consistent with experiment (Table 1). However, $A225^{5.46}$ in 2B and $A223^{5.46}$ in 2C which were reported as specific interaction for 5-HT_{2A} selectivity(Isberg, et al., 2011) shows the similar interaction at both receptors.

The superimposition of **1** in cyan and **2** in blue in Figure 2 suggest that the *N*-methyl of the cyclohexa-indole ring of 2B selective compound **2** pointing toward the upper TM 3 leads to unfavorable interactions

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*Figure 2. Superimposition of the predicted structures for the nonselective 5-HT2B/ 2C receptor antagonist (SB-206533 1) and the selective 5-HT2B receptor antagonist (***2***) at the human 5-HT2B receptor. The N-methyl of the cyclohexa-indole ring of* **2** *is closer to the upper part of TM3 leading to unfavorable interactions with bulkier Ile side chains in the 5-HT2C receptors (I132$^{3.29}$), while the methyl of the cyclopenta-indole ring in SB-206533* **1** *is pointing toward the upper TM 4.*
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with the bulkier Ile side chain in $hHT_{2C}R$ (I132$^{3.29}$), while the *N*-methyl of the cyclopenta-indole ring in the non-selective compound **1** directs toward the upper TM 4. In addition, the cyclohexa-indole ring is near the hydrophilic residue S279$^{6.58}$ in the $hHT_{2C}R$, while it interacts with hydrophobic L281$^{6.58}$ in $hHT_{2B}R$.

In the binding cavity of $hHT_{2B}R$, 13 out of 46 residues are variable compared with the binding cavity of $hHT_{2C}R$ with 72% sequence identity. Among them, L3.29I is the major contribution to the $hHT_{2B}R$ selectivity. L6.58S and M5.39V are the next important residues for the $hHT_{2B}R$ selectivity.

When compared with the recently published crystal structures of the partial agonist ergotamine-bound $hHT_{2B}R$ (4IB4),(Wacker et al., 2013) the predicted $hHT_{2B}R$ has a Ca RMSD of 2.49 Å relative to the $hHT_{2B}R$ crystal TM bundle. We observed the same TMs 1-2-7 networks among N1.50-D2.50-N7.49 and TMs 2-3-4 networks among S2.45-H3.42-W4.50 which is observed in the crystal structure. The H-bonding between D3.32 and Y7.43 which is conserved in most biogenic amine receptor was also observed near the binding site.

2.2. Subtype Selectivity of hH_3HR (Kim, Fristrup, et al., 2011)

HRs are major drug targets for the treatment of various diseases, such as schizophrenia, psychosis, depression, migraine, allergies, asthma ulcers, and hypertension. In particular, hH_3HR antagonists have been reported as specific therapeutic applications for the treatment of Alzheimer's disease, attention deficit hyperactivity disorder (ADHD), epilepsy, and obesity (Lorenzi et al., 2005) We report here the predicted lowest energy structure of all four human HR subtypes (H_1, H_2, H_3, and H_4), for each of which we have predicted the binding sites for H_3 selective antagonist: clobenpropit **3**, (*N*'-[(4-chlorophenyl) methyl]-1-[3-(3H-imidazol-4-yl)propylsulfanyl]formamidine).

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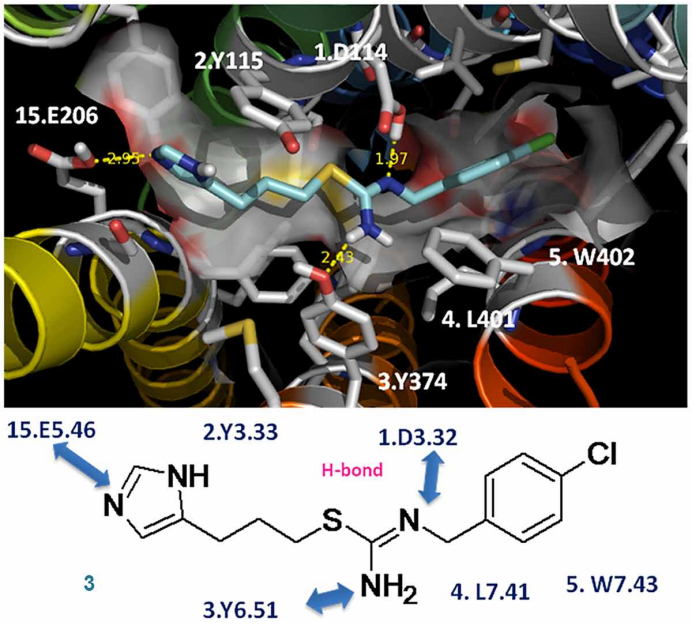
The hH_3HR shared 54.74% sequence identity with the hH_4HR, 32.15% sequence identity with the hH_1HR, and 33.08% sequence identity with the hH_2HR in the TM regions. The predicted structure of hH_3HR has an average RMSD of 0.04 Å for the hH_1HR, 1.03 Å for the hH_2HR, 1.01 Å for the hH_4HR, and 1.33 Å for the crystal structure of hH_1HR (3RZE) (Shimamura, et al., 2011). The predicted hH_1HR also has the same RMSD of 1.33 Å for the crystal structure of hH_1HR (Shimamura, et al., 2011).

We docked hH_3HR selective clobenpropit, to the lowest energy conformations of all four subtypes. From the cavity analysis of antagonist clobenpropit at hH_3HR, the major contributing amino acids are $Y115^{3.33}$ (-4.96 kcal/mol), $W402^{7.43}$ (-4.71 kcal/mol), and $D114^{3.32}$ (-3.73 kcal/mol) in Table 2. $D114^{3.32}$ and $Y374^{6.51}$ form the major H-bonding with the isothiourea group. The terminal imidazole ring forms additional H-bonding with at $E206^{5.46}$. Hydrophobic residues, $L401^{7.42}$ and $W402^{7.43}$ surround the *para*-chloro-benzyl group of clobenpropit (Figure 3).

To study subtype selectivity of HRs, the predicted best binding pose of the highly H_3-selective ligand clobenpropit at hH_3HR was matched into other three subtypes (H_1, H_2, H_4) of hHRs, and then SCREAM(V.W.T. Kam & W.A. Goddard III, 2008) was used to optimize the side chain position of residues in the binding cavity, followed by minimization and neutralization of the final ligand/ protein complex.

Table 2 displays predicted variable residues among four subtypes in the binding site (H_4: 29%, 8 out of 28, H_1: 57%, 16 out of 28, H_2: 68%, 19 out of 28). The corresponding amino acids of the same E5.46/ S5.43 in both of hH_3HR and hH_4HR are $N198^{5.46}$/ $A195^{5.43}$ in hH_1HR and $T190^{5.46}$/ $G187^{5.43}$ in hH_2HR (Figure 4) with weakened interactions in the cavity, resulting in a 2.2 to 2.4 kcal/mol favorable interaction in hH_3HR in Table 2. But, a similar interaction is observed at these two conserved residues E5.46/ S5.43

Figure 3. Predicted best structures of the H_3 selective antagonist clobenpropit **3** *bound to the human histamine H_3 receptor (hH_3HR). The H-bonding is represented by the arrows between the donor and the acceptor. The residues are numbered according to the order of unified cavity energy in Table 2. A schematic structure of the predicted binding site is displayed on the bottom figure.*
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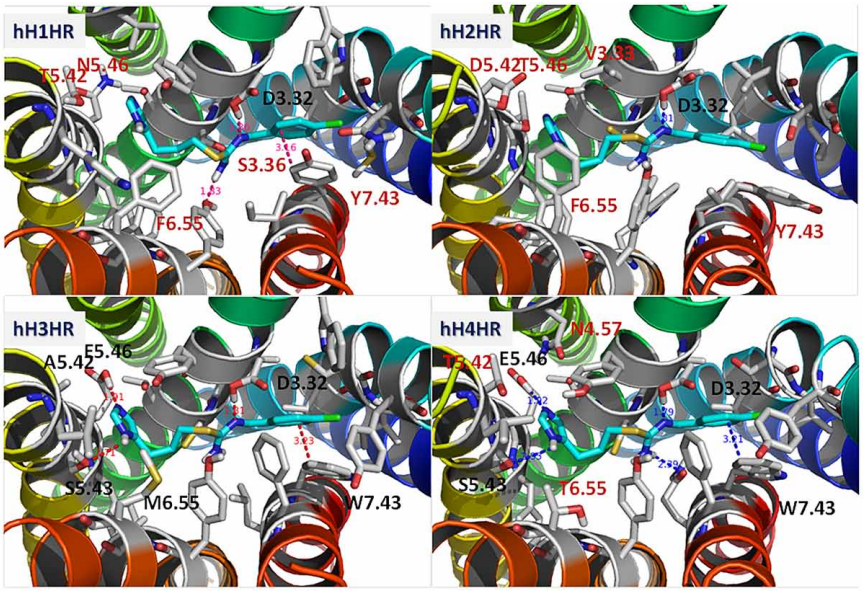
Table 2. Cavity energy of the H_3 selective antagonist clobenpropit **3** *to four human histamine receptors (HRs). Residues are ordered by total NonBond energy (H_3), which is the sum of van der Waals (vdW), Coulomb, and H-bond energy (kcal/mol) in the unified cavity. Predicted subtype residues that vary among four subtypes (H4: 29%, 8/28, H1: 57%, 16/28, H2: 68%, 19/28) are displayed in italic font. The color coding for contributions of each residue to binding of the adenosine ligand is: Dark grey: > 3 kcal/mol, Grey: 1 - 3 kcal/mol, Light grey: 0.5 - 1.0 kcal/mol. In the Ballesteros-Weinstein numbering, the most conserved residue in each of the 7 TM domains is taken as the reference and numbered as 50. This residue is designated x.50 where x is the number of the TM helix.*

Ballesteros-Weinstein #	hH_3HR (pKi: 9.4)		hH_4HR (pKi: 7.4)		hH_1HR (pKi: 5.6)		hH_2HR (pKi: 5.2)	
	Res. #	**NonBond**	**Res. #**	**NonBond**	**Res. #**	**NonBond**	**Res. #**	**NonBond**
3.33	Y115	-4.96	Y95	-5.70	Y108	-5.41	*V99*	*-3.45*
7.43	W402	-4.71	W348	-5.20	*Y458*	*-4.02*	*Y278*	*-2.78*
3.32	D114	-3.73	D94	-3.55	D107	-2.73	D98	-5.19
6.51	Y374	-2.54	Y319	-2.51	Y431	-3.65	Y250	-3.19
5.43	S203	-2.43	S179	-1.79	*A195*	*-0.53*	*G187*	*-1.21*
5.46	E206	-2.22	E182	-3.39	*N198*	*-1.92*	*T190*	*-1.07*
3.28	W110	-1.68	W90	0.00	W103	-1.60	*Y94*	*0.00*
3.36	C118	-1.66	C98	-3.11	*S111*	*-3.63*	C102	-2.80
2.57	C87	-1.58	*S68*	*-2.02*	*V80*	*-1.47*	*V71*	*-2.24*
2.53	V83	-1.33	V64	-1.26	V76	2.25	*L67*	*0.15*
6.55	M378	-1.28	*T323*	*-0.41*	*F435*	*-1.66*	*F254*	*-2.40*
2.51	Y81	-1.27	Y72	-1.35	*N84*	*-0.84*	*S75*	*-0.67*
7.39	F398	-1.20	F344	-0.12	*I454*	*-1.12*	*L274*	*-0.98*
6.48	W371	-1.14	W316	-0.58	W428	-1.51	W247	-2.83
2.58	I88	-1.03	I69	-0.78	*M81*	*0.14*	*L72*	*-1.25*
5.47	F207	-0.77	F183	-1.07	F199	-0.48	F191	-0.83
3.35	L117	-0.75	L97	-0.95	*A110*	*-0.65*	L101	-0.73
3.37	T119	-0.74	T99	-0.50	T112	-0.74	T103	-0.39
6.52	T375	-0.60	*S320*	*-0.24*	*F432*	*-1.06*	*F251*	*-0.81*
5.39	L199	-0.57	L175	-0.45	*K191*	*-0.22*	*G183*	*-0.22*
5.42	L401	-0.39	*Q347*	*0.03*	*G457*	*-0.22*	*G457*	*0.00*
3.31	V113	-0.34	*T93*	*-0.43*	*M106*	*-0.41*	*L97*	*0.00*
5.38	F198	-0.15	*I174*	*-0.14*	F190	0.00	*Y182*	*0.00*
4.57	Y167	0.00	*N147*	*0.15*	V159	-0.64	*S150*	*0.00*
4.61	I171	0.00	I151	0.00	*L163*	*-0.31*	I154	0.00
7.40	W399	0.00	W345	0.00	W455	0.00	W275	-0.75
7.46	S405	0.00	S351	0.00	S461	-0.23	S281	0.00
5.42	A202	0.13	*T178*	*0.23*	*T194*	*-1.47*	*D186*	*-0.13*
	SUM	**-36.94**	**SUM**	**-35.12**	**SUM**	**-34.11**	**SUM**	**-33.77**

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Figure 4. Predicted best structures of the H_3 selective antagonist clobenpropit **3** *bound to four human histamine receptors (hH_1HR, hH_2HR, hH_3HR, hH_4HR). H-bonding is indicated by red dots, and subtype selective residues are shown in red.*
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of hH_4HR. Thus, the final cavity energy leads to a substantial decrease in binding affinity (cavity sum = -34.11 kcal/mol in hH_1HR, -33.77 kcal/mol in hH_2HR) in agreement with the dramatically decreased experimental binding affinity at hH_1HR (PKi: 5.6) and hH_2HR (PKi: 5.2).

The final cavity energy shows a weakened binding affinity (-35.12 kcal/mol) for hH_4HR (PKi: 7.4) is a compared with the cavity energy (-36.94 kcal/mol) of hH_3HR (PKi: 9.4), Thus, this predicted binding energy is well correlated with the experimental binding affinity of H_3 subtype selective clobenpropit (Esbenshade et al., 2003).

In the binding cavity of hH_3HR, 7 (25%), 16 (57%), and 18 (72%) out of 28 residues are variable for hH_4HR, hH_1HR, and hH_2HR, respectively. The important residues for the hH_3HR selectivity are E5.46/ S5.43 in both of hH_3HR and hH_4HR which are $N198^{5.46}$/ $A195^{5.43}$ in hH_1HR and $T190^{5.46}$/ $G187^{5.43}$ in hH_2HR, respectively. When compared with the hH_4HR, M6.55T is the most important residue to overcome the hH_3HR selectivity.

Table 2 shows the unified cavity energy (UCav E) in all subtypes of hHRs, which are ordered by experimental binding affinity including. We find that the predicted binding energies for all four HRs are consistent with their experimental binding affinities with the r^2 values (correlation coefficients) ranging from 0.67 to 0.99.

2.3. Subtype Selectivity of $hAA_{2A}R$ and hAA_3R (Kim, Riley, et al., 2011)

ARs are targets for treatments of central nervous system disorders, inflammatory diseases, asthma, kidney failure, and ischemic injuries. Each agonist for 4 subtypes of ARs has various therapeutic applications. For example, the hAA_1R activation involves in cardioprotection, antilipolysis, type II diabetes,

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anti-arrhythmic, antiepileptic, analgesic/antinociceptive, ischemia, tachycardia, obesity, cachexia, while the hAA_3R activation involves in cardioprotection, cerebrovascular protection, prevention of stroke, anti-inflammatory, anti-asthmatic, neurological disease, ischemia, immune disorder, anticancer, and anti-arthritic. In addition, the $hAA_{2A}R$ agonists are useful for the target of antihypotensive, antipsychotic, and anti-inflammatory, while the $hAA_{2B}R$ agonists are useful for the target of anti-asthma cystic fibrosis, anti-inflammatory, septic shock, cardiac disease, diarrhea, impotence, and angiogenesis. The only other adenosine agonist currently in clinical use is non-selective adenosine itself, for the treatment of supraventricular tachycardia and as an aid in cardiac imaging. But the $hAA_{2A}R$-selective agonist Lexiscan (regadenoson, CV Therapeutics, Palo Alto, CA, USA), CVT-3146, recently approved for cardiac imaging in patients (K.A. Jacobson, A.M. Klutz, D.K. Tosh, A.A. Ivanov, D. Preti, & P.G. Baraldi, 2009). Selective hAA_3R antagonists are especially interesting as potential anti-glaucoma agents (Yang et al., 2005). Moreover, such selective hAA_3R agonists as IB-MECA (N^6-(3-Iodobenzyl)-5'-*N*-methylcarboxamidoadenosine) and Cl-IB-MECA (2-Chloro-N^6-(3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine), are now in Phase II clinical trials for treatment of liver cancer (K.A. Jacobson, A.M. Klutz, D.K. Tosh, A.A. Ivanov, D. Preti, & P.G. Baraldi, 2009). Thus, the development of the subtype selective ligand is critical for the specific treatment of human diseases.

To map out the binding site of A_3 subtype selective agonist, we predicted the ensemble of the 20 most stable structures for each of the four ARs. Here we used the high-resolution X-ray structure of $hAA_{2A}R$(Jaakola, et al., 2008) as the template. Because this crystal structure has $hAA_{2A}R$ bound to an antagonist, it provides little information about the structural changes involved in activation by agonist binding.

Then we predicted the binding of the above two A_3 selective agonists all 20 structures from this ensemble for each of the human adenosine subtypes (A_1, A_{2A}, A_{2B}, and A_3). In addition, we predicted the high hAA_3R selectivity for the agonist, Cl-IB-MECA and the high $hAA_{2A}R$ selectively for the endogenous agonist adenosine, validating the models for understanding the subtype selectivity. In addition we predicted binding sites for several A_3 selective antagonists to these structures for hA_3 and hA_{2A}.

The sequence identities of overall and TM region between hA_3AR and $hA_{2A}AR$ were 31.31 and 51.01%, respectively. The sequence identities of each TM region have 44.44% for TM1, 56.14% for TM2, 47.37% for TM3, 48.00% for TM4, 57.14% for TM5, 52.94% for TM6, and 51.04% for TM7. The predicted structure of hA_3AR has an average RMSD of 0.49 Å relative to the $hA_{2A}AR$ TM bundle (RMSD of TM1: 0.50 Å, TM2: 0.32 Å, TM3: 0.50 Å, TM4: 0.56 Å, TM5: 0.55 Å, TM6: 0.53 Å, TM7: 0.48 Å).

1. **$hAA_{2A}R$ selective adenosine:** We selected adenosine because of ~ 30 fold higher selectivity to $hAA_{2A}R$ than hAA_3R. The endogenous agonist adenosine was docked to the ensemble of 25 low-lying structures of apo-$hAA_{2A}R$.

All adenosine agonists show similar ribose moieties, except for the amide substitution at the 5'-position. Indeed an intact furanose moiety is present in most potent adenosine agonists previously reported (Jacobson et al., 2000). Table 3 shows the cavity analysis of the adenosine bound to $hAA_{2A}R$. Three major contributing amino acids are $N253^{6.55}$ (-7.22 kcal/mol), $F168^{EC2}$ (-6.33 kcal/mol), and $E169^{EC2}$ (-5.13 kcal/mol), based on non-bonding energies as the sum of vdW, electrostatic Coulomb with 2.5 dielectric constant and H-bond energies.

In the binding site of adenosine-$hAA_{2A}R$, the exo-cyclic amino group of the adenine ring interacts with $N253^{6.55}$ and $E169^{EC2}$ as the X-ray structure of $hAA_{2A}R$ also interacts with the amino group in the

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Table 3. Cavity energy of the endogenous agonist adenosine 4 bound to the human adenosine A_3 (AA_3R) and A_{2A} receptors ($AA_{2A}R$) () Residues are ordered by total NonBond energy (A_{2A}), which is the sum of van der Waals (vdW), Coulomb, and H-bond energy (kcal/mol) in the unified cavity. Subtype residues that are predicted to vary between A_{2A} and A_3 are displayed in bold face. The main interactions leading to subtype selectivity (underlined) in the predicted structure are E169 in A_{2A} vs. V169 in A_3, and V84 in A_{2A} vs. L90 in A_3. The color coding for contributions of each residue to binding of the adenosine ligand is: Dark grey: > 3 kcal/mol, Grey: 1 - 3 kcal/mol, Light grey: 0.5 - 1.0 kcal/mol.

Ballesteros-Weinstein #	$AA_{2A}R$ Ki: estimated 30 nM			AA_3R Ki: estimated 1,000 nM		
	Res	#	NonBond	Res	#	NonBond
6.55	ASN	253	-7.22	ASN	250	-5.82
EC2	PHE	168	-6.33	PHE	168	-4.52
EC2	**GLU**	**169**	**-5.13**	**VAL**	**169**	**0.00**
7.43	HSE	278	-2.82	HSE	272	-4.03
7.39	ILE	274	-2.72	ILE	268	-2.02
3.32	**VAL**	**84**	**-2.39**	**LEU**	**90**	**1.07**
6.51	LEU	249	-2.19	LEU	246	-3.39
7.42	SER	277	-1.78	SER	271	-1.77
3.33	LEU	85	-1.49	LEU	91	-0.96
5.38	MET	177	-1.39	MET	177	-1.34
3.36	THR	88	-1.29	THR	94	-0.80
7.35	**MET**	**270**	**-1.05**	**LEU**	**264**	**-1.27**
2.61	ALA	63	-0.49	ALA	69	-0.37
6.52	**HIS**	**250**	**-0.30**	**SER**	**247**	**0.00**
2.64	**ILE**	**66**	**-0.25**	**VAL**	**72**	**-0.18**
3.29	**ALA**	**81**	**-0.24**	**THR**	**87**	**-0.67**
6.54	ILE	252	-0.17	ILE	249	-0.12
5.35	MET	174	-0.17	MET	174	0.00
1.39	GLU	13	-0.15	GLU	19	0.00
6.48	TRP	246	0.00	TRP	243	-1.94
EC2	**LEU**	**167**	**0.00**	**GLN**	**167**	**-0.44**
2.57	**ALA**	**59**	**0.00**	**VAL**	**65**	**-0.34**
6.58	**THR**	**256**	**0.00**	**ILE**	**253**	**-0.04**
	SUM		**-37.56**	**SUM**		**-28.92**

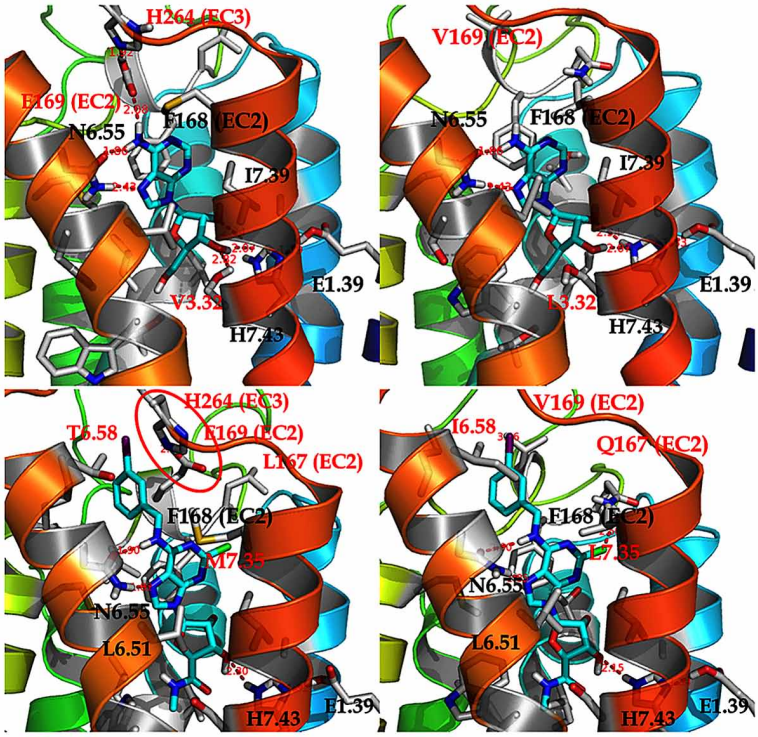
tri-cyclic ring of ZM241385 (Doré et al., 2011). We also observed an additional H-bonding interaction (-2.82 kcal/mol) between the 2' and 3'-OH group of the nucleoside ring and the side chains of the double protonated basic $H278^{7.43}$, which forms the H-bonding with $E13^{1.39}$. The mutation of $N253^{6.55}$ to alanine reports loss of binding for agonist and antagonist, while the $H278E^{7.43}$ mutation displays substantial decrease of binding for agonist and antagonist except in the case of N^6-substituted agonists (S.-K. Kim et al., 2003)

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Later this adenosine-bound $hA_{2A}AR$ was compared with the crystal structures of the agonist UK43207-bound structure (3QAK)(Xu et al., 2011) and the agonist adenosine-bound structure (2YDO) (Lebon et al., 2011) and has a Ca RMSD of 1.61 and 1.69 Å relative to the $hAA_{2A}R$ crystal TM bundle, respectively. After the TM alignment, the adenosine conformation has a RMSD of 1.33 Å relative to the crystal structure.

The predicted ligand binding structure from $hAA_{2A}R$ to hAA_3R was then matched, leading to common interactions at $N250^{6.55}$ (-5.82 kcal/mol), $F168^{EC2}$ (-4.52 kcal/mol), and $H272^{7.43}$ (-4.03 kcal/mol). However, the $V169^{EC2}$ in hAA_3R (corresponding amino acid of $E169^{EC2}$ in $hAA_{2A}R$) results in unfavorable interactions in hAA_3R because of the different loop geometry of EC2 as well as loss of the interaction with the terminal amino group (Table 3 and Figure 5A and 4B). The result is a dramatic reduced binding affinity (cavity sum = -37.56 for $hAA_{2A}R$ vs. -28.92 for hAA_3R), which agrees with the dramatically decreased experimental binding affinity of endogenous adenosine at both rat ARs ($rAA_{2A}R$ estimated K_i: 30 nM vs. rAA_3R estimated K_i, 1,000 nM) (Jacobson, et al., 2000).

Figure 5. Predicted binding sites (A) adenosine 4 to the adenosine A_{2A} receptor ($AA_{2A}R$), (B) adenosine to the adenosine A_3 receptor (AA_3R), (C) A_3 selective agonist Cl-IB-MECA **5** *at $AA_{2A}R$, (D) A_3 selective agonist Cl-IB-MECA at AA_3Rs. The high selectivity of Cl-IB-MECA for AA_3R relative to $AA_{2A}R$ is due to the salt-bridge between E169 and H264 (red circle in (C)) in AA_3R. In $AA_{2A}R$ we have V169 but there is no residue corresponding to H264 because of the gap in $AA_{2A}R$. H-bonding is indicated by red dots, and subtype selective residues are shown in red.*
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2. **hAA$_3$R selective Cl-IB-MECA agonist:** We docked A$_3$ subtype selective Cl-IB-MECA to the lowest energy conformation of hAA$_3$R. From the cavity analysis of Cl-IB-MECA at hAA$_3$R, the major contributing amino acids are F168^{EC2} (-9.79 kcal/mol), N250$^{6.55}$ (-4.44 kcal/mol), L246$^{6.51}$ (-4.28 kcal/mol), and H272$^{7.43}$ (-3.35 kcal/mol) in Table 4. The hydrophobic residues, V169^{EC2} and I253$^{6.58}$, surround the terminal benzyl group of Cl-IB-MECA (Figure 5D). The N^6-bulky benzyl group forms an additional interaction between EC2 and upper TM6, stabilizing the complex.

To study the origin of subtype selectivity, the predicted best binding pose of the highly A$_3$-selective ligand Cl-IB-MECA was matched to the hAA$_{2A}$R predicted structure, and then SCREAM(V.W.T. Kam & W.A Goddard III, 2008) was used to optimize the side chains in the binding pocket, after which the final ligand/protein complex was neutralized and minimized. In hAA$_{2A}$R, the interactions between protein and ligands were hampered by the conformationally restricted loop between E169^{EC2} and H264^{EL3} through a salt-bridge (Figure 5C). The corresponding amino acids of I253$^{6.58}$, V169^{EC2}, and Q167^{EC2} in hAA$_3$R are T256$^{6.58}$, E169^{EC2}, and L167^{EC2} in hAA$_{2A}$R with weakened interactions, resulting in a 7.31 kcal/mol favorable interaction in hAA$_3$R (Table 4). This predicted binding mode is in parallel with the experimental binding affinity of A$_3$ subtype selective Cl-IB-MECA (AA$_3$R Ki: 1.4 nM vs. AA$_{2A}$R Ki, 5,360 nM) (Jeong et al., 2007). The docking result also agrees with reported observations, where modification of the N^6-amine moiety (where large hydrophobic groups tend to produce AA$_1$R and AA$_3$R selectivity) and the C2 position (where large hydrophobic groups tend to produce AA$_{2A}$R selectivity) increase the subtype selectivity of adenosine derivatives as AR agonists for each subtype (K. A. Jacobson et al., 2009).

From the docking studies of the subtype selective antagonists, I253$^{6.58}$ and V169^{EC2} in hAA$_3$R are involved in hydrophobic interactions with the N^6-bulky group in the A$_3$ selective ligands, while E169^{EC2} in hAA$_{2A}$R (which is the corresponding amino acid of V169^{EC2} in hAA$_3$R) stabilizes the exocyclic amino group of the A$_{2A}$ selective ligands through additional H-bonding. Thus this predicted structure explains the increase of A$_3$ selectivity for the additional N^6-bulky substituent at hAA$_3$R. However, Q167^{EC2} in hAA$_3$R is located in the proximity of the C2 substituent which is oriented toward the extracellular loop 2 (EC2) in the docking model. The corresponding amino acid L167^{EC2} in hAA$_{2A}$R could explain the hA$_{2A}$AR selectivity for large hydrophobic C2 substituents which are observed in most A$_{2A}$ selective antagonists (K. A. Jacobson, et al., 2009).

From 27 residues in the binding site of hA$_3$AR, 14 residues are conserved with 52% sequence identity with the hA$_{2A}$AR. For the hA$_{2A}$AR selectivity, E169V in EC2 (Val in hA$_3$AR) is the most important residue which could form an extra H-bonding interaction. V3.32L is also important for the hA$_{2A}$AR selectivity. However, for the hA$_3$AR selectivity, Q167L (Leu in hA$_{2A}$AR) can increase the hA$_3$AR binding. T3.29A can also add a hydrophilic interaction compared with the hA$_{2A}$AR.

3. EXECUTIVE SUMMARY

The GEnSeMBLE Monte Carlo Technique was used to generate an ensemble of the 10 to 20 low energy packing of the 7-helix bundles for all subtypes of hHT$_2$Rs, hHRs and hARs to understand the origin of subtype selectivity for several subtype selective antagonists of hHT$_{2B}$R, hH$_3$HR and hA$_3$AR. Then the DarwinDock Monte Carlo Technique was performed to predict the best binding pose to each of the structures of this ensemble the

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Table 4. Cavity energy of the A_3 selective Cl-IB-MECA **5** *bound to the human adenosine A_3 (AA_3R) and A_{2A} receptors ($AA_{2A}R$). The residues are ordered by the total NonBond energy (A_3), which is the sum of van der Waals (vdW), Coulomb, and H-bond energy (kcal/mol) in the unified cavity. Subtype residues that vary between A_{2A} and A_3 are displayed in bold face. The main interactions leading to subtype selectivity are I253 in A_3 vs. T256 in A_{2A}, V169 in A_3 vs. E169 in $A_{2A,}$ and Q167 in A_3 vs. L167 in A_{2A}, leading to 3.85 of the predicted 7.31 differential binding. The other major differences, involving conserved residues are for F168, S273, M174 favoring A_3 and N250, I268 favoring A_{2A}, leading to a differential of 2.16 of the predicted 7.31 differential binding. The color coding for contributions of each residue to binding of the adenosine ligand is: Dark grey: > 3 kcal/mol, Grey: 1 - 3 kcal/mol, Light grey: 0.5 - 1.0 kcal/mol*

Ballesteros-Weinstein #	**AA_3R Ki: 1.4 nM**			**$AA_{2A}R$ Ki: 5,360 nM**		
	Res	**#**	**NonBond**	**Res**	**#**	**NonBond**
EC2	PHE	168	-9.79	PHE	168	-7.99
6.50	ASN	250	-4.44	ASN	253	-5.86
6.51	LEU	246	-4.28	LEU	249	-4.19
7.43	HSE	272	-3.35	HSE	278	-2.55
7.35	**LEU**	**264**	**-2.87**	**MET**	**270**	**-3.49**
6.58	**ILE**	**253**	**-2.87**	**THR**	**256**	**-1.29**
EC2	**VAL**	**169**	**-2.82**	**GLU**	**169**	**-1.93**
6.48	TRP	243	-2.02	TRP	246	-1.20
7.39	ILE	268	-1.91	ILE	274	-3.10
5.38	MET	177	-1.72	MET	177	-1.73
EC2	**GLN**	**167**	**-1.38**	**LEU**	**167**	**0.00**
3.29	**THR**	**87**	**-1.37**	**ALA**	**81**	**-0.75**
3.32	**LEU**	**90**	**-1.28**	**VAL**	**84**	**-1.74**
7.42	SER	271	-1.05	SER	277	0.17
5.35	MET	174	-1.05	MET	174	0.72
7.36	TYR	265	-0.67	TYR	271	-0.63
EC2	**MET**	**172**	**-0.65**	**VAL**	**172**	**-0.66**
3.36	THR	94	-0.61	THR	88	0.01
6.54	ILE	249	-0.53	ILE	252	-0.57
3.33	LEU	91	-0.48	LEU	85	-0.92
2.64	**VAL**	**72**	**-0.40**	**ILE**	**66**	**-0.42**
2.61	ALA	69	-0.38	ALA	63	-0.62
2.57	**VAL**	**65**	**-0.25**	**ALA**	**59**	**0.01**
6.47	**SER**	**242**	**-0.05**	**CYS**	**245**	**-0.15**
EC2	**ARN**	**173**	**0.00**	**PRO**	**173**	**-0.24**
6.62	**GLY**	**257**	**0.00**	**PRO**	**260**	**-0.40**
EC3				**HIS**	**264**	**0.64**
	SUM		**-46.17**	**SUM**		**-38.86**

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1. We found that the antagonist **2** is selective to HT_{2B} because of favorable vdW interaction at $L132^{3.29}$ and $L281^{6.58}$ in $hHT_{2B}R$, while become unfavorable at $I132^{3.29}$ and $S279^{6.58}$in $hHT_{2C}R$.
2. We found that clobenpropit **3** binds selective to both hH_3HR and hH_4HR because of the additional H-bonding to E5.46/ S5.43, whereas the corresponding amino acids $N198^{5.46}$/ $A195^{5.43}$ in hH_1HR and $T190^{5.46}$/ $G187^{5.43}$ in hH_2HR lead to a loss in these hydrophilic interactions.
3. We found that the A_3 selective ligand, Cl-IB-MECA **5**, has favorable vdW interactions of the N^6-bulky group with $I253^{6.58}$ and $V169^{EC2}$ in hAA_3R while $E169^{EC2}$ in $hAA_{2A}R$ (the amino acid corresponding to $V169^{EC2}$ in hAA_3R) instead interacts with the free amino group of the A_{2A} selective endogenous ligand adenosine **4** through additional H-bonding.

SOLUTIONS AND RECOMMENDATIONS

Although GPCRs are the most fruitful targets for marketed drugs, the success of developments of highly selective subtype drug candidates have been hampered by difficulty in achieving high selectivity for individual GPCR subtypes. After lead or target discovery, management of drug cross-reactivity is a crucial part in the lead-optimization process. Since a major problem of drug is cross-reactivity across members of a single GPCR subfamily because of high conservation of the orthosteric binding sites, development of subtype selective GPCR agonists or antagonists through the aid of molecular modelling is the first step of understanding for the structural requirements for subtype selectivity and the molecular properties required for activation or inactivation.

FUTURE RESEARCH DIRECTIONS

While high resolution 3D structures of $hAA_{2A}R$, hH_1HR, and $hHT_{2B}R$ represent an excellent template for homology modeling for other subtypes, the developments of subtype selective ligands has yet to be shown. Direct comparison of binding modes of subtype selective ligands at other subtypes was applied to rational design of novel subtype-selective ligands without side-effects. The non-conserved residues in the binding cavity which serve as determinants of ligand selectivity will affect overall protein conformation, leading to the different local environments in the binding cavity. Analysis of the subtype-speciđc ligand receptor interactions allowed identiđcation of the major determinants of ligand selectivity, which may facilitate discovery of more efđcient drug candidates. Application of these computational approaches is advancing the understanding of structure-function relationships among individual types, subtypes, and other family of GPCRs. Site directed mutagenesis experiments are also an effective tool to investigate the amino acid properties of specific residues for determining the subtype-selectivity. However, the issue in GPCR targeted drug discovery is the presence of multiple conformations. GPCRs exhibit several distinct low-energy conformations, each of which might favor binding to different ligands and/or lead to different downstream functions, understanding their function requires the various ensembles of low-energy configurations that might play a role in this pleiotropic functionality. Thus take-home message in this review is the activation conformation might be various among the subtypes which were regulated by different G protein, in which might be the one of the major causes of attrition in drug discovery. Accounting for 28% of cases was the lack of systemic drug efficacy because of lack of information of GPCR activation. In addition, the existence of functional dimers or larger oligomeric complexes through homo- or

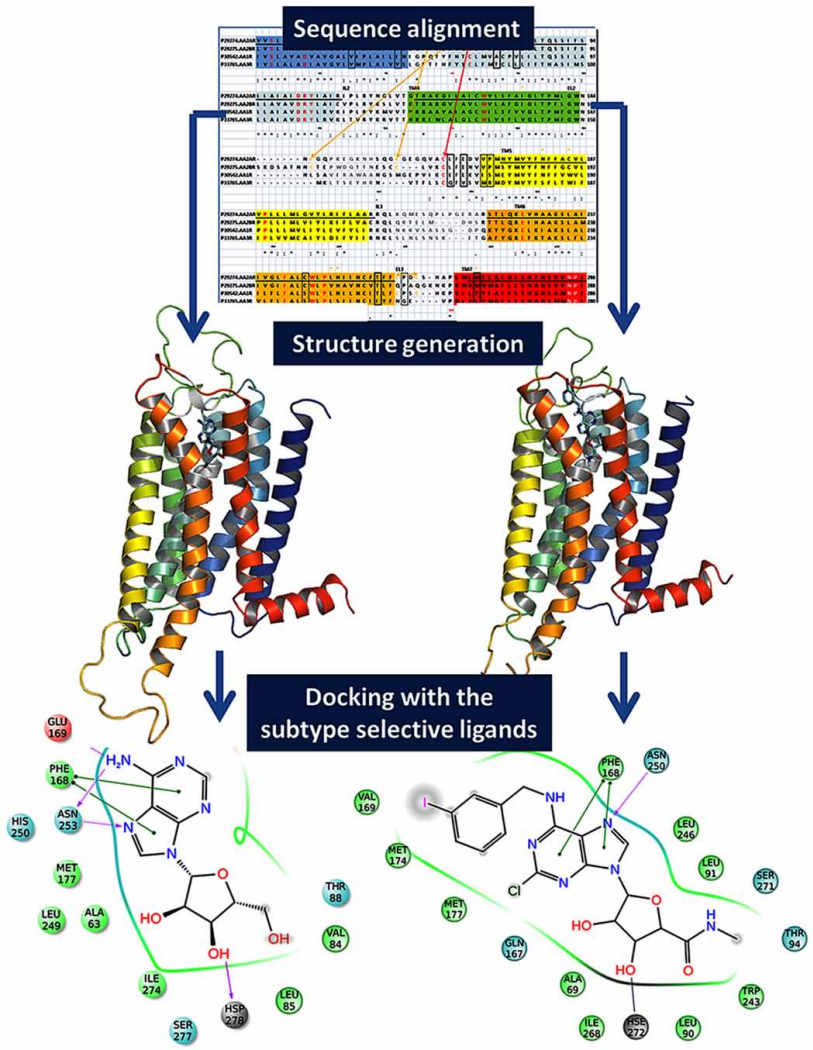
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heterodimerization makes it more difficult to interpret the experimental data. Despite all of problems, we expect that the improved understanding of the microscopic interactions at the various binding site of the various low energy conformations of the GPCRs will guide dramatically in the development of novel subtype selective ligands. Since GPCRs are important as physiological and pharmacologic targets, such results from computational predictions validated by experiment should likely provide useful new insights of broad biological and medical importance.

CONCLUSION

The availability of high resolution X-ray structures has provided new insights into the structures of GPCRs, however for distinguishing between the GPCR conformations favored by the various ligands it is

Figure 6. Diagram of rational drug design for the subtype selective GPCR ligands



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essential to determine the low energy packings of the GPCR that might be favored by different ligands for different subtypes. The GEnSeMBLE methodology provides the means to predict these low lying structures, but the accuracy of GEnSeMBLE has been dramatically improved by the availability of the many new X-ray structures. We studied the subtype selectivity of GPCRs using molecular modeling. As summarized in Figure 6, we generated the 3D-structures from the sequence alignment and docked the subtype-selective ligands. Finally, the comparison of binding site at each subtype gives the information which residues are directly involved in the subtype selectivity and how much subtype selective residues are energetically affected by ligands through the cavity analysis. This information will be the best tools for understanding the binding mechanism of each subtypes and further developing the subtype selective ligands through rational drug design to minimize the undesirable side effects because of non-specificity. Thus the computation and experiment working together can provide the information required for determining the optimum packing and binding pose for various ligands to various subtypes.

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KEY TERMS AND DEFINITIONS

BiHelix: To cover all possible rotations of all 7 helices, consider 30° increments in the rotation about each TM axis (eta η angle), leading to $(12)^7 \sim 35$ million packings.

GEnSeMBLE: GPCR Ensemble of Structures in Membrane BiLayer Environment) Monte Carlo method.

GPCR: G protein-coupled receptors.

Subtype Selectivity: The requirement to gain control of receptor selectivity.

SuperBihelix: To cover all possible rotations of all 7 helices, optimize the tilt angles: theta θ (tilting away from the z-axis) and phi φ (the azimuthal angle of the tilting from the xy plane) angle in addition to the rotation about each TM axis (eta η angle) leading to ~12 trillion packings.

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Chapter 26
Role of Molecular Docking in Computer-Aided Drug Design and Development

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ABSTRACT

Molecular Docking is widely used in CADD (Computer-Aided Drug Designing), SBDD (Structure-Based Drug Designing) and LBDD (Ligand-Based Drug Designing). It is a method used to predict the binding orientation of one molecule with the other and used for any kind of molecule based on the interaction like, small drug molecule with its protein target, protein – protein binding or a DNA – protein binding. Docking is very much popular technique due to its reliable prediction properties. This book chapter will provide an overview of diverse docking methodologies present that are used in drug design and development. There will be discussion on several case studies, pertaining to each method, followed by advantages and disadvantages of the discussed methodology. It will typically aim professionals in the field of cheminformatics and bioinformatics, both in academia and in industry and aspiring scientists and students who want to take up this as a profession in the near future. We will conclude with our opinion on the effectiveness of this technology in the future of pharmaceutical industry.

1. INTRODUCTION

Computer aided drug designing or CADD, is a strategy that harnesses state of the art technology to expedite the drug development process. Traditionally the drug development /discovery (that includes random screening, serendipitous discovery and process optimization) takes nearly a decade to complete with an

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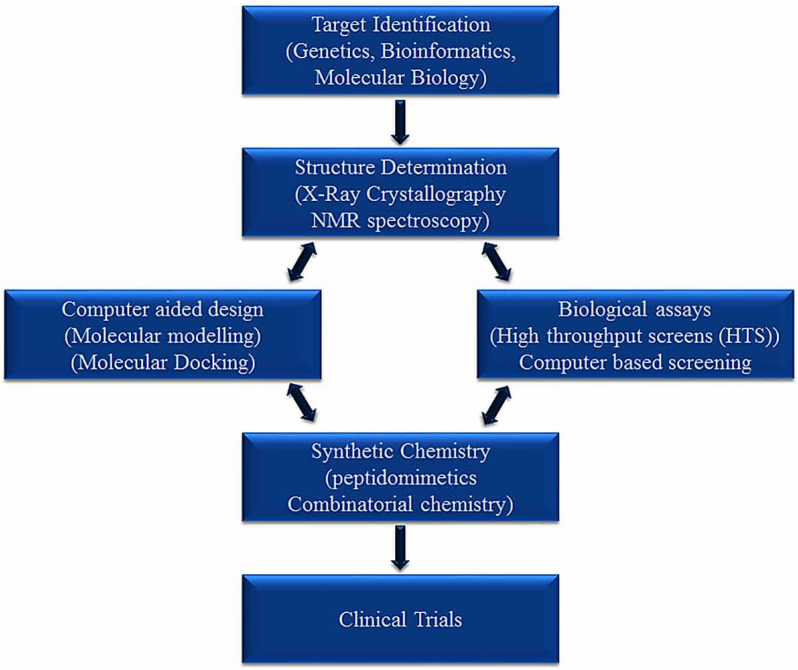
average expense of ~300 million dollar. CADD tends to curtail this expenditure and timeline by providing a holistic view of a drug discovery project and enables a SWOT (strength-weakness-opportunity-threat) analysis to evaluate the viability of the program.

Molecular docking is one of the fundamental pillars of CADD. It analyzes the binding interaction between a target protein and small molecules (Lengauer & Rarey, 1996). These small molecules also referred as ligands are the potential drug candidates that are being developed against the phenotypes and the therapeutic model for which the protein in the CADD is the target (Sliwoski, Kothiwale, Meiler, & Lowe, 2014).

2. HISTORY OF CADD

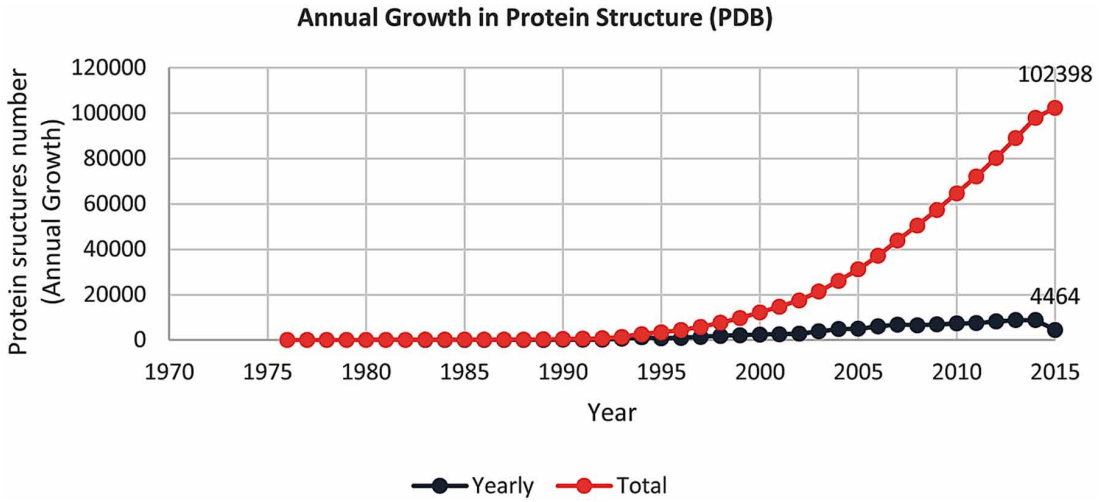
The evolution of CADD began in 1900s when Emiel Fischer (in 1894) and later Paul Ehlrich (in 1909) propagated the concept of receptors and lock and key mechanism (Fischer, 1894). Lock and key concept explains the interaction of a drug and its receptor. Just as there are innumerable pairs of lock and key, where each key inserts into a specific lock to open it, drugs are designed to be tailor made for a particular receptor. Depending on the perfection of the design, the drugs its binding capacity of the drugs with the receptor excels. But just as duplicate keys can be made for a single lock, which makes the lock vulnerable, there is a chance that multiple drugs can work on single receptor thereby giving rise to undue side effects. Additionally one drug can also interact with multiple receptors again giving rise to undue side effects. It was Paul Ehlrich, who proposed the concept of "magic bullet" the elusive drug that only bind to the chosen receptor thereby exhibiting no side effects (Elrich P, 1909,1957).

Figure 1. Flowsheet of the operations in CADD



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Figure 2. PDB protein structure growth per annum graph that displays the number of searchable protein structures per year



Nearly 70 years later with the advent of quantitative structure activity relationship (QSAR), CADD took its next leap towards advancement. At that time only 2-dimentional medchem properties were considered and it was much later that the 3-dimentional properties came into the picture. Gradually, with the evolution of the concepts of molecular biology, protein X-ray crystallography (refer Figure 1) and multidimensional nuclear magnetic resonance spectroscopy (NMR), CADD too evolved into a more reliable and viable strategy in drug discovery.

Advancements in high performance computing, availability of 3-dimensional structure of important pharmaceutical targets, has opened new possibilities for computational drug design. An article in Fortune magazine entitled "Next Industrial Revolution: Designing Drugs by Computer at Merck" (Sliwoski et al., 2014; Van Drie, 2007) clearly postulated the beginning of a new era for drug discovery methods involving a bevy of computational approaches.

3. COMPUTER AIDED DRUG DESIGN (CADD)

Computer Aided Drug Designing (CADD) broadly focuses on two major verticals; 1) Ligand Based Drug Designing (LBDD) and 2) Structure Based Drug Designing (SBDD). These two methods depend on the information available about the protein structure and the ligands binding to them. Ligand based drug designing involved structure activity relationship studies where structure of the receptor (mostly proteins) is unknown. Ligands were tested for their activity and pharmacophore generation. Ligand based drug discovery propagates that similar ligands are assumed to bind to similar proteins and thus to have similar biological activities.

In general LBDD involved four major steps:

1. Prefiltering in virtual screening: it involves Lipinski's Rule of Five (Lipinski, 2004; Lipinski, Lombardo, Dominy, & Feeney, 2001) to find out compounds with drug likeliness features

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   a. A molecular weight of less than 500 g/mol
   b. A calculated lipophilicity (log P) of less than 5
   c. Fewer than five H-bond donors
   d. Fewer than 10 H-bond acceptors
   e. Number of rotable bonds is less than 10 or one of the four rules can be violated.
2. *In silico* Absorption, Distribution, Metabolism, Elimination, and Toxicity (ADMET)
3. Similarity searches: achieved by following the principle of "similar structure- similar property"
4. Development of pharmacophore model by 3D structure alignment.

On the other hand SBDD or Structure based drug design is applied only when the 3D structure of the target of interest is known, either from X-ray crystallography, NMR spectroscopy or homology modeling. Here the known or predicted shape of the binding site is used to optimize the ligand to best fit the receptor. Existing algorithms for structure-based design can be categorized into two classes: *de novo design methods*, which builds ligands tailored to fit the target (Examples of *de novo* design include Lewis (Lewis et al., 1992) and Miranker (Miranker & Karplus, 1995) and the program LUDI (Böhm, 1992a, 1992b), and the *docking methods*, which searches for existing compounds with good complementarity to the target. In both the methods receptors are usually treated as a rigid body single conformation. There are two types of *de novo* design methods 1) *outside-in method* (Lewis & Leach, 1994), the binding sites is first analyzed to determine where specific functional groups might bind tightly. These groups were then connected together to transform into real molecules. 2) *inside-out approach* where molecules are allowed to grow inside binding site using appropriate search algorithm evaluating the each suggestion by energy functions.

4. MOLECULAR DOCKING

Molecular Docking is the central part of the structure based drug designing and is widely used for CADD applications. Fitting a ligand from a 3-D structure database into the binding site of a target protein is called docking. The docking process involves identification of "true" pose (conformation and orientation) of ligand inside receptor binding site and assessment of the binding affinity. Docking methods can be illustrated using a combined approach of search strategy and scoring function. Most docking methods generated large number of possible structures and hence require a parameter to rank the structure using scoring functions. The docking problem is linked with the generation and evaluation of structures of intermolecular complexes. To tackle docking problem, many algorithms has been developed. All the docking methods can be characterized by number of degree of freedom. An overview of docking programs is given in Table 1.

Before ligands can be docked against an enzyme or receptor, usually the binding site has to be discovered first. To minimize the search space on the receptor surface and thus minimize the degrees of freedom that has to be searched. This information can be obtained from crystal structures of ligand-bound receptors or can be predicted using binding site detection programs or online servers, e.g. GRID (Goodford, 1985; Kastenholz, Pastor, Cruciani, Haaksma, & Fox, 2000), POCKET (Levitt & Banaszak, 1992), SurfNet (Laskowski, 1995), PASS (Brady & Stouten, 2000) and MMC (Mezei, 2003). Binding site information significantly increases the docking efficiency. Generally, the largest cavity on a protein surface is considered as an active site, but this is not a universal fact.

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Table 1. List of docking programs with web availability, the key references and search algorithms

Method	Reference(s)	Website	Search Algorithm
Autodock 4.0	(Morris et al., 2009; Österberg, Morris, Sanner, Olson, & Goodsell, 2002)	autodock.scripps.edu/	GA
CDOCKER	(Wu, Robertson, Brooks, & Vieth, 2003)	N/A	MD-SA
CHARMM	(Vieth, Hirst, Kolinski, & Brooks, 1998)	www.charmm.org/	GA and MC
CLIX	(Lawrence & Davis, 1992)	N/A	RBD
DARWIN	(Taylor & Burnett, 2000)	N/A	GA
DIVALI	(Clark & Ajay, 1995)	N/A	GA
DOCK 6	(Kang, Shafer, & Kuntz, 2004; Knegtel, Kuntz, & Oshiro, 1997; Moustakas et al., 2006; Oshiro, Kuntz, & Dixon, 1995)	dock.compbio.ucsf.edu/	IC
eHiTs	(Zsoldos, Reid, Simon, Sadjad, & Peter Johnson, 2006)	www.simbiosys.ca/ehits/index.html	RBD of fragments followed by reconstruction
FITTED	(Corbeil, Englebienne, & Moitessier, 2007)	www.fitted.ca	GA
FlexX 2.2	(Rarey, Kramer, Lengauer, & Klebe, 1996)	www.biosolveit.de/	IC
FlipDock (formerly pyDock)	(Zhao & Sanner, 2007)	www.scripps.edu/Byongzhao/FLIPDock/	GA
FRED	(McGann, Almond, Nicholls, Grant, & Brown, 2003)	www.eyesopen.com/products/applications/fred.html	RBD
FTDock	(Gabb, Jackson, & Sternberg, 1997)	www.bmm.icnet.uk/docking/ftdock.html	RBD
Glide 4.0	(Richard A Friesner et al., 2004)	www.schrodinger.com/ProductDescription.php?mID ¼ 6&sID ¼ 6&cID ¼ 0	Hierarchical filters and MC
GOLD 3.1	(Verdonk et al., 2005)	www.ccdc.cam.ac.uk/products/life_sciences/gold/	GA
HADDOCK	(Dominguez, Boelens, & Bonvin, 2003)	www.nmr.chem.uu.nl/haddock/	SA
MacDOCK	(Fradera, Kaur, & Mestres, 2004)	N/A	IC
MolDock	(Thomsen & Christensen, 2006)	N/A	DE
PatchDOCK	(Schneidman-Duhovny, Inbar, Nussinov, & Wolfson, 2005)	N/A	Shape complementarity
PAS-Dock	(Tøndel, Anderssen, & Drabløs, 2006)	N/A	TS
PhDOCK	(Joseph-McCarthy, Thomas, Belmarsh, Moustakas, & Alvarez, 2003)	N/A	MA
PIPER	(Kozakov, Brenke, Comeau, & Vajda, 2006)	N/A	Fast Fourier transform
ProPose	(Seifert, 2005)	N/A	IC
PSI_DOCK	(Pei et al., 2006)	N/A	GA with TS
RiboDock	(Morley & Afshar, 2004)	N/A	MC
ROSETTALIGAND	(Meiler & Baker, 2006)	N/A	MC
SDOCKER	(Wu & Vieth, 2004)	N/A	Random walk
SLIDE	(Schnecke & Kuhn, 2000; Zavodszky & Kuhn, 2005)	N/A	IC

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Table 1. Continued

Method	Reference(s)	Website	Search Algorithm
SODOCK	(Chen, Liu, Huang, Hwang, & Ho, 2007)	N/A	Swarm Optimisation
SG-DOCK/SP-DOCK	(Fradera, Knegtel, & Mestres, 2000)	N/A	IC
Surflex 2.1	(Jain, 2003, 2007)	N/A	IC with MA

Table directly adapted from (Moitessier et al., 2008).

Abbreviation: N/A, not available.

Search Algorithms notations: DE, differential evolution; EA, evolutionary algorithm; GA, genetic algorithm; IC, incremental construction; MA, matching algorithm; MD, molecular dynamics; RBD, rigid-body docking; SA, simulated annealing; TS, Tabu search.

5. TYPES OF MOLECULAR DOCKING

Based on conformational flexibility of the ligand, molecular docking can be either rigid, flexible can be divided into two sub categories rigid and flexible docking.

Rigid Docking is the simplest algorithm that considers both the receptor and ligand as rigid entities. It explores only six degrees of translational and rotational freedom. Rigid docking approaches were being used in the initial docking algorithms for docking small molecules into the binding sites of proteins and DNA. DOCK is an excellent example of rigid docking methods (Kuntz, Blaney, Oatley, Langridge, & Ferrin, 1982). It works on identifying the small molecules with high degree of shape complementarity to the receptor binding site. The DOCK algorithm uses a geometric matching algorithm to superimpose the ligand onto a negative image of the binding pocket. The receptor spheres generated inside the binding sites of the ligand atoms is viewed in a negative image of the active site, ligand shape can be identified by looking at the negative image of the active site. Rigid docking involved docking with rigid ligands and mostly used pre-generated ligand conformers. This reduced the computational burden however was not the best docking solution provider.

Flexible Docking can be done where either ligand or receptor is flexible or both are flexible. Generally the active site or the surrounding residues of the receptor active site are made flexible rather than the whole receptor. This is done because the docking takes place in the active site or nearby active site residues. Flexible docking strategies do not require any prior knowledge of the conformation of the ligand. Hence the search space consists of 6+N translational, rotational and conformational variables. Flexible docking is useful if there is no conformational information about the ligand. Four different strategies are applied, they are a) Monte Carlo; b) in-site combinatorial search; c) Ligand building; d) site mapping and fragment assembly.

Distance geometry can also be used to perform molecular docking, however incremental construction of the ligand within binding sites is used by a number of programs.in this method a series of conformations would be generated by systematically varying dihedral angles between 0° and 360°.This involves the grid search, where all base fragments were identified within the ligand. Base fragments will be docked into the binding site and clustered to remove duplicate orientations. Each docked orientation of the base fragment then represents the starting point for the conformational analysis of the rest of the ligand.

Genetic algorithms, molecular dynamics approaches can also be used to perform molecular docking. The ideal docking method would allow both ligand and receptor to explore their conformational degrees of freedom. However such calculations are computation intensive and are more useful for refinement of docked structures.

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6. SCORING FUNCTIONS

The binding of ligand and receptor are quantum mechanical in nature, but due to computational limitations and biological complexities quantum theories becomes impractical and are not always accurate. The strength of ligand receptor interaction can be measured experimentally and is often reported as the dissociation constant, K_d or by the concentration of ligand inhibiting activity by 50% (IC 50). Most of the methods attempt to estimate binding free energy of the ligand binding to receptor, which can be given by:

$$\Delta G_{\text{bind}} = \Delta G_{\text{complex}} - (\Delta G_{\text{ligand}} + \Delta G_{\text{receptor}}) \quad (1)$$

The relationship between the binding free energy ΔG and the experimentally determined K_d or IC_{50} is defined in Equation 2

$$\Delta G_{\text{bind}} = -RT \ln K_{\text{eq}} = -RT \ln K_d = -RT \ln 1/\text{IC50} \quad (2)$$

With Equation (2) it is possible to calculate the binding free energy where K_d is defined with respect to standard state (Atkins, De Paula, & Walters, 2006). The pressure,1 atm, and the activity of the solutions, namely 1M, define standard state.

Molecular docking have played an increasing role in the functional study of proteins and structure-based drug design with the increase in experimentally determined three-dimensional protein structures. However, one important problem is the development of scoring function that can heuristically and precisely describe the protein and ligand interaction. Scoring function plays major role in ascertaining three major applications. Firstly, defining the binding mode and ligand site on a protein. For a target molecule like protein, molecular docking generates numerous putative ligand binding orientations/conformations at the active site around the protein. A scoring function is used to rank these ligand orientations/conformations by evaluating the binding tightness of each of the putative complexes. Gold standard for scoring is to rank the experimentally determined binding mode most highly. Another application is linked to first application and it involves prediction of absolute binding affinity between protein and ligand, which is very much important for lead optimization. Lead optimization is used to improvise the binding of low-affinity hits or lead compounds. An idle scoring function can significantly increase the optimization efficiency and prevent costs of ligand synthesis and experimental testing. Structure-based drug design is another important application of scoring functions and is used to screen potential drug hits (leads) for a receptor (protein target) by searching a large ligand database this is also known as virtual screening. Scoring function is commonly used for ranking known binders most highly according to their binding scores during virtual screening (Huang, Grinter, & Zou, 2010).

Large number of poses are generated by docking algorithms, few got rejected because of high-energy clash with the protein. Scoring functions will be used further to assess the left out poses. Scoring function is required to rank the ligand molecules (if using database of molecules for screening against a receptor) relative to each other. Moreover, scoring functions are essential to identify the docked orientation that could be the real representation of 'true' structure of the intermolecular complex or docking mode.

Molecular docking is used to predict how a ligand will interact with the receptor binding site. The ligand is mostly a small molecule but can be a protein also. Likewise, receptor is mostly a protein of pharmacological significance however it could be a DNA or RNA molecule as well. The next step in docking is consists of scoring functions which will distinguish among the generated binding modes and

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the optimal solution that is fairly matches to actual binding. The most promising predicted protein-ligand complex is considered to be the biologically relevant one. A large number of different scoring functions have been developed to model protein-small molecule docking which can be divided into three types or classes: 1) Force-field based 2) empirical and 3) knowledge-based scoring functions.

Force-field Scoring Functions: Force field (FF) based scoring tries to quantify internal ligand energy as well as ligand-receptor binding energy. Molecular mechanics force fields based scoring functions are based on different parameter sets, like AutoDock is based on the AMBER force field (Kitchen, Decornez, Furr, & Bajorath, 2004).

The ligand-receptor binding interactions can be best described by using van der Waals (VDW), electrostatic energy terms, bond stretching/bending/torsional energies, etc. Intermolecular forces are calculated using "6-12" Lennard-Jones potential functions representing van der Waals interactions however the interactions between atomic charges can be calculated using Coulomb's equation. Examples of force field based scoring functions include D-Score, G-Score, GOLD, AUTODOCK, DOCK. Following equations shows the use of distance-dependent dielectric constant ε (*rij*) force field scoring function in DOCK:

$$\Delta G_{bind} = \sum_{i}^{lig} \sum_{j}^{rec} \left[\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}} + \frac{q_i q_j}{\varepsilon(r_{ij}) r_{ij}} \right] \tag{3}$$

where *rij* stands for the distance between protein atom *i* and ligand atom *j*, *Aij* and *Bij* are the VDW parameters, and *qi* and *qj* are the atomic charges. *ε* (*rij*) is usually set to *4rij*, reflecting the screening effect of water on electrostatic interactions.

Force field scoring functions is widely used but faces some challenges which involves the explanation of solvent effect and entropy effect.

Empirical Scoring Functions: Emperical scoring functions uses weighted energy terms to estimate the binding affinity of a complex. Ludi empirical scoring functions conceptualized that binding energies can be estimated by addition of individual unrelated terms.

These unrelated terms can be obtained from receptor-ligand crystal structure or training the binding constants of thousands of protein-ligand complexes. In empirical scoring function simple scalable contributions like hydrogen bonds, metal ligations, hydrophobic effect rotable bonds evaluated for estimating the energetics of ligand- receptor binding.

$$\Delta G_{bind} = \Delta G^{0} + \Delta G_{HB} \sum_{HB} f(\Delta r) f(\Delta 1\alpha) + \Delta G_{met} \sum_{met} f(\Delta r) + \Delta G_{lipo} \sum_{lipo} f(\Delta r) + \Delta G_{tot} N'_{rot} \tag{4}$$

Or it can also be given as,

$$\Delta G = \sum_{i} Wi \,.\Delta \mathrm{G_i} \tag{5}$$

In Equation 5 (Huang et al., 2010), ΔG_i represents different energy terms such as Van Der Waal energy, electrostatic energy, hydrogen bond, desolvation, entropy, hydrophobicity, etc. Here, *Wi* are determined by fitting the binding affinity data of a training set of protein–ligand complexes with known three-dimensional structures.

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Because of their simple energy terms, empirical scoring functions are much more faster in binding score calculations compared to force field scoring functions.

LUDI, F-score, Chemscore, Score, x-score are some examples of empirical based scoring function.

Knowledge based scoring functions: Knowledge based scoring functions (also referred to as statistical-potential based scoring functions). Knowledge based scoring functions recruits energy potentials directly derived from structural information of experimentally determined atomic structures. Knowledge based scoring functions for protein- ligand complex are modelled using simple atomic interaction-pair potentials. This scoring functions is based on the potential of mean force, which is defined by the inverse Boltzmann relation. The GOLD fitness function is a measure of Chemscore or GOLD score, represented by $\sigma_{o,}$ which is measure of water contribution $\sigma_{1,}$

Is loss in the entropy of the water molecule and σ_w is the intrinsic binding affinity of the water.

$$\text{Fitness} = \sigma_o + \sum_w O\left(w\right)\left(\sigma \mathrm{p} + \sigma \mathrm{i}\left(w\right)\right) \tag{6}$$

Drugscore, ITScore, DFIRE, GOLD/ASP,Kscore, PMF and SMoG, BLEEP, MScore are examples of knowledge based scoring functions.

Compared to the force field and empirical scoring functions, the knowledge-based scoring functions provide more balanced option between precision and heuristics. Because the potentials in Equation (6) are extracted from the structures rather than from attempting to reproduce the known affinities by fitting, and because the training structural database can be large and diverse, the knowledge-based scoring functions are quite robust and relatively insensitive to the training set (Huang et al., 2010).

7. APPLICATIONS OF MOLECULAR DOCKING

Molecular docking has been one of the most extensively used technique in CADD (Computer Aided Drug Designing) and is the central dogma of Structure-Based Drug Designing (SBDD). SBDD is applied when the structure of target macromolecules generally, protein is known. The main aim of the SBDD is to identify the molecules which favorably interact with the target protein at its binding site. Molecular docking fulfills this objective by predicting the binding orientation of one molecule with the other in 3D space thereby identifying the lowest free energy complex structures. Another aim of the SB-CADD is the lead identification in the drug discovery process. In general, lead identification is done by two methods: High Throughput Screening (HTS) and Virtual High Throughput Screening (vHTS). HTS is the traditional technique which is slow and expensive compared to vHTS. In vHTS, a database containing large number of small molecules are screened against a protein target using molecular docking techniques and then ranked hits for lead generation. Previously, molecular docking was also used in SBDD pipeline for screening large compounds database and to identify the binding orientation with the target. With the advancement of the docking methodologies, presently molecular docking is used for various other applications viz. identification of differential binding of a ligand to different target biomacromolecules, de novo design for lead generation, adverse drug reaction prediction in the drug development and in some cases molecular docking is also used for non drug related projects like identification of putative pollutants for biodegradative enzymes. This exhibits the diversity of application of molecular docking.

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In the following section we will discuss major applications of molecular docking along with case studies.

7.1 Virtual Screening

Virtual screening is a tool to screen large compound libraries against a drug target that can be a protein or an enzyme or any other biomacromolecules. It is used to find the compounds from a large library which exhibited good binding interaction with the drug target. It is one of the most useful technique in the drug development process.

Case Study (Virtual Screening): Identification of BCR-ABL Tyrosine Kinase Hits Using Molecular Operating Environment (MOE) Software

In this case study (Rahul & Ashutosh, unpublished) we discuss how virtual screening is performed using MOE (Molecular Operating Environment (MOE), 2015). We screened an in-house database of ~2000 compounds against BCR-ABL tyrosine kinase, durg target for chronic myeloid leukemia (CML) treatment. The crystallographic co-ordinates of BCR-ABL is available on Protein Data bank (Berman et al., 2000) (PDB ID: 3OXZ, (Zhou et al., 2011)). The coordinates pdb file was downloaded and prepared using MOE Strucuture preparation wizard (Molecular Operating Environment (MOE), 2015). During struture prepration the hydrogens was added to the protein structure (Note: at times the pdb file has some missing side chains or missing residue which could be fixed by various tools like MOE (Molecular Operating Environment (MOE), 2015) or chimera (Pettersen et al., 2004)). Appropriate charges were added and water molecules which were more than 4.5 A^0 apart from the receptor were removed. Finally the energy was minimized using MMFF94x forcefield (Halgren, 1996) (There are various other force field are available like Amber (Cornell et al., 1995), OPLS (Jorgensen & Tirado-Rives, 1988), CHARMM (MacKerell et al., 1998) etc. with different versions here we are using the default).

In order to prepare the ligands the conformations of the 2000 compounds structure database were generated using MMFF94x forcefield (Halgren, 1996). A limit of 4.5 kcal/mol strain energy was imposed with a 500 conformation limit for each molecule. Duplicate conformations were removed with a heavy atom RMSD tolerance of 0.6 A^0 (0.75 A^0 for conformations with strain greater than 3.5 kcal/mol). The fact that a ligand was already bound at the active site of the protein, enabled us to define the active site. Next step involved generation of possible poses from the various ligand conformations. Diverse placement methods are available viz. Alpha Triangle, Alpha PMI, Flex, GOLD, Proxy Triangle, Template Forcing, Template Searching, Triangle Matcher. Here we used the default triangle matcher for the placement step. Next rescoring, refinement and again rescoring took place using London DG methods (other methods such as ASE, Affinity dG, Alpha HB, Electron Density, GBVI/WSA dG are also available). Further refinement is done using forcefield method and again rescoring was done using GBVI/WSA dG method. Finally 30 poses were kept and total 46,129 conformations were generated which were docked into the active site of BCR-ABL tyrosine kinase. MOE (Molecular Operating Environment (MOE), 2015) gave an output in form of a table having 10 fields which are depicted in Table 2 and the output of top 10 compounds on the basis of final score S was given in Table 3. These 10 compounds having score in order of (-9.2595) to (-8.8398) were obtained. All the top 10 compounds were docked into active site, the results are shown in Figure 3. These compounds could be used further for invitro assays.

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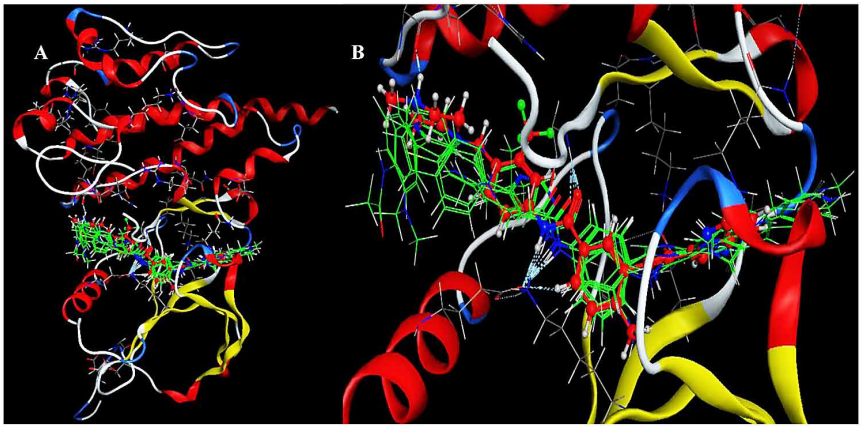
Table 2. Fields with description of MOE output (Molecular Operating Environment (MOE), 2015)

Field(s)	Description
Mol	An output pose.
receptor	The receptor after the refinement stage.
Rseq	The receptor sequence number.
Mseq	The molecule sequence number.
S	The final score, which is the score of the last stage that was not set to None.
Rmsd	The root mean square deviation of the pose, in Å, from the original ligand. This field is present if the site definition was identical to the ligand definition.
rmsd_refine	The root mean square deviation between the pose before refinement and the pose after refinement.
E_conf	The energy of the conformer. If there is a refinement stage, this is the energy calculated at the end of the refinement. Note that for Forcefield refinement, by default, this energy is calculated with the solvation option set to Born.
E_place	Score from the placement stage.
E_score1 E_score2	Score from rescoring stages 1 and 2.
E_refine	Score from the refinement stage, calculated to be the sum of the van der Waals electrostatics and solvation energies, under the Generalized Born solvation model (GB/VI).

Case Study (Virtual Screening): Identification of BRD4(1) Inhibitors Using Protein-Ligand Docking and Structure-Guided Design.

Bromodomains (BRDs) are recently discovered as a potential new drug targets for cancer, inflammation, diabetes and cardiovascular therapeutics. BRD-containing proteins choses epigenetics route for binding against acetylated lysines (KAc) on chromatin structures. Duffy et al., in 2015 employed a virtual screening & X-ray crystallography along with other cheminformatics techniques to identify several scaffold

Figure 3. 3D view of bound ligands with the BCR-ABL protein. A) Full view of the BCR-ABL protein with ligand. B) zoomed view of the ligands present into the active site of BCR-ABL protein. Red is the ligand already present in crystal structure while green are the top 10 compounds which are screened using docking studies.



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Table 3. Top 10 compounds docked on the basis of MOE dock final score S (Molecular Operating Environment (MOE), 2015)

Mol	Rseq	Mseq	S	rmsd_refine	E_conf	E_place	E_score1	E_refine	E_score2
O=C(NCC[NH+](C)C)c1cc(ccc1)-c1nc(N2CCN(CC2)c2cc[nH+]cc2)cnc1	1	1299	-9.25949	2.229406	85.04276	-52.6258	-12.1489	-11.9827	-9.25949
O=C(Nc1ccc(Nc2ncnc(c2)-c2cc3c(cc2)cccc3)cc1)c1ccc(N)cc1	1	505	-9.15959	1.383814	-6.63474	-86.3349	-12.6617	-21.7742	-9.15959
O(C(C)(C)C)C(=O)Nc1ccc(Nc2ncnc(c2)-c2ccc(N(C)C)cc2)cc1	1	550	-9.13703	1.297704	-55.6844	-108.026	-11.3118	-14.6355	-9.13703
O=C(Nc1ccc(Nc2ncnc(c2)-c2cc(N(C)C)ccc2)cc1)c1ccc(N)cc1	1	510	-9.02742	1.506958	-27.5484	-102.505	-11.7205	-30.7333	-9.02742
o1c2c(cc1-c1ncnc(Nc3ccc(NC(=O)c4ccc(N)cc4)cc3)c1)cccc2	1	507	-8.93877	0.817132	-22.9512	-130.471	-13.7038	-17.7379	-8.93877
S(C)c1ccc(cc1)-c1ncnc(Nc2ccc(NC(=O)c3ccc(N)cc3)cc2)c1	1	508	-8.92662	1.648885	-18.9101	-88.5567	-11.9491	-23.8481	-8.92662
O=C(Nc1ccc(Nc2ncnc(c2)-c2ccc(NC(=O)C)cc2)cc1)c1ccc(N)cc1	1	515	-8.91988	2.205891	-49.7061	-64.7249	-11.8485	-31.4016	-8.91988
O=C(Nc1cc(ccc1)-c1nc(N2CCN(CC2)c2cc[nH+]cc2)cnc1)C	1	1294	-8.8837	1.633233	105.6261	-59.2689	-12.4821	-11.4932	-8.8837
O=C(Nc1ccc(Nc2ncnc(c2)-c2cc3c(cc2)cccc3)cc1)c1ccc(N)cc1	1	505	-8.88196	2.717045	-7.07057	-77.8603	-12.1259	-23.2881	-8.88196
O=C(Nc1cc(ccc1)-c1nc(N2CCN(CC2)CCN(C)C)cnc1)C	1	1441	-8.83977	1.08584	150.4833	-60.5674	-10.335	-11.6034	-8.83977

clustershits binding to BRD4(1) (Duffy et al., 2015). Database of approx 25 million compounds from various publicly available sources were filtered on the basis of common drug-like property. Approximately 230,000 filtered compounds were selected through multiple rounds of random selections followed by diversity confirmation *via* 2-D atom-pair similarity calculations. These were docked against two X-ray crystal structures of BRD4(1) with different co-crystallized ligand using Glide (Richard A. Friesner et al., 2004). The binding pose was considered if potential hydrogen bond is present with the Asn140 sidechain amide, that mimics the native histone acetylated lysine binding location. Finally, 153 available compounds were selected on the basis of hydrogen bond requirements & Glide score. These compounds represented 82 scaffolds and were purchased from commercial vendors and evaluated in vitro screening. These 153 compounds were tested by HTRF binding inhibition to BRD4(1) and BRD2(2) at 50 μM concentrations. Compounds having 50% or more binding to BRD4(1) were submitted for dose-response testing and IC_{50} calculations, while hits in the 30–49% binding range at 50 μM were grouped by scaffold. The compound showing highest activity was retest for IC50 calculation and also co-crystallized in BRD4(1) to identify the actual binding pose. A small series of compounds analogs of the highest active compound were prepared and tested. The most active compound in this series, shows greater activity. The molecular docking with other experimental method process allowed to identify BRD4(1) inhibitors also helps in improving the activity of a compound in BRD4(1) inhibition.

7.2 Identifying the Binding Orientation ('True' Pose) of Ligand with the Drug Target

Molecular docking can be used as a tool for virtual screening if screening against ligand database or if single or few ligands are searched for identification of true pose inside receptor binding site. This information is extremely advantageous to identify the binding sites or groups which are involved in binding. This will also help in designing the new potent compounds by knowing the binding pattern of the existing compounds. There are two steps involved in identifying the binding orientation of ligand withn the drug target. They are preparation of the protein and the liagnd and then the final docking. Preparation of the protien involves the searching the protein structure in PDB database for the availability of their crystal structure. If the protein crystal structure is not available then the protein structrue is modelled using bioinformatics methods. While the preparation of ligand involves the generation of energy minimized 3D structure of the lignad.

The docking depends on individual cases which is depicted below.

Case Study: True Pose Identification of Acarbose with α-Glucosidase Enzyme

This study which was conducted in our lab (unpublished results), involved the step wise procedure to identify the binding orientation of Acarbose with α-Glucosidase enzyme. In this example α-Glucosidase protein model was built using I-Tasser (Yang et al., 2015) while docking analysis was done using Autodock (Morris et al., 2009). α-Glucosidase is a proven drug target for Diabetes. α-Glucosidase inhibitors decrease the absorption of carbohydrates from the digestive tract, thereby lowering the aftermeal glucose levels. Crystal structure of α-Glucosidase enzyme is not available on PDB (Berman et al., 2000). So, α-Glucosidase protein structure was modelled using bioinformatics methods. Modelling of the protein structure involved few steps and primarily depends on the identity of sequence similarity with the template sequence. Here we had 44% sequence identity with two human maltase-glucoamylase templates

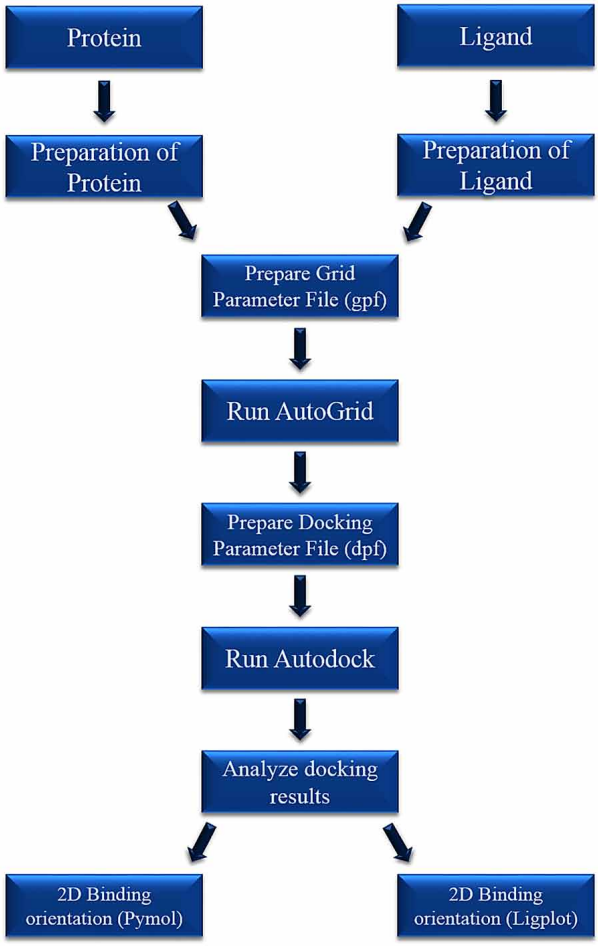
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(PDBID: 2QLY and 3L4T) with query coverage 90% each. On this sequence identity we used threading method to model the protein. I-Tasser (Yang et al., 2015) was applied to model α-Glucosidase protein structure. The modelled protein structure was validated through ramachandran plot. Since there was no ligand bound to the protein we identified the active site residues in the protein structure *via* centroid co-ordinates of the complex ligands in the crystal structure (PDB ID: 3L4T). Finally docking was performed using Autodock program (Morris et al., 2009). It is one of the widely used freely available docking tool. The docking procedure for this molecule is shown in Figure 4.

After successful execution of autodock program docked log file (dlg) was generated in which the docked conformation co-ordinates of ligand with their binding energy were present. Total 10 docked conformation were obtained and the best conformation is having -4.45 Kcal/mol binding energy. This docked acarbose with protein target can be visulized using pymol (The PyMOL Molecular Graphics System) as shown in Figure 5 and save as complex pdb file. This complex pdb file is used to analyze the 2D and 3D binding orientation of Acarbose with protein using Ligplot as shown in Figure 6 & 7 resp.

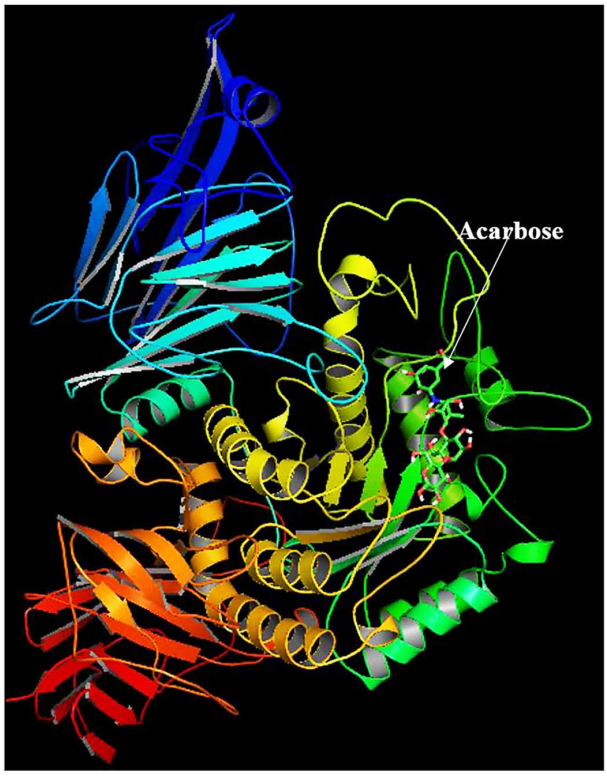
The major limitation of this case study is the quality of the alpha-glucosidase protein structure because the whole docking is depends on the receptor structure. While performing docking studies using predicted structure one have to ensure that the modelled protein structure quality is upto the mark or not.

Figure 4. The procedure followed to perform this study



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Figure 5. Full view of the Acarbose docked in the modelled alpha-glucosidase active site. Acarbose are shown as sticks model while the protein are shown as cartoons.
This image is viewed using Pymol (The PyMOL Molecular Graphics System).



7.3 Hydrated Docking

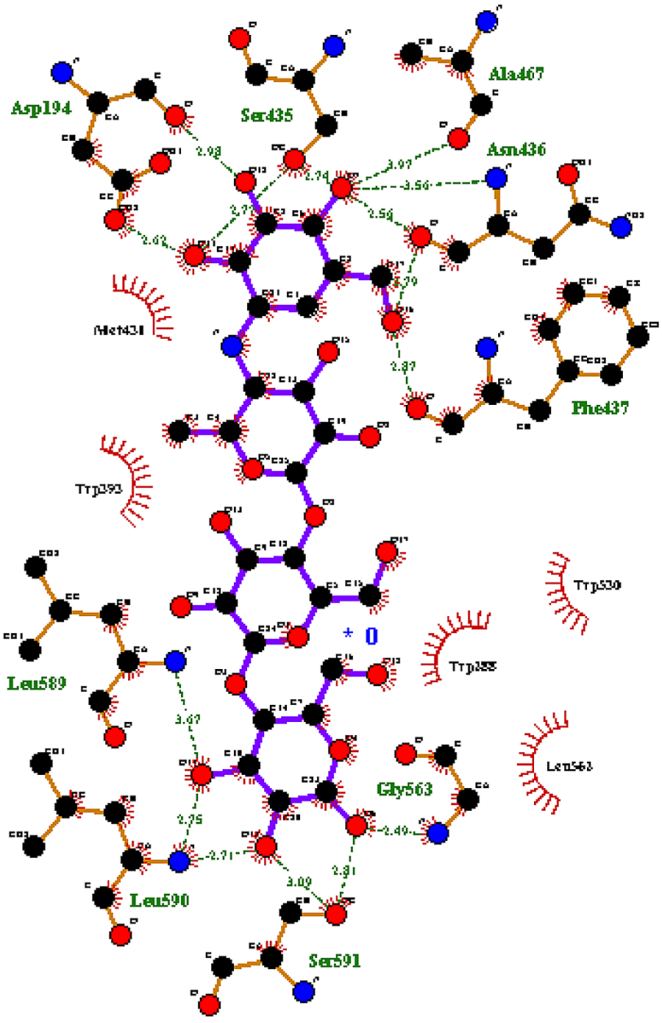
Hydrated docking involved docking of protein-ligand in presence of water molecules. Water molecules play an important role during protein-ligand interaction. Forli & Olson in 2012 developed a force field with discrete displaceable water and desolvation entropy for studying hydrated ligand docking (Forli & Olson, 2012). Autodock tool provided a script for implementing hydrated docking.

Case Study: Identification of Noncamptothecin Topoisomerase I (Top1) Inhibitors Using Hydrated Docking

Taliani et al. in 2013 used hydrated docking to identify the binding orientation of their noncamptothecin Topoisomerase I (Top1) inhibitors (Taliani et al., 2013). To begin with, the binding pose of HTOP1 (human topoisomerase 1) with inhibitor Topotecan (inform the readers about Topotecan) was observed from X-ray diffraction technique and Autodock docking method (Morris et al., 2009) (as shown in Figure 8 below). Here, hydrated docking between HTop1 and Topotecan provided useful information about the binding pattern of the compound in presence of water molecules. This information was used to design novel compounds with diverse architectures that resulted in the improvement of Top1 inhibitory activity.

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Figure 6. 2D interaction of modelled alpha glucosidase with the Acarbose inhibitor. Acarbose is majorilly interacted with Asp194, Ser435, Asn436, Phe437, Ala467, Gly563, Leu589, Leu590, Ser591.
Image is created by Ligplot (Wallace, Laskowski, & Thornton, 1995).



The problem with hydrated docking is the difficulty in comparing the results with the crystal complex having water interactions which is not able to distiguish whether water is really involved or it is apart of crystal effect.

7.4 Reverse Docking

Reverse docking is a technique that identified the target proteins of known biological active compounds. In this method small compounds or ligands are docked into the active sites of multiple drug targets, their binding orientation were analysed and ranked on the basis of their binding interaction strength. It is very useful when target information is unknown.

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Figure 7. 3D view of modelled alpha glucosidase with the Acarbose inhibitor. This image shows the 3D binding pattern of Acarbose with alpha glucosidase enzyme.
The image is created by Pymol (The PyMOL Molecular Graphics System) and Ligplot (Wallace et al., 1995).

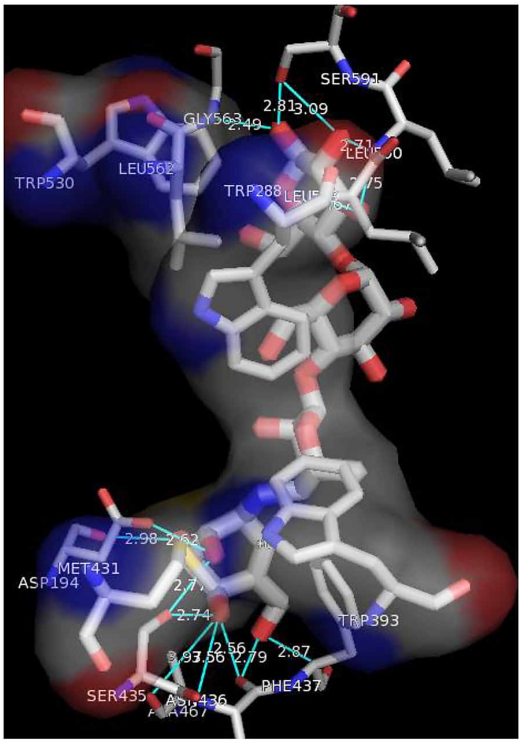
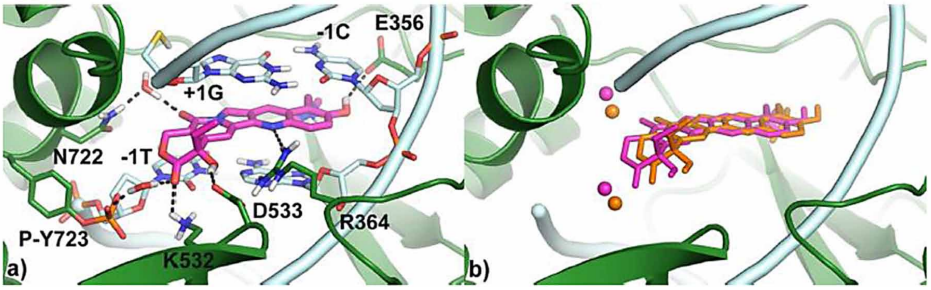


Figure 8. a) X-ray binding pose of Topotecan (II) within the Top1 DNA cleavage site (PDB code: 1K4T). The ligand is shown as magenta sticks. The enzyme and the substrate DNA are represented as green and cyan cartoons, respectively. Residues important for ligand binding are highlighted as sticks. H-bonds are depicted as dashed black lines. b) Superimposition of the X-ray binding pose (magenta sticks) of II and the docking conformation (orange sticks) as predicted by AD4. The enzyme and the substrate DNA are represented as green and cyan cartoons, respectively. Crystal and AD4 water molecules bridging between the ligand and the enzyme are shown as magenta and orange spheres, respectively.
Source: (Taliani et al., 2013).



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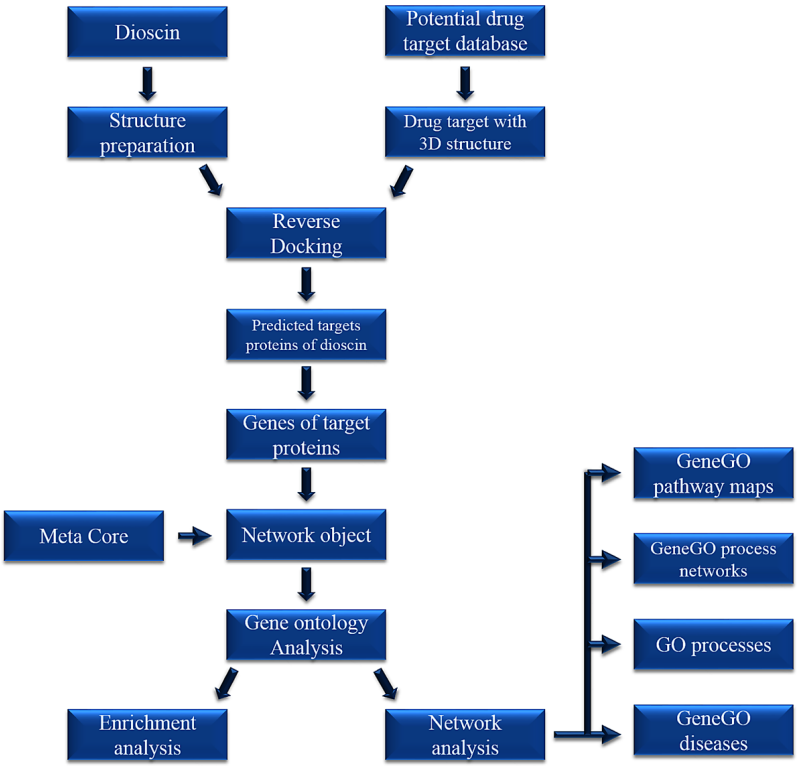
Case Study: Identification of Targets which are binding to Dioscin

Recently Yen et al. described a workflow in which they coupled reverse docking with other bioinformatics methods to predict drug targets, biological activities, signal pathway and regulating networks of Dioscin that could be implemented on other available biological active compounds (Yin et al., 2015). Dioscin is a natural steroidal saponin present in many Chinese medical herbs (like, Dioscorea nipponica Makino, Dioscorea zingiberensis) and known to posses many medical pharmacological properties including anti-tumor, anti-hyperlipidemic, anti-fungal and antivirus activities. Yen et al. screened the available potential protein targets from humans (71), rats (7) and mice (8). The reverse docking indicated that most probable targets of dioscin were cyclin A2, calmodulin, hemoglobin subunit beta, DNA topoisomerase I, DNA polymerase lambda, nitric oxide synthase and UDP-N-acetylhexosamine pyrophosphorylase, etc. The workflow used by (Yin et al., 2015) is depictedin Figure 9.

7.5 Adverse Drug Reaction Prediction

Prediction of Adverse drug reactions (ADRs) is essential for a pharmacologically active small molecules because adverse drug reaction produces effect which could be extremely harmful,unusual and could alter

Figure 9. The analysis process of the prediction of drug targets, biological activities, signal pathway and regulating networks
Source:(Yin et al., 2015).



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the biochemical pathways (LaBute et al., 2014). Nearly hundred thousand fatalities are reported in US every year because of ADRs. Prediction of adverse drug reactions (ADRs) by lead compounds is very useful in drug discovery process.

Case Study: Adverse Drug Reaction Prediction Workflow

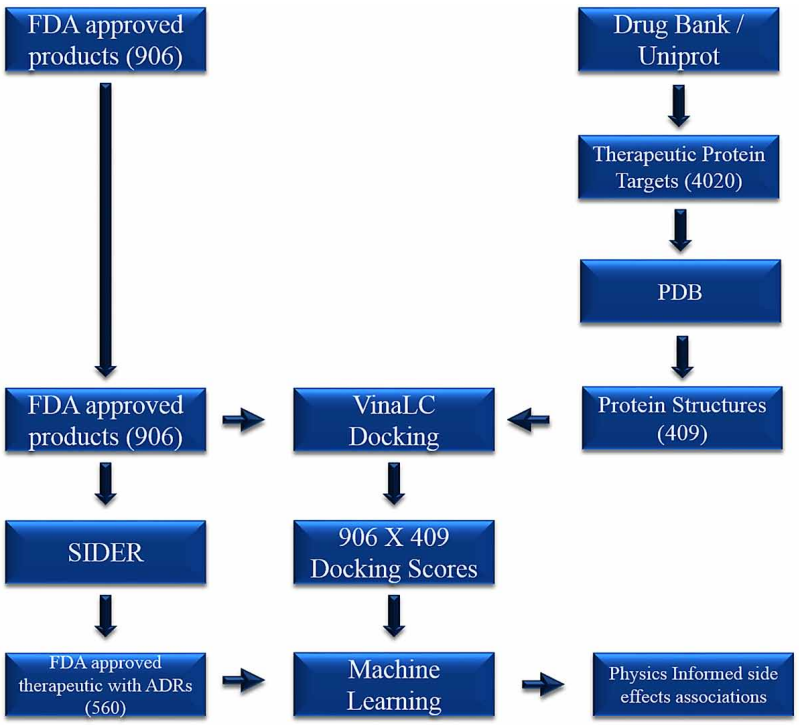
In 2014, LaBute et al. developed a computation process that enabled prediction of ADRs using molecular docking and known ADR information from DrugBank and SIDER. The workflow is shown in Figure 10.

The drug target information were obtained from Uniprot (Consortium, 2015). Nearly four thousand proteins were identified and extensive search of their crystal structure led to the discovery of 409 of them in Protein Data Bank (PDB) (Berman et al., 2000). They were used as a virtual panel. All 906 FDA-approved drugs (till date) were docked in this virtual panel using the VinaLC program (Zhang, Wong, & Lightstone, 2013). Side effect of 560 compounds were present in SIDER database which were used for building logistic regression models for ADR prediction.

This type of study immensely helped in drug development process by which the lead ADRs is accessed at earlier stage of drug discovery and could be dropped or redesigned.

This study depends on the machine learning methods which may provide various false positives and false negatives.

Figure 10. Data integration/analysis workflow scheme



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7.6 De-Novo Drug Design

De novo drug design is generally used to generate new drug-like compounds by linking fragments. De novo design uses library of building blocks that also contain single atoms, functional groups, and small molecular fragments. De novo drug design is widely used in the drug development process to generate new novel and potent compounds by using the drug target active site information.

Case Study: De Nove Design

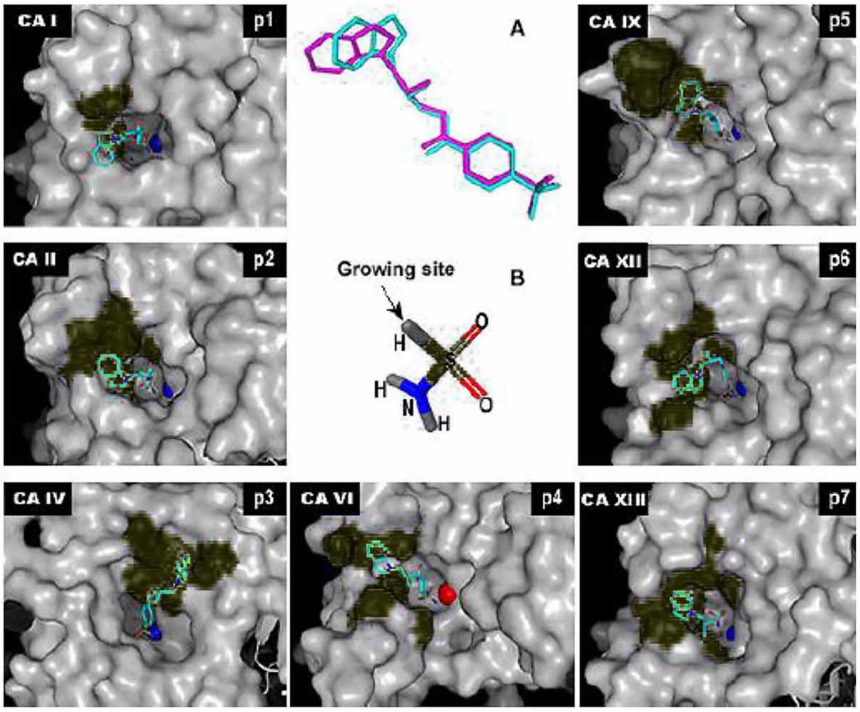
Xiang et al. used this approach to discover the isozyme-selective inhibitor scaffolds of human carbonic anhydrases (CAs) (Xiang, Xiang, Fang, Zhang, & Li, 2014). CAs are known drug targets for the treatment or prevention of disease like altitude sickness, epilepsy, glaucoma, obesity, tumor, acid–base disequilibria, and other neuromuscular diseases. Experimental procedure included the alignment of available 9 PDB structures of CAs where one of them was complexed with SBR ((R)-N-(3-indol-1-yl-2-methyl-propyl)-4-sulfamoyl-benzamide). The ligand (SBR) was docked to all 9 structures using Autodock Vina (Trott & Olson, 2010). POCKET module of LigBuilder (Yuan, Pei, & Lai, 2011) was used to identify the interaction residues and molecules were constructed for the target protein using GROW module. GROW module required a seed structure on which fragments were added to generated new hit compounds. Figure 11 depicted the interaction of SBR ligand with different CAs and seed struture with growing site. Total 200 top candidates were selected after Lipinski filter of maximal molecular weight and logP value being 500 and 5, respectively. Further top hits having multiple rings were used for further docking analysis. These four molecules are naphthalenesulfonamide, N-sulfonamide-phthalimide, 3-sulfonamide-acridan, and fluorenesulfonamide derivatives, these scaffold structures can be the potential isozyme-selective inhibitors against CA II, IV, and IX.

8. CHALLENGES OF MOLECULAR DOCKING

Identification of binding pattern between proteins and small molecules face substantial challenges. Majority of the docking programs are sensitive enough to predict known protein bound poses with averaged accuracies close to about 1.5–2 Å and with reported success rates in the range of 70 – 80%. However, significant improvement beyond this range seems for now unachievable. One of the fundamental challenges are scoring in which various assumptions are used in order to make calculations computationally more efficient but by using these assumptions, some very important aspects of molecular recognition like solvent effect and entropy effect got neglected. Along with this the incorporation of water molecules in the active site having interaction with ligand and receptor is also a challenge. Molecular docking process also facing limitations on the availability of the crystallographic structure of the protein. Besides all these limitations and challenges molecular docking is still a method of choice and widelly used for the prediction of ligand-receptor pose.

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Figure 11. The poses of SBR in the active sites of carbonic anhydrase (CA) I, II, IV, VI, IX, XII, and XIII predicted by program AutoDock Vina, named as p1, p2,p3, p4, p5, p6, and p7, respectively. The hydrophobic half of the active site cleft is shown in yellow, while Zn(II) in blue and Mg (II) in red. (A) The superposition of SBR (in magenta) and p2 (in cyan). (B) The seed structure derived from p1-7 with the hydrogen attached to sulfur as the growing site.
Source: (Xiang et al., 2014).



9. CONCLUSION

Herein we have provided a complete overview of diverse docking methodologies that are used in drug design and development. Our discussion typically aimed at professionals in the field of cheminformatics and bioinformatics, both in academia and in industry and aspiring scientists and students who want to take up this as a profession in the near future. We discussed several case studies, pertaining to each method. In few cases we provided step wise information to conduct the experiments. This discussion highlighted various application of molecular docking in drug discovery. We apprehend that in the years to come molecular docking in conjunction with variety of state of the art techniques *viz.* molecular biology, computer graphics and protein x-ray crystallography will be an indispensable tool in drug discovery.

List of Abbreviations

ADMET: Absorption, Distribution, Metabolism, Elimination, and Toxicity
ADRs: Adverse Drug Reactions
CADD: Computer Aided Drug Designing
CML: Chronic Myeloid Leukemia

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FF: Force Field
HTS: High Throughput Screening
LBDD: Ligand Based Drug Designing
MOE: Molecular Operating Environment
NMR: Nuclear Magnetic Resonance Spectroscopy
SBDD: Structure Based Drug Designing
SWOT: Strength-Weakness-Opportunity-Threat
QSAR: Quantitative Structure Activity Relationship
VDW: Van Der Waals
VHTS: Virtual High Throughput Screening
VS: Virtual Screening

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Chapter 27
Molecular-Docking-Based Anti-Allergic Drug Design

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ABSTRACT

Allergens are foreign proteins that when come in contact of part(s) of human body stimulate the production of immunoglobulin types of proteins (antibodies). These allergens react with antibodies (immunoglobulin type E or IgE) produces allergic reactions, also known as immediate-type hypersensitivity reactions. As much as 20% of the general population may be affected by grass pollen as a major cause of allergic disease. EXPB class of proteins are known in the immunological literature as group-1 grass pollen allergens Molecular docking method can be used to identify the predicated the interaction of pollen allergen EXPB1 (Zea m 1), a beta-expansin and group-1 pollen allergen from maize with IgE molecules of human. The World Health Organization recognised allergen immunotherapy, as therapeutics for allergic diseases. RNA Interference (RNAi) is a biological process in which RNA molecules e.g. Small Interfering RNAs (siRNAs) inhibit gene expression, by cleavage and destruction of specific mRNA molecules. Use of Small Interfering RNA (siRNA) is a novel method in the induction of RNA Interference (RNAi), which is a potent method for therapeutics of allergic reactions. Due to various effects of STAT 6 proteins during hypersensitivity reactions caused by pollen allergens, mRNA of STAT6 gene is selected as target gene for allergy therapeutics via Post-Transcriptional Gene Silencing (PTGS). Using molecular docking study a specific sense siRNA is identified as anti allergic drug to treat allergic asthma during immediate type of hypersensitivity reaction, caused by Zea m 1 pollen allergen.

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INTRODUCTION

Allergens are foreign proteins that when come in contact of part(s) of human body stimulate the production of immunoglobulin types of proteins (antibodies). These allergens react with antibodies (immunoglobulin type E or IgE) produces allergic reactions, also known as immediate-type hypersensitivity reactions (Kay, 2008). As much as 20% of the general population may be affected by grass pollen as a major cause of allergic disease. An evolutionary conserved protein in plant developmental biology, expansins play an important role in cell wall expansion by slippage or rearrangement of matrix polymers during plant cell growth (Basu, 2013). But among the four classes of expansin protein family, EXPB class of proteins are known in the immunological literature as group-1 grass pollen allergens (Anderson, 2003). These EXPBs proteins cause hay fever and seasonal asthma in humans (Ball, 2005). Allergy symptoms are caused by these proteins when these allergens come into contact with the moist surface of the human respiratory tract (Knox, 1996). Molecular docking method can be used to identify the predicated the interaction of pollen allergen EXPB1 (Zea m 1), a beta-expansin and group-1 pollen allergen from maize with IgE molecules of human. Antibodies, expressed on the surface of B –cell of human immune system, recognize antigenic determinants, also called epitopes on their antigen. The interacting part of the antibody involved in the antigen-antibody interaction, is called the paratope (Stave, 2013). Paratope is formed in combination of different amino acids in the complementarity Determining Regions (CDRs) of antibody or immunoglobulin (Collis, 2003).

At present drugs such as antihistamines, leukotriene receptor antagonists, and corticosteroids are used for symptomatic treatment, but they do not prevent the allergic response (Natt, 2011). The World Health Organization recognised allergen immunotherapy, as therapeutic vaccines for allergic diseases, also known as desensitisation or hyposensitisation. The use of chemically altered allergens, allergoids, recombinant allergens, and relevant T-cell epitope peptides are some common approaches for immunotherapy.RNA interference (RNAi) is a biological process in which RNA molecules e.g. small interfering RNAs (siRNAs) and microRNAs (miRNAs) inhibit gene expression, by cleavage and destruction of specific mRNA molecules. Small interfering RNAs (siRNAs) are 21-25 nucleotide single-stranded RNAs containing 2 nucleotide 3′ overhangs produced by cleavage of longer double-stranded RNAs by the enzyme Dicer. After cleavage by Dicer the 21-25 nucleotide double-stranded product is loaded into an Argonuate protein (humans contain 4 Argonautes) and rendered single-stranded.

Small interfering RNAs (siRNAs) can regulate eukaryotic gene expression when transcribed endogenously or preformed, synthetic siRNA introduced into cells. Among two strands of siRNA, the guide strand pairs with a complementary sequence in a messenger RNA molecule and induces cleavage of mRNA molecule with the help of catalytic component of the RNA-induced silencing complex (RISC) complex. The siRNA component guides RISC to mRNA molecules containing a homologous antisense sequence, resulting in cleavage and degradation of that mRNA. RISC is composed of dicer protein, the double-stranded RNA binding protein TAR RNA Binding Protein (TRBP), and Argonaute2 (AGO2) and does not required ATP for its activity. Dicer protein converts long double-stranded RNAs into siRNAs by the endonuclease activity and Argonaute protein performs the cleavage of the mRNA. At first the process of RNA interference is initiated through Dicer protein by converting double-stranded RNA into small interfering RNA (siRNA). Then the siRNA guide strand after binding with the Argonaute protein in RISC recognizes its complementary sequence in mRNA and targets mRNA for its cleavage.

RNA interference or RNA silencing occurs due to endonucleolytic cleavage of the mRNA specifically by AGO2 protein, when RISC complex binds with a perfectly complementary mRNA (Kandeel, 2013).

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The 'minimal RISC' complex composed of AGO2 bound to a short interfering RNA (siRNA). Among two domains of AGO2, The PIWI domain may perform RNA cleavage by a mechanism similar to that of RNase H and the PAZ domain is an RNA-binding module (Song, 2003). It has been already established that PAZ domain of AGO2 protein in RISC anchors the 2-nucleotide 3'-overhang of the siRNA duplex causing two types of effects (Lingel, 2004). The first effect is the binding the 7-nucleotide phosphodiester backbone of the overhang-containing strand and the second one is the capping the 5'-terminal residue of the complementary strand in a sequence-independent manner. In RISC, PAZ acts as an anchoring site for the 3'-end of guide RNA within silencing effector complexes. Similarly the PAZ domain of AGO2 serves as a siRNA-end-binding module for siRNA transfer in the RNA interference method.

Use of small interfering RNA (siRNA) is a novel method in the induction of RNA interference (RNAi), which is a potent method for therapeutics of allergic reactions (Bantounas, 2004). Effect on human immune system can be modulated by siRNAs in immunotherapy of pollen allergen Zea m1. Structures of Zea m1 allergen (PDB ID 2HCZ), human immunoglobulin E (PDB ID 4J4P) and human Argonaute2 protein (PDB ID 4OLA) have been extracted from RCSB PDB web server. Protein- protein molecular docking between pollen allergen Zea m 1 and human immunoglobulin E was done using PatchDock protein- protein docking server. In our present work we propose a strategy for vaccine preparation for Plant pollen allergen Zea m 1 by RNA Interference by using In Silico approaches such as molecular docking and designing siRNA. Analyzing the docking results for pollen allergen Zea m1 with human immunoglobulin E protein, we can identify the paratopes present in that specific antibody, which is essential for preparation of vaccine for that pollen allergen. Findings from the molecular docking study of AGO2 protein with siRNAs would help us to guide the future design of modified siRNA analogues as anti allergic drug in immunotherapy of pollen allergen Zea m 1.

BACKGROUND

Zea m 1 as Pollen Allergen

Allergens from various sources (e.g. pollen, house dust mites, pets, moulds, food and insects) and are mainly proteins or glycoproteins (Valenta, 2008). When allergen comes in contact to human body the production of allergen-specific IgE antibodies increases and that bind to their receptors on immune cells. That binding causes allergic inflammatory response. Cross-linking of IgE antibodies that are bound to the high-affinity receptors for IgE (i.e. FcεRI) on mast cells and basophils causes degranulation within a few minutes.The release of inflammatory mediators, proteases and pro-inflammatory cytokines after degranulation is known as immediate hypersensitivity reaction and that is the cause for the majority of allergic symptoms.

Inhaled pollen allergens encounter antigen presenting cells (APC) that are present in the lining of respiratory tract. After recognition as antigen allergenic proteins are presented by APC, leads to cell differentiation of naive T cells into TH2 cells. Activated TH2 stimulate the formation of IgE by B cells. IgE molecules bind to IgE receptors located on mast cells. The cross linking of mast-cell-bound IgE by allergens leads to the release of biologically active mediators (histamine, leukotrienes) by means of degranulation and, so, to the immediate symptoms of allergy as shown in Figure 1. Mast cells also release chemotactic factors that contribute to the recruitment of inflammatory cells, particularly eosinophils, whose proliferation and differentiation from bone marrow progenitors is promoted by IL-5.There are

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Figure 1. Immediate-type hypersensitivity reactions. A. Recognition of specific epitope of allergen by paratope of Ig E on B cell, B. Complementary binding of epitope and paratope between allergen and Ig E, C. Histamine release after Ig E binding with the receptor on mast cell, causes allergic symptoms.

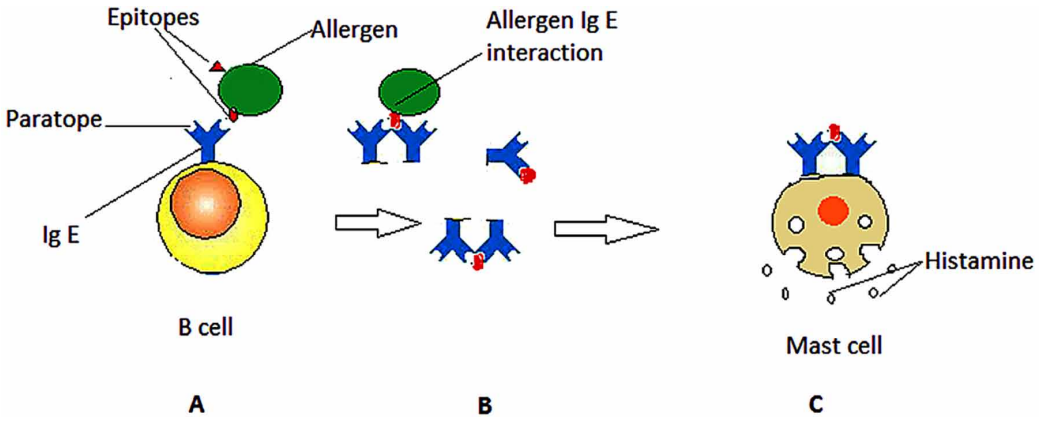
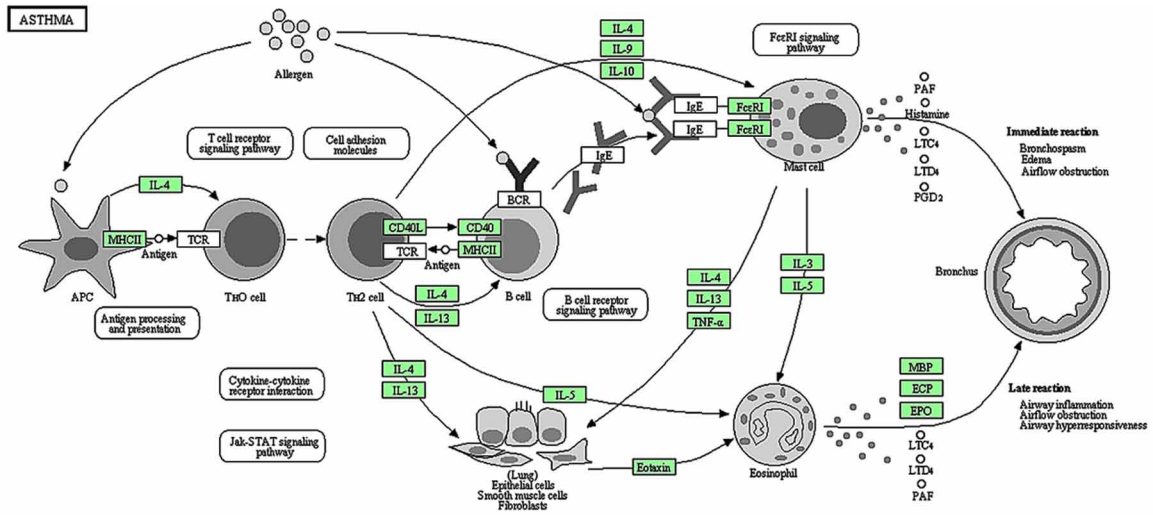


Figure 2. KEGG pathway for allergic response due to allergen in human
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several other protein factors are involved in airflow obstruction, bronchial hyperresponsiveness, and airway inflammation.These are some important symptoms of immediate type of hypersensitivity reaction, caused by pollen allergens.

Molecular docking study with Zea m1 allergen (PDB ID 2HCZ) and human immunoglobulin E (PDB ID 4J4P) using PatchDock web server (Duhovny 2002; Schneidman-Duhovny, 2005) with clustering RMSD 4.0 and score 10504 is shown in Figure 3. PatchDock is an algorithm for molecular docking. The algorithm has three major stages:

- Molecular Shape Representation

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Figure 3. Molecular docking structure of Zea m1 with human IgE



- Surface Patch Matching
- Filtering and Scoring

After refinement of docking results with FireDock web server (Andrusier, 2007; Mashiach, 2008) the following figure is obtained as shown in figure 4. The FireDock web server solves the refinement problem of protein-protein docking solutions. The method simultaneously targets the problem of flexibility and scoring of solutions produced by fast rigid-body docking algorithms. Here the candidate solutions for FireDock are generated by rigid-body docking method, such as PatchDock as shown in table 2. Each result from PatchDock is subsequently refined by restricted interface side-chain rearrangement and by soft rigid-body optimization. The side-chain flexibility is modelled by rotamers and the obtained combinatorial optimization problem is solved by integer linear programming. Following rearrangement of the side-chains, the relative position of the docking partners is refined by Monte Carlo minimization of the binding score function. The refined candidates are ranked by the binding score. The binding free energy is the change in the free energy of the system, which occurs upon complex formation, which combines the free energy of the receptor-ligand complex and the free energies of the uncomplexed receptor and ligand, respectively. The desolvation free energy is estimated by the ACE potential. The electrostatic contribution is approximately calculated by the atomic pair-wise partial Coulomb energy. The torsion energy, contributions of the hydrogen bonds, disulfide bonds, van der Waals interactions and Π-Stacking and aliphatic interactions are also considered to calculate binding score (Andrusier,2007). Interacting amino acid Thr 12 of Zea m1 with heavy and light chains of human immunoglobulin E are shown in Figure 4. Molecular docking result proves that Zea m 1 allergen binds with human IgE and causes immediate-type hypersensitivity reaction and allergic symptoms.

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Figure 4. Molecular docking result from FireDock

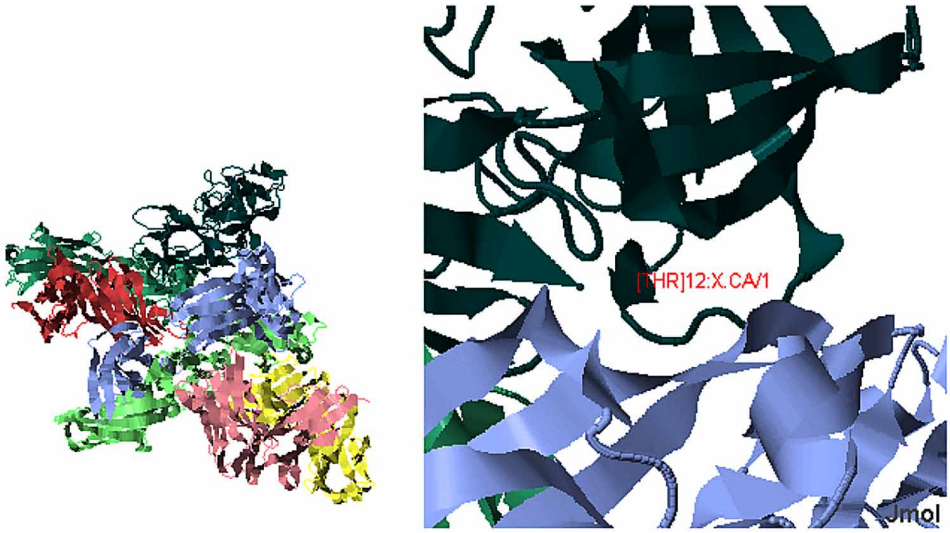


Table 1. Result from FireDock

Rank	Solution Number		Attractive VdW	Repulsive VdW	ACE	HB
1	5	-21.47	-28.30	15.74	10.06	-2.56

Where,

- **Global Energy:** The binding energy of the solution.
- **Attractive and Repulsive VdW:** The contribution of the van der Waals forces to the global binding energy.
- **ACE:** The contribution of the atomic contact energy (ACE) to the global binding energy. The ACE (Atomic Contact Energy) function is a statistical contact potential for all (non-hydrogen) atom pairs less than 6 Å apart,
- **HB:** The contribution of the hydrogen bonds to the global binding energy.

Antiallergic Drug Design for Pollen Allergen Zea m 1

Compounds, Histamine, Prostaglandins and Leukotrienes are involved in allergic reactions in human as shown in Figure 2. Nowadays drugs such as antihistamines, leukotriene receptor antagonists, and corticosteroids are used for symptomatic treatment, but they do not prevent the allergic response. In order to eradicate allergic reaction due to any allergen, allergen specific immunotherapy can be applied. Allergen-specific immunotherapy is based on the repeated lower dose administration of disease-causing allergens to modify the allergen-specific immune response in patients so that higher doses of the allergen can be tolerated during hyper sensitivity reaction. (Valenta, 2012) reviewed several approaches for

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allergen specific immunotherapy using T-cell epitope-containing peptides, Recombinant hypoallergens and chemically modified allergens. In first method allergen-derived synthetic peptides containing T-cell epitopes are used to treat sensitivity reactions. Because these peptides which are synthesized from linear sequences of small allergen fragments, that are epitopes of T cells. These peptides can bind with the receptor of allergen-specific T cells and show no reactivity with IgE antibodies of B cells. The T-cell epitope-containing peptides can be used to reduce the IgE-mediated allergic reactions for that allergen. In second method recombinant hypoallergenic allergens, developed in Escherichia coli, by introducing mutations into the allergen sequence, are used to reduce IgE reactivity. Combination of allergens with DNA sequences containing CpG motifs as immunomodulatory components are used to activate the innate immune system through toll-like receptors (TLRs).This method is applied to inhibit Th2 immune responses and finally later stages of hypersensitivity reaction.

RNA interference (RNAi) technologies (Ball, 2004) are very new approach in the development of allergy therapeutics via post-transcriptional gene silencing (PTGS). From one mRNA molecule about 5,000 copies of that protein can be produced. So, it is a wise decision to target mRNA molecules related to allergic reaction rather than blocking different proteins involved in hypersensitivity reactions. (Popescu, 2005) identified different types of protein molecules as target for potential anti-mRNA drugs in asthma and other allergic disorders. They are shown in Table 2.

Miller et al (1998) suggested that the regulation of class switching to IgE by cytokines is mediated by STAT transcription factors, by analyzing the cytokine signalling in patients with extrinsic asthma and hyper immunoglobulin E. IgE production in asthma and hyper immunoglobulin E usually is associated with elevated levels of IL-4in the peripheral blood. The protein encoded by STAT6 is a member of the STAT family of transcription factors. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein plays a central role in exerting IL4 mediated biological responses. Walford and Doherty (2013) elaborately discussed about the various functions of STAT6 protein, which play crucial roles in the pathogenesis of allergen-induced airway inflammation, mucus production, and airway hyper responsiveness (AHR). In asthma patients different structural cells (airway epithelium, smooth muscle, and fibroblasts) and immune cells

Table 2. Different types of protein molecules as target for potential anti-mRNA drugs in asthma and other allergic disorders

Serial Number	Type of Protein Molecule	Examples
1.	Cell surface receptors	adenosine A1 receptor, high-affinity receptor Fc-ε RI-α, cytokine receptors
2.	Adhesion molecules and ligands	ICAM-1, VLA-4
3.	Ion channels	calcium-dependent chloride channel-1
4.	Cytokines and related factors	IL-4, IL-5, IL-13, SCF, TNF-α, TGF-β1
6.	Intracellular signal transduction molecules	tyrosine-protein kinases (Syk, Lyn, Btk),
7.	Serine/threonine-protein kinases	p38 α MAP kinase, Raf-1
8.	Non-kinase signaling proteins	RasGRP4
9.	Transcription factors involved in Th2 differentiation and allergic inflammation	STAT-6, GATA-3, NF-κB

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(B and T cells, macrophages, dendritic cells, and innate lymphoid cells) are involved in many pathological conditions e.g. Th2 cell differentiation and production of cytokines (IL-4, IL-5, IL-9, and IL-13), IgE production from B cells, airway eosinophilia, epithelial mucus production, change in contractility of smooth muscle. Effects of STAT6 protein on different types of cell are summarized in Figure 5.

Due to various effects of STAT 6 proteins during hypersensitivity reactions caused by pollen allergens, mRNA of STAT6 gene is selected as target gene for allergy therapeutics via post-transcriptional gene silencing (PTGS). For drug designing using siRNA for STAT6 mRNA, following steps are performed:

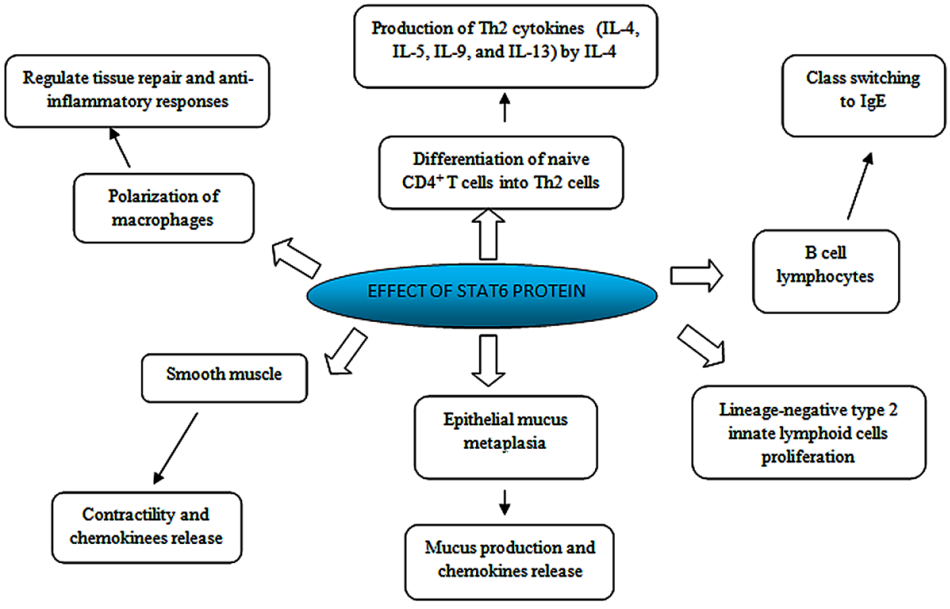
- Search for siRNA for signal transducer and activator of transcription 6, interleukin-4 induced (STAT6) for human.
- Comparative modelling of siRNA 3D structures.
- Secondary structure determination of siRNAs.

Search result for siRNA for signal transducer and activator of transcription 6, interleukin-4 induced (STAT6) for human in NCBI, shows six siRNA sequences as shown in Table 3.

Using ModeRNA (Rother, 2011) a program for comparative modelling of RNA 3D structures, three dimensional structure for all six siRNAs can be elucidated. But this program requires a pairwise sequence alignment and a structural template to generate a 3D structural model of the target RNA sequence. But template finding results for those six siRNAs show that only fifth and sixth sequences have structural templates with PDB Ids (shown in Table 4) and their models were built using ModeRNA server (Rother, 2011).

Secondary structure determination of Probe|16741853| sense and antisense siRNAs was carried out using RNAstructure, Version 5.7 (Mathews, 2014) which is a complete package for RNA secondary structure prediction and analysis including facility to predict base pairing probabilities. Results from

Figure 5. Role of STAT6 protein on various types of cells tissues during allergic reaction



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Table 3. siRNA sequences for STAT6 genes in human from NCBI

NAME OF siRNA PROBES	SEQUENCE OF siRNA	POSITION OF siRNA FEATURE OF SEQUENCE
>Probe|16741855|SIRNA_SENSE siRNA sense sequence (21b)	AGACCUGUCCAUUCGCUCATT	1..19 sense sequence [rna] 20..21 sirna overhang [dna]
>Probe|16741855|SIRNA_ANTISENSE siRNA antisense sequence (21b)	UGAGCGAAUGGACAGGUCUTT	1..19 antisense sequence [rna] 20..21 sirna overhang [dna]
>Probe|16741854|SIRNA_SENSE siRNA sense sequence (21b)	GGUGCCUUCUUAUGACCUUTT	1..19 sense sequence [rna] 20..21 sirna overhang [dna]
>Probe|16741854|SIRNA_ANTISENSE siRNA antisense sequence (21b)	AAGGUCAUAAGAAGGCACCAT	1..19 antisense sequence [rna] 20..21 sirna overhang [dna]
>Probe|16741853|SIRNA_SENSE siRNA sense sequence (21b)	AGAACCUGCUUCUCAAGAATT	1..19 sense sequence [rna] 20..21 sirna overhang [dna]
>Probe|16741853|SIRNA_ANTISENSE siRNA antisense sequence (21b)	UUCUUGAGAAGCAGGUUCUTG	1..19 antisense sequence [rna] 20..21 sirna overhang [dna]

Table 4. Template finding results for Probe|16741853| sense and antisense siRNAs respectively

Rfam Family	PDB ID	Alignment	Identity	Similarity
RF00005	1UOB_A	>Target - - - - - - - - - - A - - - - - - - - - G – A - - - - - - - - - - - - - - - - - A C - - - - - - CU GCUUC - - - UCAA - - - - GAA - - - - - - - - - - - - - > 1 U0B A GGCGCGUUAACAAAGCGGUUAUGUAGCGGAUUGCAAAUC CGUCUAGUCCGGUUCGACUCCGGAACGCGCCUCCA	0.162	0.189
RF00005	2DXI_D	>Target - - - - - - - - - - UUCUUG - - - - - - - -AGAAG - - - - - - - - - - -C- - - - - - - - AGG- - - - - - - -UUC- - -U- - - - - - - - - - - - - - - - - - - >2DXI D GGCCCCAUCGUCUAGCGGUUAGGACGCGGCCCUCUCAA GGCCGAAACGGGGGUUCGAUUCCCCCUGGGGUCACCA	0.16	0.18

RNAstructure for Probe|16741853|SIRNA_SENSE siRNA sense sequence (without overhang) and Probe|16741853|SIRNA_ANTISENSE siRNA antisense sequence (without overhang) shown in Figure 6. Predicted 3D secondary structures of two siRNAs can be visualized as shown in Figure 7 by using UCSF Chimera software (Petersen, 2004). UCSF Chimera is a highly extensible program for interactive visualization and analysis of molecular structures and related data e.g. molecular docking results.

Since the 'minimal RISC' complex composed of AGO2 bound to a short interfering RNA (siRNA), a docking server for nucleic acid and protein was identified. NPDock (Nucleic acid-Protein Dock) is a web server (Tuszyńska, 2015) for modelling of RNA-protein complex structures. It combines (1) GRAMM for global macromolecular docking, (2) scoring with a statistical potential, (3) clustering of best-scored structures, and (4) local refinement. PDB files for sense and antisense siRNA obtained from RNAstructure (Mathews, 2014) and AGO2 (PDB ID 4OLA) were used as input files. Using NPDock web server (Tuszyńska, 2015) 'minimal RISC' structures for sense and antisense siRNAs with AGO2 proteins are built and visualized by UCSF Chimera software (Petersen, 2004) as shown in Figure 8 where sense and antisense siRNA were shown in green colour. The distance and orientation-dependent knowledge-based potentials (DARS-RNP and QUASI-RNP) were generated for both sense and antisense RNAs when bound with AGO2 protein using reverse Boltzmann statistics.

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Figure 6. Predicted secondary structures of two siRNAs

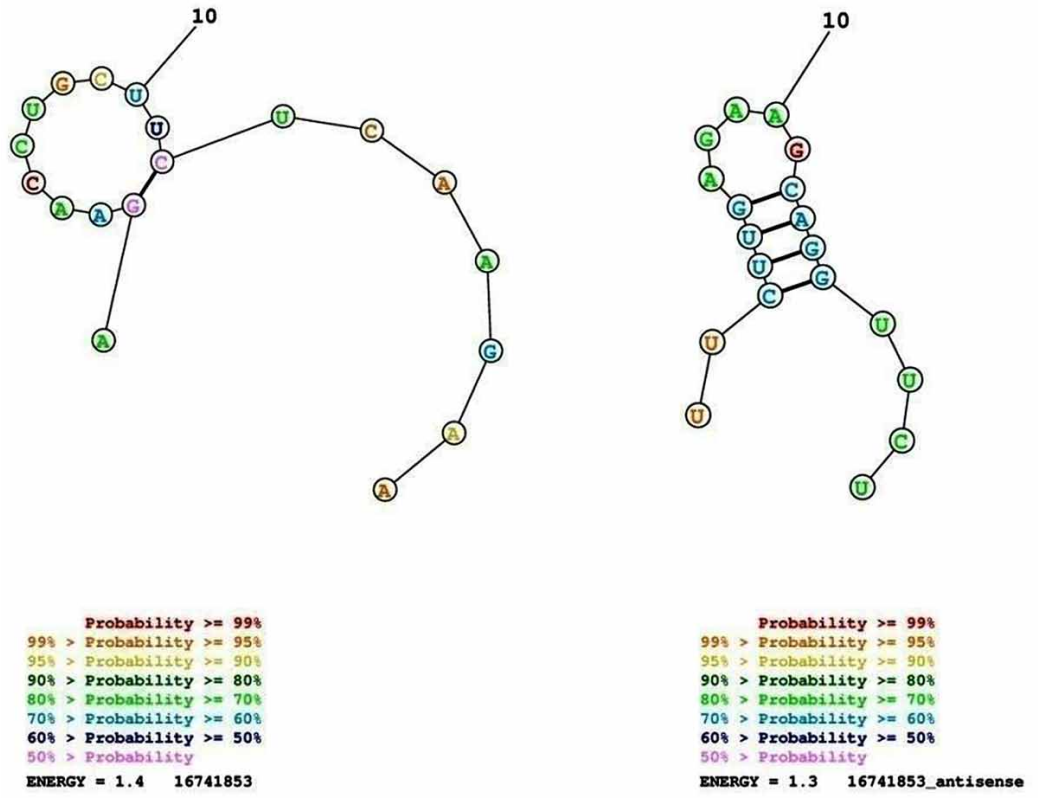
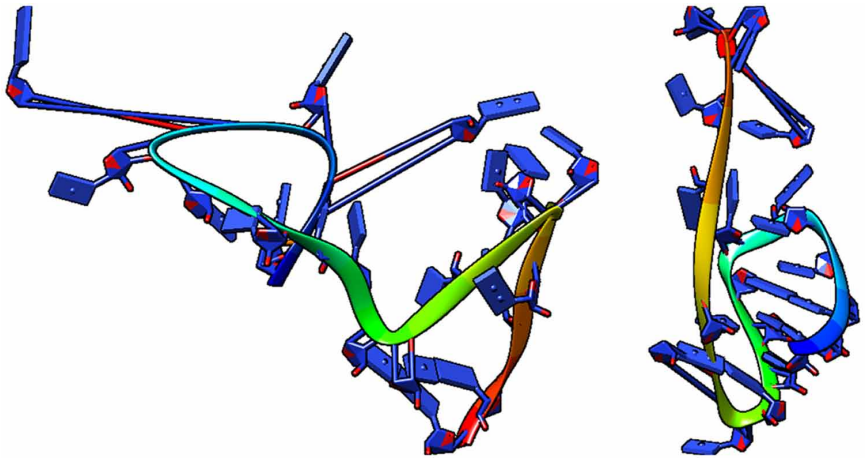
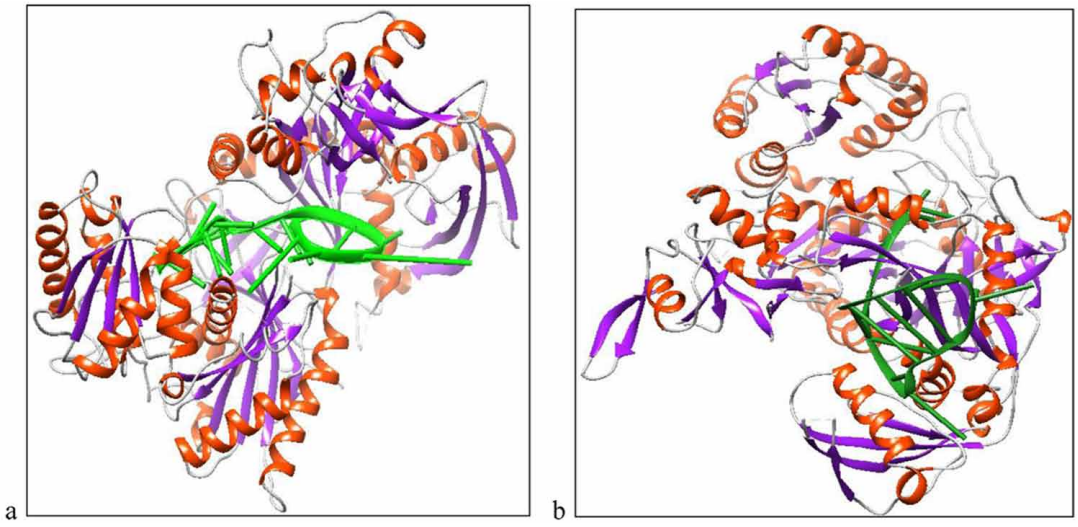


Figure 7. 3D structures of sense and antisense siRNAs



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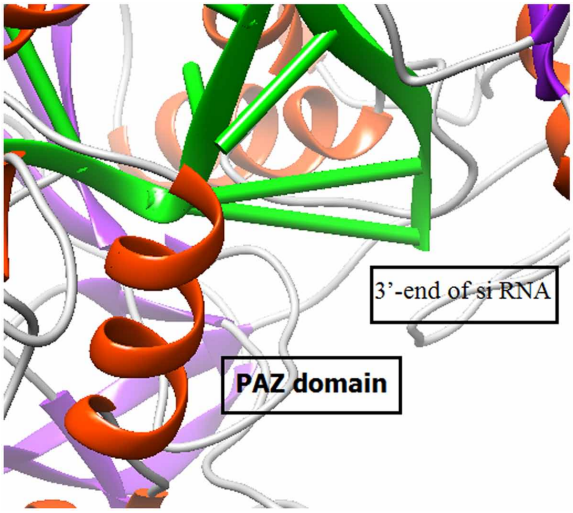
Figure 8. Binding structures of siRNAs with AGO2. a. For sense strand b. For antisense strand.



From the best scored docking results, after visualizing with UCSF chimera software (Petersen, 2004), it can be concluded that both sense and antisense siRNA strands bind with PIWI domain of AGO2. PIWI domain is present in AGO2 protein from 517-818 amino acids sequence. Since binding energy score was more negative for sense siRNA, so it has been selected among two binding structures for sense and antisense siRNAs with AGO2 protein.

In RISC, PAZ acts as an anchoring site for the 3'-end of guide RNA within silencing effecter complexes. For sense siRNA the 'minimal RISC' complex composed of AGO2 bound to a siRNA as shown in Figure 9 where PAZ domain of AGO2 protein shown in red and sense siRNA in green.

Figure 9. Interaction of siRNA with PAZ domain of ARO2 protein



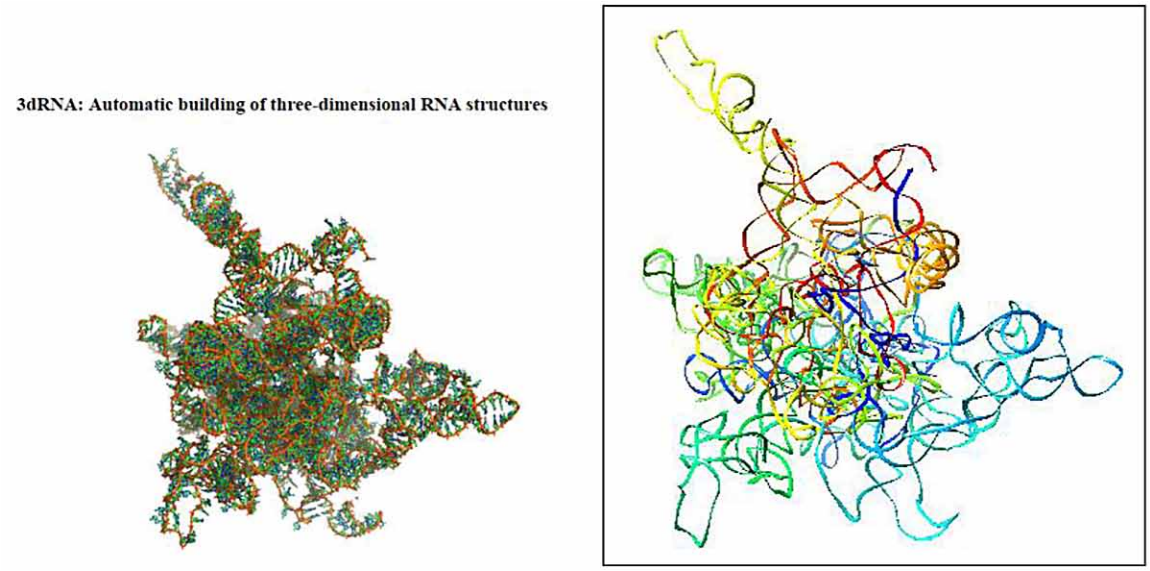
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STAT6 Gene as Target Gene for Allergy Therapeutics

After retrieving gene sequence from NCBI for Homo sapiens STAT6 mRNA for signal transducer and activator of transcription 6 nirs variant 2, complete cds (GenBank Accession number AB103089.1) containing 2022 bp, mRNA sequence was obtained from Transcription and Translation Tool (www.attotron.com/cybertory/analysis/trans.htm). From RNAfold web server (Gruber, 2008), which can predict secondary structures of single stranded RNA sequences, Minimum free energy (MFE) secondary structure of that mRNA molecule was built. The MFE structure of an RNA sequence is the secondary structure that contributes a minimum of free energy (Zuker, 1981). The optimal secondary structure of STAT6 mRNA for human was modelled with minimum free energy -1584.20 kcal.mol using RNAfold web server (Gruber, 2008). For molecular docking study required three dimensional structure of the same molecule was formed from 3dRNA web server (http://biophy.hust.edu.cn/3dRNA/3dRNA.html) as shown in Figure 10 and visualized with UCSF chimera software (Petersen, 2004).

Using web server LigandRNA (Philips, 2013) molecular docking was performed with 'minimal RISC ' complex (formed between AGO2 protein and sense siRNA, shown in Figure 4a) and mRNA of STAT6 transcript2 gene (shown in Figure 10). Since this mRNA is 2022 nt long, its 3' end was selected (red coloured molecule in Figure 11 A). The molecular docking study results in following conformer with LigandRNA score -61.34, shown in Figure 11 B. In this figure mRNA molecule shown in black, different domains of AGO2 protein in red, blue, cyan, yellow colour and sense siRNA bound with AGO2 protein in dark blue colour. From the Figure 11 B it is clear that minimal RISC complex (AGO2 and sense siRNA) interacts with STAT6 mRNA through PIWI domain of AGO2 protein (red in colour) and may perform RNA cleavage by a mechanism similar to that of RNase H. Similarly in RISC, PAZ acts as an anchoring site for the 3'-end of guide RNA within silencing effecter complexes. Similarly the PAZ domain of AGO2 serves as a siRNA-end-binding module for siRNA transfer in the RNA interference method. RNA interference or RNA silencing occurs due to endonucleolytic cleavage of the mRNA specifically

Figure 10. 3D structure of mRNA of STAT6 transcript2 gene



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by AGO2 protein, when RISC complex binds with a perfectly complementary mRNA. Finally it can be concluded that the sense siRNA can act as antiallergic drug to treat allergic asthma during immediate type of hypersensitivity reaction, caused by Zea m 1 pollen allergen.

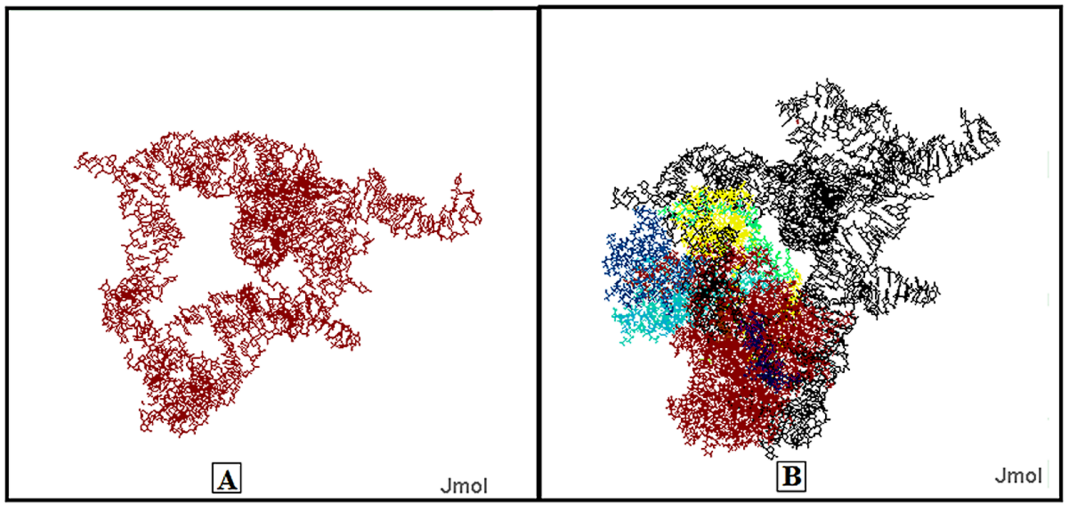
CONCLUSION

Molecular docking is an important technique in computational biology. By using this technique we first of all identified Zea m 1 as allergen after analyzing the binding mode of Zea m1 allergen (PDB ID 2HCZ) and human immunoglobulin E (PDB ID 4J4P). Resulting immediate-type hypersensitivity reaction and allergic symptoms can be treated with the concept of RNA interference (RNAi) technologies. In this work Homo sapiens STAT6 mRNA was selected as target gene for allergy therapeutics. Drug designing for allergy was done by selecting, modelling and binding of sense siRNA with STAT6 mRNA in presence of AGO2 protein. Molecular docking study reveals that in RISC, PAZ acts as an anchoring site for the 3'-end of guide RNA within silencing effecter complexes and RNA silencing occurs due to endonucleolytic cleavage of the STAT6 mRNA specifically by AGO2 protein. Thus crucial roles of STAT6 proteins in the pathogenesis of allergen-induced airway inflammation, mucus production, and airway hyper responsiveness (AHR) can be avoided by using siRNA of STAT6 mRNA.

FUTURE RESEARCH DIRECTIONS

For future work plan we propose the study on 'off target binding' of that specific sense siRNA, which is essential for rational therapeutic use of this siRNA in allergic reaction.

Figure 11. RISC complex



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KEY TERMS AND DEFINITIONS

Argonaute Proteins: Argonaute proteins (AGO) play a central role in RNA silencing processes, as essential catalytic components of the RNA-induced silencing complex (RISC). Argonaute proteins bind different classes of small non-coding RNAs, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs). Small RNAs guide Argonaute proteins to their specific targets through sequence complementarity (base pairing), which then leads to mRNA cleavage or translation inhibition.

RISC: The RNA-induced silencing complex, or RISC, is a multiprotein complex, specifically a ribonucleoprotein. RISC complex is responsible for the gene silencing phenomenon known as RNA interference (RNAi), which incorporates one strand of a double-stranded RNA (dsRNA) fragment, such as small interfering RNA (siRNA) or microRNA (miRNA).

Small Interfering RNA (siRNA): siRNA is a class of double-stranded RNA molecules, 20-25 base pairs in length. siRNA interferes with the expression of specific genes with complementary nucleotide sequences in RNAi or RNA interference technique. siRNA functions by cutting mRNA into pieces after transcription, resulting in no translation.

STAT Protein: Signal Transducers and Activators of Transcription (STAT) family proteins contains transcription factors that are specifically activated to regulate gene transcription when cells encounter cytokines and growth factors; hence they act as signal transducers in the cytoplasm and transcription activators in the nucleus. STAT proteins play a critical role in regulating innate and acquired host immune responses.

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Chapter 28
Molecular Docking Technique to Understand Enzyme-Ligand Interactions

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ABSTRACT

Molecular docking has advanced to such an extent that one can rapidly and accurately identify pharmaceutically useful lead compounds. It is being used routinely to understand molecular interactions between enzyme and ligand molecules. Several computational approaches are combined with experimental work to investigate molecular mechanisms in detail at the atomic level. Molecular docking method is also useful to investigate proper orientation and interactions between receptor and ligand. In this chapter we have discussed protein-protein approach to understand interactions between enzyme and amyloid beta (Aβ) peptide. The Aβ peptide is a causative agent of Alzheimer's disease. The Aβ peptides can be cleaved specifically by several enzymes. Their interactions with Aβ peptide and specific enzyme can be investigated using molecular docking. Thus, the molecular information obtained from docking studies might be useful to design new therapeutic approaches in treatment of Alzheimer's as well as several other diseases.

BACKGROUND

Large numbers of three-dimensional structures of biomolecules are being uploaded in the various structural databases. These 3-D structures along with molecular models may be used in the docking process to understand proper interactions between receptor and ligand molecules. Molecular docking method is useful to investigate proper orientation and interactions between protein-protein, protein-ligand and DNA-protein molecules. In this chapter we have discussed protein-protein approach to understand interactions between human cathepsin B and *Aβ* peptide a causative agent of Alzheimer's disease with the help of AutoDock. Docking methodology and procedure has been discussed with proper figures.

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INTRODUCTION

Molecular docking has become a useful technique in structure-based drug design (Sousa, 2006). We can predict the preferred orientation of receptor and ligand bound to each other to form a stable complex (Lengauer & Rarey, 2006). The molecular docking is a multistep process which starts with the representation followed by docking algorithms to identify best matched conformation to receptor structure. The docking algorithms complemented by scoring functions which is used for evaluation of interactions between ligand and receptor based on shape and electrostatic complementarities (Kitchen, et al., 2004). The two main features of molecular docking technique are conformation search algorithm and scoring function estimates the binding affinity between molecules. There are three types of docking such as protein-protein (Sonawane & Barage, 2004; Tseng, et al., 2007; Barage, et al., 2014), protein-ligand (Jalkute, et al., 2014; Jalkute, et al., 2015) and DNA-protein (Parulekar, et al., 2013) which may apply depending upon the receptors and ligands used in the docking experiments. There are two types of docking approaches used such as rigid docking and flexible docking. In rigid docking approach both the protein and ligand are considered as rigid bodies whereas in the flexible-ligand docking, receptor and ligand kept flexible. Most of the molecular docking programs treat protein as a rigid molecule and ligand as a flexible molecule. For molecular docking studies several docking programs such as AutoDock (Morris, et al., 2009), HEX (Macindoe, et al., 2010), PatchDock (Duhovny, et al., 2002; Schneidman-Duhovny, et al., 2005), Ludi (Böhm, et al., 1992), Glide (Halgren, et al., 2004), etc. are being used with appropriate docking algorithms and scoring functions.

In this chapter to understand the protein-protein docking we have taken an example of docking between cathepsin B from human with *Aβ* peptide (Musil, et al., 1991; Crescenzi, et al., 2002; Dhanavade, et al., 2013; Dhanavade, et al., 2014). Amyloid plaque formation is the pathological hallmark in the Alzheimer's disease (Hardy & Selkoe, 2002; Glenner & Wong, 1984). The Aβ peptides (1–42) are the main constituent of amyloid plaques in Alzheimer's disease (Gouras, et al., 1998; Masters et al., 1985; Tanzi, et al., 2005). There are various enzymes such as IDE, NEP, ECE, ACE, MMP-9, and plasmin are known to degrade Aβ peptides (Iwata, et al., 2001; Qui, et al., 1998; Tucker, et al., 2000; Yin, et al., 2006; Zou, et al., 2007; Cimerman, et al., 1999). Similarly, certain bacterial enzymes like aminopeptidase from *Streptomyces grieus* KK565 (SGAK), Nattokinase from *Bacillus subtilis* Natto, and Angiotensin Converting Enzyme (ACE) from *Stigmatella aurantiaca* has potential to degrade Aβ peptide (Jalkute, et al., 2015; Yoo, et al., 2010; Hsu, et al., 2009). The Aβ peptide degradation is well studied experimentally but their cleavage mechanism is yet to be understood properly. Hence, in such situation molecular docking studies between respective enzyme and ligand plays an important role to understand molecular mechanism in detail at the molecular level. The detailed procedure of docking is explained and then the results are analyzed to understand the degradation of Aβ peptide by cathepsin B from human. Finally the importance of molecular docking studies is discussed.

MATERIALS

Downloading AutoDock and AutoGrid

Download freely available version of AutoDock and AutoGrid software (Morris, et al., 2009) for Windows operating system from the AutoDock webpage (http://autodock.scripps.edu/downloads/autodock-

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registration/autodock-4-2-download-page/). Install the AutoDock and AutoGrid as per the installation instructions.

Downloading the AutoDock Tools (Version 1.5.4, Version 3)

The AutoDock Tools (ADT) (mgltools_win32_1.5.6) is a tool required for the preparation and visualization of molecules along with AutoDock and AutoGrid. This mgltools_win32_1.5.6 has been made for Windows which can be downloaded from link: http://mgltools.scripps.edu/downloads. After downloading it can be installed as per the installation instructions (Morris, et al., 2009).

Other useful software for molecular docking studies:

- **MODELLER 9v7:** This software is developed by Sali and Blundell in 1993 (Sali & Blundell, 1993) and being used routinely to predict three-dimensional structures of proteins using homology modeling technique.
- **AutoDock:** This is developed by Morris and coworkers in 2009 (Morris, et al., 2009) and being used in the docking studies.
- **RasMol:** This is useful to visualize and analyze the 3D structures. This software is developed by Roger Sayle and coworkers in the year 1995 (Sayle, & Milner-White, 1995).
- **Chimera:** The Chimera is developed by the Pettersen and coworkers in the year 2004 (Pettersen, et al., 2004) and is useful for the structure visualization, analysis of models as well as to prepare attractive images of docked complex.

The above discussed software's are freely available and may be used for the basic study of molecular docking but besides this various online servers are also available for homology modeling process.

METHODS

- **Homology Modeling:** In order to start molecular docking process, three dimensional structures of receptor and ligand must be downloaded from RCSB and PUBCHEM respectively. If the 3D structure of receptor is not available then it has to be modeled by using MODELLER 9V software. In this current chapter we have considered human Cathepsin B (Musil, et al., 1991) as a receptor. Its 3-D structure (2IPP.pdb) has been directly extracted from RCSB Protein Data Bank (Musil, et al., 1991). For protein-protein docking, the amyloid beta protein, 1IYT.pdb (Macindoe, et al., 2010) has been taken as a ligand and retrieved from RCSB. If ligand is a chemical compound then its structure may be extracted from NCBI PUBCHEM.
- **Virtual Screening:** If receptor is an enzyme and its 3-D structure data available in Protein Data Bank (PDB) then one can use structural information of active site residues of receptor to search suitable drug molecules from ZINC database (Irwin, et al., 2005) which can bind effectively to the receptor. Dockblaster program has a flexible-ligand sampling algorithm which superimposes atoms of the docked molecule as per the supplied binding site which represent positions to bind ligand atoms (Irwin, et al., 2009). Basically the selection of ligand was performed by bin size; distance tolerance up to 2.0 Å, each ligand configuration was scored for electrostatic, van der Waals

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interaction complementarity and corrected for ligand desolvation. The screening of ligand is done in ZINC database of commercially available molecules, which contains 3 million compounds available ready to dock (Irwin, et al., 2005).

Molecular Docking

Molecular docking study mainly aims to predict the intramolecular interactions formed between two or more molecules. Molecular docking has been described as a useful tool in structure-based drug design and discovery (Sousa, et al., 2006). Earlier, significant amount of work has been made in the molecular modeling and drug discovery (Blaney & Dixon, 1993; Jones & Willet, 1995; Lybrand, 1995; Rosenfeld, et al., 1995; Gschwend, et al., 1996; Lengauer & Rarey, 1996). Molecular docking is a method to predict preferred orientation of receptor and ligand interacting to each other to form a stable complex (Lengauer & Rarey, 1996). Docking method considers only translational and orientational degrees of freedom of the ligand with respect to receptor (Kuntz, et al., 1982). However, the molecule's geometry may change upon binding into the active site of an enzyme, so now most of the method considers ligand as a flexible molecule. Proteins are not rigid structures therefore ideally it should be flexible during the docking simulations. However, introducing flexibility in the macromolecular proteins during docking procedure is quite difficult because of the requirement of the huge computational power for such processes. Hence, docking studies are performed using a flexible ligand and a rigid receptor protein.

Docking is multistep process which starts with molecular representation followed by docking algorithms to identify best matched conformation of receptor and ligand complex. The docking algorithms based on shape and electrostatic complementarities with scoring functions are used to evaluate the interactions between receptor and ligand (Kitchen, et al., 2004). The AutoDock (Morris, et al., 2009) was used to predict protein-protein and protein-ligand interactions with an appropriate docking algorithms and scoring functions. The search algorithms are classified into three types such as systematic, stochastic and deterministic search algorithms (Brooijmans & Kuntz, 2003). The grid value for all degrees of freedom in molecule can be explored by systematic algorithms in a combinatorial way. The incremental construction and conformational search are carried out by some systematic search algorithms (Halperin, et al., 2002). The random changes to single ligand or to the population of ligand can be done by stochastic search algorithm operations. The algorithms such as Monte Carlo methods, genetic algorithms and tabu search are included in stochastic algorithms. The deterministic search algorithms started with known initial state up to the state of lesser energy at which the termination criteria is met (Halperin, et al., 2002). Monte Carlo methods, Genetic algorithms and Lamarckian genetic algorithms have been adopted in AutoDock, a flexible docking program (Morris, et al., 2009).

Molecular Docking Procedure

The molecular docking methodology can be divided into three steps: file preparation, docking, and analysis. Before preparing the receptor, the bound ligand and water molecules must be deleted from the crystal structure. Firstly, the addition of polar hydrogen's, computing the partial charges, and assigning of AutoDock atom types has to be performed. After that two files such as rigid.pdbqt and flexible.pdbqt are generated from the receptor file. The flexible.pdbqt file is generated by keeping the active site residues of receptor as flexible residues while remaining residues are taken as a rigid in rigid.pdbqt file. Then the parameter file must be prepared to run the AutoGrid. The docking grid should be specified to

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constrain the search area for the docking. The grid parameter file (gpf) will be generated to show the macromolecule location, size and atoms types to compute grid map. While running AutoGrid, the parameters will be taken from the gpf file and generated grid map will be used further in AutoDock. AutoDock will generate a docking parameter file (dpf) for the ligand and then modified with the parameters for Lamarckian genetic algorithm. When all the files such as rigid.pdbqt, flexible.pdbqt and ligand.pdbqt which are needed for the docking process are generated, then one can start actual docking process. The time required for docking depends on various factors like size and number of rotatable bonds in the ligand, the docking grid size, the genetic algorithm parameters, and the number of runs. After the completion of docking process, AutoDock will give output in docking log file (dlg) which contains docked structures and binding free energies. The pose of the ligand, structural coordinates, cluster size and binding free energies can be obtained after analyzing the dlg file. The docked complex can be further converted into pdb file format and analyzed by different 3D structure visualizing software such as RasMol (Sayle & Milner-White, 1995) and Chimera (Pettersen, et al., 2004). In order to understand structural stability of docked complex molecular dynamics (MD) simulations can be carried out using available packages such as GROMACS (Spoel, et al., 2005), CHARMM (Brooks, et al., 2010) and AMBER (Case, et al., 2005).

Preparing the Macromolecule

For the preparation of macromolecule the stepwise procedure is given below.

- **Start AutoDock Tools:** Open the macromolecule structure in ADT: Click on File-----Read Molecule and then get your interested molecule e.g. 2IPP.pdb in the working area of ADTviewer.

Add hydrogens to the receptor file: Click on Edit-----Hydrogens-----Add. This will add hydrogen atoms in your interested PDB file. Then save pdb file with hydrogen atoms in the same folder; File-----Save-----Write PDB-----Receptor.pdb.

- **Preparing the Rigid and Flexible Residue File:** In this part the rigid and flexible residue files are prepared which has to be saved in the PDBQT file format. The active site residues of receptor are kept as flexible residues while the other residues are considered as rigid; Click on Flexible Residues-----Input-----Choose Macromolecule. A window will appear then select Receptor.pdb-----Select Molecule (Figure 1). This will compute Gasteiger partial charges, assigning AutoDock4 atom types to receptor and merged nonpolar hydrogens (Figure 1).
- **Selecting and Saving Flexible Residues:** The information about the active site residues are required to prepare the flexible residues file. The active site residues are considered as flexible residues.

Flexible Residues-----Input-----Choose Macromolecule. Then the receptor is selected. Then select flexible residues by clicking on Select-----Select From String-----Molecule List in which we have to select molecule (Receptor) then in the Residue column enter the names of active site residues (CYS29, HIS110, HIS111) then click on Add and then Dismiss (Figure 2). After that for setting torsions, click on Flexible Residues-----Choose Torsions in Currently Selected Residues. This will give the information about the torsion count in a separate window (Figure 3). Finally, the Flexible residue file (Flexible.pdbqt) and rigid residue file (Rigid.pdbqt) are saved as;

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Figure 1. Selecting the receptor molecule

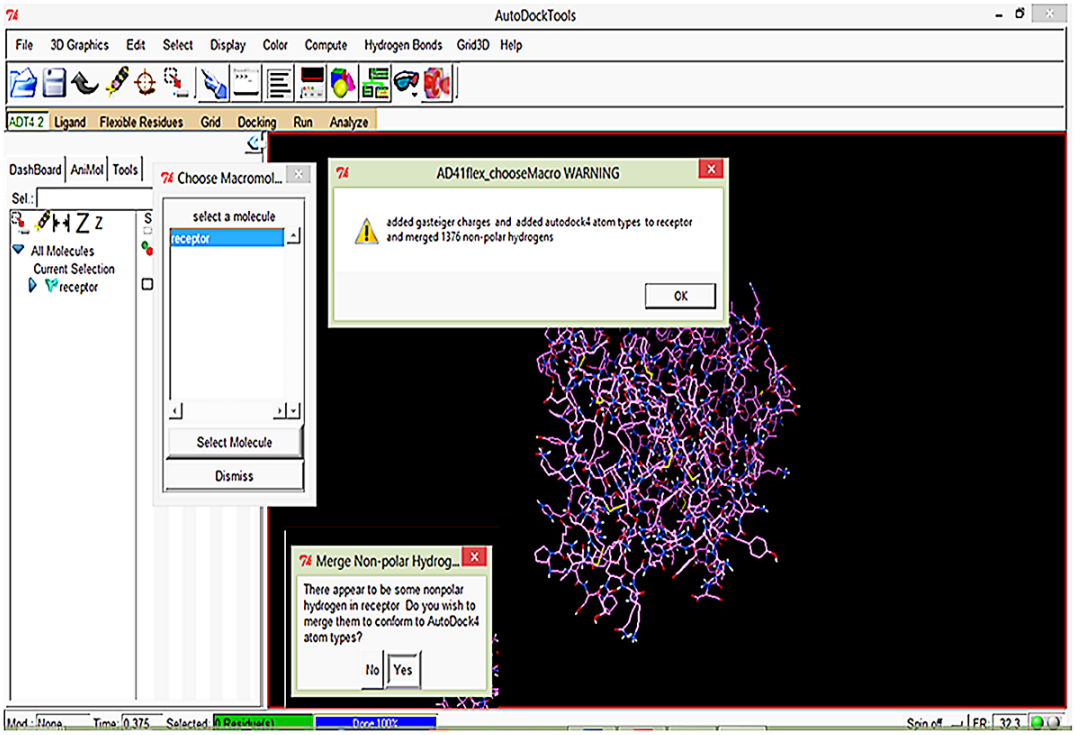
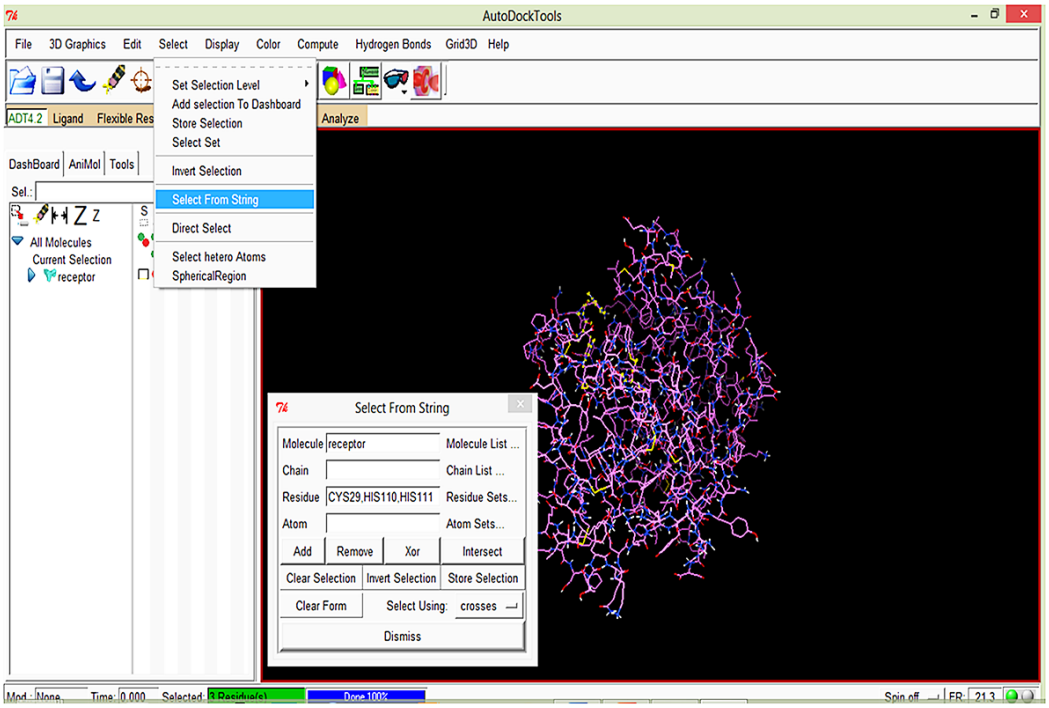
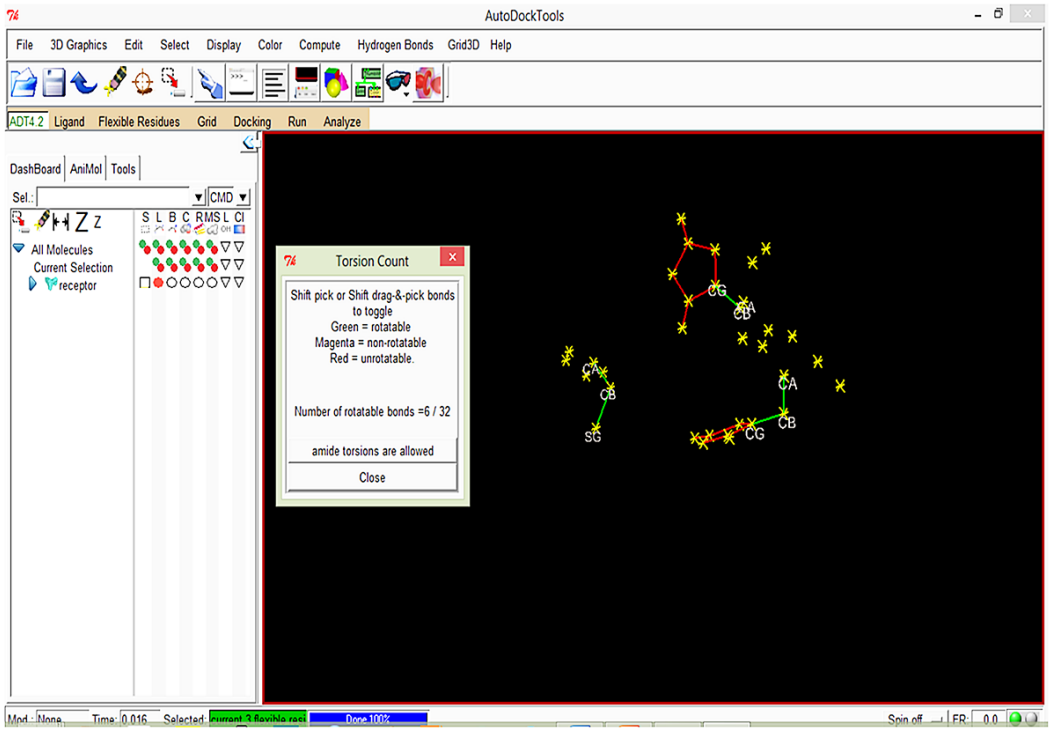


Figure 2. Selecting the active site residues



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Figure 3. Choose torsions in active site residues



Flexible Residues-----Output-----Save Flexible PDBQT and
Flexible Residues-----Output-----Save Rigid PDBQT (Figure 4).

- **Preparing the Ligand File for AutoDock:** In AutoDock the ligand files are written in special keywords which can be recognized by AutoDock. The keywords such as ROOT, ENDROOT, BRANCH, and ENDBRANCH establish a "torsion tree" object or *torTree* which has a *root* and *branches*. The root is nothing but the rigid set of atoms, whereas the branches are rotatable groups of atoms connected to the rigid root. The keyword TORSDOF signals the number of torsional degrees of freedom in the ligand. The TORSDOF for a ligand is the total numbers of possible torsions in the ligand minus the number of torsions that only rotate hydrogens. TORSDOF is mainly used during calculation of the change in free energy caused due to the loss of torsional degrees of freedom upon binding. In the current example the ligand which is a patch of Aβ peptide (1IYT.pdb) is used for the docking. For the preparations of ligand.pdbqt following steps can be performed.

Click on Ligand-----Input-----Open to choose ligand PDB file i.e. IYT.PDB as shown in Figure 5. After choosing ligand.pdb click on Select Molecule for AutoDock4. Then the window will appear, in which the summary for ligand.pdb will be displayed with the ligand information as can be seen in Figure 5 and then click on OK.

Then following steps are carried out to detect root.

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Figure 4. Save rigid and flexible files

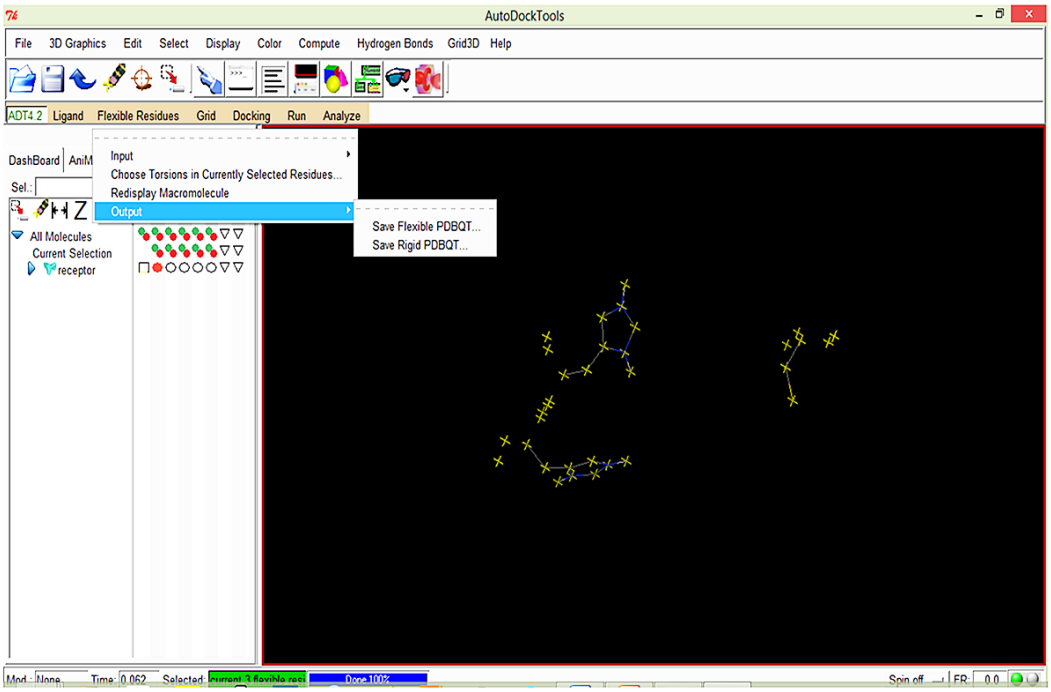
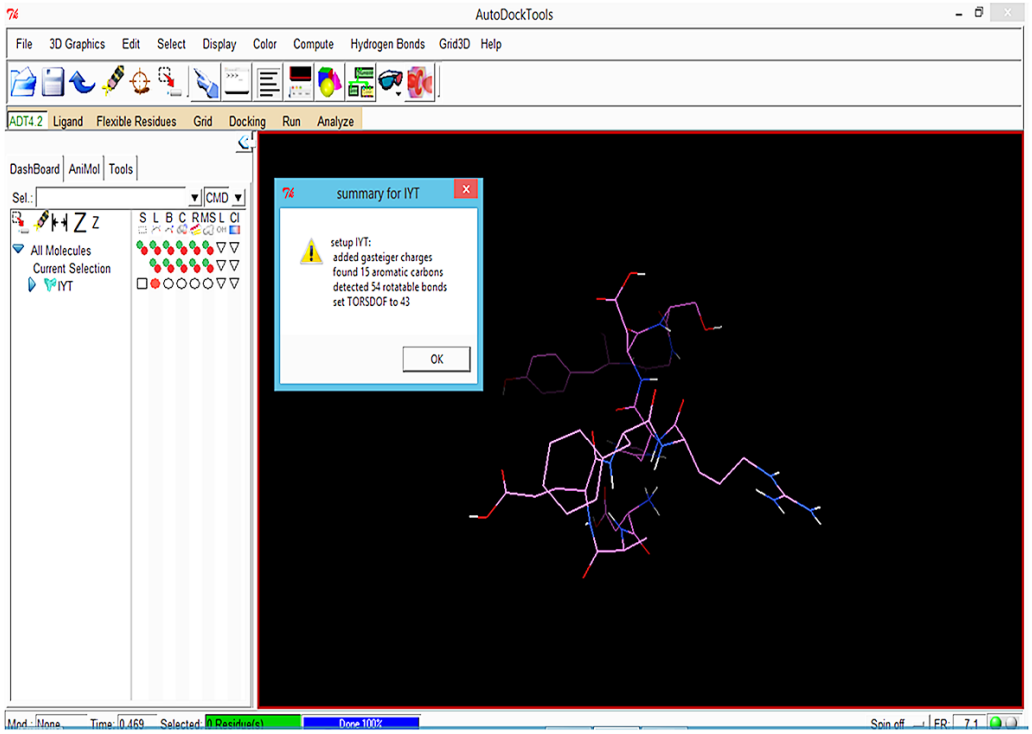


Figure 5. Selecting the ligand Molecule



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Ligand-----Torsion Tree-----Detect Root

Ligand-----Torsion Tree-----Hide Root Expansion

Ligand-----Torsion Tree-----Show/Hide Root Marker

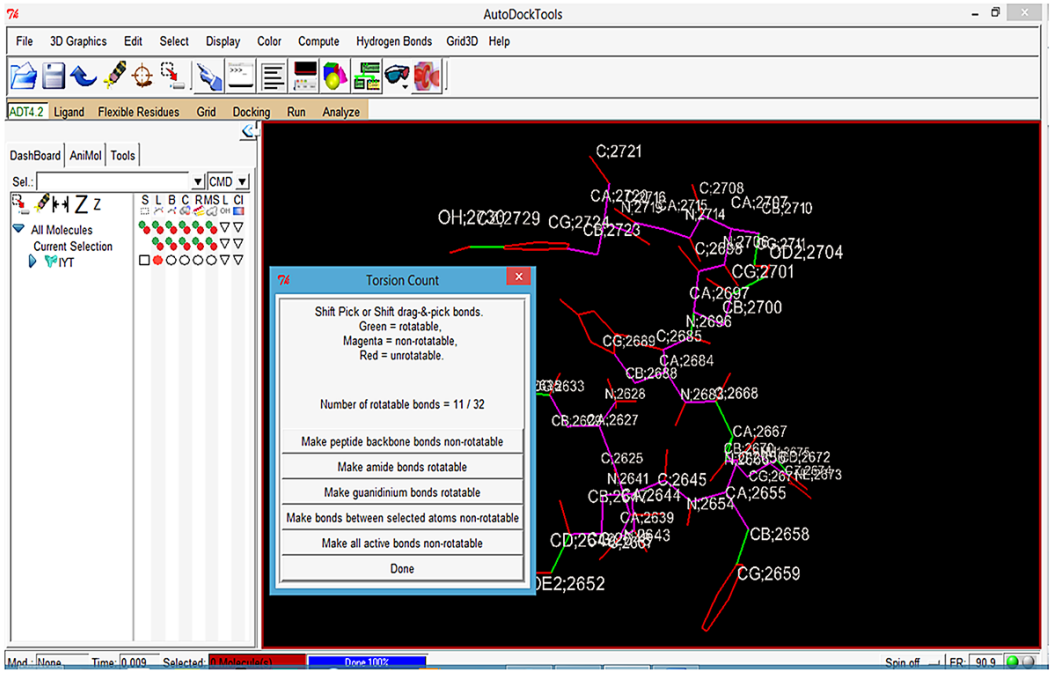
ADT determines the atom which fits its idea of the best root and mark it with a green sphere. This best root is nothing but the atom in the ligand having small largest subtree.

Ligand-----Torsion Tree-----Choose Torsions

These operations will open the Torsion Count widget which displays the number of currently active bonds showing; Red-non rotated bonds, Purple-currently marked as inactive, Green- Currently active. Those bonds which are in cycles cannot be rotated. ADT determines the bonds which could be rotated ('possibleTors'). In ADT one can see rotatable bonds ('activeTors') by inactivating the other bonds. We can also toggle the activity of a bond or group of bonds by picking them in the viewer. Alternatively, buttons on this widget let you toggle the activity of a type of bonds like 'peptide bonds', 'amide bonds', 'bonds between selected atoms' or 'all rotatable bonds'. To do this we can click on Make all active bonds non-rotatable then click on Make all rotatable bonds rotatable. It also states that 11 out of 32 bonds on the ligand are rotatable. Click on Done in the Torsion Count window to exit (Figure 6).

- **Saving the Ligand PDBQT (IYT.pdbqt):** To save ligand PDBQT file go to Ligand-----Output-----Save as PDBQT----- ligand.PDBQT. After these steps a ligand is prepared for the further docking procedure. This ligand.PDBQT file is then used for the actual docking process.
- **Preparing the Grid Parameter File:** Grid parameter file (gpf) is required to compute the types of maps, the location and extent of those maps as well as specific pair-wise potential energy parameters using AutoGrid. One map is calculated for each element present in the ligand with an electrostatic map. The following steps are performed for the preparation of gpf file.

Figure 6. Choose Torsion count in ligands



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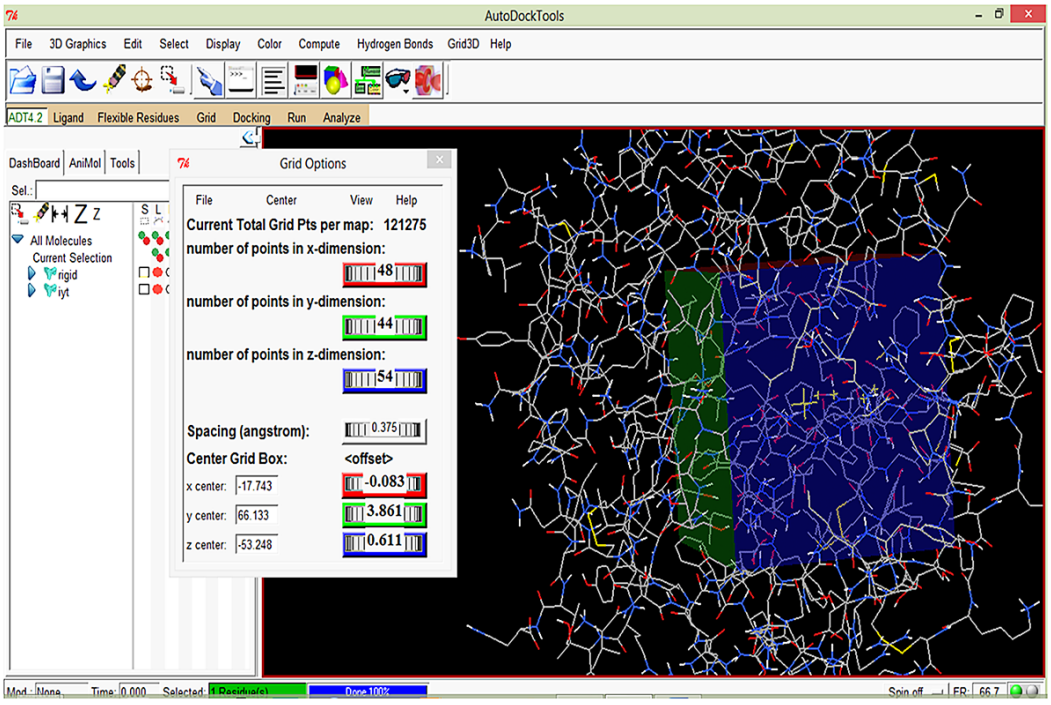
- **Open the Macromolecule File RIGID.PDBQT in ADT:** Click on File-----Read Molecule. This will open a file browser in the directory where ADT was started. Select the file, RIGID.PDBQT, and click on Open. Then Click on Grid-----Choose Molecule-----Choose the RIGID.PDBQT file. After choosing the RIGID.PDBQT a window will appear asking to preserve input receptor charges instead of adding Gasteiger charges. Then Click on Yes to keep the charges. Also a WARNING box will then pop-up, showing the initialization of the pdbqt file, stating that the autodock_elements and gasteiger charges have already been added. Then click on OK.
- **Selecting the Ligand and Generate Maps:**

Click on Grid-----Set Map Types-----Open Ligand-----ligand.PDBQT.

Then Grid-----Set Map Types-----Choose Ligand. Choose Ligand window will appear. Click on ligand.PDBQT and click on Select Ligand (assuming the ligand file is already open). After selecting the ligand, the gpf will specify AutoGrid to generate maps for atom types present in the ligand.

- **Specify the Grid Box Dimensions, Spacing and Center:** The grid box can be constructed by using this step; Click on Grid-----Grid Box. The window will appear with Grid Options. Specify the grid box center where ligand is located and the grid Box size such as the active site residues should be in the box. In the Grid Options window, click on the Center tab and select Center on ligand. In the current window the grid box center is now located with dimensions such as -17.743, 66.133, -53.248. The size of the grid box, as shown in Fig 19, is 48 × 44 × 54 using a 0.375 Å resolution grid. For saving the modified grid option parameters, click on file and select close saving current (Figure 7).

Figure 7. Specifying the grid box dimensions



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Save the grid parameter file (grid.gpf): Click on-----Grid-----Output-----Save GPF. A file browser will appear in which file name like grid.gpf should be saved.

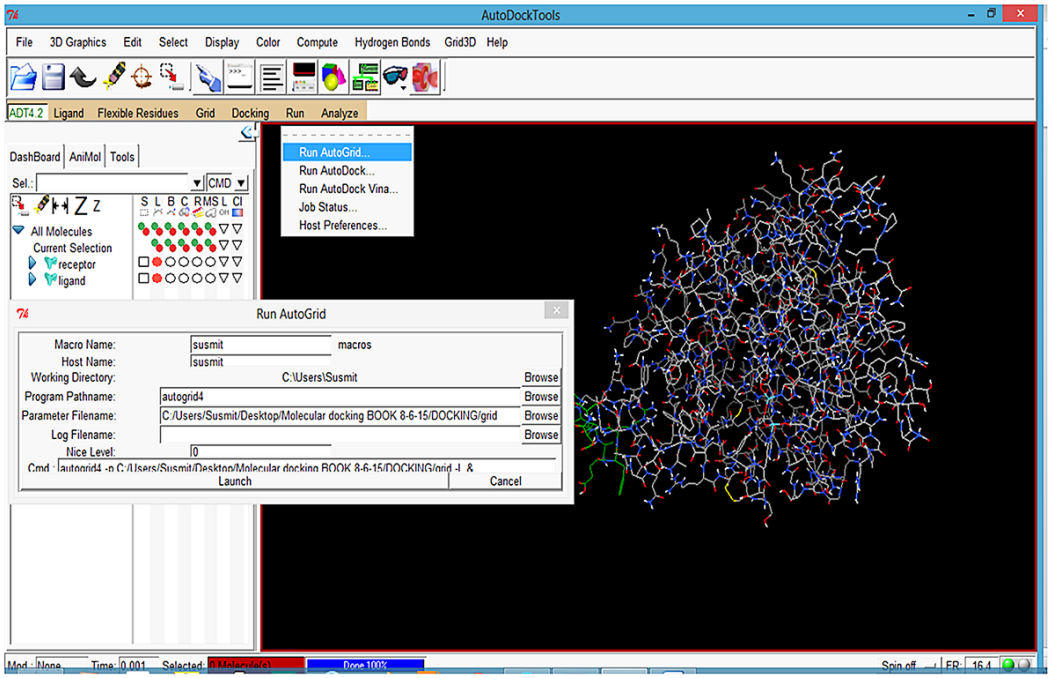
Edit the grid parameter file in a text editor. The Edit option is used to change required parameters of gpf file.

- **Running AutoGrid to Create Grid Maps:** To create the grid map for macromolecule run the AutoGrid: The AutoGrid must run in the same directory in which the ligand, macromolecule and grid parameter files are located. AutoGrid can be accessed by using ADT or command line. For running in ADT, click on

Run-----Run AutoGrid. After that a window will appear. The Macro Name and Host Name will specify your local machine by default. The location of AutoGrid executable must be specified by the program Pathname. The grid parameter file (gpf) specifies the parameter filename. The Log Filename is specifying the grid log file for the AutoGrid run. By default the name of the log file will be same as the grid parameter file, except with the extension.glg. The nice level for the remote jobs is also specified. The commands can be executed by running in cmd for windows. Click on Launch to start the job. We can see the status of job in the AutoDock Process Manager window which will appear after running the job. Alternatively, AutoGrid can be run via command line by typing: autogrid4 –p grid.gpf –l grid.glg& (Figure 8).

After completion of AutoGrid, the grid maps will be created for each specified atom type, electrostatic potential and desolvation energies. Also, other files created such as xyz file which specifies the grid box size, and the fld file which has other information about grid. The summary from the AutoGrid run is given in a grid log file (glg).

Figure 8. Running AutoGrid command



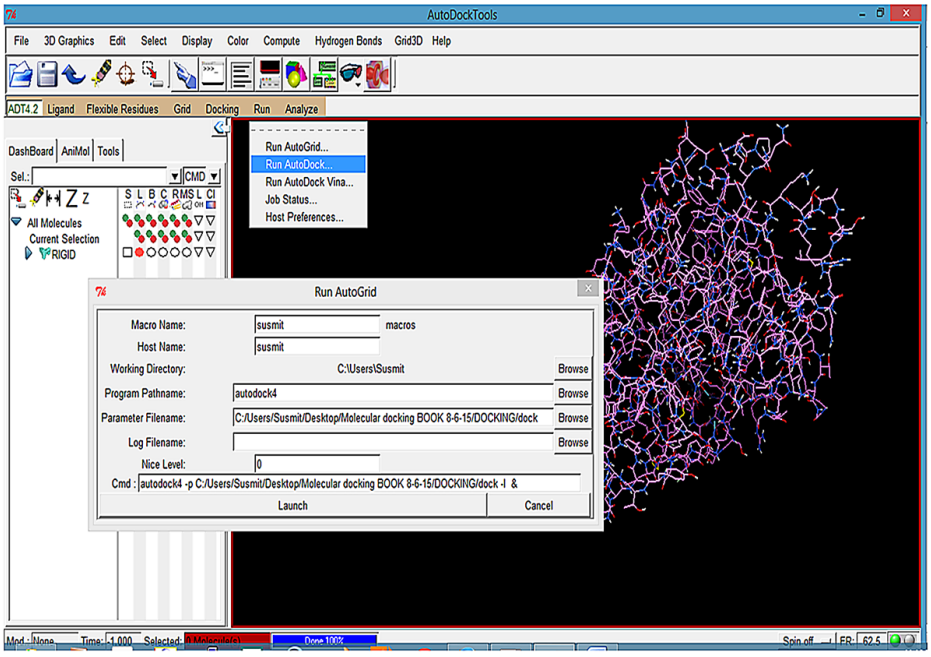
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- **Creating the Docking Parameter File and Running AutoDock:** The docking parameter file (dpf) is necessary to run the final docking step. The files such as ligand.pdbqt, rigid.pdbqt and flexible.pdbqt are needed for docking should be placed in same directory. To prepare docking parameter file (dpf) firstly open all the files required for docking in ADT.

Click on-----Read Molecule-----Select RIGID.PDBQT-----Open.
Click on-----Read Molecule-----Select FLEXIBLE.PDBQT-----Open.
Then for selecting the rigid and flexible files for docking
Click on Docking-----Set Rigid Filename
Click on Docking-----Set Flexible Residue Filename
Finally open the ligand in the ADT
Click on Docking-----ligand-----Open
The search parameters are selected as;
Docking-----Search Parameters-----Genetic Algorithm
The default docking parameters are used
Docking-----Docking Parameters-----Use Defaults-----Accept
Finally saving the docking parameter file (dpf)
Docking-----Output-----Lamarckian GA (4.2) -----dock.dpf
Save the docking parameter file (dock.dpf) in the same directory where all other files are generated.

- **Run the AutoDock:** AutoDock can be accessed by using ADT or command line. For running in ADT, click on Run-----Run AutoDock (Figure 9). Alternatively, AutoDock can also be run via command line by typing: autodock4 –p dock.gpf –l dock.dlg&

Figure 9. Running AutoDock command



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- **Analyzing Docking Results:** In AutoDock the docking results will be written in the docking log file (dlg).
  Firstly open the docking log file (dlg) in the ADT.
  Click on Analyze-----Docking-----Open-----dock.dlg. This will open the docking file.
  Open macromolecule in ADT
  Click on Analyze-----Macromolecule-----Open-----RIGID.PDBQT.
  Selection of lowest energy conformation

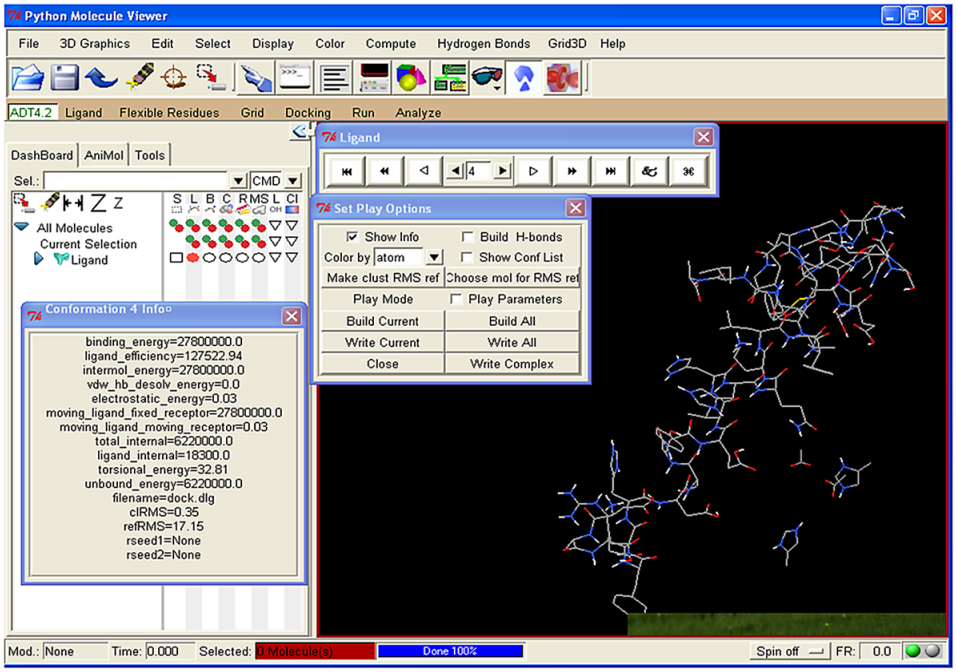
The lowest energy conformation must be selected from generated docked complexes. In this current procedure we have carried out 10 runs in which 10 different docked conformations were generated so we can see the energy of each conformation and then select the lowest energy conformation (Figure 10).

Click on Analyze-----Conformation-----Play

When this operation is performed a Conformation Player (CP) gets opened in which one can examine the docked conformations. The Conformation Player has a current conformations list (its sequence) and a current ID list. The sequence list consists of all the docked conformations, ordered by run. The ID list is [0, 1, 2, 3…10]. Then select the conformation on the basis of energy.

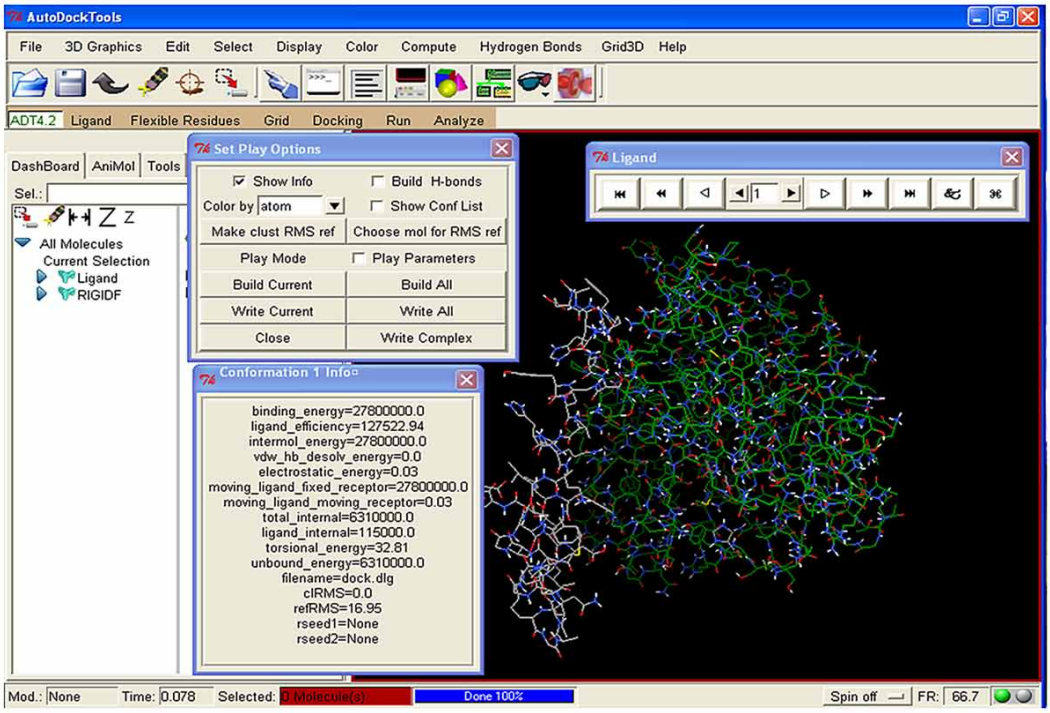
- **Saving the Docked Complex:** Finally, the docked complex with lowest energy can be saved by clicking on Write complex, and then the browser window will appear. After that select the location to save the docked complex and also change the file type and save the file in pdb file format as Docked.pdb (Figure 11). Similarly, other ligand types such as DNA and chemical compounds can

Figure 10. Selection of the lowest energy conformation



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Figure 11. Saving the docked PDB file

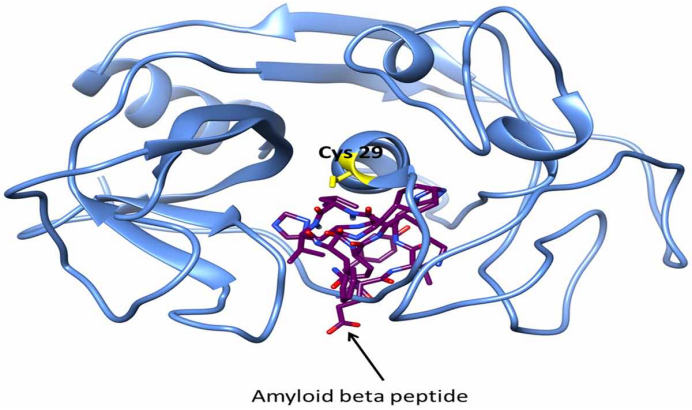


be used for molecular docking studies as per the same procedure as explained above. The detailed methodologies for such docking studies have been given in (Sonawane, & Barage, 2014; Tseng, et al., 2007; Barage, et al., 2014; Jalkute, et al., 2014; Jalkute, et al., 2015; Parulekar, et al., 2013; Dhanavade, et al., 2013, Dhanavade, et al., 2014).

- **Visualizing the Docked Complex in Various Visualizing Software:** The docked complex can be visualized in the different visualizing software such as Chimera and RasMol. The images can be saved in the different image formats like TIFF, JPEG and PNG etc. The final docked complex between Cathepsin B and amyloid beta peptide which is a causative agent of Alzheimer's disease can be seen in the Figure 12. Then the docked complex can be taken further for MD simulation study to understand structural stability as explained in earlier studies (Dhanavade, et al., 2013).
- **Analyzing the Final Docked Complex:** For the analysis of docked complex various software having molecular graphics such as RasMol (Sayle & Milner-White, 1995), Chimera software (Pettersen, et al., 2004), etc. can be used. The interactions between the receptor and ligand can be investigated which would be helpful to make final conclusions. In this chapter we have considered a case study done by Dhanavade and coworkers in 2013 (Dhanavade, et al., 2013) in which docking has been carried out between cathepsin B (receptor) with *Aβ* peptide (ligand) as an example. The receptor cathepsin B (2IPP.pdb) which is a human enzyme has ability to degrade Aβ peptide. Hence, the molecular docking was carried out between the cathepsin B (receptor) with Aβ peptide (ligand) to find the active site residues of cathepsin B (receptor) involved in the interactions with the Aβ peptide (ligand).

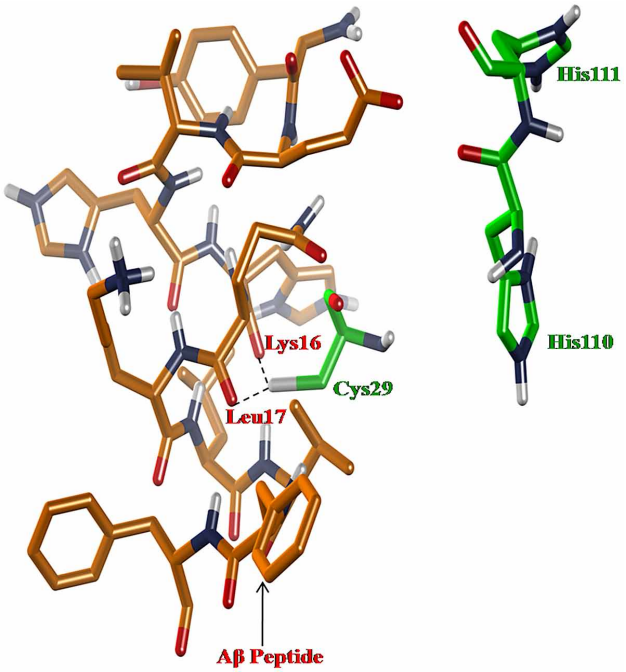
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Figure 12. The docked complex between Cathepsin B and amyloid beta peptide



Finally, on the basis of interactions it is easy to find the active site residues of cathepsin B (receptor) which take part in the cleavage of Aβ peptide. The docked complex was analyzed by using Chimera (Pettersen, et al., 2004) and the results revealed that there is strong hydrogen bonding between the cathepsin B (receptor) with Aβ peptide (ligand) (Figure 13) (Dhanavade, et al., 2013). This hydrogen bonding between active site residue Cys 29 of cathepsin B with Lys 16 and Leu 17 of Aβ peptide indicate that cathepsin B can cleave Aβ peptide in between Lys 16-Leu 17 (Dhanavade, et al., 2013). There is experimental report available which confirm the degradation of Aβ peptide by human cathepsin B (Mueller-Steiner, et al., 2006). Hence, to study the exact cleavage mechanism of Aβ peptide (ligand) by cathepsin B the

Figure 13. Showing the hydrogen bonding between the cathepsin B (receptor) with Aβ peptide (ligand)



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molecular docking technique has been found useful. Likewise, interactions between protein-protein, protein-ligand or drug, and protein-DNA can be studied using molecular docking technique.

CONCLUSION

The docking procedure and methodology discussed in this chapter would be useful for beginners to get into the field. The molecular modeling studies along with experimental findings could be useful to understand exact cause of several diseases in detail at the molecular level. Such studies might be useful to discover new lead molecules to combat neurological and several other diseases.

Future Work

The docking results must consider as a good starting point for further experimental design. MD simulations studies are must to check the stability and dynamic behavior of docked complex. Then binding free energy calculations of docked complex might be useful to get exact idea about the interacting residues. The continuous increase in computational power can be helpful to expand the practical applications of molecular models. These results are then compared with experimental findings. In future, experimental studies coupled with docking and MD simulations would be more promising to discover new lead molecules.

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Chapter 29
Molecular Modelling, Dynamics, and Docking of Membrane Proteins: Still a Challenge

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ABSTRACT

Computational tools and techniques are now most popular and promising to progress the research at rapid rate. Molecular modelling studies contribute their maximum role in wide variety of disciplines especially in proteomics and drug discovery strategies. Molecular dynamics and molecular docking algorithms are now became an essential part in daily research activities of every laboratory throughout the world. These strategies are now well established and standardised to study any specific protein of interest and drug molecule. But still there exist considerable drawbacks in a special concern with membrane proteins as the presently available tools and methods cannot be applied directly to them. Modelling, dynamics and docking studies of membrane proteins need a special care and attention as several challenges are to be crossed with an intensive care to produce a reliable result. This chapter is aimed to discuss such challenges and solutions to handle membrane proteins.

INTRODUCTION

Membrane Proteins

Cell membranes are the most indispensable attributes of any living organisms that safeguard the integrity of a cell by wrapping around. It acts as a potential barrier to perform several physiological functions by offering complex molecular machineries. These multifarious functions and mechanisms of membranes are facilitated and regulated by an inimitable group of proteins called as membrane proteins which are

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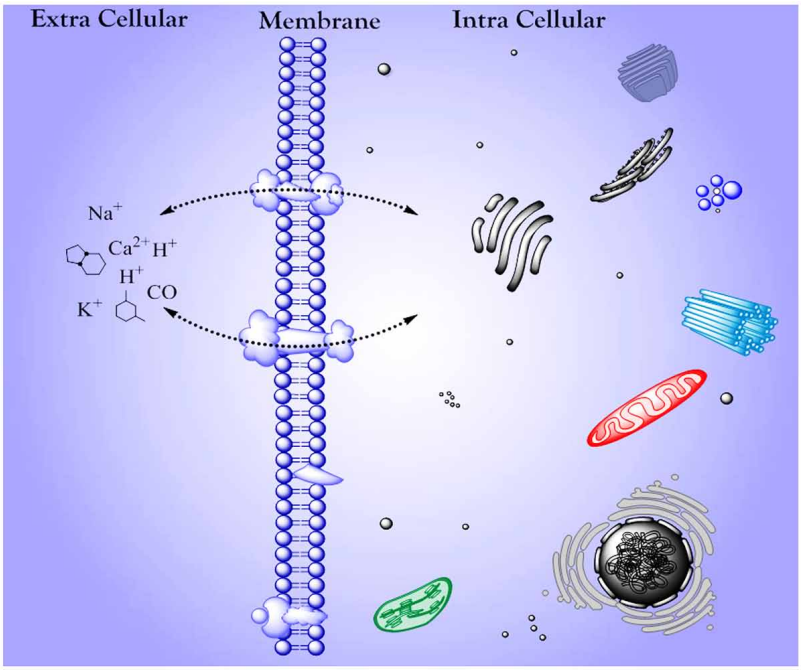
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of both surface and integral types (Figure 1). They play a vital role as channels for the transport of ions and molecule to meet the regular demands of biochemical functions (Cooper, 2000).

The structural and functional characterization of membrane proteins is typically a thorny task as the difficulties are associated with providing membrane environment that limits the purification and crystallization. Recent advancement in the technologies has led to the structural and functional characterization of several membrane proteins by providing their experimental structural information (Tatulian, 2000). But still it is not meeting the required demand as presently only *2%* of membrane protein structures were solved. Such demanding situations paved a spectacular way for the construction of membrane protein models through computational modelling methods (Arinaminpathy et al., 2009). There exist very narrow traditions to construct the protein models but providing an immense support to the structural biologists (Lacapère et al., 2007). Such ways even relies on the experimental data of pre-existing proteins (Ash et al., 2004). Such methods are incredibly known as homology modelling methods; nevertheless homology modelling also fails many times with membrane models as the availability of experimental data for them is very limited. Construction of such models remains as a challenge when the subject of protein is very demanding and necessitous. This chapter is going to describe how such situations will overcome. Stepwise recurrent methodologies with unequivocal and particularized algorithms can be followed where the propensities of amino acids can be utilized to construct the membrane protein models where *de novo* modelling is one of the methods comes under this technology. Even, this also never solve the problem entirely, as this idea works out well with the small proteins and still leavening the challenge unsolved as the membrane proteins usually bigger in size. This chapter will further deals, how the larger size membrane protein models are constructed. The constructed models must represent or resemble a natural conformation so as to mimic the experimental model. How it can be made? Still another chal-

Figure 1. Membrane and membrane proteins; their significant role as transporters and signal transducers



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lenge remnants, whether the protein model fits into the membrane structure in line or aligns properly in the lipid bilayer. What would be thickness of the membrane for a specified protein? This chapter will explain detailed procedural solutions for all these challenges to obtain an ideal theoretical model for any membrane protein irrespective of its size.

A membrane structure with its phospholipid bilayers and component proteins resembles a disordered environment. Several types of interactions such as electrostatic, hydrophobic, covalent and hydrogen bonding etc., contribute to the dynamic moments and functions of membrane proteins. Such interactions will drive the proteins to function dynamically. Characterization and monitoring of such behaviour of channels is really challenging task through experimental techniques but can make easy by *in silico* means applying molecular dynamics (MD) simulation studies. Beyond the static conformation of a membrane protein, addition of membranes, orientation of model in the membrane and finally providing the membrane like environments can be done so as to mimic the *in vivo* environment. Simulation of such atomic models plays a vital role in shaping the perfect view to characterize the function of a membrane protein (Chang et al., 2005). Molecular dynamics simulations, irrespective of theoretical or experimental structures, offer a distinctive direction for the interpretation and correlation of structures with their function (Lindahl & Sansom, 2008). The most essential physiological functions of membrane proteins such as molecular recognition, ion regulation and energy transduction are regulated by their dynamic moments which are offered by the internal dynamic folding and orientation of several conformations of the proteins. Inspection, characterization and interpretation of such dynamic motions are highly convoluted and challenging task through experimental means and further are solved by computational simulations. Such characterizations works well with small and cytosolic proteins and when come to large and membrane proteins it will prone to several challenges. The membrane proteins will behave differently when they are present in lipid environment as they are embedded in lipid bilayer their folding is majorly influenced by the membrane. The dynamic motions of the membrane can be monitored in presence of simulated peripheral forces that duplicate some aspect of the environment, such as its voltage and surface tension. This chapter deals with the challenges associated with the membrane protein dynamics especially how the simulation of ions/molecules conductance works out which is the most challenging exertion in computational studies of membrane proteins because of the complexity of the potential energy surface, alternate hydrophilic and hydrophobic environments that finally makes the passage of ions/molecules more specific.

Membrane Proteins as Transporters/Ion Channels

The small molecules and ions possessing charge cannot pass through the cell membrane on their own and they require transport proteins that are available in the membranes. The transporter proteins in the transmembrane regions are of two types (1) Channel proteins that functions as selective pores that opens and closes in response to an electro-chemical gradient and (2) Carrier proteins that use energy processes for the translocation of a substrate against concentration gradient (Saier, 2000). Human cell membranes contain several kinds of transporters for transporting glucose, amino acids, ions, neuro transmitters etc. Some transporters are also involved in the multi drug resistance. Such multi drug resistance is the particular limitation in the cancer chemo therapy and antibiotic treatments. Hence the drug molecules that inhibit drug efflux transporters are required to use as a complement to the existing therapy in order to overcome multidrug resistance (Dantzig et al., 2003). Usually drugs show their effect on transport

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system by binding to transporter proteins and either inhibits their activity so as preventing the passage of ions/molecules or by acting as false substrate for the transport process.

So far only a few transporter proteins from some bacterial species have been crystallized to determine their structure. There is only less structural data is available on the membrane transporter proteins that limits the development of new leads that could interfere with the structure and functions of transporter proteins. Such situations demand the molecular modelling studies for the bio computation of alternative methods for the structural studies of membrane transporters (Dahl et al., 2004). However, these methods depend both on the computational studies and experimental interference for the perfect molecular modelling studies.

Membrane Proteins as Potential Drug Targets to Discover New Drugs

The three dimensional information of a membrane structure and its interaction with a specific drug reveals several critical factors about the topology of the complex and their interacting surfaces between the membrane proteins and drug molecules (Klebe, 2006). Such information provides several possible ways for the virtual screening experiments and helps to develop novel potential lead molecules that specifically bind with a defined target membrane protein (Blundell et al., 2002). Several docking softwares can be utilised to obtain the orientation and binding affinities of the drugs against a membrane protein. This information will help a chemist to optimize the potential lead molecule by incorporating several functional groups in different orientations finally getting an improved molecule with high potency and selectivity (Hajduk & Greer, 2007).

Several drugs came into existence into the market in the present days which are developed based on the concept of structure based drug design. Some of them include HIV drugs Agenerase and Viracept developed based on the structure of HIV proteinase (Tomasselli & Heinrikson, 2000; Stoffler et al., 2002), Flu drug Zanamivir developed based on the structure of neuraminidase (Monto et al., 1999) and the inhibitors of angiotensin-converting enzyme (Cushman et al., 1987; Fournie et al., 1996). The structural information obtained from molecular and biophysical studies when combined with bioinformatics and computational approaches followed by the amalgamation of knowledge from synthetic chemistry will paves a pivotal role in the discovery of potential drug molecules which could be effective and safer.

The structural information of the membrane proteins which are involved in the cellular networking is very essential to understand the cellular machineries. The information about the structure of membrane proteins, their regulation and interacting partners forms an important basis to discover new therapeutic targets. Membrane proteins play a crucial role as transporters and also acts as co-players in the cellular metabolic activities. Such membrane transporter proteins can be targeted for the development of novel drugs.

Most of the protein targets where the drugs are supposed to bind are classified into four different types such as (1) Receptors, (2) Enzymes, (3) Ion channels and (4) Transporters. Majority of these targets includes the membrane integral proteins only rather than the cellular components. Such membrane integral proteins are involved in wide variety of metabolic networks and processes and represent themselves as plethora of molecular targets for pharmacological interference. The structural information of such membrane proteins with high resolution in the cell membranes is highly significant to develop novel drugs with high potential and specificity. Such information also provides an insight into the understanding of molecular mechanisms involved in cellular networks and functions.

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Therefore the structural biology concepts such as theoretical predictions and three-dimensional structural information are becoming more significant criteria in present days.

Drugs and Membrane Proteins

Several transporter membrane proteins are targeted so far and the drugs were discovered based on their structural information and the most important target among them are the ABC transporters (Leslie et al., 2005). There are justifications why the drugs are specifically to be targeted on membrane proteins and primary reason is that, several diseases are caused by mutations in the membrane proteins such as neurological disorders; schizophrenia and depression are due to mutations in the iron transport channels. Mutations in the ABC transporter protein system cause cystic fibrosis. The next important reason is more than 50% of known drugs binds to the membrane proteins on the surface of the membrane. If better drugs are to be discovered by aiming a disease, actual molecular structure of the drug molecule and its intermolecular interactions are required. Such structural information assists to develop better drug molecules with improved activity. Hence membrane proteins could be the major determinants of pharmacokinetic, safety and efficacy profiles of drugs (Giacomini et al., 2010).

Drugs will have their own specific way in the membrane proteins and according to their solubilities they distribute in the hydrophobic regions of the structure. Not only this, several questions also to be justified for a successful drug discovery such as selection of specific membrane transporter protein that should be clinically important in drug absorption and disposition. This will also clarify what kind of *in vitro* mechanisms are to be chosen. Specific attention has to be paid for the transporters that are confined to the epithelia of the intestine, liver and kidney, and in the endothelium of the blood–brain barrier.

MODELLING OF MEMBRANE PROTEIN STRUCTURES

Molecular modelling is not only a robust method for generating the theoretical models of proteins it is also an integral part of crystallography and Nuclear Magnetic Resonance (NMR) structure determination techniques. Experimental determination of membrane protein structures and their complexes is extremely a difficult task and more over the problem hinders with the size of the membrane protein also. The Membrane proteins represents approximately one third of the total encoded proteins of human and other genomes, but the currently available crystallographic structural information of membrane proteins is very limited (Frishman & Mewes, 1997; Wallin & Von Heijne, 1998). Many important puzzles in biology, chemistry and medicine remain unsolved because of limited understanding of the membrane structures, behaviour and their molecular interactions.

Usually the integral membrane proteins have two types of conformations such as (1) α−helices and (2) β−barrels. Till now it has been observed that the plasma membrane and reticulum membranes of eukaryotes contain α−helices whereas the β−barrel conformations are observed mainly in the proteins of outer membrane of gram-negative bacteria, mitochondria and in chloroplast membranes (Basyn et al., 2003). The α−helical conformations contain usually long hydrophobic helical structures and densely packed together. β−barrel conformations are very huge proteins possessing anti parallel β−sheets those folds into a barrel shape and closed by the first and last β−strands (Oberai et al., 2006; Elofsson & Heijne, 2007). In a protein with known amino acid sequence and unknown structure and function, the long run hydrophobic residues that represent spanning regions of membrane can be recognised easily in the

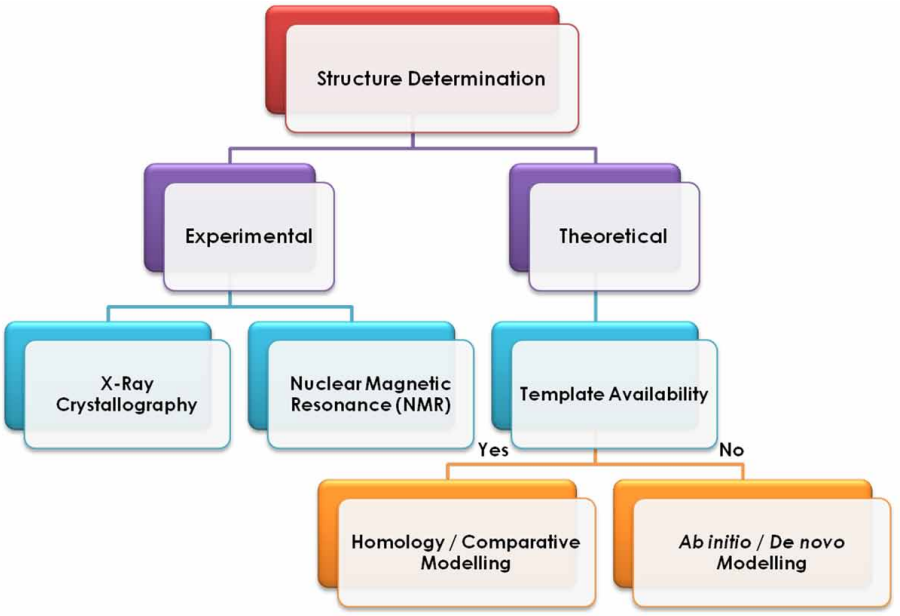
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sequence than the less hydrophobic trans-membrane β-strands. *In silico* approaches works best for the prediction and identification of such membrane spanning α−helical bundles and β−sheet barrels. As it is difficult to determine the 3D-structure of an integral membrane protein experimentally, prediction of such secondary conformational elements from the sequence information helps best for the annotation of protein sequences to membrane protein families. Such information when combined with the recognition of structural motifs by bioinformatics approaches provides valuable structural information that will also helps to determine the function (Amico et al., 2006; Granseth et al., 2006). *In silico* methods offers robust methods and algorithms for the accurate construction of protein models.

The molecular structure of a membrane protein contains very essential information such as architecture of active sites on the surface of membrane, possible binding sites for ligands and antigens and evolutionary determinants. Such information from membrane protein structure is utilised for several proteomic approaches and designing of successful experiments. The electrostatic properties and the orientation of the membrane protein structure are essential to understand its interaction with its interacting partners which are all involved in the regulation of metabolic activities. Hence it is very essential to acquire a three dimensional structural information of a membrane protein.

Experimentally the membrane structures are determined and solved by either X-ray crystallography or by NMR method. Computationally, there are two main popular ways to construct the membrane models and even applicable universally for all the proteins. The first one is the homology or comparative modelling and the other is *Ab initio* or *de novo* modelling. Homology modelling is strictly based on the scaffold of the existing structure with homology, nothing but a template and the *de novo* modelling is based only on the sequence information where the propensities of each amino acid is taken into consideration that tends to form a specific kind of conformation (Figure 2). There will be other consideration such as prediction of secondary structure and membrane spanning regions before constructing the final three dimensional model.

Figure 2. Criteria and methods involved in the protein structure determination



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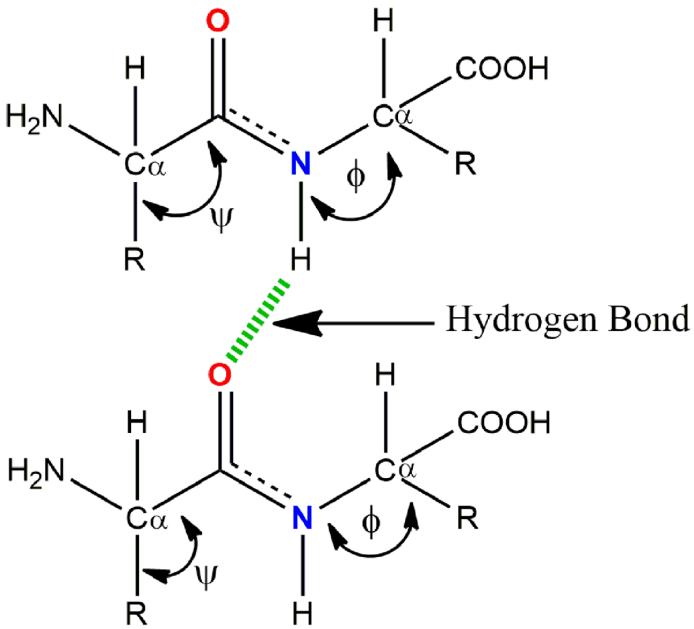
Irrespective of the model chosen for the prediction of theoretical models for a specific protein, certain preliminary analysis is required to be done to acquire a reliable and accurate model without any obligations. Such criteria are discussed in detailed as follows.

Secondary Structure Prediction

Before proceeding for the construction of a three dimensional conformation of a protein, it is mandatory to determine their secondary conformations. These secondary conformations only further folds into a three dimensional conformations producing a functional protein. Hence the secondary conformational elements such as helices, sheets, turns and coils are the basements to build a perfect conformation of any protein. The multiple conformations of a protein are possible due to the rotation of CΨ and CΦ angles of peptide bond. Each amino acid with its specific R-group has its own role to form a specific kind of secondary conformation. The secondary structure is popularly said to be a local conformation of a polypeptide chain that is strictly formed and stabilised by hydrogen bonding. A hydrogen bond always forms in between the carbonyl oxygen of one peptide bond and amide hydrogen of another peptide bond by means of a hydrogen atom (Figure 3). These are the responsible factors for the stability of any secondary conformation of a protein.

A α-helical conformation forms when n^{th} peptide bond forms H-bond with $n+4^{th}$ peptide bond whereas a β-conformations forms when H-bond forms between a normal of 5–10 sequential amino acids in one bit of the chain with another 5–10 more remote down the chain. Loops are the locales of a protein chain that are between α helices and β sheets, of different lengths and three-dimensional arrangements, and on the surface of the structure. Hairpin loops that cause a complete turn in the polypeptide chain joining two anti parallel β strands may be as short as two amino acids long. A locality of auxiliary structure that is not a α-helix, a β-sheet, or a conspicuous turn is ordinarily eluded as a coil. All these types of

Figure 3. Formation of Hydrogen bond between two peptide bonds



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conformational elements are taken into consideration while prediction of a full pledge secondary conformation from a given amino acid sequence. This will paves a reliable way for the next step to predict accurate three dimensional conformation of a protein.

Several secondary structure prediction methods were introduced in the early 1960 itself (Guzzo, 1935; Kotelchuck & Scheraga, 1969; Lewis et al., 1970; Prothero, 1966; Schiffer & Edmundson, 1967) relying on the recognition of likely α-helices and were constructed principally in light of helix-coil conversion models. More precised expectations that included β-sheets were presented in the 1970s and depended on measurable evaluations in view of likelihood parameters got from known comprehended structures. The transformative conservation of secondary structures can be abused numerous homologous sequences in a diverse sequence alignments, by computing the net optional structure inclination of an adjusted segment of amino acids. Working together with bigger databases of known protein structures and cutting edge machine learning routines, for example, neural networks and support vector machines, these strategies can accomplish up to 80% accuracy rate (Dor et al., 2006).

Algorithms in Secondary Structure Prediction

Chou-Fasman Method

The Chou–Fasman system is an experimental method for the prediction of secondary conformations in proteins, initially grew in the 1970s by Peter Y. Chou and Gerald D. Fasm (Chou & Fasman, 1974, 1978). The technique is in light of examinations of the relative frequencies of every amino acid in alpha helices, beta sheets and turns taking into account known crystal structures of proteins. From these frequencies an arrangement of likelihood parameters were inferred for the presence of every amino acid in every secondary structure, and these parameters are utilized to anticipate the likelihood that a given succession of amino acids would shape a helix, a beta strand, or a turn in a protein. The parameters discovered some solid propensities among individual amino acids to incline toward a specific kind of structural conformation over others.

GOR Method

The GOR method (Garnier-Osguthorpe-Robson) is an information theory-based method developed in the late 1970s (Garnier et al., 1996). This system is in light of likelihood parameters got from exact investigations of known protein tertiary structures from X-ray crystallography. Nonetheless, not like Chou-Fasman, the GOR technique considers not just the propensities of individual amino acids to frame specific secondary structures, additionally the contingent likelihood of the amino acids to shape a specific secondary structure given that its quick neighbours have effectively framed that structure. This technique dissects successions to anticipate alpha helix, beta sheet, turn, or arbitrary coils at every position in view of 17-amino-acid sequence windows. The unique explanation of this method is, it includes four scoring matrices with a size of 17×20, where the columns correspond to the log-odds score that reflects the likelihood of judging a certain amino acid at each position in the 17-residue sequence. The four matrices imitate the probabilities of the vital, ninth amino acid tends to be in a helical, sheet, turn, or coil conformation. This method was considered as finest requiring at least four neighbouring amino acids to attain alpha helices to organize the region as α-helical, and at least two adjoining residues for a β-sheet.

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Machine Learning

Such methods popularly include Artificial Neural Networks (ANNs) and Support Vector Machines (SVMs). ANNs are a group of statistical learning models enlivened by biological neural networks and are utilized to estimate functions that can rely upon large quantity of inputs and are usually anonymous. They use the training set formed from experimental structures and investigate the similar sequence motifs connected with certain arrangements of secondary elements. SVMs have been demonstrated especially helpful for predicting the areas of turns, which are hard to identify using statistical methods. All machine learning strategies endeavour to predict the more fine-grained nearby properties of proteins, for example, backbone dihedral angles in unassigned areas (Kuang et al., 2004; Zimmermann et al., 2006).

Servers for Secondary Structure Prediction

A wide variety of servers are available for the prediction of secondary structures from a given protein sequence among which some are listed as in Table 1.

Transmembrane Prediction

The most important task in the membrane protein modelling is the identification of transmembrane regions. Several factors are to be considered for predicting membrane spanning regions. The free energies are summed over residues of 10-15 and the regions with high hydrophobicity are considered to be in the membrane. This idea works well with helical structures as all the residues present in the helix will have same kind of hydropathy scales. But this type of prediction is unable to work out to predict the membrane spans beta conformations. Utilising the evolutionary information from multiple sequence alignments and using machine learning approaches helps to predict transmembrane regions accurately. Several tools and servers exist based on these approaches that can predict the perfect topology of the membrane protein. The perfect orientation of membrane protein i.e. external regions, spanning regions and inside regions will be predicted based on the propensities, artificial neural networks (ANN), hidden markov models (HMM) and support vector machines (SVM).

Some of the online tools available for the prediction of transmembrane regions are given in Table 2.

Once the secondary structure and transmembrane spanning regions are predicted from a given sequence, the next step is to move towards the prediction of tertiary structure. It can be done in two methods i.e. homology modelling and de novo modelling.

Table 1. Secondary structure prediction tools

Tool	Web Link
APSSP	http://imtech.res.in/raghava/apssp/
CFSSP	http://cho-fas.sourceforge.net/
GOR	https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html
Jpred	http://www.compbio.dundee.ac.uk/jpred/
PROF	http://www.aber.ac.uk/~phiwww/prof/
SOPMA	https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html

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Table 2. Transmembrane prediction tools

Tool	Web Link
TMpred	http://www.ch.embnet.org/software/TMPRED_form.html_
HMMTOP	http://www.enzim.hu/hmmtop/_
Phobius	http://phobius.sbc.su.se/_
TMHMM	http://www.cbs.dtu.dk/services/TMHMM-2.0/_
DAS	http://www.sbc.su.se/~miklos/DAS/_
TOPCONS	http://topcons.cbr.su.se/_

Homology Modelling

Homology modelling otherwise called as comparative modelling is a most widely used popular method to construct the protein models based on the template similarity. This method is very important in case of membrane proteins as the experimental determination of their structures is prohibitively time-consuming and tough task (Wieman et al., 2004). Homology modelling found very important for membrane proteins as many of them such as ion channels are the primary drug targets for wide variety of diseases.

Unfortunately there are very less reports on the homology modelling of the membrane proteins which is due to several factors such as lacking of proper template, unambiguous prediction methods, poor resolutions, high rate of errors in model validation etc. Recently, there is an extensive increase in the number of structures in the PDB database that helps to analyse the hydrophilic surface areas and hydrophobic interiors based on the structural information but rather than the sequence information alone. Although the number of crystal structures is being increased in the PDB, membrane proteins are found to have poor resolution where 10% of available membrane structures have a resolution ranging from 3-4 Å. This situation made the homology modelling as an exceptionally significant task to construct their 3D models and also for the refinement of existing crystal structures with poor resolution (Sanchez et al., 2000). There are several steps for the prediction of membrane protein structures which are explained as follows.

Template Selection and Alignment

A template is the mandatory element to construct a homology model of a protein. The template should have high similarity to the target so that high quality model will be produced. Sequence coverage is also an important point under consideration during template selection. A template assuring a minimum of 98% coverage is responsible to produce the overall reliable quality of the model. A template with a similarity of 70% produces a model with an RMSD of 1-2 Å where as the low similarities of templates ranging around 25% will produce a model with an RMSD of 3-4 Å.

The quality of sequence alignment between the target and template sequence will decide the quality of the final model where the quality of the alignment is improved by BLOSUM and PAM matrices. But for the membrane protein alignment there is specific matrix for the improvements of alignments such as PHAT (Ng et al., 2000) that utilises the membrane protein specific substitutions and PRALINETM (Pirovano et al., 2008) is an example that utilises such matrix. JSUBST (Shi et al., 2001) is another membrane specific substitution matrix which is amalgamated with MP-T (Hill & Deane, 2013) tool that is a sequence-structure alignment tool. This tool also considers other environment factors such as

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secondary structure, membrane spanning region and solvent accessibility regions such as intra cellular and extra cellular regions.

In many instances membrane proteins does not have any suitable templates for the entire sequence and in such cases discontinuous templates may be available. Discontinuous templates have similarity with the target protein at multiple locations rather being at a confined location and such templates may be from different sources. So such cases demand the use of multiple templates to construct the final model. Such methods are called as threading methods that utilises the alignment between target and multiple templates. Further sequence-structure alignment followed by scoring functions which are based on the factors such as secondary structure, residue pairing and mutatability produces a quality model.

Model Construction

Once the improved alignment has been generated, it can be used for the construction of homology model of the target using any homology modelling tools. Several tools are available for the homology modelling and some of them are given as follows.

- **RosettaMembrane:** This is the most reliable and oldest tool available for the membrane protein homology modelling. This tool is also able to model the open and closed conformations of the ion channels (Yarov et al., 2005).
- **MODELLER:** This is the most popular tool in the present days both in the modelling and experimental areas. This tool constructs the models by satisfying the spatial constraints derived from sequence alignment (Eswar et al., 2006).
- **MEDELLER:** This is a coordinate generation tool especially used for the membrane proteins. This tool works in conjunction with other tools like MEMOIR, HHPred and SWISS Model.

The overall outline of the homology modelling is represented in Figure 4.

De Novo Modelling

De novo modelling is an alternate to homology method where it is preferred when no suitable template is available for the construction of reliable model. De novo modelling works with the propensity of each amino acid in a protein sequence and build a specific kind of secondary structural conformation and produce the model. De novo methods able to predict the tertiary structure of a protein based on the sequence principles which are basis for protein folding energetics and statistical tendencies of conformational characteristics that experimental structures possess and never rely on template information. Amino acid sequence is the best and sole starting material for the de novo predictions.

De novo prediction algorithms usually predicts the structure initially by producing the multiple conformations of the protein and chose the best amongst based on the thermodynamic stability and energy levels. Most of the de novo prediction servers considers the following factors, (1) Accurate energy function that filters thermodynamically stable conformation and resembles to native structure (2) A potential search method for the quick identification of low energy conformation (3) Ability to select most near native conformation among multiple conformations.

The outline of de novo modelling method is represented in Figure 5.

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Figure 4. Outline of homology modelling of membrane proteins

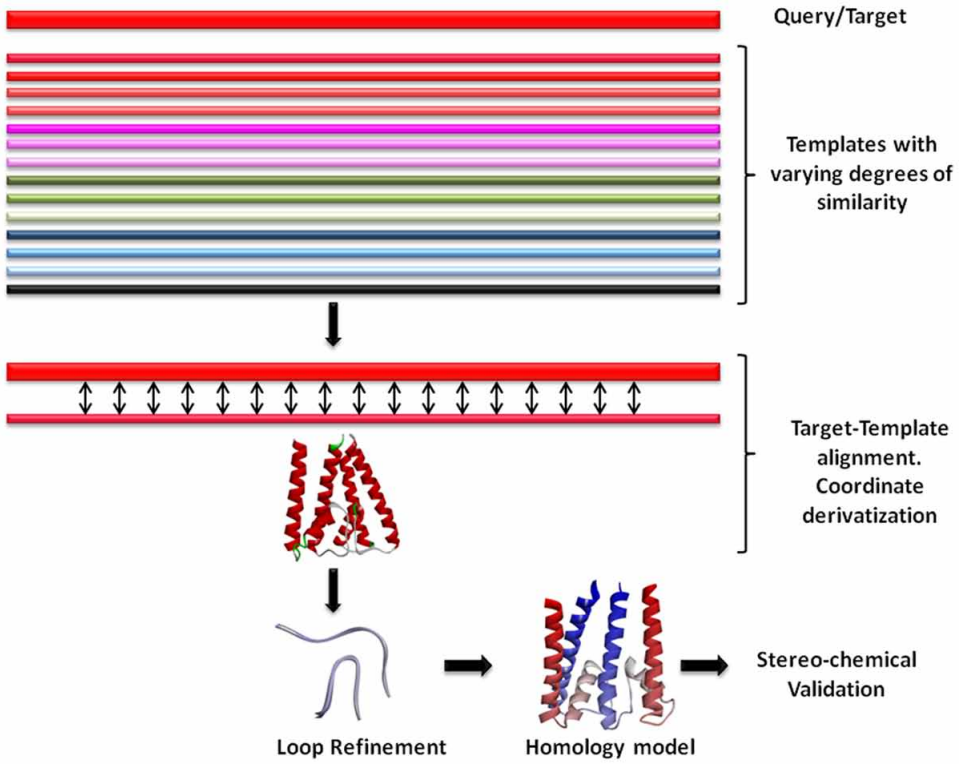
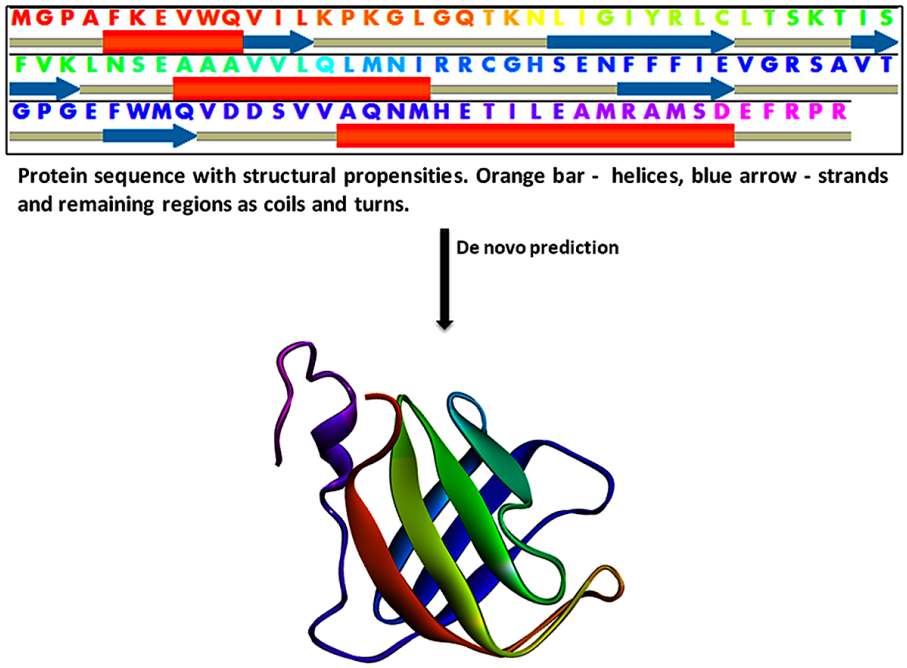


Figure 5. Outline of de novo modelling



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De novo prediction uses the strategies such as,

- **Ab Initio:** Initially the secondary structural propensities such as helices, sheets, coils and turns are elucidated from the primary sequence based on the physico chemical properties and neural net algorithms. Based on this information the tertiary folding will be determined.
- **Fold Prediction:** This method also initially predicts the secondary structure information and compare with the existing protein fold libraries such as CATH and SCOP databases.
- **Threading:** The fold recognition process is progressed to threading where the interaction of residue pairs is used based on the empirical energy functions and placed onto a putative backbone of the protein utilising the gaps and gap penalties.

The main disadvantage of de novo prediction methods is they require huge amount of efforts and computational time for the successful prediction of near native conformation of the protein. Few examples of de novo modelling servers are given in Table 3.

MOLECULAR DYNAMICS OF MEMBRANE PROTEIN MODELS

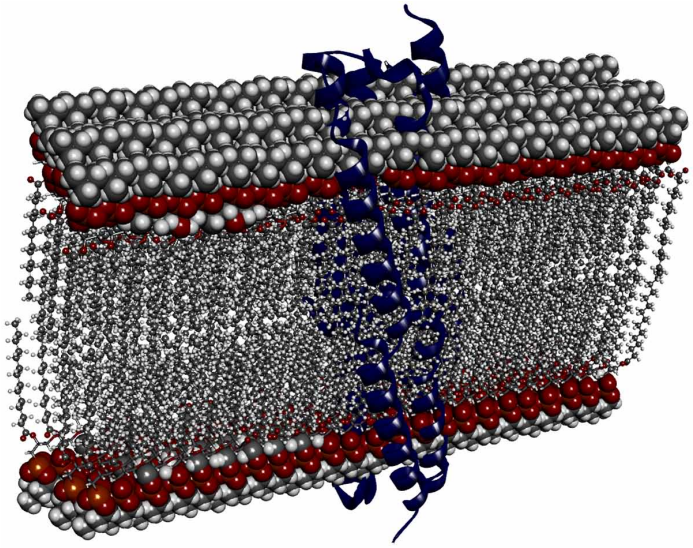
The majority and comprehensive information about the theoretical models of ion channels well provides by Molecular Dynamics (MD) simulations. The present available MD methodologies are capable to generate the trajectories that are very close to the realistic atomic models of typical biological membrane channel systems. MD simulations can be used to discover detailed atomic information about the molecular mechanisms behind the function of a specific membrane protein such as ion transport, selective entry and voltage gating of channel proteins. Although there is a vast improvement in the MD simulations of proteins of different sizes for the past two decades, still it remains as a challenge with the membrane protein simulations. The heavy size of the membrane proteins limiting the success rates of the simulations and several approaches are being put forward to solve the critical hindrance arising with membrane proteins. One such a solutions is limiting the size of protein to the short possible fragments and proceed for simulations. The complex membrane structure may be broken down into smaller fragments of specific importance such as function, domain, and structural elements so on. Such significant fragments are then added with membrane layers around and subjected to simulations to observe their behaviour in an isolated environment (Figure 6). Another important solution is the time scale of production phase, where the simulations can be carried out for a longer period rather than the traditional time

Table 3. De novo modelling servers

Server	Web Link
Robetta	http://robetta.bakerlab.org/_
CABS	http://biocomp.chem.uw.edu.pl/CABSfold/index.php_
Phyre2	http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index_
RaptorX	http://raptorx.uchicago.edu/StructurePrediction/predict/_
Pep-Fold	http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/_
I-TASSER	http://zhanglab.ccmb.med.umich.edu/I-TASSER/_

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Figure 6. Alignment and orientation of a membrane protein into the lipid bilayer



boundaries where a steady state is keep on monitored. The production phase may be terminated when a steady state is attained. It is applicable for the simulation of all proteins irrespective of their size, but the limitation could be the time factor needed for the computation. This situation will also demand the high end computing systems for the successful simulations.

Force Fields and Softwares

The folding of membrane proteins at the atomic levels in explicit membrane proteins proves challenges and basic problems with the specific force fields and simulation algorithms (Nymeyer et al., 2005; Ulmschneider & Ulmschneider, 2009). Employing new lipid force fields by the concern of the detailed atomic partitioning and folding of individual transmembrane regions into explicit lipid regions helps to achieve successful unbiased long time MD runs (Ulmschneider et al., 2011). Coarse-grained MARTINI force field is the most acceptable method developed specifically to model explicit lipid bilayers (Marrink et al., 2004). This force filed is used extensively to study the membrane bilayer parameters such as raft formation, membrane joining and membrane interactions. This force filed is more popular to study the large scale MD systems especially drug delivery strategies through lipid bilayers. To study the molecular mechanism of membrane folding, partitioning and it's inter molecular interactions requires detailed representation of hydrogen bonds at atomic level. This force filed will uncover the mechanisms behind ion transport, specificity, selectivity and gating properties of membranes. Several most common force fields are also available such as AMBER (Wang et al., 2004), OPLS (Jorgensen & Tirado, 1988) and CHARMM (Karplus, 1983) for the past few years. Highly accurate lipid parameters are added for these force fields in the recent years that made them to utilise for the simulation of membrane proteins with a variety of atomic models. But all these force fields are supplemented with MARTINI force field that has coarse-grained representations of proteins and lipids. Finally all the kinds of force fields are available with several kinds of software packages. The best example of such package is GROMACS (Van et

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al., 2005), fastest and most popularly used package. Other best examples count with NAMD (Phillips et al., 2005) and Desmond (Shaw, 2005) which are also fastest but not used as popularly as GROMACS.

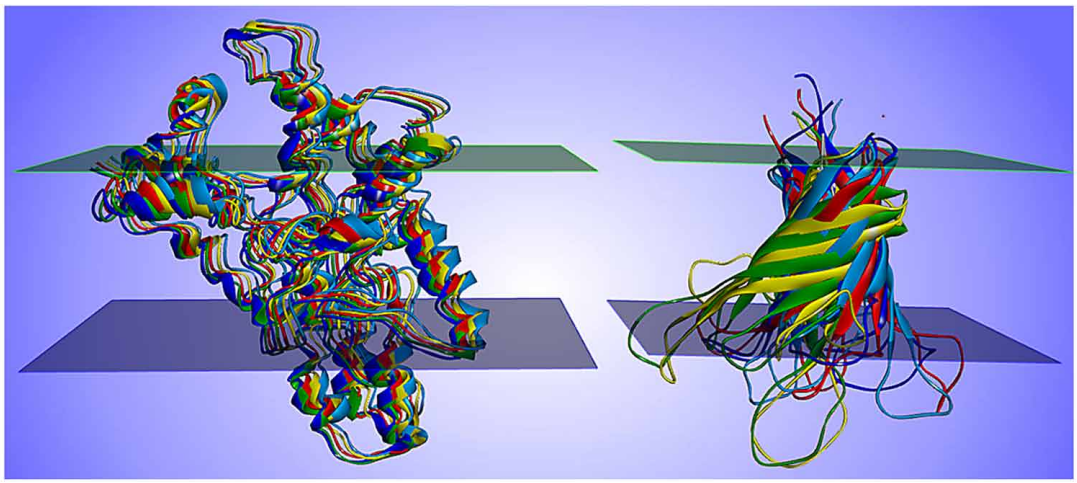
Several methods are in progress for the acceleration of MD simulations at faster rates in addition to the general approaches discussed above. One such popular method is replica exchange sampling or parallel tempering that creates indistinguishable replicas of an MD system which are simulated in parallel manner with an exponential ladder of temperature. The replicas are swapped at regular intervals using Monte Carlo approach and speed up the transitions towards near native state. Another related method to accelerate the MD process is using the elevated temperature ranges. This method is preferred during the unbiased partitioning of peptides into membranes. But the experimental confirmation of high thermo stability of the peptide is required. Equilibrium MD method is best suitable to accelerate the MD simulations of low energy conformations.

Conformational Analysis

Although the structure of a membrane protein is determined, it is not sufficient to predict its function. A single protein structure represents only a discrete conformational state and it never represent itself as a sole maker of its function, interaction with other molecules and its regulation by other elements in the bilayer. To understand the function of a membrane protein, different conformational states are needed which is hard to capture (Figure 7). The structure of a particular conformation can be characterized by altering the variable interactions and a specific snap shot of interest could be trapped. The function of membrane protein can be modulated by its properties such as energy levels and charge distribution. In addition the spring constant of the lipid bilayer also induce the changes in conformation and properties of a membrane protein. These specific considerations help to trace the required conformation of the protein that can be used for the interpretation of the function.

These computational studies of membrane protein modelling even though a challenging effort, a prospective and massive progress has been made in the field of computational modelling. So far the major efforts were put on the homology models whereas de novo modelling with a special concern with

Figure 7. Membrane proteins with multiple conformations represented in multiple colours



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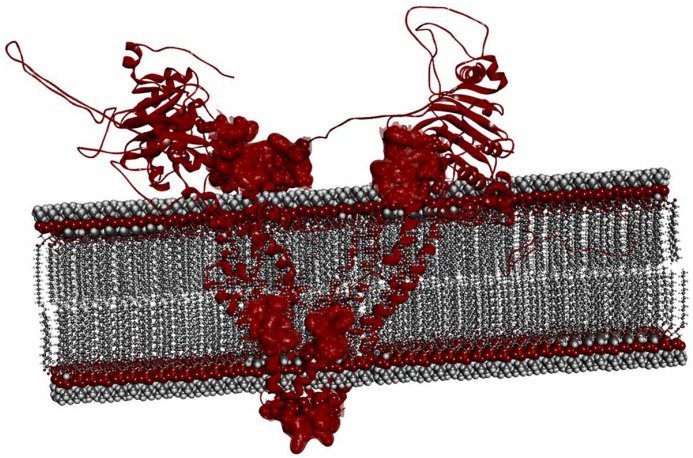
membrane proteins has yet to be progressed to a maximum extent. As of now we are able to model the membrane proteins in a coarse grained process further research is likely to focus on predicting the function of a membrane protein from multiple functional states of the protein. In addition the structures are needed to represent at high resolution both for protein and lipid bilayers.

PREDICTION OF BINDING SITES

The next challenging task with the membrane proteins is accurate prediction of binding sites that offers sites of interactions and molecular passages. Prediction and blocking of specific binding sites makes them efficient targets for several drugs especially the membrane proteins of potential pathogenic organisms. The ideal membrane protein models with explicit water molecules both inside and outside and with added phospholipid bilayer are the only perfect starting materials to find out such potential sites (Bordner, 2009). Like cytosolic proteins, it is not an easy task to predict the binding sites based on the cavities information obtained from the three dimensional models. Membrane proteins have a peculiar compartmentalized composition and orientation. The initial challenge is associated in solving the different regions of the membrane proteins, where such region/segment of the membrane protein has its own function. Then those regions with functional significance can be used to find out the binding sites. The actual challenges are concurrent with the accurate prediction of transmembrane, extra and intra cellular regions. Extra cellular regions are associated with protein-protein interactions especially involved in signal transduction and also offer specific sites as pores where the ions/molecule come and sits then enters the pore channels. Membrane regions are to be monitored to find out such pore regions where the channels are formed especially by beta barrel structures. Further the intracellular regions are to be concentrated where the pores opened into the cytosolic regions and also offers sites for the interactions with intracellular components that may be either proteins or small molecules. All these criteria are to be considered for the perfect prediction of binding sites (Figure 8).

Membrane proteins forms complexes with other molecules to perform their functions where the structural information about such interactions is helpful to generate experimentally acceptable hypoth-

Figure 8. Predicted binding sites in a membrane protein highlighted as surface clouds



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eses which also further helps for structure based drug designing methods. The computational methods can address to predict the appropriate residues on the surface of membrane proteins which are involved in the interactions. Such predicted residues can also be used as constraints for the molecular docking processes such as protein-ligand or protein-proteins interactions. Presently available computational tools are able to predict the binding sites on the membrane proteins based on two approaches; (1) sequence based, and (2) structure based.

Sequence based approaches are applied only when no structural information is available and even no template structure is available. Structure based methods provide additional information such as solvent accessibility and residue proximity in the three dimensional orientation of the molecules. Structure based methods relies on the scoring functions, ANNs and SVMs that were trained on various properties for the efficient prediction of binding sites. But the problem lies with the membrane proteins especially with the intra membrane regions. The above said methods are well works with non-membranous proteins as they are trained on more experimental structures available. This situation now can demand the separate predictors that are trained on the specific class of membrane proteins. Random forest prediction methods came into existence that made for individual surface residues which then are averaged above a local surface region and a final prediction set is prepared.

MOLECULAR DOCKING OF MEMBRANE PROTEINS

The final great challenge is, finding the perfect intermolecular interactions with protein partners and small molecules. If the above steps are followed stringently, then this task will be facile and the result will be veracious (Deepshikha, 2002). There will be bit variations in the parameters to be chosen while setting a docking process for membrane protein. First, the site of interaction, nothing but the binding site and its location whether it is present in water or lipid environment. The interaction will be completely different as the hydrophillicity and hydrophobicity greatly alter the interaction mechanisms. Some sites will be spanning both membrane-intracellular and membrane-extracellular regions. Such situations are crucial to specify what kind of environment or force-fields. Membrane protein docking is more trivial as the length of transmembrane regions is not conserved and structural elements occasionally contain bulges and kinks that will challenge the perfect molecular interactions. Another challenge exists with numerous and diverse conformations with near similar energies and RMSD values (Nygaard et al., 2013). Choosing a correct one from thousands of likely conformations is of greatest challenge to proceed for docking. The next provoking challenge exists with multiple loops which are also involved in the generation of kinks in the structure and disturb the local conformations and interrupting the ensemble stabilization (Klco et al., 2005). These investigations and technical considerations may probably provide immense opportunities to understand the basic signalling that are useful to develop pharmacological tools and drugs that modulate function of membrane proteins which could be therapeutic effects. The molecular models of docking complexes provide vast opportunities for the fundamental understanding of signalling processes and help to develop pharmacological agents and drugs that modulate function of membrane proteins.

The docking methods such as DOCK, LibDock, CDOCKER and GOLD with their different scoring functions are used to predict small molecule interactions in the binding sites of membrane proteins both in intra and extra cellular regions. The binding affinity estimations relies on the scoring functions whereas the binding orientations on the docking methods. Regression-based scoring functions are used to estimate the binding affinities using the interaction terms derived from experimental complex struc-

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tural parameters. Regression methods give weights by fitting predicted and experimentally determined affinities to the training set complexes (Bohm & Stahl, 1999). The size and composition of the training set also a major concern to derive the weights. But the drawback is any new molecular scaffolds will be ignored that were absent in the training set complexes (Tame, 1999). Principle based approaches also calculate binding free energies based on the individual contribution of intermolecular interactions between the protein and ligands that are derived from physico-chemical theory and are not based on experimental data. The implicit solvent methods like Possion-Boltzmann and surface generalized born methods are used to calculate the salvation energies (Shoichet et al., 1999).

The flexibility of the integral membrane regions of the protein and their inter molecular interfaces influences the binding affinities and specificities of the ligands and their recognitions. The stabilities and flexibilities of the membrane structures during the molecular recognitions interfere with the binding energies and contact surface areas that could be linked with several functions such as specificity and permissiveness in recognition. All such points complicate the correct orientations of the protein-ligand complexes especially in the instance where the flexible binding sites in the membrane are unknown. Hierarchical approaches solve such problems to an extent by the consideration of the flexibilities of both the ligand and membrane proteins. Such approaches include multistage docking methods and hierarchical energy functions.

CONCLUSION

Membrane proteins have major problems especially construction of theoretical models, binding site prediction and molecular docking that are complicated by the flexible moments and multiple conformations of the huge membrane structures. Application of computational methods to solve the membrane protein structures and their conformational analyses were discussed in detail in a technical manner so as to give a clear glance to the readers. The methods were explained to construct the complete structure of huge membrane protein models in fragment based methods, combining and analysing them and choosing the possible reliable model. A range of molecular dynamics simulations were explained specific to membrane proteins to study the relevant moments and multiple conformations in the membrane regions. Further coarse grained methods are discussed to model, simulate, analyze and to predict probable binding sites. Such a specific analysis helps to predict the function of several membrane receptors in a less time.

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Chapter 30
Molecular Docking Challenges and Limitations

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ABSTRACT

Today, the development of new drugs is a challenging task of science. Researchers already applied molecular docking in the drug design field to simulate ligand- receptor interactions. Docking is a term used for computational schemes that attempt to find the "best" matching between two molecules in a complex formed from constituent molecules. It has a wide range of uses and applications in drug discovery. However, some defects still exist; the accuracy and speed of docking calculation is a challenge to explore and these methods can be enhanced as a solution to docking problem. The molecular docking problem can be defined as follows: Given the atomic coordinates of two molecules, predict their "correct" bound association. The chapter discusses common challenges critical aspects of docking method such as ligand- and receptor- conformation, flexibility and cavity detection, etc. It emphasis to the challenges and inadequacies with the theories behind as well as the examples.

INTRODUCTION

Molecular recognition plays a key role in promoting basic biomolecular experiences such as drug-protein, enzyme-substrate and drug-nucleic acid interactions. Detailed understanding of the general principles that administrate the nature of the interactions (van der Waals, hydrogen bonding, electrostatic) between the ligands and their protein or nucleic acid targets may present a conceptual support for designing the desired potency and specificity of potential drug leads for a given therapeutic target. Practical application of this knowledge requires structural information for the target of interest and a route for evaluating

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candidate ligands. To this end, a variety of computational docking methods are available(Mohan, Gibbs, Cummings, Jaeger, & DesJarlais, 2005)

Among the vast studies, there are several reports that represent molecular docking as worthwhile strategy to predict 3D structures of complexes. Molecular docking can overcome to experimental complications surrounding the structure determination of complexes, and can give valuable structural insights on biomolecular interactions. However, the process of binding a small molecule drug to its protein target is not simple; several factors affect on the interaction between two molecules, so these methods can be improved as a solution to docking problem. Despite great developments and achievements, the widespread application of docking methods, and the accurate and rapid prediction of protein–ligand interactions is still a challenging area to explore and some downsides still exist. The flexibility or mobility of both ligand and target, the effect of the protein environment on the charge dispersal over the ligand, and their interactions with the surrounding water molecules, complicate the quantitative description of the process.

This chapter reviews the literature in the last decades to account for the various abilities, limitations and challenges of the currently widespread applied algorithms which presently characterize this methodology. It introduces some efforts of drug developers for the improvement of algorithms to overcome the shortcomings and to enhance the computations for the incorporation of both ligand and receptor flexibility in the docking process, careful exploration of the ligand conformation within the binding site and improving complementarity between them, refinement and stability evaluation of the final complexes and thus accounting for induced fit. The discussion will further be limited to protein-small ligand complexes, omitting macromolecular complexes; however, much of what is presented here is also valid for that class of complexes.

The chapter handles some critical problems of docking calculation such as the lack of speed, accuracy, protein flexibility and ligand sampling with somewhat aspects of fundamental basic concepts such as movements of side chains and the biased selection of ligands as a result of using ligand-bound protein structures during a docking process or mis-docking ligand. As mentioned before, something of the recent advances provide on the ligand sampling, protein flexibility, and scoring functions as important features in protein-ligand docking. Three types of ligand sampling algorithms will be discussed; shape matching, systematic search, and stochastic algorithms. Computational efficiency for each algorithm is represented according to their main advantages and disadvantages. Covalent docking is another issue of molecular docking which is to be considered in the last part. Authors hope that the recently published papers on covalent docking give insights on biomolecular interactions to the readers.

BACKGROUND

Mutual molecular recognition is the initial point for approximately all processes in biological systems. Today, molecular docking as a very demanding computational and algorithmic tool plays a fundamental and advanced role in structural molecular biology and drug design. These computational tools help us for understanding molecular interactions of two molecules such as protein–protein or protein–ligand that is a key for the understanding of chemical process in diseases and other life issue occurrence. Molecular docking has a wide range of potential uses and can be applied in the following fields of drug discovery:

- Structure–activity studies
- Lead optimization

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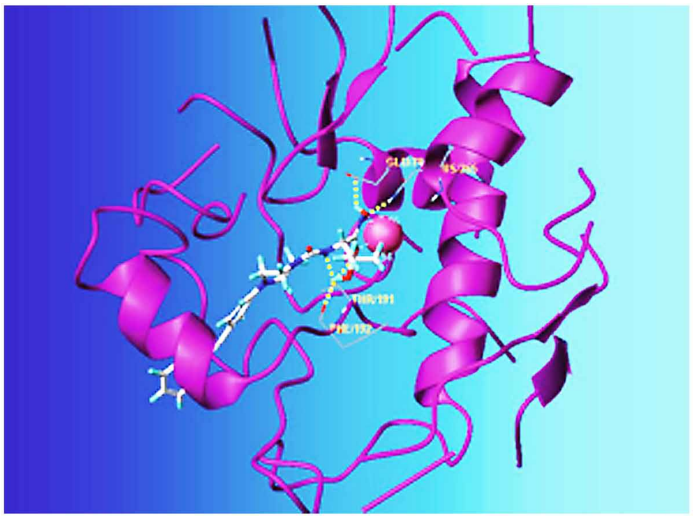
- Finding potential leads by virtual screening
- Providing binding hypotheses to ease predictions for mutagenesis studies
- Helping x-ray crystallography in the fitting of substrates (Figure 1 shows a x-ray crystallography structure as PDB) and inhibitors to electron density
- Chemical mechanism studies
- Combinatorial library design

Molecular docking techniques are designed to aid the development of therapeutic agents via predicting of positioning of a small molecule (ligand) when it is bound to a protein receptor or enzyme, and to find most stable conformation of a ligand and its target macromolecule or receptor. However, in a docking process study, both molecules are flexible and may alter each other's structure as they interact:

- Hundreds to thousands of degrees of freedom
- Total possible conformations are astronomical

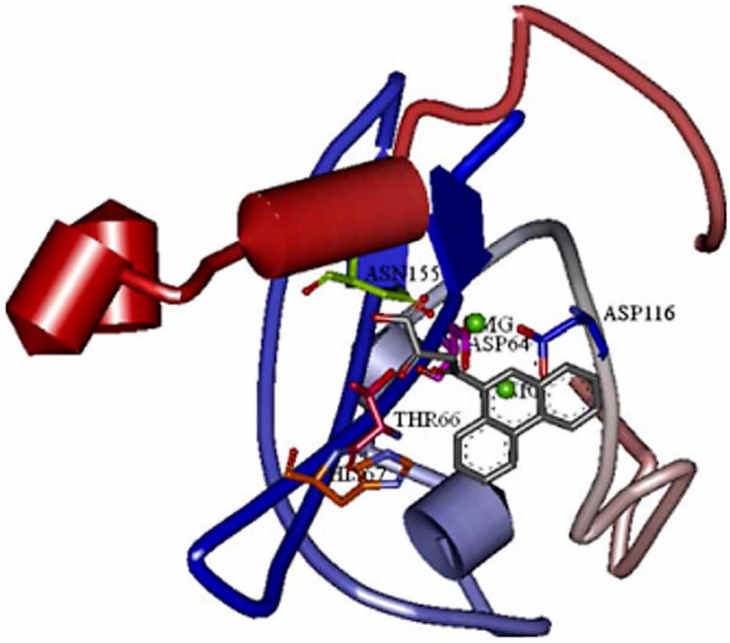
Docking is an important *in silico* method widely used for recognizing drug led by 3D docking ligand to the three dimensional structure of a pre-choose target site continued with the optimization of binding configurations and assessment of binding possibility based on paired molecular interactions (Gozalbes *et al.,* 2008; Kitchen, Decornez, Furr, & Bajorath, 2004). Docking process is a combination of a search algorithm and a scoring function that attempt to find the "best" matching between two molecules: a ligand and a receptor. Now a relatively big and ever growing number of search algorithms and scoring functions are accessible. Reasonably, the best solution would be to associate the ideal searching algorithm with the ideal scoring function. Figure 2. Indicates an example of the best docking corresponding to the ideal scoring function. However, numerous studies have shown that the performance of docking is greatly depend on the specific features of both the binding site and the ligand to be explored, and that

Figure 1. X-ray crystallography structures deposited as PDB file: A typical PDB structure (PDB code: 3PS2)
Ghasemi, Safavi-Sohi, and Barbosa (2012)



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Figure 2. The best docked conformation of one of the most active compounds, (compound 141) in the active site of IN
Ardakani and Ghasemi (2013)



establishing which method would be more appropriate in a precise context is nearly difficult (Sousa, Fernandes, & Ramos, 2006). The molecular docking problem can be defined as follows: Given the atomic coordinates of two molecules, predict their "correct" bound association. In its most general form, no additional data are provided.

The present field mainly adapts to dock small molecules into macromolecule target especially protein molecule and its use is increasing year by year (Pei, Yin, Ma, & Lai, 2014; Sliwoski, Kothiwale, Meiler, & Lowe, 2014; Warren, Do, Kelley, Nicholls, & Warren, 2012; Zheng *et al.*, 2013). The goal to run a docking simulation of ligand–protein is to calculate the main binding mode(s) of a ligand with a protein of known 3D structures of its own components. Ligand molecule uses its action by binding to specific molecular parts of the cell such as modulating biochemical processes in an illness adapting manner. Considering the protein-protein docking and protein-small molecule docking, the following types of problems occurs:

- **Protein-protein docking**
  - Bound docking ("rigid redocking problem"):
  - Unbound docking: side chain flexibility
- **Protein-small molecule docking**
  - Rigid receptor, rigid ligand
  - Rigid receptor, flexible ligand
  - Flexible receptor, flexible ligand

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The docking process is divided into two main stages: first, the correct situation of the ligand at the protein binding-site and, second, the estimation of the ligand affinity by a scoring function. Figure 3 shows more details of the key steps in docking that are used in all protocols commonly. To determine the structure of a biomolecular complex, this technique creates a broad set of conformations of the receptor complex, and then ranks them according to their stability (Morphy & Harris, 2012).

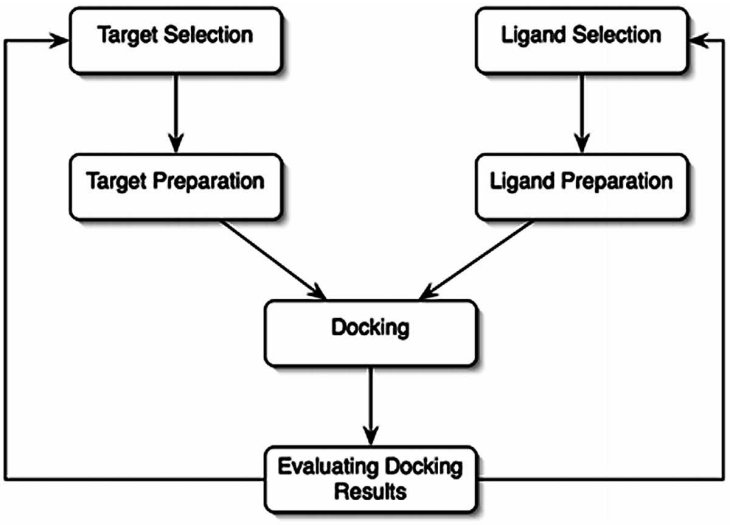
One key area for improvement in docking systems is in the methods for scoring putative protein-ligand interactions. Scoring is central to the docking problem. Development of protein structures for potential drug leads by molecular docking is critically dependent on methods for scoring putative protein-ligand interactions. An ideal function for scoring must exhibit predictive accuracy and high computational speed, and must be tolerant of variations in the relative protein-ligand molecular alignment and conformation.

In comparison to the rapid virtual screening (VS) methods similar to pharmacophore modeling, the performance of docking methods is a trade-off between computational demands and accuracy. The extent and specificity of binding depends on the complementarity between the ligand and its target in terms of shape, polarity, and chemical functionality (Reymond & Awale, 2012). Effective docking methods search high-dimensional spaces well and use a scoring function that properly ranks candidate dockings.

The success of small molecule or ligand roots from the following facts;

1. Matches of their size for most biologically relevant binding sites;
2. Sufficient existing of the structural and functional diversity in small molecules to reach strong and specific binding to most of these binding sites (see Figure 4.); and
3. The pharmacokinetics of ligand molecule can be adjusted while target binding to support efficacy and safety *in vivo* (Bon & Waldmann, 2010; van der Horst *et al.*, 2011).

Figure 3. Common key steps in typical docking protocols
A: *Choose of 3D structures of ligand and the target macromolecule.*
B: *Preparation of two structure considering the docking method being used.*
C: *Analysis of docking results and selecting the binding modes with the best scores.*



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Figure 4a. Compound docked in the binding site of target factor Xa

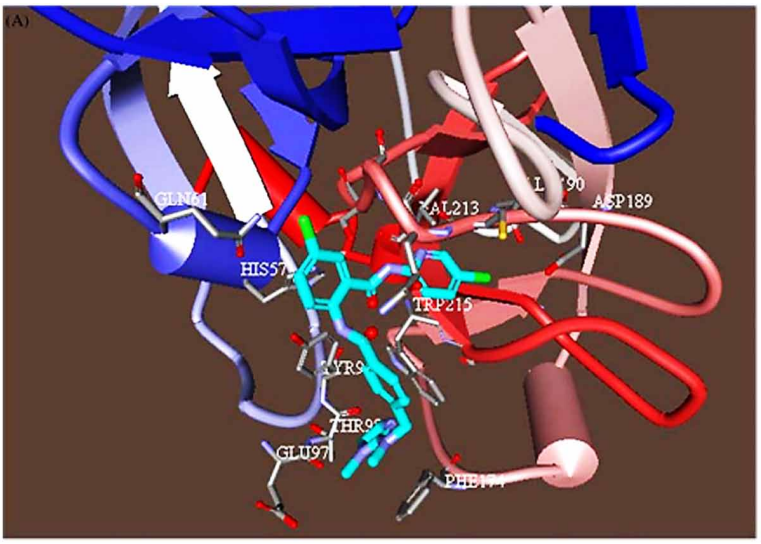
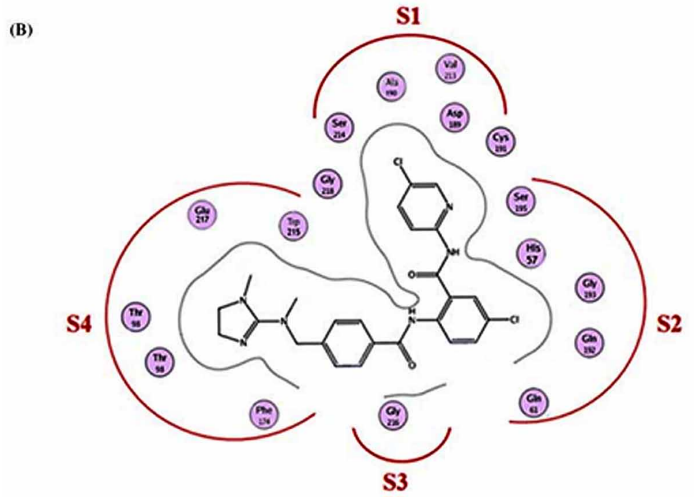


Figure 4b. The residues and pockets of the active site of factor Xa
Ghasemi and Hooshmand (2013)



Docking method is simple and has a variety of applications but some difficulties limit its ability. Over the last decade a wide effort has been directed toward developing efficient docking methods and for the identification of lead compounds. Considerable progress has been made in the computational prediction of ligand-target binding modes. A number of review articles in this emerging area of research have been recently published (Mohan et al., 2005). Some of the problems of docking calculation have been considered in the present text and a number of problem solutions considering some efforts have been introduced.

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COMMON DOCKING COMPLEXITY

For docking method, the equipment necessity and information of the structural aspects and molecular frameworks of active compounds is low (Alonso, Bliznyuk, & Gready, 2006; Chen, 2015), but its ability may have limited to the accounting of target flexibility (Hou *et al.,* 2013) and certain physicochemical features are vital for drug binding. These limitations arise from insufficient sampling of target conformational space, the use of gas-phase algorithms and parameters in scoring, poor modeling of solvation and entropic effects, and the use of simplified cut-off distances for non-bonded interactions. Careful calculation shows that accuracy is a major problem with docking studies, unless the docking is not approached with precision (Chen, 2015).

The complexity of computational docking increases in the following order:

1. Rigid body docking, where both the receptor and small molecule are treated as rigid.
2. Flexible ligand docking, where the receptor is held rigid, but the ligand is treated as flexible; and
3. Flexible docking, where both receptor and ligand flexibility is considered.

Thus, the most regularly used docking algorithms use the rigid receptor/flexible ligand model (Mohan et al., 2005)

Several factors influence the process of binding a small molecule to a target protein and make it quite complex; for example, the motion or flexibility of the small molecule drugs and receptor which affect the possible interactions solvent-receptor-ligand-solvent present depends on the molecular distribution of charges. There are common problems such as incorrect binding site of the target protein, the screening use of an improper small-molecule database, the selection of docking pose, and high dock score (binding affinity) but failure in molecular dynamics (MD) simulation, deficiency of clarity whether the compound is an inhibitor or agonist, or the docking results are unreliable with bioassays (Morphy & Harris, 2012). These problems in the elucidation of docking should be considered with caution and concern before performing docking (Chen, 2015).

The context covers joint critical aspects in all key steps with major emphasis to the general strengths and shortcomings. Enough theory behind these challenges is provided by avoiding many unnecessary equations, and details of calculation algorithms. Some literature within the last decades reviews to account the various capabilities, limitations and challenges of the currently widespread applied algorithms that presently characterize this methodology. Some efforts have been made by drug developers for improving algorithms by examples of its applications in drug design to overcome such shortcomings and computational enhancing for incorporation of both ligand and receptor flexibility in the docking process, extensive exploration of the ligand conformation within the binding site and improving complementarity between them (Figure 4 indicates the binding site of a case study and an active site in the same target), refinement and stability evaluation of the final complexes and thus accounting for induced fit. Discussion will further be limited to protein-small ligand complexes, omitting macromolecular complexes; however, much of what is presented here is also valid for that class of complexes. Future directions are included in the protein flexibility, ligand sampling, and scoring functions as important features in protein-ligand docking.

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LIGAND CONFORMATION AND SAMPLING

Ligand - protein interaction complexes are the most important structures to get information about the mechanism of action of the chemicals compounds. A ligand molecule that can bind strongly to the target receptor should have energetically favorable interactions with the receptor with an appropriate relative geometry. In docking simulation, the problem of finding such geometry between ligand and target molecules is too difficult to be accomplished only by computational methods. To find a likely stable geometry and conformation, the ligand molecule is subjected to a series of interactive three-dimensional manipulations (rotation, translation, and bond rotation) inside the ligand binding site of the protein on the 3D-computer graphics display.

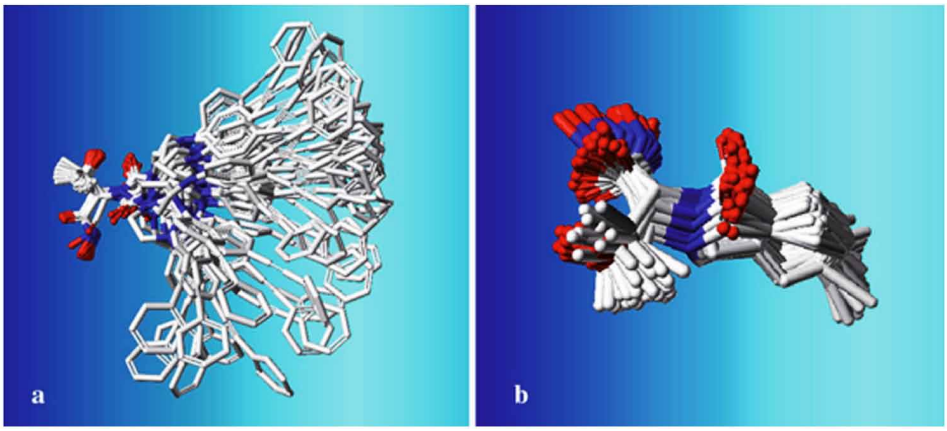
Ligand flexibility is one feature of docking simulation process. Small ligand-oriented docking schemes are either semiflexible or flexible. Small ligands are often highly flexible, with a broad range of populations, adapting their surface to optimally complement the receptor pocket. Therefore, one of the main features of drug docking schemes is enhanced ligand flexibility (Halperin, Ma, Wolfson, & Nussinov, 2002). Simulating ligand flexibility is also a computationally expensive process. Lomber and Shoichet reported that the number of possible ligand conformations increases in proportion to the power of the number of rotatable bonds (Lorber & Shoichet, 1998). They calculate that for an organic molecule with ten rotatable bonds, the number of possible conformations is 59049, if only three minima are considered per bond. However, allowing six minima per bond yields 3.48×10^9 conformations. To address the problem, one approach involves Monte Carlo simulation and simulated annealing to sample the ligand flexibility. In Goodsell et al., the binding site is rigid, whereas in Stoddard and Koshland some binding site flexibility is allowed (Halperin et al., 2002).

Rigid docking calculations are chosen when time is critical, i.e., when a large number of compounds are to be docked. Nevertheless, flexible docking methods are quite required for refinement and optimizing of poses found in an initial rigid docking process. With the development of computational resources and their efficiency, flexible docking approaches are commonly suitable. Three general algorithms are planned to give ligand flexibility: systematic methods; random or stochastic methods; and simulation methods (Sousa et al., 2006). Systematic search or systematic enumerations of conformations, and stochastic algorithms (such as genetic algorithms and Monte Carlo search method with Metropolis criterion (MCM)), shape matching and MD simulations (Figure 5 represents a typical MD calculation) are the most popular approaches for the improvement of efficiency. Systematic algorithms include ligand flexibility through a full search of a molecule's degrees of freedom. In this method, the current state of the system determines the next state. Starting from the same exact state and same set of parameters, systematic methods will yield exactly the same final state. Systematic methods can be categorized into (1) fragmentation algorithms and (2) exhaustive search algorithms (Sliwoski *et al.,* 2014).

A MD protocol was defined for docking small flexible ligands to flexible targets in water by Mangoni *et al.* (1999). They disjointed the center of mass motion of ligand from its inside and rotational motions (Mangoni, Roccatano, & Di Nola, 1999). Independent control of the different motions was conducted to permit flexible or rigid ligand and/or receptor. ROSETTALIGAND uses a knowledge-based scoring procedure with a Monte Carlo-based energy minimization scheme that reduces the number of conformations that must be sampled while providing a more rapid scoring system than offered through molecular mechanics force fields. ROSETTALIGAND through a Monte Carlo-based sampling of torsional angles includes ligand flexibility and side-chain during a high-resolution refinement stage and total torsion angles of ligand and protein are optimized (Sliwoski *et al*., 2014).

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Figure 5. Resulting from MD simulations to the most active compound, with common atom fit (a) and cluster alignment method (b)
Ghasemi et al. (2012)



Molecular flexibility in genetic algorithms (GA) was achieved through recombination of parental conformations with child conformations and the "fittest" or best scoring conformations was retained for another round of recombination. Genetic Optimization for Ligand Docking (GOLD) discovers full ligand flexibility with limited target flexibility by a GA (Jones, Willett, Glen, Leach, & Taylor, 1997).

Docking tools samples conformations of small molecules in protein binding sites; scoring functions are used to judge which of these conformations best matches the protein binding site. Scoring functions that predict the possibility of a compound being a ligand, mainly derived from three key methods based on their characteristics:

- Knowledge based scoring methods
- Force-field or physics based scoring methods
- Regression or empirical based methods

The main interest aspect of empirical scoring functions is the typically easy computation of the numerous terms. The key disadvantage of these methods is their dependency on the experimental data set used in the parameterization process (Leach, Shoichet, & Peishoff, 2006; Sousa et al., 2006).

Venkatachalam *et al*. have described a shape-based method, LigandFit, for accurately docking ligands into protein active sites. In their study a grid-based method for assessing protein–ligand interaction energies have been used for minimizing candidate poses in the context of the active site. They reported combination of LigandFit, with LigScore, as an internally scoring function, to the thymidine kinase receptor yields very good hit rates for a ligand pool seeded with known actives (Venkatachalam, Jiang, Oldfield, & Waldman, 2003).

Another problem of a ligand during a docking process is the biased selection of ligands as an outcome of using ligand-bound protein structures that was undoubtedly revealed in a series of cross-docking analyses. Displacement of particular portions of the protein backbone, movements of side chains, and mobility of metal atoms found within the active site produce most failure (see Figure 6 shows an active site in a case study). It was observed that the movements of side chains were usually linked to those of

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the backbone Ca atoms and, as a result, it was essential to consider more than sidechain flexibility to avoid mis-docking of ligands.

Therefore, careful preparation of the structure of the receptor before the docking process is very important. While structures of ligand-bound protein may offer the highest enrichments, the final consequences might be biased towards certain types of ligands.

Erickson *et al.* have evaluated the effect of some key factors on docking accuracy with CDDOCKER, DOCK, FlexX, GOLD programs. In particular, they have studied the significance of ligand flexibility. Their studies showed that the accuracy of docking considerably decreased for ligands with 8 or more rotatable bonds: DOCK (14%); FlexX (28%); GOLD (19%); CDOCKER (71%). This indicates that the capacity to differentiate between correct or incorrect conformations may decrease for the most public docking programs for ligands with large numbers of probable low-energy conformations (Erickson, Jalaie, Robertson, Lewis, & Vieth, 2004).

RECEPTOR CONFORMATION AND FLEXIBILITY

The 3D structure of both ligand and protein are essential for the application of docking process. The 3D structure of a target obtained by NMR technique or X-ray crystallography (Clore & Gronenborn, 1991; Siegal, Van Duynhoven, & Baldus, 1999) is the best starting point for docking (Sliwoski *et al.*, 2014). Docking algorithms can search the enormous conformational space of small molecule in a reasonable cost and short time. While several ligand conformational structures may be relatively easy to predict,

Figure 6. Active site of the CC-chemokine receptor 5 (CCR5)
Ghasemi and Nouri (2013)



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the lowest energy conformation obtained may not match that of the bound ligand. Compared to ligand, the structures of protein is a bigger challenge. While X-ray and NMR analysis are currently routine, in some cases intrinsic difficulties exist in the planning high-throughput process. The structure of many targeted proteins in drug design is not determined by above methods experimentally and, therefore, docking studies cannot be performed straightforward. In some cases, the structure of a closely related protein homolog is predicted through computational techniques.

Other significant drawbacks for docking process is the lack, or poor flexibility of the protein, which is not allowed to adjust its conformation during ligand binding (Sgrignani *et al*., 2009). This problem often ignored in great virtual screening (VS) calculations because of the high computational cost, but it can be managed by more focusing of docking process, and the consistency of the predicted poses can be improved (Lexa & Carlson, 2012).

Variations of existing docking program mostly treat the receptor as (more or less) rigid which is unlike of the biological facts and the ligand as flexible (Leach et al., 2006). Induced-fit docking and ensemble generated from MD simulation snapshots have been used for inducing receptor flexibility. Induced-fit algorithms agree small overlap between the ligand and receptor along with side chain movements, subsequent in elasticity. Another way to incorporate receptor flexibility in docking algorithms, known as ensemble-based screening is applied multiple fix receptor conformations (Sliwoski *et al.*, 2014). Other methods such as umbrella sampling, metadynamics, accelerated MD, etc. are used as sampling method (Sinko, Lindert, & McCammon, 2013).

ENTROPY IN BIOMOLECULAR INTERACTIONS

Binding affinity is a function of the free energy of binding which is composed of entropy and enthalpy terms dependent on the properties of an ensemble of structures. In a computational manner to scoring drug affinity, entropy of fixation can be expressed in terms of the number of ways in which a rotatable bond can be incorporated, $\Delta S = RTlnW$. If we assume that there are 3 orientations per rotatable bond then $\Delta G = RTln\ (3^N) = \sim N$ kcals/mole. Quantitation of these effects requires temperature dependent measurements of the free energy of complex formation.

The processes involving ligand binding are extremely complicated, both ligand and protein are flexible molecules, and the energy inventory between the bound and unbound states must be considered in aqueous solution. The most common handling of receptor flexibility in structure-based drug design is to ignore it and to design ligands to bind to more rigid regions of the receptor (Morton & Matthews, 1995). This has the advantage that the entropic costs of immobilising a receptor on binding a ligand are minimized.

There is an entropic cost when two molecules associate in any biomolecular interaction process. The entropic cost is a result of degrees of freedom of motion that lost when two molecules are strictly constrained within a complex. If the binding process involves capturing a flexible molecule, whose internal rotations about single bonds must also be restricted, the consequence is a further adverse entropic penalty that results in a reduction in binding constant ***K***, according to the classical relationships

$\Delta G = \Delta H - T\Delta S$ and $\Delta G = -RTlnK$.

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If we are to begin to predict binding constants for bimolecular associations in a manner that might develop useful rules for understanding the forces that drive molecular recognition,' then we need to be able to semiquantitate the adverse cost in free energy of generating conformational order from associations of flexible molecules(Searle & Williams, 1992).

Approximate methods can be used to estimate the loss of configurational entropy of the ligand by counting the number of torsional angles that have become restricted upon binding to the protein, and if desired, this entropy loss can be subtracted from the total energy change(van den Berg et al., 1995) (Burgen, Roberts, & Feeney, 1975).

The estimation of the entropic cost is difficult for several reasons.

- The entropies of molecules in solution are subject to uncertainties.
- The amount of residual motion present in a complex formed by association in solution is also uncertain.
- This amount of residual motion in a complex may vary as a function of the nature and extent of the intermolecular forces (enthalpy term) involved in binding.

Moreover, the issue is complicated by the fact that the accounting of the balance of entropy changes may be done in different ways. For example, it may be considered that the degrees of vibrational freedom associated with residual motion in the complex are translational and rotational entropy of the separate components which are not lost upon association. Otherwise, this vibrational freedom can be regarded as a benefit of the association, appearing after the interacting components have lost all of their translational and rotational entropy (Searle & Williams, 1992).

STRUCTURAL WATER MOLECULES

The handling of structural water molecules is a particular problem in molecular docking. Recently, structural water molecules in ligand protein recognition have intensely been studied and its importance is a well-known topic. However, handling the difficulty of structural water molecule in docking designs is still problematic and different guidelines have been suggested for managing water molecules during docking (Sgrignani, Novati, Colombo, & Grazioso, 2015). Now, some methodologies are applied to tackle this problem including: penalizing the existence of water molecules, awarding rational situation of structural water molecules in an active location or cavity and no amendment for water. Single water molecules in the interface may be particularly important in small ligand docking, mediating hydrogen bonds. The water solvent is described by a soft sphere Langevin dipole model, with discrete dipoles that interact with the electric field of the protein but subject to random thermal fluctuations that reduce the effective electric field at the dipole itself. van der Waals and field-dependent hydrophobic terms are also included(Halperin et al., 2002).

A number of currently available programs suffer the lack of satisfying to account for structural water molecules which can be either displaced in ligand binding or mediate protein-ligand interactions. Some of research aims is data generation to enable the worldwide work of evolving scoring functions in docking programs to account for contributing of structural water molecules. This is done by validating the performance of docking using a simple model system (such as cytochrome C peroxidase (CCP) W191G) containing four well-arranged structural water molecules which are known to either interact with a li-

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gand or to be moved upon ligand binding. The goal of working on molecular dynamics simulations of Sotriffer's group is estimation the enthalpic and entropic influences of the structural water molecules in CCP W191G to ligand binding affinity (Z. Li & Lazaridis, 2003; Olano & Rick, 2004). In a similar study on molecule docking in the framework of structural water molecules, the performance was certified by the following methods:

- Comparison of the binding modes with the docked ligands and the crystal structures,
- Comparing docking scores with the ligands in the different situations,
- Improving of the ligands from a database of decoys
- predicting new ligands from the decoy database (Wahlström, 2009).

The GOLD docking program lets water molecules to be "changed on and off" with a penalty for enabled waters thus preferring water movement by working with the first approach *(*Verdonk *et al*., 2005). Sgrignani *et al* in study of boronic acid have reported, GOLD 5.2 provides a correct computational process for management water molecules in binding sites, permitting to alteration water placing during docking calculations (Sgrignani *et al.*, 2015). The FlexX program puts the second tactic by awarding interactions of the ligand with the water molecule and penalizing for unoccupied interaction sites at the water molecule (Kramer, Rarey, & Lengauer, 1999). In an extension to DOCK 3.5, interactions of structural water molecules with a ligand were scored in similar mode as interactions with protein atoms (Huang & Shoichet, 2008). For the entropically disapproving fixation of water molecules in the active site, no penalties were added.

Recent surveys pay attention more carefully to account selected *water molecules* in docking calculations (Roberts & Mancera, 2008; Weill, Therrien, Campagna-Slater, & Moitessier, 2014). These reports show the undiscriminating inclusion of all the water molecules placed in or around the binding site can lead to a noticeable decrease of the predictive performances of the docking simulation process (Kumar & Zhang, 2013). Sgrignani *et al*. have evaluated the significance of the protein flexibility and catalytic site hydration during docking simulations in order to tune up a robust covalent docking practice for boronic acids. They set out to identify the solvent molecules more often present in the Amp/ boronic acid crystal structures, for identifying the minimum number of water molecules to provide accurate results. Their results show a reasonable cooperation between computational speed and structural accuracy (Sgrignani *et al*., 2015).

This difficulty of predicting and scoring the above parameters, binding modes unambiguously generates false negative and positive scoring compounds as it has been made obvious previously. One example was Warren *et al.* who docked 1303 known ligands into 7 receptors using 10 different docking programs and compared the docking poses to the structurally determined binding modes. The result for the different algorithms performance ranged from 0% to 90% satisfactorily predicted docking modes (Wong *et al*., 2006).

CAVITY DETECTION

A variety of algorithms have been proposed with the aim to identify cavity detection. The vast majority of cavity detection algorithms have been developed to treat static structures like crystal structures of proteins available in the protein data bank (PDB). However, this represents a serious limitation to ac-

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count for the intrinsic plasticity of the binding pocket. Protein dynamics act on a multitude of aspects in protein function. For instance, side chain flipping or domain motions can obstruct or free internal cavities or channels that allow migration of ligands and reshape the binding sites (Bidon-Chanal et al., 2006; Carrillo & Orozco, 2008; Spyrakis et al., 2011). In turn, these findings raise challenging questions about the impact of protein flexibility on the topological features of cavities and their binding properties.

Few works have tried to account for the dynamical behavior of proteins in the identification of binding cavities and tunnels. The interplay between protein dynamics and ligand migration pathways can be characterized by tools that rely on prior molecular dynamics (MDs) simulations and further post-processing of the trajectory. VOIDOO (Kleywegt & Jones, 1994) allows internal cavity and volume calculations, but it is rather time consuming and its use is not straightforward. CAVER (Beneš et al., 2010) is a PyMOL plugin that allows internal channel detection on MD trajectories. CAVER was improved to a software called MOLE using computational geometry principles instead of grid-based calculations (Petřek, Košinová, Koča, & Otyepka, 2007). Wolfson and coworkers have proposed a method called MolAxis for detection of channels from the interior of the protein to the bulk solvent (Yaffe, Fishelovitch, Wolfson, Halperin, & Nussinov, 2008). These methods are designed to detect channels on static structures or conformational ensembles of the protein. MDpocket is another generic pocket detection program. The aim of MDpocket is to identify and characterize binding sites and channels that might be transiently formed in the protein from the analysis of conformational ensembles generated by MDs or other sources. The core of this program is the recently published open-source platform Fpocket (Le Guilloux, Schmidtke, & Tuffery, 2009), which is a very fast geometry-based cavity detection algorithm.

Although many softwares and algorithms to discern different types of cavity are introduced and guidelines for assessing the accuracy and improving the comparability of cavity calculations are given, we need an accurate protocol to find the best cavity.

COVALENT DOCKING

Many methods of target-based drug design have been established for prediction of the protein/ligand complex structure and, they are used to this aim, (Warren *et al.*, 2006; Warren *et al.*, 2012; Weill *et al.,* 2014; Wong *et al.,* 2006). In the docking field, a number of methods mainly focus on the studying of docking between the two molecules through non-covalent interactions (the electrostatics interaction, van der Waals interaction and hydrogen bonding) or non-covalent complexes, while covalent docking is a crucial aspect in the docking field. Only a few methods are accomplished to predict the 3D structure of complexes in which the ligand is covalently bound. However, except non-covalently drugs, there exist other classes of drugs, i.e. the covalent drugs that bind non-covalently to the active site. Covalent ligands are not given significant attention in the traditional drug discovery process due its off-target reactivity besides of toxicity profile. However two FDA-approved covalent inhibitors targeting HCV, namely telaprevir and boceprevir highlighted the focus on covalent inhibitors. *GOLD* and *Autodock* software support feature of covalent docking, but they have major limitations. However, this problem has been addressed in *CovDock* and *CovalentDock* by automatic preparation of ligand files.

In the frame of these considerations, Sgrignani *et al.* have assessed the importance of catalytic site hydration and the protein flexibility during docking calculations for boronic acids with GOLD program. GOLD permits water molecules to be rotated round their three principal axes (to optimize hydrogen bonding) during docking. Moreover, GOLD can switch automatically whether a water molecule should

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be bound or displaced by the ligand during docking. To certify the accurate geometry of the bound ligand, GOLD 5.2 applies the angle-bending potential from the Tripos Force Field, merged in the fitness function during covalent docking process (Sgrignani et al., 2015).

SOLUTIONS AND RECOMMENDATIONS

Some of drug developers focused their attention on the enhancement of computational protocols. These improvements include several methods. A number of target flexibility approximation methods have been used to tackle the risks connected with some searching methods to take a proper ranking of the final complexes in order to improve docking performance. For performance increasing, the molecular dynamics-enhanced docking approach has been used to try to account for the target flexibility (Morphy & Harris, 2012). The most common of approximate methods include:

- Combining docking and MD simulation
- Soft docking
- Side chains flexibility
- Rotamer-based flexible docking (conformation of binding-site residues are tested by rotamer libraries as backbone-dependent or backbone-independent)
- Molecular relaxation
- Joined protein grid docking (several structures and conformations are combined into an energy-weighted or geometry-weighted average depiction)
- Protein ensemble docking
- United description of receptor docking (multiple structures and conformations are overlaid and an average structure is made from conserved features)
- Conformational sampling of receptor side-chain
- Conformational sampling of combined ligand, and
- Receptor backbone energy minimization

More details on the some popular methods of the above list have been presented in the next sections.

Currently, *in silico* structure-based methods have lower throughput and poor consideration of solvation and conformational flexibility. Then, ligand-based virtual screening is considered as more robust hit generation for multi-target drugs in future development of improving of the speed and fidelity of docking-based methods. Conformational flexibility in both ligand and protein can adopt different conformations in binding the same ligand (Wong *et al.*, 2006) and the same ligand can bind to different targets in very different conformations (Smid *et al.,* 2005).

The development of search algorithms for better and efficiently sampling from conformational space of the protein–ligand and inclusion of solvation and rotational entropy contributions in the scoring function are attempts to improve the level of success of docking (Sousa *et al.,* 2006).

There are three key properties in an ideal scoring function:

1. Accuracy in predicting binding affinities
2. Speed
3. Tolerance to inaccurate ligand pose.

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The first two are obvious, but the end requires some discussion. A geometric search engine attempting to generate suitable poses for a ligand given a protein binding site will create the 'correct' pose within some tolerance with some probability within some amount of time. As the tolerance increases, so does the probability of generating a good enough pose quickly. So, the degree to which a scoring function is able to compute an accurate binding affinity given an inaccurate pose will have a significant impact on its utility in such a system.

The deficiencies in the scoring function are the main limiting factor. Scoring functions in docking program routinely make some assumptions and simplifications to let a more computationally efficient assessment of ligand affinity, but logically at the cost of accuracy. (Sousa *et al.,* 2006). Unfortunately, current scoring functions achieve predicting binding free energies poorly. Improved scoring functions would increase the capability to distinguish binders from non-binders. However, the enhancement of scoring functions has been stuck by the lack of a hard formalism for obtaining binding free energies from molecular docking.

COMBINED DOCKING AND MD SIMULATION

In recent years in order to improve docking performance, and have a reliable model of the complex ligand-receptor docking methods have been joined with MD simulations. Docking is applied to search for conformations of the ligands, and in the long run contains the screening of giant libraries of drug-like compounds in a short period of time. The problem is that the conformation of the receptor is not permitted to change in the binding process. Here, MD is a tool that can augment some flexibility to the receptor, ligand allow some modification upon the binding experience. Different procedures have been established for the MD insertion before or after the docking process. Multiple conformations of the receptor can be produced during the receptor structure preparation and then subjected to docking as an ensemble (Alonso *et al.*, 2006). MD simulations can help us to refine the resulting docking complexes. The dynamics can augment some flexibility to the ligand and receptor, refine their assembly and improve the intermolecular interactions between two molecules. Furthermore, the stability of the complex structure during a simulation path shows that the complex is true and it was appropriately docked. The interaction of the solvent with the complex is another significant feature of MD running after docking that the solvent molecules influence on the stability of the ligand's docked pose. MD simulations can be used to improve a docking procedure for the most of docking algorithms do not consider the contribution of the mobility of the receptor or the interactions with the molecules in the explicit solvent (Carlson, Masukawa, & McCammon, 1999).

SOFT DOCKING

Soft docking is a simpler procedure for dealing with protein flexibility in an indirect way. Despite handling the receptor as a rigid object, the repulsive expressions of the Lennard-Jones potential can be diminished by making a *soft* interaction. Thus, this method involve allowing the small molecular drug to partially penetrate the binding site surface of the protein in order to account for localized changes that could occur in a more flexible environment (Morphy & Harris, 2012).

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Soft docking describes the molecular surface and volume as a "cube representation". This cube representation mentioned implicit conformational variations by way of size/shape complementarity, close packing and, most importantly, liberal steric overlap. Soft docking method containing of two steps, geometric docking and interaction scoring, is able to accommodate conformational charges associated with the molecular complexing process, and to identify the correct complex model or a small number of complex models, which can be further screened visually or based on other experimental results to recognize the correct complex model(Jiang & Kim, 1991). The soft docking concept has evolved primarily toward use in protein-protein docking (Fernández-Recio, Totrov, & Abagyan, 2002; C. H. Li, Ma, Chen, & Wang, 2003; C. H. Li, Ma, Zu Chen, & Wang, 2003; Palma, Krippahl, Wampler, & Moura, 2000; Schneidman-Duhovny et al., 2003) and protein-receptor modeling combined with experimental NMR data (X. Morelli, Czjzek, et al., 2000; X. Morelli, Dolla, et al., 2000; X. J. Morelli, Palma, Guerlesquin, & Rigby, 2001).

SIDE CHAIN FLEXIBILITY

The mobility of some residues mainly those within an enzyme active site can be managed in a different and more comprehensive tactic either during the docking process or after the ligand has been approximately placed. The receptor is generally held rigid in studies in which docking is required to be fast. However, some methods permit explicit treatment of side-chain (and a few selected backbone) torsions, e.g. by Monte Carlo (MC) (Abagyan, Totrov, & Kuznetsov, 1994) or Conformational search (CS)(Leach, 1994) procedures, and more computationally demanding molecular dynamics approaches allow binding site back-bone and side chain mobility to be simulated.

In study of receptor-ligand interactions, commonly two methods to treat side-chain flexibility during docking have been examined:

1. Systematic CS of discrete side-chain rotamers. The number of possible states is defined and therefore partition functions and free energies can be computed.
2. Stochastic MC exploration of a continuous probability distribution of side-chain rotamers.

A set of rotamer libraries can be used to discover the conformational space of selected side chains. Side-chain flexibility increases the computational cost but it lets localized protein movement and improved fit of the ligand in outcomes (Källblad & Dean, 2003).

COMBINED PROTEIN GRID

Some alternative structures of the protein receptor can be combined into a single representation of the ensemble to account for bigger conformational changes that may be critical for the binding process. The averaging can be done over atom coordinates, to generate a final average structure, or over the grid representation of all receptor conformations to produce an average docking grid.

In a similar approach, Wei *et al*. (Wei, Weaver, Ferrari, Matthews, & Shoichet, 2004) used a modified version of the program DOCK to incorporate receptor flexibility during the docking process. In this case, the interactions between a given configuration of the ligand and different flexible parts of the receptor

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were calculated independently. Then, those flexible regions that present the best interaction energies with the ligand were recombined into a final representation of the protein receptor.

The inclusion of protein flexibility during the docking procedure using a united description of the protein with rigid conserved regions and alternative flexible parts can improve the screening process and produce new ''hits'' in a shorter time than simple sequential docking against each protein structure. For obtaining an adequate ranking of the final complexes, the internal energy of the receptor must be taken into account, as high-energy conformations of the protein may lead to unrealistic low-energy positioning of a ligand.

Briefly, docking methods that have combined multiple protein structures, whether from NMR, X-ray complexes, or MD simulations, into a single grid representation of the target molecule have provided better results than grids from single structures. The choice of combination procedure, however, has an even greater impact on the final outcome, with weighted-average protocols providing better results than simple average combination of structures.

FREE ENERGY CALCULATIONS

For a docking process to be successful, it is necessary that both the right conformation of the ligand–receptor complex is predicted, and that the ranking of final structures will be correct. The procedure needs to be able to differentiate among similar conformations of the same system, as well as to predict the relative stability of different complexes.

As most contain empirically fitted parameters, their performance on any particular problem will depend on the set of structures used for the calibration. So far, no scoring function has proven to be reliable for every docking case tested. The main constraint on their improvement rests with the need for speed; when ranking hundreds, if not thousands, of complexes a compromise in accuracy must be made. Knowledge-based functions used in the ranking of molecular interactions may not be general and accurate enough, because of the limited number of interactions that can be inferred from crystal structures and the inadequate description of repulsive forces.

Molecular dynamics simulations present an attractive alternative for structural refinement of the final docked complexes. They incorporate flexibility of both ligand and receptor, improving interactions and enhancing complementarity between them, and thus accounting for induced fit.

Recently developed approaches called MM/PBSA and MM/GBSA improves ligand ranking and, marginally, binding affinity prediction (Yuriev, Agostino, & Ramsland, 2011; Yuriev & Ramsland, 2013). The correlation between MM/GBSA energies and experimentally determined binding affinities was found to degrade with greater variability in the affinity data and/or increased structural faults in the complex structures, either experimental or computational. These findings demonstrate that improving pose prediction methods could improve the computation of binding affinities using implicit solvation methods.

Some of drug developers have focused their attention on the enhancement of computational protocols. These improvements include: incorporation of protein flexibility in the docking process, extensive exploration of the ligand conformation within the binding site, refinement and stability evaluation of the final complexes, and estimation of the binding free energies. The refinement of docked complexes deals with the applications of MD for the optimization and validation of the final complexes.

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SUMMARIZES

This chapter handles some of crucial aspects such as the lack of accuracy, speed, ligand sampling, protein flexibility and basic concepts such as movements of side chains, displacement of particular portions of the protein backbone, and mobility of metal atoms within the active site and the biased selection of ligands as a result of using ligand-bound protein structures during a docking process or mis-docking ligand. Meanwhile, it describes approximate methods such as soft docking and side chains flexibility and protein ensemble docking to tackle the risks associated with some searching methods to obtain an adequate ranking of the final complexes. The solvent effect and the direct contribution of water molecules in protein–ligand interactions, the restricted resolutions of crystallographic receptors, and obviously, protein flexibility, both in terms of intrinsic structural flexibility and in terms of conformational alterations upon ligand binding, are some of the most crucial of docking calculations (Sousa *et al.,* 2006).

Here, briefly three types of ligand sampling algorithms are discussed; shape matching, systematic search, and stochastic algorithms. Computational efficiency for each algorithm represents according to their main advantages and disadvantages.

CONCLUDING REMARKS

Molecular docking is a computational method for predicting the location of ligands in the binding sites of their receptor(s). Ruling principle of molecular docking is essential in drug discovery process. Despite considerable advances and success the widespread application of docking methods, accurate and rapid prediction of protein–ligand interactions is still a challenge area to explore and still holds several hidden weaknesses. The most docking algorithms do not explanation for receptor flexibility or the effect of explicit water molecules, MD simulations can be used to complement and improve a docking protocol. In order to improving docking performance most common of approximate methods including combining docking and MD simulation, soft docking, side chains flexibility, rotamer-based flexible docking, molecular relaxation, joined protein grid docking, protein ensemble docking, conformational sampling of receptor side-chain and conformational sampling of combined ligand are applied.

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Chapter 31
Different Types of Molecular Docking Based on Variations of Interacting Molecules:
Variations of Molecular Docking

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ABSTRACT

Molecular docking plays an important role in drug discovery research by facilitating target identification, target validation, virtual screening for lead identification and lead optimization. Depending upon the nature of the disease of interest, targets can be either protein or DNA while drugs are mostly organic small molecules. Different types of molecular docking techniques like protein-protein or protein-DNA or protein-small molecule or DNA-small molecule are employed for achieving the above mentioned objectives. This chapter provides a clear idea of the position of molecular docking in drug discovery with detailed discussion on different types of molecular docking based on the varieties of interacting partners. Subsequently the authors provide a detailed list of tools that can be used for docking in drug discovery and discus some examples of molecular docking in drug discovery before concluding with a remark on future areas of improvement in molecular docking related to drug discovery.

1. INTRODUCTION

Present day drug discovery faces several major issues like rising cost, high development time and high failure rate at different stages of development. On average it takes 10-12 years and around one billion US dollar to market a drug but less than 5% of the compounds which are selected to undergo the drug-discovery pipeline, only end up being marketable. This high failure rate is associated with every stages of drug development starting from initial screening to final patient trial. Since the marketed drugs are

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only available for revenue generation which has to cover the development cost of all the trials, including the ones which failed at different developmental stages. Thus it is easily understandable that optimizing strategies that save both time and money are of significant interest to the drug-discovery world. It should also be kept in mind that subsequent stages of drug development are also associated with exponential increase of cost. To counteract these issues, computer aided drug development (CADD) has played significant role to reduce cost and time for the last two decades and has established itself as an essential part of modern day drug discovery.

Combined with the above mentioned issues, the ever rising compound library has imposed another challenge in terms of selection of proper compounds which can be used for initial screening. While a few thousand compounds can be screened using standard high throughput screening (HTS) methods, the challenge remains to find these few thousands from the immensely large compound library which houses several tens of thousands of compounds, sometimes in the ranges of hundreds of thousands. CADD plays an important role not only in this aspect but also in two other ways – a) by computationally generating secondary modifications of compounds which saves both time and money in comparison to synthesizing them in real world, b) by analyzing the quantitative structure-activity relationship (QSAR) of the compounds of interest. All the three above mentioned ways, in which CADD plays an important role in drug discovery, require the association of the compound with the target of interest which can be either DNA or protein and the technique which facilitates this association in a computational way is known as 'Molecular Docking'. However the benefit of molecular docking extends beyond this small molecule–DNA/protein interaction as it facilitates the docking of DNA with proteins as well as of proteins with proteins.

The benefit of molecular docking is exhibited at several stages of drug-discovery. Even before the beginning of drug-discovery pipeline, molecular docking facilitates the proper understanding of several potential target molecules by facilitating DNA-protein and protein-protein interactions. Later this technique facilitates virtual screening which enables screening of compound libraries to find the potentially promising ones as well as helps in understanding of the best suitable secondary modifications. Thus it can be clearly envisioned that different types of molecular docking depends on the types of interacting molecules. Keeping in mind the scope of this chapter, further discussion on molecular docking with respect to drug discovery will be limited to different types of molecular docking.

As we have previously discussed, small molecules or ligands, DNA, proteins and short peptides are four different individual components with which molecular docking can be performed. Based on the combinations of participating components, drug discovery can be of several types. In case of protein-ligand docking, molecular docking is performed with a small molecule ligand and a protein receptor. Interaction of 'dorzolamide', used for treatment of glaucoma, with carbonic anhydrase is an example of protein-ligand docking. It should be highlighted at this point that most of the known or developed drugs fall in to this category. Platinum containing cancer drugs interact with DNA and thereby results in cell death. Studies of interactions of such drugs with DNA are facilitated through DNA-ligand type molecular docking. Azacitidine and decitabine containing drugs which are used for treatment of myelodysplastic syndrome and acute myeloid leukemia act through several mechanisms that ultimately inhibit DNA-methyltransferases. Understanding the mechanism of actions of such drugs require proper knowledge of interaction of DNA, it's bases and base analogues with DNA-methyltransferases.DNA-protein docking analyses such interactions. The tyrosine protein kinase BCR-Abl when mutated can become constitutively active and thereby causes uncontrolled target phosphorylation which ultimately causes abnormal cell growth. Imatinib effectively blocks this constitutive BCR-Abl phosphorylation activity by binding to

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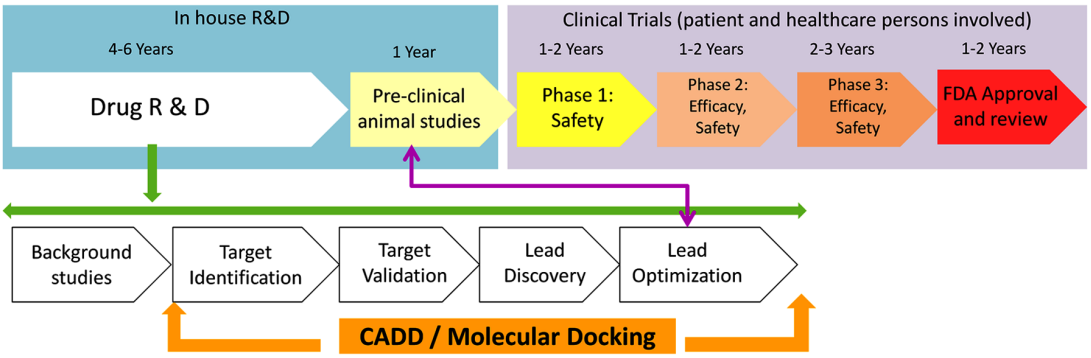
the enzyme which also blocks the binding of target proteins. Effective understanding of imatinib actions require proper understanding of BCR-Abl and its target proteins interactions, both in the form of wild type and mutant enzyme. Protein-protein docking facilitates such studies while the interactions of imatinib with BCR-Abl kinase as well as with the enzyme-target protein complexes can be analyzed through protein-ligand and protein complex-ligand types of docking respectively. This chapter provides a detailed discussion on these several types of molecular docking based on the variations of interacting molecules.

The chapter begins with very brief discussion on basic molecular docking with an introduction to ligand, receptor and inhibitor concept. Then detailed discussions are followed on different types of molecular docking that are mentioned above. Subsequently different tools that can be used to perform molecular docking are listed to aid readers in order to make them free from the tedious work of finding out the right tool to perform their job. Thereafter this chapter discusses on case studies i.e. different known drugs which have been reported to be significantly benefitted from molecular docking. Towards the end an emerging aspect of highly potential alternative therapeutic approach, termed as 'peptidomimetics' is introduced. Finally the chapter concludes with highlighting the present day challenges which are required to overcome in order to make molecular docking and CADD more effective in drug discovery.

2. BACKGROUND

The aim of rational drug discovery is to find a small molecule which when made available within the body, reacts with its target molecule of interest to achieve some desired modification of the target molecule and thereby exert a positive effect toward disease cure. Historically drug discovery was a trial and error type of work. Although several lifesaving drugs were discovered in this way, modern day drug discovery is a much more systematic effort (Figure 1). As shown in Figure 1, present day drug discovery is broadly grouped in to two parts – 1. in-house drug research and development (R&D) and 2. clinical trials. Both of these two parts constitute of several sub parts (Figure 1). Success at the previous sub part only ensures progress towards the next sub part. It is true that several drugs have been develop by this systematic developmental pipeline but hundreds of disease are yet to find a cure even when underlying

Figure 1. Involvement of CADD and molecular docking in the drug discovery pipeline; drug discovery primarily constitutes of two parts – in-house R&D and clinical trials. Virtual screening and QSAR works for lead identification and optimization part of in-house R&D takes the advantage of molecular docking for better management of time and cost of drug discovery.



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molecular reasons are known (Bhattacharya, Das, Dasgupta, & Bagchi, 2014). Thus there is an ever growing need for development of new drugs for un-treatable disease as well as development of more efficient drugs for those diseases with some level of existing treatment. To address these urgent requirements, potential drug molecules are required to be identified which can be sent for clinical trials. In house R&D is responsible for generation of these potential drug molecules. As detailed in Figure 1, the process of generation of these molecules starts with background data analysis for target identification and validation, followed by screening of compound library to find lead molecules and subsequent lead optimization which runs in conjugation with pre-clinical animal model studies.

As discussed in previous sections, drug design is a very time and resource intensive process. Thus there was always a need to apply as much automation as possible. Initially HTS proved to be a significant step forward by allowing automatization of the screening process. However preparing the resources required for HTS along with assay development is another challenge. Hence computers were brought in to this drug design and optimization arena to make the whole scenario faster, cheaper and more streamlined. Figure 1 highlights the role of CADD in this aspect. Molecular docking is an integral part of CADD and has found its application at almost all levels of in house drug R&D (Kapetanovic, 2008; Meng, Zhang, Mezei, & Cui, 2011; Shoichet, McGovern, Wei, & Irwin, 2002). Molecular docking has made it possible to find out suitable target, screen initial compound set to find a handful of lead molecules along with enabling QSAR analysis of optimized lead molecules. However different types of docking are required at different level of drug R&D. The following section provides a detailed discussion on different types of molecular docking, their underlying principles, advantages and fundamental limitations.

3. DIFFERENT TYPES OF DOCKING

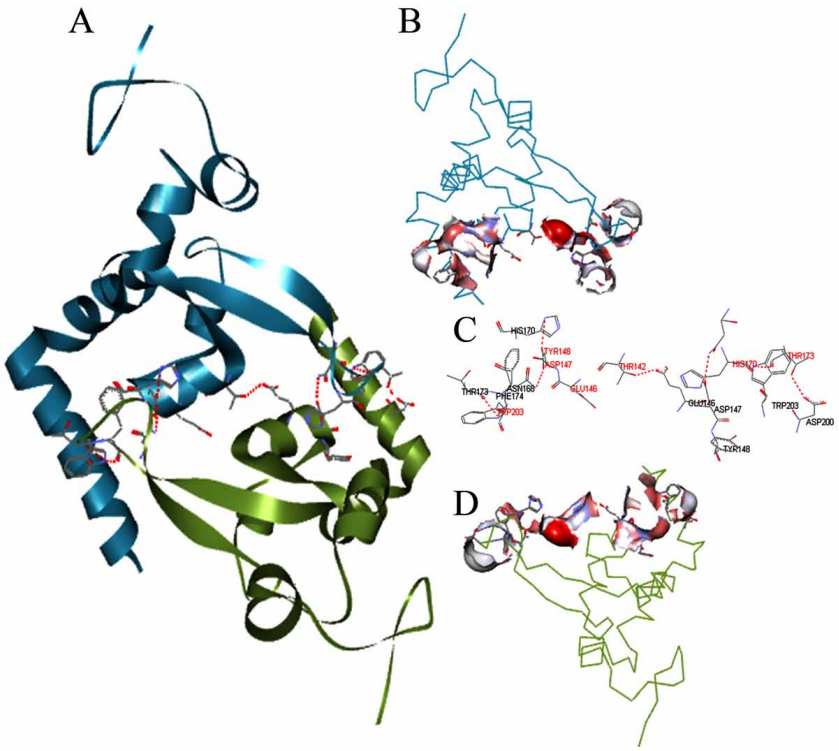
Molecular docking requires two interacting partners. In biological, especially drug discovery context, these two partners can either be proteins or DNA or small organic molecules. Based on the type of two interacting molecules, molecular docking can be of 4 different types which are discussed in details in the following sections.

3.1 Protein-Protein Docking

As the name implies, protein-protein docking is the type of molecular docking in which both the interacting partners are proteins in nature (Figure 2). Proteins in this context are a three dimensional stable conformation of an amino acid chain. Essentially protein-protein docking algorithms do not specifically discriminate between peptides, truncated proteins, protein domain structures and full proteins (Bhattacharya, Das, Ghosh, Dasgupta, & Bagchi, 2014; Das, Bhattacharya, Bagchi, & Dasgupta, 2013) which allows uniformity of tools and algorithms. The main challenge associated with protein-protein docking is the inherent flexibility of proteins and its constituting bonds. However protein-protein docking being the major point of interest across different types of docking, several tools have been designed to take care of these issues. However the speed and output of these tools are essentially a tradeoff between the computing resources and time against the degree of flexibility allowed. Thus it is fairly easy to understand that rigid body protein-protein docking is significantly faster and less resource intensive than flexible docking while the later ensures better quality of output with a time penalty.

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Figure 2. Schematic illustration of protein-protein docking; two protein molecules (one in blue, another in green) were docked with each other (A). The binding surface of blue color protein is shown in figure 2B while 2D shows the binding surface of green color protein. Intermolecular hydrogen bonds are shown in red dotted lines (A and C). Elucidation of contact surface and its properties along with residues of significance often come handy in drug discovery research.



In the drug discovery context protein-protein interaction plays major role in target identification and validation i.e. at the very beginning of drug discovery pipeline (Figure 1). Before the screening of compounds for a potential lead molecule, it is essential to identify a target molecule which in most cases a protein like enzyme, receptor or membrane associated pump or similar other proteins of significant functional interest. Protein-protein docking helps in understanding the various interactions of protein molecules with their interacting partners (Figure 2B-2D). With the increase in protein structure data, protein-protein docking is becoming more and more important in unraveling significant details of bimolecular interactions which are very essential for proper target identification. Not only target identification, protein-protein docking also helps in identification of potential drug binding pocket and elucidation of several characteristics of binding surfaces and interacting residues (S. Grosdidier & Fernández-Recio, 2012; Sable & Jois, 2015).

Several tools that can be used for protein-protein docking with different degree of flexibility are discussed in a later section.

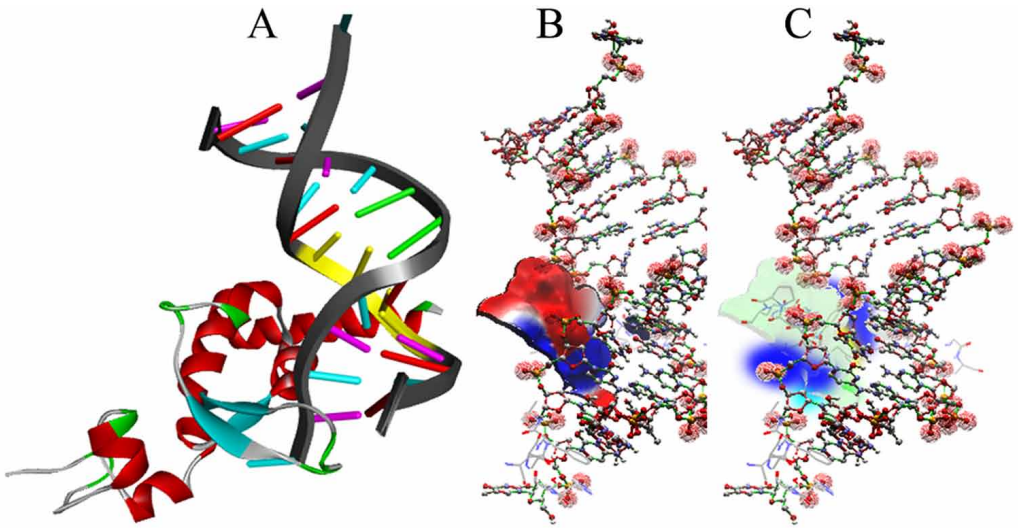
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3.2 Protein-DNA Docking

DNA carries the genetic information of a cell whereas proteins are the molecules of execution. DNA-protein interactions hence are an important hub to regulate the mechanisms of replication, transcription (or gene expression) and recombination. Proteins with helix-turn-helix motif, zinc binding sites, leucine zipper form a large cluster of proteins that can bind DNA. Several transcription factors bind to DNA at promoter regions to regulate the expression of certain genes. Mutation in the promoter region or in the DNA binding region of the transcription factor can result in the improper interaction between these two partners, resulting in improper regulation of the concerned genes. A number of diseases have been associated with mutation in either of the two or in both cases (Heidenreich, Rachakonda, Hemminki, & Kumar, 2014; Songdej & Rao, 2015). However in many cases the underlying molecular mechanism either unknown or poorly understood. Protein – DNA docking can play a significant role in unraveling these unknown issues. With the progress in researches that showed the importance of protein DNA interaction in living cell environment, the research on DNA-protein docking has gained new attention (Gao & Skolnick, 2009; Takeda, Corona, & Guo, 2013; van Dijk & Bonvin, 2008).

As the name implies, in case of protein-DNA docking, there is no receptor-ligand complex since both the interacting partners are bio-macromolecules (Figure 3). However depending upon the nature of algorithm or the requirements of the docking program, sometimes it may be necessary to declare either of the molecules as a receptor and the other as a ligand but theoretically this should not make much difference in to the output. The main challenge associated with protein-DNA docking is the size of the interacting partners and their associated molecular and structural flexibility. DNA has its inherent

Figure 3. Protein-DNA docking: (A) schematic illustration of docking between a protein and a DNA molecule. The region of significance of the DNA molecule is highlighted in yellow. Figure 3B highlights the binding surface of the protein while Figure 3c also shows the energy map of those residues. Identification of DNA interacting residues and their surface properties of a protein are of vital interest from drug discovery point of view while the binding region of the DNA (yellow in Figure 3A) provides the consensus sequence required for proper protein binding.



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flexibility and also there remains a lack of proper information for the particular interacting residues on DNA structures. These have made the situation unfavorable to generate effective docking methods for proper docking of DNA with protein. Computationally it is a huge task to take into account all the rotational and vibrational flexibility of each and every atom and bond during the search for the best docking pose. While some docking algorithms take the advantage of associated information to counteract this issue (Takeda et al., 2013), few other programs only allow partial flexibility (Banitt & Wolfson, 2011). However some docking tools take the advantage of advanced computing architecture to allow greater flexibility and more parameters like explicit addition of water (van Dijk, Visscher, Kastritis, & Bonvin, 2013; Wu, Hong, Takeda, & Guo, 2012).

The structural basis of protein DNA recognition process has been deciphered with X-Ray crystallography, NMR and many other biochemical and biophysical experiments. With the increasing availability of structural data on protein-DNA interaction, it can be expected that molecular docking will play greater role in discovering the underlying mechanisms of disease causing mutations of transcription factors, DNA promoters and other DNA binding proteins. Such knowledge will help to develop new drugs for several diseases.

3.3 DNA-Small Molecule Docking

Molecular interaction of ligand to its targeted macromolecules plays pivotal role in designing sensible therapeutic agents. Being the genetic material, DNA has always been the subject of interest and have been targeted with small molecules or drugs to inhibit replication, transcription, in other words, the central dogma to kill cancerous cells or tumor cells, auto-immuned cells as well as fungal, viral and pathogenic microbial growths (Rohs, Bloch, Sklenar, & Shakked, 2005). DNA is negative in charge distribution and hence it attracts molecules with positive charges (Figure 4). Based on structural scaffolds and orientation flexibility, small molecules bind to either major or minor groove of DNA molecule and therefore interfere with replication and transcriptional machineries which in turn mostly activates the cellular apoptotic pathway (Pandya, Islam, Kumar, Jayaram, & Kumar, 2010). The biological activity of these molecules depend on slow rate of dissociation from DNA complexes which affects intra and intermolecular hydrogen bonds, planar interaction between DNA bases and small molecules and several other van der Waal interactions.

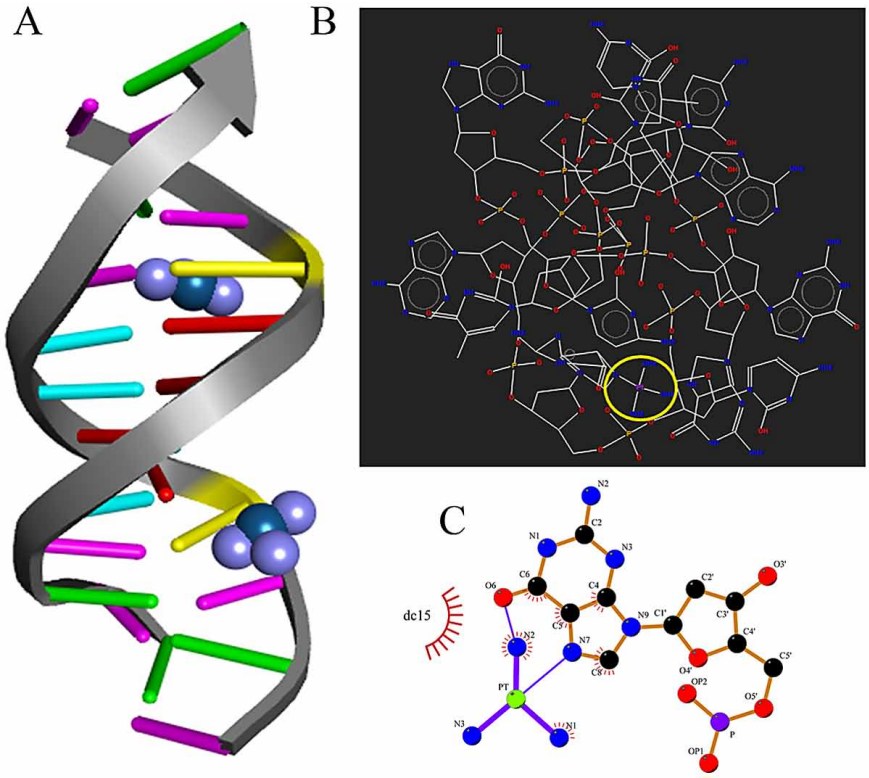
Molecular docking between DNA and small molecule takes in to account the above mentioned fundamental aspects of DNA-small molecule interaction during drug development and optimization. Selection of cation carrier molecules, their orientations (cis-trans), poses, DNA binding groove, binding affinity etc can be analyzed from DNA-small molecule docking (Figure 4B-4C). Similar to Protein-DNA docking, availability of proper tools is also a challenge with DNA-small molecule docking.

3.4 Protein-Small Molecular Docking

Proteome world in a cell depends on the central dogma: sequence, structure and function. The last trail of this pattern requires the knowledge whether a structurally fit protein can interact successfully with its partner proteins. If the former question gets its answer then determining the active sites and residues responsible for interaction play central role to build up the stage to study protein interaction world. To cure diseases, medical chemistry needs drugs or small molecules to be delivered successfully to the ailed system. Their bio-activity, stability and reduced toxicity form the key parameters for proper development

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Figure 4. DNA-small molecule interaction: (A) schematic diagram of two platinum containing small molecules after being docked to a DNA molecule. The small molecules are shown in blue spheres while the interacting guanine residues of the DNA are highlighted in yellow. (B-C) Two different representations of the Ligand map of one the small molecules with DNA. Understanding the interaction map of small molecules with DNA is of prime importance for the development of drugs that target DNA.



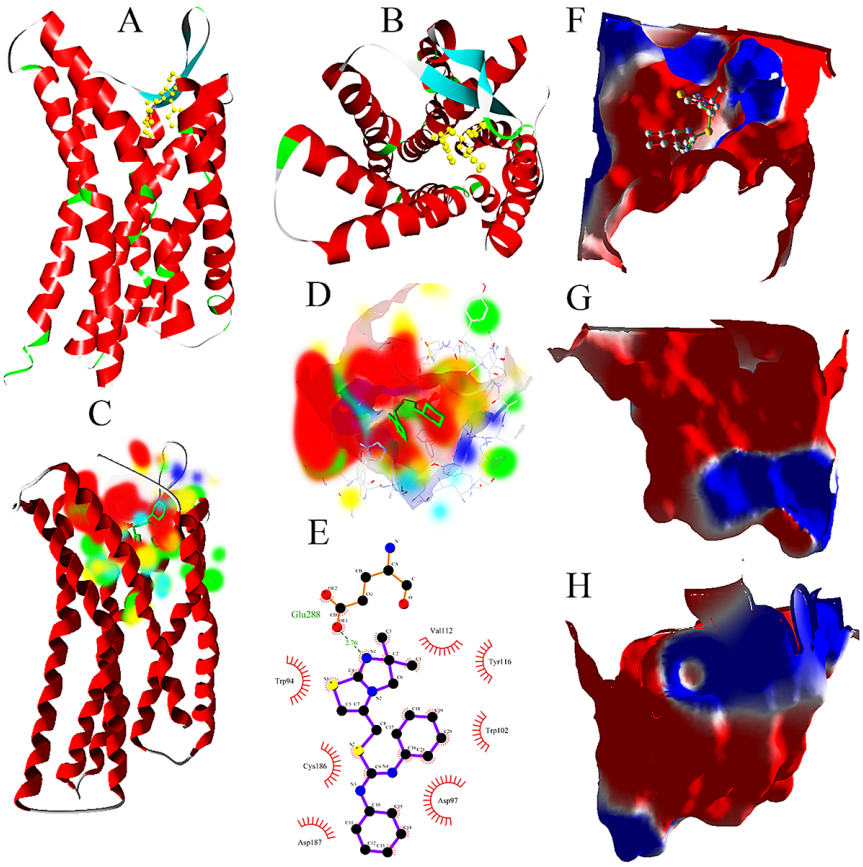
of a lead compound. But at the same time, the mode of interaction of these small molecules with their respective cellular receptor or target protein form the golden stone to pave the path in curing diseases. Not only working as therapeutics, protein small molecule interactions are key factors in enzymatic reactions and signaling cascades.

Protein-small molecule docking takes the center stage of modern day drug discovery as most of drugs available at market are organic small molecule in nature and bind to protein targets like enzymes, cell receptors, membrane pumps etc (Figure 5). This form of docking is associated with virtual screening, lead discovery, QSAR studies and lead optimization. Thus several tools are available to dock proteins with small molecules as well as to analyze different parameters of their interactions (such tools are mentioned in a later section).

In general, protein-small molecule docking is classified into two categories: bound docking where a complex is reproduced based on the template known complex and unbound/ de-novo docking where scoring functions and probability estimation generate the idea to dock the residue to target protein (Taylor, Jewsbury, & Essex, 2002). All small molecules docking program have three principal components: understanding the molecular architecture, generating search algorithms and assigning scoring functions for energy evaluation. While variety of search algorithms have been developed to study protein small

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Figure 5. Example of protein-small molecule docking: (A) an organic small molecule is docked to a multi pass trans-membrane protein (A) side view, (B) top view. The small molecule is shown in yellow ball and stick while the protein is colored according to its secondary structure. C-D. Energy plot of this interaction (C-side view, D-top view). E. Interaction map between this small molecule and the interacting residues of the protein. F-H. Ligand interaction surface of the protein (F) top view, (G-H) two different side views. Deciphering the ligand interacting residues of a protein and characteristics of these important residues have pivotal role in optimization of drug molecules. Also the characteristics of this binding pocket determine the nature of the interacting ligand.



molecule docking, less attention has been given to the relative side chain flexibility of the proteins involved in these interactions (Meiler & Baker, 2006). Side chain conformational changes frequently occur upon small molecule (ligand/drug) binding. But recent studies have come up with plausible consideration to simulate the real world interactions which has brought the protein-small molecule research at its ease. Moreover, ligand flexibility has also been taken into account with the application of Monte Carlo sampling of torsion angles in small molecules. Search algorithms have dealt with the scoring functions majorly, but de-solvation and entropy functions remain the two major problems to solve. Finally an effective benchmark system for the evaluation of the robustness in scoring functions also needs be taken care of (Huang & Zou, 2010).

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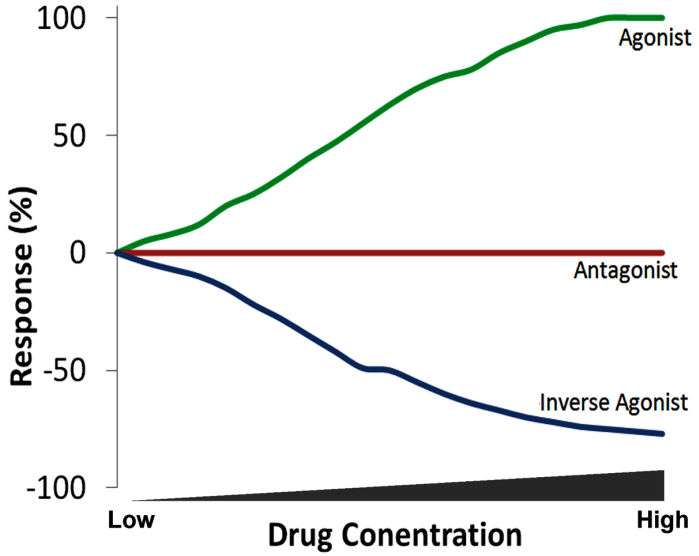
Beyond the computational and algorithmic view of protein-small molecule docking, there lies another physiological view of understanding protein-small molecule docking. Based on the nature and outcome of an interaction between a protein and a small molecule, small molecule ligands can be broadly classified in to two groups – agonists and antagonists.

Agonists are nature identical small molecules that upon binding to its receptor, activates it while inverse agonists exert opposite effect (Figure 6). For example a drug meant for vasodilation would be acting as an agonist if upon administration it dilates blood vessels while an inverse agonist will cause vasoconstriction. Both agonists and inverse agonists bind to the same receptor binding site and its effects can be either full or partial.

Antagonists are small molecules that attenuate the effect of an agonist (Figure 6). They can be either competitive or non-competitive in nature and in each case can be reversible or irreversible in nature. Competitive antagonists bind to the same agonist binding site of a receptor while non-competitive ones bind to some other allosteric site of the receptor. Reversible antagonists bind non-covalently and can be out-competed in a concentration dependent manner while non-reversible antagonists form covalent bonds and often exert permanent effects on the receptor. For example Naloxone acts as an opiate antagonist by binding to the same receptor binding site of opiate agonists.

Information available on drug agonists and antagonists have been widely used by researchers for different purposes (Kim, Gao, Jeong, & Jacobson, 2006; Kortagere et al., 2010; Lu et al., 2010; Ng et al., 2014). Not only basic research usage, drug agonist and antagonist information are of equally high importance in the drug discovery scenario. During screening of molecules as well as during lead development and optimization, agonist and antagonist information (pose, rotamers, binding affinity, nature of interaction etc.) are taken in to consideration to select and optimize a potential drug molecule base on its intended purpose.

Figure 6. Relationship between physiological effects of agonists, antagonists and inverse agonists in a concentration dependent manner; while agonists help in activation of its receptor, inverse agonists exert opposite effects. Antagonists attenuates the effect of any agonist.



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4. TOOLS FOR DIFFERENT TYPES OF MOLECULAR DOCKING

To facilitate molecular docking, several tools have been developed by researchers and companies over the years. Table 1 provides a detailed list of such tools which have proven to be reliable and have been popular across wide range of researchers which include basic science scientists as well as researchers or experts specifically involved in drug discovery projects. It should be noted that not all tools facilitate all the previously mentioned types of molecular docking. Table 1 also highlights the type of docking that can be performed by each tool. Most of these tools are online where the users can either provide their custom receptor and ligands (mostly in the.pdb format) or can select the receptors or ligands from standard known databases like the protein data bank (PDB, available at www.rcsb.org). Online tools or servers enable the users to make the most of the facility without any added cost (both hardware and software wise) along with enabling multiple users to be able to submit their jobs which can be run simultaneously. However several online servers are only accessible to academic users and thereby require an academic affiliation (in most cases an academic email id is sufficient). Standalone programs either free or paid are required to be installed or compiled on local computers, often a very high end desktop computer or a standard workstation. These programs provide the users the freedom to modify it as per their requirements as well as ensure data security.

Table 1. List of docking servers and softwares

Sl. No.	Server / Software Name	Type of Docking Performed	Online Server / Standalone Program (O/S)	Free/Paid (F/P)	Remark	Reference
1	AutoDock / AutoDockVina + AutoDock Tools	Protein-Ligand	S	F	Flexible docking	(Morris et al., 2009; Trott & Olson, 2010)
2	FlipDock	Protein-ligand	O	F	Flexible docking	(Zhao & Sanner, 2007)
3	HADDOCK	Protein-protein, protein-DNA	O & S	F	Data / information driven flexible docking	(de Vries et al., 2007; Dominguez, Boelens, & Bonvin, 2003; van Dijk, van Dijk, Hsu, Boelens, & Bonvin, 2006)
4	ClusPro	Protein-protein	O	F	Best outputs are filtered from several poses generated by the program.	(Comeau, Gatchell, Vajda, & Camacho, 2004a, 2004b)
5	PyDock	Protein-protein	O	F	Rigid body docking, require academic email	(Cheng, Blundell, & Fernandez-Recio, 2007; Gabb, Jackson, & Sternberg, 1997; Jiménez-García, Pons, & Fernández-Recio, 2013)
6	Patchdock	Protein-protein, protein-ligand	O	F	Rigid body docking, outputs can be further refined by Firedock	(Andrusier, Nussinov, & Wolfson, 2007; Duhovny, Nussinov, & Wolfson, 2002; Mashiach, Schneidman-Duhovny, Andrusier, Nussinov, & Wolfson, 2008; Paquet & Viktor, 2013)

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Table 1. Continued

Sl. No.	Server / Software Name	Type of Docking Performed	Online Server / Standalone Program (O/S)	Free/Paid (F/P)	Remark	Reference
7	Hex	Protein-protein, protein-ligand, DNA-ligand, DNA-protein	O & S	F	Rigid ligand	(Macindoe, Mavridis, Venkatraman, Devignes, &Ritchie, 2010)
8	GRAMM-X	Protein-protein	O	F		(Tovchigrechko & Vakser, 2006)
9	SwarmDock	Protein-protein	O	F	Flexible docking	(Torchala & Bates, 2014; Torchala, Moal, Chaleil, Fernandez-Recio, & Bates, 2013)
10	rDock	DNA/RNA-ligand, protein-ligand	S	F	Can be used for virtual screening	(Ruiz-Carmona et al., 2014)
11	GOLD	Protein-ligand	S	P	Can be used for virtual screening, lead optimization	
12	ArgusLab	Protein-ligand	S	F	Can also be used for molecular modeling and graphics purposes	http://www.arguslab.com/ arguslab.com/ArgusLab.html_ (last accessed June 2015)
13	DockingServer	Protein-ligand	O	P	Restricted usage with free registrations	http://www.dockingserver.com/ web_(last accessed June 2015), (Bikadi & Hazai, 2009)
14	1-Click docking/mcule. com	Protein-ligand	O	P	Choice of ligand can be drawn or created, limited free usage	
15	EADock DSS/ SwissDock	Protein-ligand	O	F		(A. Grosdidier, Zoete, & Michielin, 2011a, 2011b)
16	Molsoft ICM Docking (Pro package)	Protein-ligand, protein-protein	S	P	Additional features like structure analysis, 3D editor, virtual screening, structure prediction etc.	
17	Glide	Protein-ligand	S	P	Flexible docking, virtual screening possible.	(Friesner et al., 2004; Repasky, Shelley, & Friesner, 2007)
18	Molegro Virtual Docker	Protein-ligand	S	P	Flexible docking, virtual screening possible.	
19	FlexX-HYDE	Protein-ligand	S	P	Flexible docking, virtual screening possible, can integrate metal ions and flexible water molecules	
20	FRED	Protein-ligand	S	P	Rigid docking, virtual screening, command line interface	

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Table 1. Continued

Sl. No.	Server / Software Name	Type of Docking Performed	Online Server / Standalone Program (O/S)	Free/Paid (F/P)	Remark	Reference
21	Surflex-Dock (Tripos SYBYL-X package)	Protein-ligand	S	P	Virtual screening possible.	(Jain, 2003)
22	MOE (from CCG)	Protein-ligand	S	P	Combined with several other packages for drug discovery.	
23	Pardock	Protein-ligand	O	F	Requires some initial molecule editing like hydrogen addition	(Gupta, Gandhimathi, Sharma, & Jayaram, 2007)
24	Discovery Studio – Biovia (formerly Accelrys)	Protein-protein	S	P	Combined with several other packages for drug discovery, uses Z-DOCK algorithm for molecular docking	
25	RosettaDock/ ROSIE	Protein-ligand, protein-protein	O	F		(Lyskov & Gray, 2008; Lyskov et al., 2013)
26	Paradock	Protein-DNA	O	F	Rigid protein, flexible DNA	Not yet reported
27	iGEMDOCK v2.1	Protein-ligand	O	S	Purely flexible or Hybrid mode	(Yang & Chen, 2004)
28	Lead Finder	Protein-ligand	P	S		
29	LigandFit	Protein-ligand	O	S	Flexible ligand	(Terwilliger, Adams, Moriarty, & Cohn, 2006; Terwilliger, Klei, Adams, Moriarty, & Cohn, 2006; Venkatachalam, Jiang, Oldfield, & Waldman, 2003)
30	LigDockCSA	Protein-ligand				(Shin et al., 2011)
31	MADAMM	Protein-ligand			Flexible docking	(Cerqueira, Bras, Fernandes, & Ramos, 2009)
32	PLANTS (Protein-Ligand ANT System)	Protein-ligand	O (for academic users)	S	Side chain flexibility, additional facilities like explicit water, virtual screening etc.	(Korb, Möller, & Exner, 2010; Korb, Stützle, & Exner, 2007, 2009)
33	SODOCK	Protein-ligand	O	S	Flexible docking	(Chen, Liu, Huang, Hwang, & Ho, 2007)
34	3D-Garden	Protein-protein	O	F	Flexible docking	(Lesk & Sternberg, 2008)
35	GPU.proton. DOCK	Protein-protein	O	F	Flexible docking	(Kantardjiev, 2011)

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As we have already described, molecular docking, in drug discovery context, is significantly involved in virtual screening of compounds. Several docking programs have been developed with this added screening facility. Among the softwares or Standalone programs mentioned in Table 1, some of them feature this added facility. Such programs are mentioned as 'virtual screening' enabled in the remarks section of Table 1. There are some other programs like MSDOCK (Sauton, Lagorce, Villoutreix, & Miteva, 2008), EUDOC (Pang, Perola, Xu, & Prendergast, 2001) etc. which have been specifically designed to aid drug discovery by facilitating virtual screening and drug interaction site identification.

A list of useful resources related to docking and virtual screening is available at the Click2Drug repository which can be accessed via http://www.click2drug.org/bibliography/docking/webservices.html and http://www.click2drug.org/bibliography/screening/webservices.html.

5. INDUCED FIT DOCKING: OPTIMAL BALANCE BETWWEN FLEXIBILITY AND RESOURCE COST

In principle, to obtain the best possible ligand receptor pose (in the form of complex or docked structure), an explicit solvent MD simulations with the presence of ligand and receptor structures would prove to be best solution. However it is quite evident from the previous discussions that significant time and resource cost would implemented to obtain such level of precision. Additionally different force field parameters, optimization of simulation environment add further level of complexity. To reduce such complex setup without much sacrifice in the quality of docking outcome, induced fit docking has been established as an optimal solution (Xu & Lill, 2013).

The biological principle behind induced fit docking is that in real life, protein, unlike computer structures (for example pdb structures), are flexible molecules and their active site residues often incorporate certain degrees of rotational freedom to accommodate the ligand in most effective conformation. Even there are certain receptors which only attain ultimate stable confirmation when bound to ligand. So impact of presence of ligand on receptor structure and conformation (especially of active site) is beyond doubt.

Induced fit docking takes in to account the above mentioned ligand impacts to figure out best ligand and receptor active site poses to facilitate the following benefits:

1. Generation of active ligand conformation that cannot be simply docked in to a rigid body receptor.
2. Rescue of poorly scoring true ligands (false negatives) during virtual screening experiments.

An induced fit docking algorithm primarily undertakes following steps:

1. Preparation of receptor molecule,
2. Identification of flexible residues, including potential ones,
3. Rotamer sampling of those flexible residues,
4. Interaction analysis and sampling,
5. Docking and complex/pose refinement,
6. Scoring of complexes,
7. Selection of best outputs

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There have been numerous studies taking advantage of induced fit docking (Nabuurs, Wagener, & de Vlieg, 2007; Zhong, Tran, & Stang, 2009) and now a day it has become a pretty common feature of many softwares (example: Schrodinger Glide) or servers (example: Fleksy) that facilitate virtual screening to incorporate these protocols for generation of better outputs.

6. DOCKING AND DRUG DISCOVERY

Since molecular docking is an integral part of present day drug discovery procedure, almost all the drugs that come to market or clinical trial are designed by CADD to a significant extent. Docking being an essential part of CADD, contributes to a great extent in the development of such drugs. Notably development of several anti-HIV drugs has been associated with CADD and molecular docking (Esquieu et al., 2003; Liao & Nicklaus, 2010). A number of drugs like Dorzolamide, Captopril, Oseltamivir, Aliskiren, Saquinavir, Zanamivir, Rupintrivir, Boceprevir, Nolatrexed etc have been designed through CADD and progressed to the level of clinical trials or marketed after regulatory approval (Talele, Khedkar, & Rigby, 2010; Zhang et al., 2013). These molecules are very well representatives of protein-small molecule docking.

With the advent of multi-drug resistant (MDR) varieties of *Mycobacterium tuberculosis* and other MDR type causative agents for visceral leishmaniasis and several other pathogenic diseases; development of new drugs are becoming essential for the survival of a significant portion of human population. Not only these few diseases, several cancers often develop MDR properties after a few doses of standard treatment. The ever changing influenza viruses are also causing greater number of causalities with each passing year. The two main benefits of molecular docking and CADD are its abilities to save time and money and these two apparently seems to be the best weapon in hand to deliver new drugs to address the above mentioned challenges within a foreseeable future.

7. FUTURE RESEARCH DIRECTIONS

Drug discovery, especially in today's context, is becoming even more dependent on computational techniques that ensure best utilization of time and money. But it should be acknowledged that neither computers can alone deliver a magic bullet nor computers, however advanced it may be, can take in to account all parameters at a time. All the biological interactions of a cell cannot be simulated by any computer in a reasonable time frame. Thus the entire process of a drugs entry to a human body till it is metabolized and subsequently excreted can only be mathematically modeled, not simulated (with the consideration of all degrees of freedom). Thus the future areas of development which bear the potential of efficient use of molecular docking in drug development can be broadly classified in to two parts – 1. technological advancement and 2. methodological changes.

Technologically there are two areas of development. First efficient use of present day computer architecture, especially the general purpose graphics processing unit (GPGPU) promises a significant level of advancement in the speed of molecular dynamics (MD) simulations. It is beyond any point of argument that flexible docking with MD simulations ensures best output. However additional time requirements and hardware associated costs have often been limiting factors in flexible docking and MD simulations. To counter these issues, rigid body docking was the choice of many. However with the advent of advanced computing facilities like GPGPU, MD simulation jobs have become significantly

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faster. These present day multi-core (often in the range of several thousands) GPGPUs can significantly accelerate MD simulations by offloading a huge portion of load from the computer/workstation CPU (with essentially run in serial mode and only contain a few cores, often at a maximum number of 20 in high end standalone workstations). Present day MD simulation programs like GROMACS or NAMD (Phillips et al., 2005) are intrinsically equipped to harness the power of a computer architecture created by pairing of GPGPU with CPUs. These GPGPU-CPU combinations (both as single node and cluster) not only significantly lowers the simulation time but also enables users to take in to account greater number of molecular parameters without much time penalty and added cost. GPU based algorithms of molecular docking should be developed to generate better quality of outputs compared to rigid body docking. The added benefit of all atom flexible docking with MD simulations which can easily be performed at a GPGPU-CPU combined system, is that it can be employed to docking studies with all kinds interacting molecules i.e. DNA, protein and small molecules or ligands. If technological advancements (mainly in the form of efficient algorithms) can be directed to harness the power of upcoming computing architectures, molecular docking would be able to provide greater in-sights in to the interaction patterns of biological macromolecules and/or their interacting small molecules in much greater details and that too within a reduced time frame.

Methodologically docking in drug discovery has been traditionally associated with virtual screening, lead discovery and optimization steps. While the contribution of molecular docking in saving valuable time and billions of dollars cannot be ignored in any way, it should also be acknowledged that the much hyped benefit of CADD, QSAR or docking has failed to a significant extent to keep up to their expectations. However it is very important to understand the reason behind such gap of expectations and achievements of molecular docking in drug discovery. One fundamental point in this aspect is what to dock with which i.e. what molecule to dock to which molecule. Much like the exponential increase in protein or DNA structural data, compound libraries have also been increasing exponentially over the years. Despite such remarkable achievements, not many drugs with significant potentials have made it to the market in recent years. Thus it can be clearly understood that there lies some issues with the best utilization of this increased structural and chemical data. Several factors like administrative, financial, intellectual property etc. can be linked to the causes of such issues. It is very essential to overcome such factors in order to make best use of present day and upcoming computational facilities and docking algorithms to achieve increased success with molecular docking in drug discovery. Greater collaboration between industry and academia as well as sharing of molecule libraries are potentially the avenues of achieving higher success with molecular docking based lead discovery and optimization aspects. Further distributed computing architecture can be employed globally to increase the rate of docking with higher degree of flexibility.

Finally it should be noted that the greatest benefit of molecular docking has been in the area of virtual screening which has significantly reduced the time, effort and expenditure of assay development, result interpretation and infrastructural requirement of standard high throughput screening. However it should be noted that to make more reliable outputs with maximum specificity, both virtual screening and HTS should be employed to a reasonable level while virtual screening should be allowed to use as many parameters as possible (Ferreira et al., 2010; Gschwend, Good, & Kuntz; Okimoto et al., 2009)

Beyond such apparently traditional solutions of making molecular docking more efficient in drug discovery, another approach which promises much greater potential is the use of peptidomimetics. It makes great use of protein-protein docking during its design, optimization and efficacy analysis part.

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7.1 Peptidomimetics and Drug Discovery (Role of QSAR and Molecular Docking)

A part of present day drug discovery aims at developing modified peptides with increased stability and better pharmacokinetic trends. In short this technique is known as peptidomimetics and can be defined as synthetic molecules that are developed by modifying the natural peptides or proteins. Peptidomimetics are applied in targeted chemotherapy, in mitochondria apoptosis, as antimicrobial and antiviral agents as well as in cosmetics production (Walensky et al., 2004). Peptidomimetics can be synonymous with Molecular mimicry to a certain extent (Vagner, Qu, & Hruby, 2008). Recently, bioactive peptides are used as powerful tools for initial investigation in disease biology. However, these natural peptides are associated with certain problems: 1. physiological stability, 2. lack of oral bio-availability, 3. poor DMPK properties and 4. prone to peptidase digestion and susceptibility to proteolysis (Vagner et al., 2008). Peptidomimetics is employed to broadly circumvent these problems associated with natural peptides. The pharmacophores (hotspots which are necessary for the molecular recognition of a ligand by a biological macromolecule) of peptidomimetics retain the ability to interact with the target proteins and help to mediate the natural biological activities.

Various peptide bond modifications are designed and synthesized to obtain natural peptide analogues with improved pharmacological properties. These added advantages in these compounds come from significant differences in their structures. Such modifications contribute to protect the peptidomimetics from protease cleavage and significantly improve their metabolic stability. Structure activity relationship (SAR) is the founding step in designing a peptidomimetic compounds. It defines minimal active region and key residues required for identical biological effects. The binding relationships of target molecule and the peptide analogues are evaluated and this evaluation is done majorly through amino acid scan, truncations and N- and C- terminal modifications. This is followed by stable bioactive conformation generation. The stepwise structural modifications require computational approaches, whereas the confirmation for bioactive set generation largely depends on experimental evaluation. In all these steps of peptidomimetic design and optimization, molecular docking plays crucial role as it technically eliminates the experimental part of synthesizing each and every modifications and performing their binding assays. By enabling virtual screening and in-silico analysis, molecular docking allows fast and efficient screening of peptidomimetic compounds (Hruby, al-Obeidi, & Kazmierski, 1990; Kharb, Meenakshi, Sharma, & Yar, 2011; Singh, Dolphin, Razkin, & Dumy, 2006).

8. CONCLUSION

CADD has been well established as an approach to accelerate in house R&D related to drug discovery. Among several aspects of CADD, target identification and validation as well as lead identification and optimization are the most important parts for couple of reasons. First maximum numbers of failures occur at these steps of drug discovery. Secondly these steps being the early steps of drug discovery pipeline, failure at these steps incur less time and economical penalty. Computerization and automatization of these steps to a significant extent using molecular docking techniques have further reduced the above mentioned penalties in case of failures. Depending upon the type of disease of interest, protein-protein or protein-DNA or protein-ligand or DNA-ligand types of docking programs can be employed for proper identification of the potential drug target. Thereafter in-silico mapping techniques can be employed to

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figure out potential drug binding pockets which in turn save lot of experimental time and other resources. Subsequently computer based virtual screening, which essentially employs protein-ligand / DNA-ligand docking algorithms, can be used to screen tens of thousands of molecules to pick up only a few lead molecules. Such lead molecules can be used, in conjunction with limited experimental studies, for efficacy analysis using QSAR. Based on the outputs of QSAR and other efficacy analyses, in-silico secondary modifications of lead molecules can be generated which can further be subjected to molecular docking with its receptor of interest. Jointly these molecular modeling and docking techniques enable faster generation of all possible secondary modifications as well as analyses of potential impact on efficacy due to the introduced changes. All these steps, if were performed in traditional ways, would have posted several challenges like – synthesis and purification of all the modified lead molecules and their subsequent storage and structural validation using NMR or similar techniques, experimental analyses of their efficacy and manual comparison of efficiency of all the incorporated modifications. Thus it is fairly easy to understand the benefit of different types of molecular docking techniques on different segments of the R&D part of drug discovery. Especially the ability to virtually interact all possible types of bio-macromolecules with each other and other organic ligands has made molecular docking and indispensable part of present day drug discovery. However time has come to explore more efficient docking algorithms for better accuracy of output prediction as well as identification of newer avenues of implementations of various docking techniques to further accelerate drug discovery.

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KEY TERMS AND DEFINITIONS

Computer Aided Drug Design: Computer aided drug design or CADD means the use of computer programs or softwares or online resources for the purpose of identification and optimization of potential drug molecules. The functionality of CADD also extends to identification of potential drug targets.

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Docking: The process of finding a thermodynamically and physiologically relevant pose of interaction between two biological macromolecules or between a small molecule and a biological macromolecule. Surface complementarities and energetic parameters are often taken in to account to find out such poses.

HTS: HTS stands for High Throughput Screening, a process which can be automated to a very large extent to facilitate the screening of molecules of interest without much human intervention. In present day, often robotic liquid handling systems facilitate HTS.

Ligand: In molecular docking, ligand is referred to a organic small molecule that can bind to another large biological macromolecule like a DNA or a protein and upon binding, often causes structural and functional alterations of the concerned macromolecule.

Online Server: An online facility that hosts certain website or program and makes it available to global users. In the context of this chapter, several online docking servers enable the users to dock their molecules of interest by making the docking algorithm available in the form of a website.

QSAR: QSAR stands for Quantitative Structure Activity Relationships. QSAR is the process of quantitative identification of relationship between a chemical structure and its functional activity, primarily pharmacological. QSAR is often used in drug discovery for the efficacy analysis of the molecule of interest.

Receptor: A biological macromolecule (mostly a protein) to which a ligand or small molecule binds.

Small Molecule: In drug discovery or docking context, small molecules are synonymous with ligand.

Standalone Program: A computer program that is required to be installed or compiled in a local computer or workstation to make it usable. In docking terms, several docking programs are available as source codes or binaries which the users can download from the web and thereafter can either compile or install on their local systems. Depending upon the program type it can be either GUI (graphic user interface) based or command line based.

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Chapter 32
The Comparison of Docking Search Algorithms and Scoring Functions: An Overview and Case Studies

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ABSTRACT

This chapter, composed of two parts, firstly provides molecular docking overview and secondly two molecular docking case studies are described. In overview, basic information about molecular docking are presented such as description of search algorithms and scoring functions applied in various docking programs. Brief description of methods utilized in some of the most popular docking programs also applied in our experimental work is provided. AutoDock, CDOCKER, GOLD, FlexX and FRED were used for docking studies of the DC-SIGN protein, while GOLD was further used for docking studies of cathepsin K protein. Methods and results of our studies with their contribution to science and medicine are presented. Content of the chapter is therefore appropriate for public of Medicinal and Organic Chemistry as an overview of docking studies, and also for Computational Chemists at the beginning of their work as the introduction to application of molecular docking programs.

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INTRODUCTION

Those who devote their time to molecular science are well aware of the amount of molecular data available that has rapidly increased in the past few decades due to breakthrough in structural biology. Since 1975 development of high-throughput protein purification, crystallography, and nuclear magnetic resonance spectroscopy techniques predominantly contributed to structural details of macromolecules and complexes with ligands. In 2014, after less than four decades, the number of structures exceeded the 100.000 with 10.000 structures per year being deposited in Protein Data Bank (PDB). PDB is the single worldwide repository of information about the 3D structures of large biological molecules, including proteins and nucleic acids (Berman et al., 2000; Berman, Kleywegt, Nakamura, & Markley, 2014).

These advances are responsible for computational methods to become indispensable in all aspects of drug discovery including the structure-based drug design and computer-based analysis of molecular interactions. Interactions between biomolecules are fundamental to biological processes; using these interactions, living organisms maintain complex regulatory and metabolic mechanisms responsible for the life course. Experimental research work is fundamental for a better understanding of biological processes; however, computer simulations and analysis are of mayor importance to extract information from large amount of multivariate experimental data. Application of these scientific methods provides exploration of molecules that can be utilized as bioactive substances able to modify and control molecular interactions. For bioactive substances, molecular recognition of the constituents is imperative. Binding mode, binding affinity, and kinetics play the crucial role in molecular recognition as they characterize intermolecular interaction.

Due to the limitations of experimental methods for determining the structure of molecular complexes, as they are expensive, time consuming and not always feasible, computational methods are preferable for predicting putative binding modes and binding affinities. Nowadays, the main advantages of rational drug discovery approaches, such as virtual screening, are low costs and effectiveness when compared to high-throughput screening. Molecular docking is one of the virtual screening methods used since 1980's. It is based on modeling of interactions between two molecules, a small molecule (ligand) and its receptor/protein (target). The predicted interactions allow us to characterize the binding mode and binding affinity of the ligand-target complex.

Currently there are more than 50 molecular docking programs available, which all have similar implementations. This chapter provides explanation and comparison of computational approaches, specifically different algorithms enclosed in software for molecular docking, considering several of the most commonly used program tools (AutoDock, FRED, CDOCKER, FlexX and GOLD). Authors also present the use of these algorithms in the comparative study for docking of small ligands to specific protein, lectin Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) and covalent docking of cathepsin K.

BACKGROUND

Molecular docking, in the case of small molecules also known as ligand docking, can be defined as computational method for prediction of the structure of ligand-receptor complexes. Ligand docking process involves two basic steps: prediction of ligand conformation and orientation (the pose) by search algorithms and assessment of binding affinity by scoring functions.

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By search algorithms as mentioned above different ligand conformations and orientations are searched and therefore different modes (poses) are generated. Over time different search algorithms, all being defined by set of rules and parameters applied to predict conformations, were developed and became available for users. Algorithms differ regarding to constituent molecules flexibility consideration, therefore several approaches can be utilized that are classified as: methods for "rigid-body" docking and methods for "flexible docking". Depending on how much conformational space is covered, speed and effectiveness are two critical elements in search algorithms. Rigid-body docking methods use either a single conformation or multiple conformations implemented by user to consider ligand flexibility. Search algorithms here consider only geometrical complementarities between molecules and that is limiting factor concerning specificity and accuracy of prediction. Methods for flexible docking consider various conformations of ligand or receptor (or both molecules at the same time) generated by program itself, what makes prediction more effective at higher computational time cost. So far, molecular docking software generally uses one or more methods for binding mode prediction: matching algorithms, incremental construction, Monte Carlo simulations, genetic algorithms, etc.

Assessment of the different orientations and conformations of a ligand is the second step in molecular docking, where scoring functions delineate the correct poses from incorrect poses or binders from inactive compounds. It is necessary to mention that scoring function involve estimation adopting various assumptions and simplifications, rather than calculating accurate binding affinity between constituent molecules. Scoring functions can be applied for ligand binding mode identification, binding affinity prediction, and consequently virtual database screening. There are numerous available scoring functions that can be classified into three main categories: force-field based, empirical and knowledge-based. Force-field based scoring functions calculate the sum of intermolecular interaction and atomic forces for assessment of binding energy, whereas empirical scoring functions estimate binding energy of a complex by summing up the weighted energy terms. Knowledge-based scoring functions employ statistical analysis of molecular complexes crystal structures. Moreover there has been additional strategy introduced; consensus scoring combines several different scores from multiple scoring functions to assess a docking conformation/orientation.

There are lots of reviews about molecular docking and since the beginning of the new millennium practically every year new publications are available that report recent advances and challenges in the field, compare programs available and outline growing number of applications of molecular docking in drug discovery (Table 1).

SEARCH ALGORITHMS

Search algorithms for molecular docking originate from computer analysis studies of intermolecular interactions. Calculations of protein-protein interactions led to the development of first algorithms for computing molecular interactions starting from the structure of two components. These algorithms utilize approximate shapes of two molecules represented as rigid bodies. As these algorithms essentially consider the geometrical complementarities between molecules, they are termed shape matching algorithms. Although this approach does not take into account the conformational space of molecules it is still convenient for the applications in drug design and discovery, such as for initial stages of virtual screening studies and as a part of strategies of other more sophisticated docking algorithms.

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Table 1. Molecular docking review publications outlined according to the year of publication

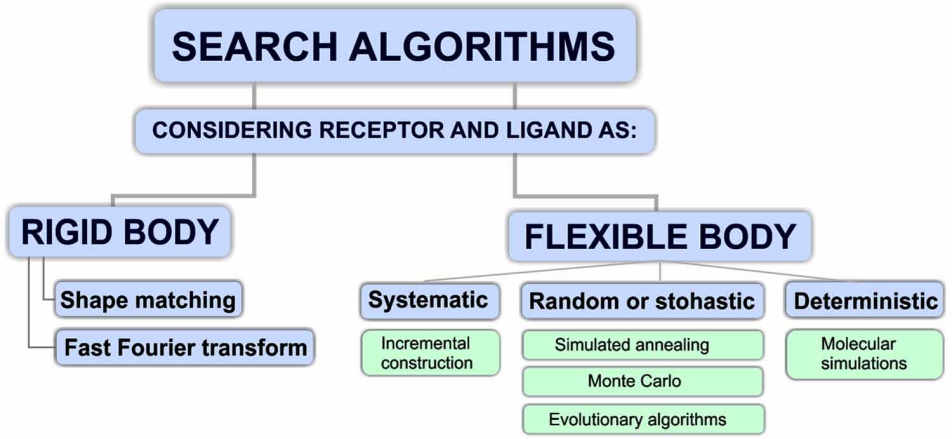
Year of Publication	Reference
1995	Jones & Willett, 1995
1995	Lybrand, 1995
1996	Gschwend, Good, & Kuntz, 1996
1996	Lengauer & Rarey, 1996
2002	Halperin, Ma, Wolfson, & Nussinov, 2002
2002	Taylor, Jewsbury, & Essex, 2002
2003	Brooijmans & Kuntz, 2003
2003	Kroemer, 2003
2004	Kitchen, Decornez, Furr, & Bajorath, 2004
2006	Sousa, Fernandes, & Ramos, 2006
2007	Kroemer, 2007
2008	Moitessier, Englebienne, Lee, Lawandi, & Corbeil, 2008
2010	Huang, Grinter, & Zou, 2010
2010	Youriev, Agostino, & Ramsland, 2010
2011	Meng, Zhang, Mezei, & Cui, 2011
2012	Mihasan, 2012
2013	Bello, Martinez-Archundia, & Correa-Basurto, 2013
2013	Sousa et al., 2013
2013	Youriev & Ramsland, 2013
2014	Grinter & Zou, 2014
2015	Chen, 2014
2015	Ferreira, dos Santos, Oliva, & Andricopulo, 2015
2015	Wang et al., 2015
2015	Yuriev, Holien, & Ramsland, 2015

Development of sophisticated docking algorithms practically coincided with the increasing power of computers, allowing the integration of more parameters into search algorithms. Considering more degrees of freedom a new methodology emerged. Flexible docking accounts for conformational space of molecules that is responsible for molecular dynamics essential for all biological systems. Since the molecular complex is composed of two molecules, it would be optimal to consider both of them as flexible. Even though, due to the computational limitations, predominate algorithms considering only flexibility of the ligand, there are several examples of specialized algorithms and computational strategies that account for partial flexibility of the receptor.

Computational tools for molecular docking use different methods to deal with ligand or receptor flexibility. There are three categories of algorithms that consider ligand flexibility: systematic, random or stochastic and deterministic (Figure 1) and some of these are appropriate and applied also for exploring receptor flexibility. Difference between docking software depends on the nature of algorithm implementation. This subchapter describes basic search algorithm approaches that progressed from the time of first intermolecular interaction studies.

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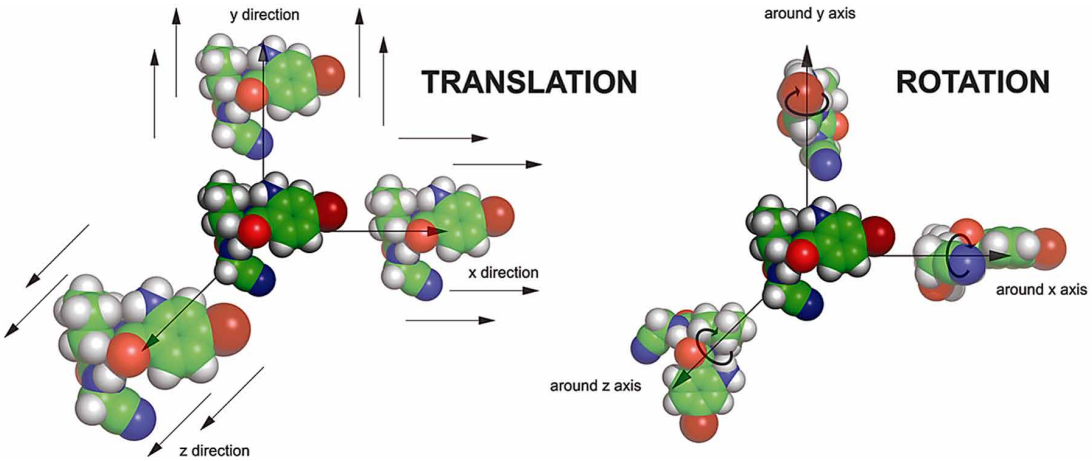
Figure 1. Search algorithms that consider receptor and ligand as rigid/flexible bodies



Search Algorithms that Consider Ligand and Receptor as Rigid Bodies

All primer algorithms calculating intermolecular interactions of molecular complexes considered molecules as rigid bodies. Rigid body, according to physics, is an idealization of a solid body whose deformation is neglected and its position determined by the position of its center of mass and by its altitude, taking into account 6 basic parameters referred as degrees of freedom. There are 3 rotational and 3 translational degrees of freedom that represent possible movements of rigid body in three-dimensional space (3D) (Figure 2). These algorithms consider geometry match between molecules and are based on Shape Matching (SM) and Fast Fourier Transform (FFT).

Figure 2. Demonstration of rigid body movement in 3D space considering 6 degrees of freedom (three translational and three rotational); molecule – ID 3 represents rigid body in 3D space
Borišek et al., 2015.



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Shape Matching

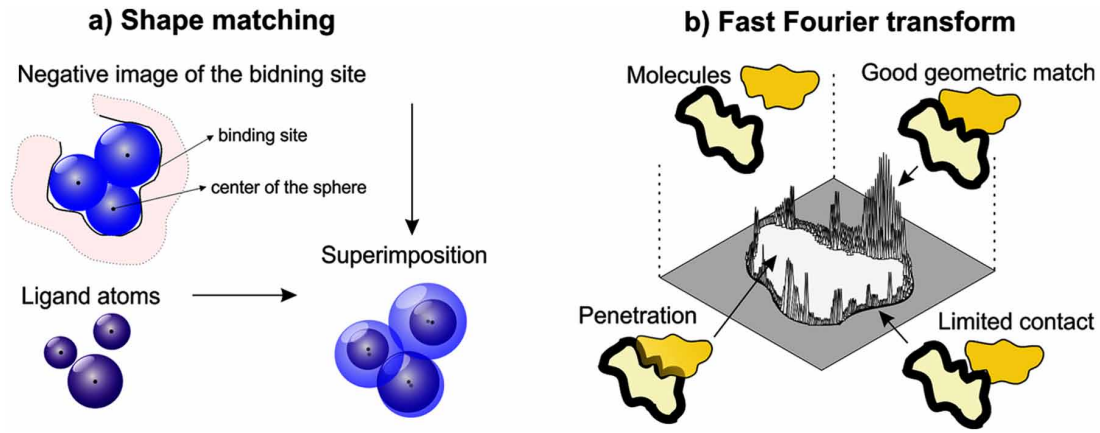
First calculations and structural analysis of protein-protein interactions (Levinthal, Wodak, Kahn, & Dadivanian, 1975; Selemme, 1976; Wodak & Janin, 1978) led to development of shape matching algorithms applied to search for shape complementarities between two molecules. Kuntz et al. (1982) first presented a method to explore geometrically feasible alignments of ligands and receptors of known structure, where negative images of the receptor were generated. Negative images are represented by spheres that fill the binding sites and algorithm attempts to superimpose the center of these spheres onto ligand atoms (Figure 3a). Several programs such as DOCK (Ewing, Makino, Skillman, & Kuntz, 2001; Allen et al., 2015) and MSDOCK (Sauton, Lagorce, Villoutreix, & Miteva 2008), utilize this approach as part of their search strategies of flexible docking algorithms.

Fast Fourier Transform

Ten years later, new geometric recognition approach was developed by Katchalski-Katzir et al. (1992). Algorithm involves atomic coordinates for the digital representation of molecule by 3D discrete functions that distinguish between surface and the interior of the molecule. Algorithm also calculates correlation function that assesses the degree of molecular surface overlap and penetration considering 6 degrees of freedom using Fourier transformation (Figure 3b). This approach, in comparison with shape matching algorithms, more rapidly evaluates possible associations between molecules. FTDOCK (Gabb, Jackson, & Sternberg, 1997) and ZDOCK (Chen & Weng, 2002, 2003) are examples of docking programs that apply Fourier transformation.

Algorithms that consider ligand and receptor as rigid bodies were initially developed for intermolecular interaction analysis. Latter these methods were optimized for searching binding sites and appropriate ligand pose prediction in ligand-receptor complex where 3D structures of constituent molecules were known. These algorithms, however, are not successful if there is conformational change upon binding of the ligand. Currently, practically all molecular docking software utilizes different approaches for

Figure 3. Schematic representation of shape matching algorithm and fast Fouirer transform



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considering conformational space of ligand (and in some cases receptor) molecule. Therefore, it can be suggested that nowadays there are no limitations concerning rigid body consideration in molecular docking since several approaches to cope with molecular flexibility are available (details discussed below).

Search Algorithms that Consider Ligand and Receptor as Flexible Bodies

Flexible bodies with consideration of rotational, translational and conformational degrees of freedom are somehow more realistic imitation of biological molecules. Therefore, molecular docking with flexible ligand and flexible receptor is rather accurate approximation of *in vivo* complexes formation, especially when conformational changes upon formation are present. At first flexible-ligand docking algorithms emerged, which considered partial or full flexibility of just ligand and afterwards, due to importance of 'induced fit' paradigm, some specialized algorithms were developed considering also partial flexibility of the receptor/protein. Depending on which molecule of the complex (ligand or receptor) is considered as flexible, the molecular docking is categorized in flexible-ligand docking and flexible-receptor/protein docking methods. Former consider only ligand (partial or full) flexibility, meanwhile latter consider ligand and partial receptor/protein flexibility.

This section of chapter provides description of search algorithms available that consider ligand and/or receptor as flexible bodies and their advantages and limitations. There are three categories of flexible-ligand docking methods (Brooijmans & Kuntz, 2003; Bras, Cerqueira, Sousa, Fernandes, & Ramos, 2014): systematic, random or stochastic, and deterministic.

Systematic Methods

Example of systematic search algorithm is incremental construction or "anchor and grow procedure" approach, (DesJarlais, Sheridan, Dixon, Kuntz, & Venkataraghavan, 1986; Leach & Kuntz, 1992) where ligand is split into several fragments that are separately docked in the receptor site and covalently linked to reassemble the ligand. This method is one of the most applied and employed by several docking programs such as DOCK (Ewing et al. 2001; Allen et al., 2015), FLOG (Miller, Kearsley, Underwood, & Sheridan, 1994), LUDI (Böhm, 1992), etc. FlexX (Rarey, Kramer, Lengauer, & Klebe, 1996; Rarey, Wefing, & Lengauer, 1996) uses this strategy, where chemical interactions are basis for placing the fragments. Therefore the generation of a large set of geometrically feasible solutions is avoided. For placing the base fragment into active site of the protein, FlexX uses pose clustering, where algorithm merges initial placements into clusters. Another systematic search algorithm is database search approach, where libraries of multiple conformations of each ligand structure are constructed. FLOG is the program that constructs Flexibases (Kearsley, Underwood, Sheridan, & Miller, 1994) for ligand flexibility consideration. OMEGA (Hawkins & Nicholls, 2012) is program that generates multi-conformer structure databases used by docking program FRED (McGann, 2011).

Random or Stochastic Methods

There are several types of docking programs that apply random approach: Monte Carlo, simulated annealing, evolutionary algorithms, particle swarm optimization, etc.

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Monte Carlo (Caflisch, Niederer, & Anliker, 1992; Hart & Read, 1992) is method that searches for global energy minimum of the conformers. It generates random changes in the ligand conformation, thermodynamically explores different states and selects energetically favorable states to create more reliable protein-ligand complexes. Monte Carlo is standard minimization method and tends to be trapped in local minimum, therefore simulated annealing has been applied to avoid this issue. Simulated annealing (Kirkpatrick, Gelatt, & Vecchi, 1983; Goodsell & Olson, 1990; Yue, 1990) is a technique, where changes in temperature are involved during the run for every conformation and therefore more detailed conformational state (flexibility) at different thermodynamic states is considered. MCDOCK (Liu & Wang, 1999) is a program that utilizes Monte Carlo method for global minimum searching, while AutoDock (Morris et al., 2009) uses simulated annealing approach for more accurate conformations exploration.

Evolutionary algorithms (Morris, Olson, & Goodshell, 2000; Thomsen, 2003), same as Monte Carlo methods, generate and optimize low-energy conformations and orientations of the ligand and thus search for global energy minimum. They have the ability to simultaneously explore the search space with a population of candidate solutions represented by artificial "genes" and form new solutions by combining existing ones. Evolutionary algorithms encompasses a range of algorithms that are based on the principles of biological evolution and the most popular variant of these algorithms are genetic algorithms (GA) (Jones, Willet, & Gleen, 1995; Jones, Willet, Glen, Leach, & Taylor, 1997; Oshiro, Kuntz, & Dixon, 1995) implemented in docking program GOLD (Verdonk, Cole, Hartshorn, Murray, & Taylor, 2003).

Genetic algorithms mimic the process of evolution by manipulating a collection of data-structures called chromosomes. Chromosomes represent randomly generated population that are different ligand poses, where each ligand pose is characterized by a set of state variables that describe translation, orientation and conformation of the ligand in relation to the protein. Each state variable corresponds to a gene, ligand's state corresponds to the genotype and atomic coordinates of ligand correspond to the phenotype. Each of the chromosomes is evaluated with appropriate fitness score, where fitness represents the total interaction energy of the ligand and the protein. Genetic operators like mutations and crossovers, are applied to the population for offspring generation or, in the case of molecular docking, pose space sampling. Selection of the offspring of the current generation occurs based on the fitness of the individual, where the best chromosomes are saved, chromosomes with good scores are selected for propagation into new generation, whereas chromosomes with poor scores perish. The survival of the best chromosomes enables evolution of optimal solutions that represents the correct binding mode. AutoDock uses a hybrid of a genetic algorithm search method together with local search method, named Lamarckian genetic algorithm (LGA) (Morris et al., 1998), which has enhanced performance relative to genetic algorithm alone. This is due to the concept of local search during optimization, which can help to escape local minimum. Although this method is superior to standard genetic algorithm there are still several million evaluation steps needed for the prediction of a reliable protein-ligand pose.

Another stochastic method is particle swarm optimization (Eberhart & Kennedy, 1995), a bio-inspired evolutionary approach to model swarm intelligence. It simulates the social behavior among individuals (particles), with a population (swarm) moving through a multidimensional search space. Each particle of the swarm represents a possible solution described by its location in the search space. As particle between each other share their knowledge about their own best position, the swarm moves towards the best overall solution. In the case of molecular docking swarm represents population of the ligands and particles represent an individual pose, which are determined by translation, orientation and conformation. Each ligand pose moves through the search space and if one of them recognizes a region with low energy the rest of the particles will follow quickly and the best solution is memorized. This

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method is implemented in AutoDock program (PSO@AutoDock) (Namasivayam & Günther, 2007) as it outperforms default LGA and standard GA in terms of speed, robustness and accuracy of the predicted protein-ligand complex.

Deterministic Methods

Third category that considers ligand as flexible body includes deterministic methods, such as molecular simulations. Simulations provide fine detail that concerns the motions of individual particles as a function of time. Molecular simulations for example numerically solve Newton's equation of motion and therefor allow observation of structural fluctuations with respect to time. Therefore simulations can quantify the properties of a system at a precision and on a time scale that is otherwise inaccessible. Molecular dynamics, energy minimization and also Monte Carlo (Caflisch et al., 1992; Hart & Read, 1992) are methodologies that can be used to investigate and study the dynamics of the system with known atom coordinates (Adock & McCammon, 2006). AMBER (Case et al., 2005; Salomon-Ferrer, Case, & Walker, 2013) and CHARM (Brooks et al., 1983) are example of programs using molecular simulations for different poses generation. Molecular simulations computations are very demanding and require long computation time, which is the reason this method is not popular for virtual screening of large compound libraries. With more powerful computers, there are more and more docking programs that incorporate this method, for example CDOCKER (Wu, Robertson, Brooks, & Vieth, 2003).

SCORING FUNCTIONS

After the first step in molecular docking, where different ligands and ligand poses are determined or generated by various search algorithms, the assessment with scoring function follows, where correct poses from incorrect poses are delineated. The main goal of scoring function is the calculation of the energy that estimates the binding affinity between the protein and the ligand, which can be expressed by the binding free energy (ΔG_{bind}):

$$\Delta G_{bind} = G_{complex} - G_{protein} - G_{ligand} = \Delta H - T\Delta S \tag{1}$$

where ΔG_{ligand} is the free energy of the ligand, $\Delta G_{protein}$ is the free energy of the protein and $\Delta G_{complex}$ is the free energy of the complex formed by the constituent molecules. Binding free energy, also known as Gibbs free energy can be measured by determining the equilibrium binding constant K_{eq}. ΔG_{bind} is related to the K_{eq} by the following equation:

$$\Delta G_{bind} = -RT \ln\left(K_{eq}\right) \tag{2}$$

where R is the perfect gas constant and T is the temperature.

There are numerous methods that are capable to computationally predict the binding free energy of a molecular complex. They significantly differ in accuracy and speed. Scoring function has to follow several criteria for being appropriate for molecular docking applications. Despite being sufficiently fast,

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it must be capable to estimate interaction between molecules that is proportional to the binding free energy, correctly rank the poses, discriminate binders from nonbinders or active compound from inactive compounds, all in comparison with experimentally determined 3D structures of complexes. Free energy perturbation (Zwanzig, 1954) and thermodynamic integration (Straatsma & McCammon, 1991) are methods, which enable very accurate calculation of binding free energies with very high calculation time consumption and that is the reason these are not applied for molecular docking. Other less accurate and therefore less time consuming methods rely on several assumptions and simplifications for binding free energy estimation. These scoring functions are generally classified into four main categories: force-field based (first principle based approach), empirical, knowledge-based and consensus scoring (Gohlke & Klebe, 2001; Brooijmans & Kuntz, 2003; Huang et al., 2010, Meng et al., 2011; Bras et al, 2014) (Figure 4). Below, brief description of every category is provided.

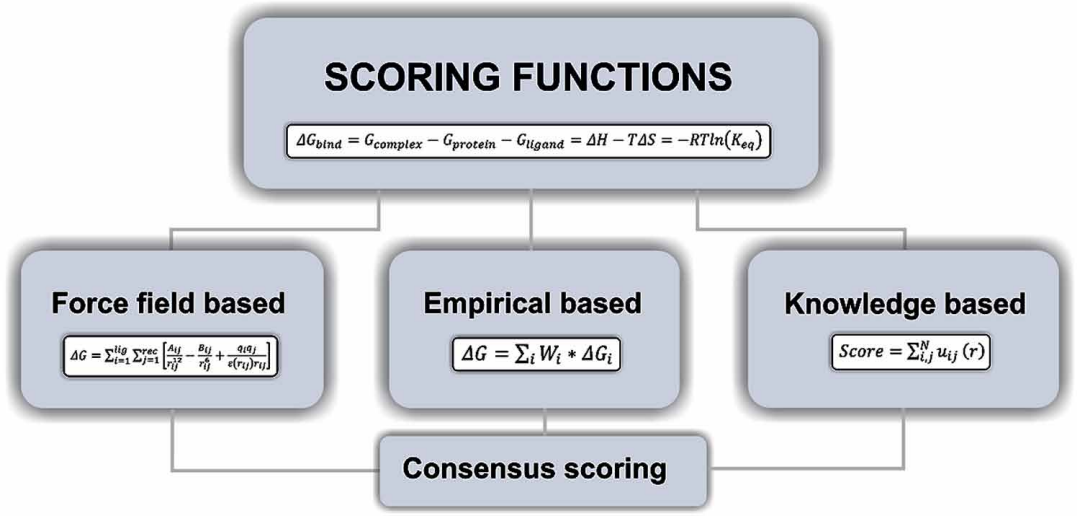
Force Field Based Scoring Functions

Force field based scoring functions basically take into account the non-bonded terms of the molecular mechanics force field, which means they estimate the interaction energy between the protein and the ligand. Non-bonded terms in this case represent electrostatic and van der Waals interactions, where electrostatic term is estimated by a Columbic formulation and van der Waals term by a Lennard-Jones potential function (Mehler & Solmajer, 1991; Meng, Shoichet, & Kuntz, 1992):

$$\Delta G = \sum_{i=1}^{lig} \sum_{j=1}^{rec} \left[\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}} + \frac{q_i q_j}{\mu\left(r_{ij}\right) r_{ij}} \right] \tag{3}$$

where r_{ij} is the distance between protein atom i and ligand atom j, A_{ij} and B_{ij} are Lenard-Jones parameters, q_i and q_j are atomic charges and ε is distance dependent dielectric constant in the Coulombic term.

Figure 4. Scoring functions applied for assessment in molecular docking



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DOCK (Ewing et al., 2001; Allen et al., 2015) and AutoDock (Huey, Morris, Olson, & Goodsell, 2007) are based on force field scoring functions.

Besides non-bonded forces there are forces present in protein-ligand binding, such as ionic bonds and hydrogen bonds and also conformational changes after ligand binding, which have to be considered for free energy estimation. Empirical force field scoring function used in AutoDock 4.0 for example (Huey et al., 2007) applies thermodynamic model of the binding process which includes intramolecular terms for the free energy estimation and also a full desolvation model, that includes terms for all atom types (favorable energetics of desolvating carbon atoms and unfavorable energetics of desolvating polar and charged atoms). This semiempirical approach estimates the energetics of the complex formations using pair-wise terms to evaluate interaction between molecules and an empirical method to estimate the contribution of surrounding water. In this aspect semiempirical approach differs from traditional molecular mechanics force field, which uses explicit water molecules for evaluation of solvation contributions and does not include entropic terms that are important for the interaction energy assessment. In some cases implicit solvation models are used for the estimation of desolvation energies of the ligand and the receptor. Poison-Boltzman-surface (Grant, Pickup, & Nicholls, 2001) and generalized Born-surface area (Still, Tempczyk, Hawley, & Hendrickson, 1990; Hawkins, Cramer, & Truhlar, 1995) are examples of models that treat water as continuum dielectric medium.

Empirical Based Scoring Functions

Approximation of binding free energy with empirical based scoring functions is composed of a sum of various energetic terms (Huang et al., 2010; Bras et al., 2014):

$$\Delta G = \sum_i W_i * \Delta G_i \tag{4}$$

where ΔG_i represents different energy terms such as van der Waals energy, electrostatics, desolvation, hydrogen bond, entropy, etc. The coefficients W_i that correspond to specific energy term are derived from regression analysis of known (protein-ligand) complex structures binding affinities.

Böhm (1994) developed first empirical scoring function, implemented in docking program LUDI, which takes into account hydrogen bonds, ionic interactions, lipophilic protein-ligand contact surface and the number of rotatable bonds in the ligand. Afterwards various docking programs implemented similar scoring functions. ChemScore (Eldridge, Murray, Auton, Paolini, & Mee, 1997) for example is parametrized form 82 complexes and is implemented in program GOLD (Cole, Nissink, & Taylor, 2005; Liebeschuetz, Cole, & Korb, 2012) and is also one of the functions available in FRED (McGann, 2011). In comparison with Böhm's scoring function ionic interactions term is absent in ChemScore, whereas metal-ligand binding contributions are taken into account. FlexX (Rarey et al., 1996) docking program also uses similar scoring function with additional aromatic group interactions term consideration. Various docking programs, despite applying similar energy terms typical for empirical scoring functions, implement different computations of specific term.

Empirical scoring functions are generally simpler than force filed based functions, therefor binding score calculations of former are faster. As these functions are dependent on experimental data set used for parameterization it is not necessary that the binding affinity will be predicted correctly for structurally

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different ligands from those exploited in the training set. With the increasing number of protein-ligand complexes with known 3D structures, functions can be trained with higher number of known binding affinities, which leads into development of scoring functions appropriate for various ligands.

Knowledge Based Scoring Functions

Knowledge based scoring functions are statistical methods, where the parameters of the potential functions are extracted from the structural information of experimentally determined atomic structures. This approach implicitly incorporates physical interactions (electrostatic, van der Walls, cation-π interactions), where high frequency of occurrence/non-occurrence of specific atom-atom interactions is assumed to represent favorable/unfavorable contact (Brooijmans & Kuntz, 2003; Li & Liang, 2006; Huang et al., 2010; Bras et al., 2014). Potential function is the weighted linear sum of pairwise contacts, presented in following formula:

$$Score = \sum_{i,j}^{N} u_{ij}\left(r\right) \tag{5}$$

where i and j represent protein and ligand atom type, r is the distance between atom pair, N is the number of all possible atom pairs in the system and u_{ij} corresponds to the pairwise potentials between atom types, obtained from the occurrence frequency of atom pairs in a database using inverse Boltzman relation:

$$u\left(r\right) = -k_B T\, ln\left(\frac{\acute{A}_{ij}\left(r\right)}{\acute{A}_{ref}\left(r\right)}\right) \tag{6}$$

where k_B is the Boltzmann constant, T is the absolute temperature of the system, $\rho_{ij}(r)$ is the normalized probability of protein-ligand atom pair to be in contact at distance r, and $\rho_{ref}(r)$ is the reference probability - the state where atomic interactions between protein and ligand are zero.

First ideas of statistical potentials originate from the field of protein folding and structure prediction (Huang & Zou, 2006). Tanaka and Scheraga (1976) were first that discussed about the statistical mechanical basis for obtaining the parameters for interactions between amino acids in proteins for prediction of the 3D structure. Since then, knowledge based approaches have been extensively used to develop the scoring functions for protein-ligand interactions and nowadays such scoring functions are implemented in various docking programs, e.g. DrugScore^{CSD} (Velec, Gohlke, & Klebe, 2005), AutoDock Vina (Trott & Olson, 2009) and also GOLD, where knowledge based Astex Statistical Potential (Mooij & Verdonk, 2005) is used as one of the scoring functions for docking. Another interesting method for scoring protein-ligand interaction is knowledge based quantitative structure-activity relationship (QSAR) (Ballester & Mitchell, 2010), where distance-dependent atom pair occurrences are utilized as descriptors for QSAR. Here is machine learning used instead of reverse Boltzman relationship and advantages of this methods are application of a very large training set of binding affinities besides structural data (traditional knowledge based methods use only structural data).

Knowledge based scoring functions, in comparison with force field based and empirical based, offer good balance between accuracy and speed, since the computation is relatively simple and the potentials

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are extracted from the known structures of protein-ligand complexes. These functions are also able to implicitly capture the binding effects that are difficult to model explicitly (e.g. sulfur-aromatic and cation-π interactions). The disadvantage of these functions is the accuracy of the reference state, where different methods were developed with attention to solve this problem, for example the adjustments of the pair potentials with iterative method (Huang & Zou, 2006).

Consensus Scoring

Consensus scoring combines results from various scoring functions to improve the performance of scoring in molecular docking. Charifson et al. (1999) first presented the results, where consensus scoring provided reduction in the number of false positives identified by individual scoring functions, leading to a significant enhancement in hit rates. Since then, several studies were published where consensus scoring that indicate improved quality of the results, was applied for evaluating ligand-protein interactions for molecular docking and ligand based virtual screening (Stahl & Rarey, 2001; Clark, Strizhev, Leonard, Blake, & Matthew, 2002; Baber, Shirley, Gao, & Feher, 2005). Improvements depend on the applications, the consensus method and the conditions of the computational tests. However, it should be mentioned that blind combination of arbitrarily chosen scoring functions will not necessarily lead to better results (Feher, 2006). For this purpose Oda et al. (2006) compared the performance of consensus scoring strategies, where multiple scoring functions were used simultaneously and demonstrated that choosing the most appropriate type of consensus score is essential for model selection in computational docking.

CASE STUDY: COMPARATIVE EVALUATION OF SEVERAL DOCKING TOOLS FOR DOCKING SMALL MOLECULE LIGANDS TO DC-SIGN

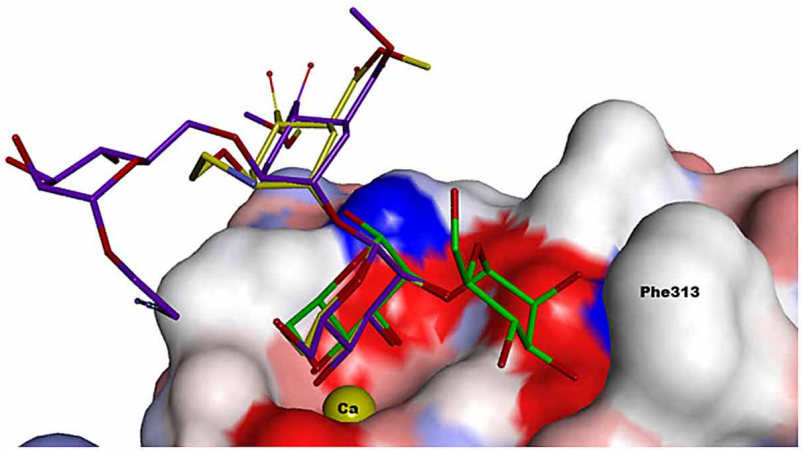
DC-SIGN: How to Dock Non-Drug-Like Saccharide Ligands into a Non-Druggable Target?

DC-SIGN or Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin is a C-type lectin located specifically on dendritic cells (DCs). Several pathogens like HIV-1 enter DCs using DC-SIGN as an entry receptor, but escape the normal lytic pathway thus avoiding the host immune system (Anderluh, Jug, Švajger, & Obermajer, 2012). Potential anti-infectives that would inhibit DC-SIGN-pathogen interaction as the very first step in pathogen infection would act via completely new mechanism of action as compared to the current mode of anti-infectives' action. Therefore, the main scope of the research was the design of new DC-SIGN carbohydrate recognition domain (CRD) antagonists that could act as novel anti-infectives (Anderluh et al., 2012; Obermajer et al., 2011; Tomašič et al., 2014). Authors have envisaged docking studies as a great help in the design of new DC-SIGN antagonists to virtually preselect potential antagonists to be synthesized and characterized thus avoiding a vast effort in synthetic and biochemical laboratory (Jug, Anderluh, & Tomašič, 2015; Obermajer et al., 2011; Tomašič et al., 2014). Unfortunately, DC-SIGN proved to be a hard target for docking studies for several reasons and therefore authors decided to seek a docking tool that would address the below listed issues to a highest possible extent (Jug et al., 2015).

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- As a lectin, DC-SIGN specifically binds glycosylated proteins, oligosaccharides and monosaccharides. The main determinant of its binding site is the hexacoordinated Ca^{2+} ion that forms two complex bonds with water molecules in the unbound state (Figure 5). Similar interactions are formed with the hydroxyl groups of the bound monosaccharide at positions 3 and 4 alone (i.e. D-mannose or L-fucose) or as a part of glycoconjugate with aglycone part attached to increase binding affinity. Accordingly, a key question was whether the docking algorithm can predict the coordination bonds between the core mannose and DC-SIGN and whether the scoring functions are able to evaluate the coordination bonds accurately.
- Desolvation is expected to be of paramount importance in docking studies of highly solvated ligands like (oligo)saccharides (Kroemer, 2007). DC-SIGN binds oligosaccharides and non-drug-like glycoconjugates that undergo massive desolvation of saccharide moieties upon binding, so different docking algorithms were tested to correctly evaluate their binding, even if desolvation effects are not considered explicitly or ignored.
- Docking of small molecules to a flat/shallow binding site is likely to result in a number of different (either correct or wrong) docked poses (Gowthaman, Deeds, & Karanicolas, 2013). Accordingly, the values of assigned binding energy could be of similarly diverse values because scoring functions are not able to accurately estimate all of the possible interactions and desolvation of the target protein and ligand poses. DC-SIGN CRD binding site is rather flat and shallow, and hence non-druggable, so there is a high probability of docking protocol failing to correctly predict the binding pose of a ligand.

Figure 5. Binding site of DC-SIGN with bound ligand(s) from several crystal structures of DC-SIGN CRD; Ca^{2+} ion and flexible side chain of Phe313 are explicitly named. The protein is presented as a surface colored according to the electrostatic potential and the Ca^{2+} ion is presented as a yellow sphere. Ligands shown on this figure were taken from the above stated crystal structures and are presented as sticks in following color: ligand from 2IT6 (dimannoside Manα1-2Man) is presented in green, ligand from 2XR5 (pseudo-dimannoside mimic) is presented in yellow, and ligand from 2XR6 (pseudo-trimannoside mimic) is presented in violet (Jug et al., 2015)



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Selected Docking Programs and Crystal Structures

Five docking tools that differ in the search algorithms and scoring functions have been validated in the study: FlexX (Rarey et al., 1996; Rarey et al., 1996) as available in LeadIT 2.1.2 (BioSolveIT GmbH), CDOCKER (Wu et al., 2003) as available in Accelrys Discovery Studio 3.0 (Accelrys Inc.), FRED 2.2.5 (McGann, 2011) (OpenEye Scientific Software, Inc.), AutoDock 4.2 (Morris et al., 2009) and GOLD 5.2 (Verdonk et al., 2003) (The Cambridge Crystallographic Data Centre). GOLD and AutoDock use a genetic algorithm (Kitchen et al., 2004), FRED uses a docking algorithm based on Gaussian function (McGann, 2011), CDOCKER uses a molecular dynamics simulated annealing-based docking algorithm (CHARMm) (Wu et al., 2003) and FlexX uses an incremental construction algorithm (Rarey et al., 1996). Re-scoring was performed with HYDE (Schröder et al., 2013) (BioSolveIT).

Re-docking of ligands from selected crystal structures has been performed to allow high ligand diversity. The 3D structures of all ligands used for docking validation were extracted from selected crystal structures of D-mannose containing ligands in complex with DC-SIGN CRD (Figure 5) (PDB codes: 2IT6, 2XR5 and 2XR6). Description of specific ligand preparation and docking protocols is beyond the scope of this chapter and may be found in the original publication (Jug et al., 2015).

RMSD (root-mean-square deviation) was selected as the key parameter for evaluation of docking success (Jain, 2008). Ligand poses from the above mentioned crystal structures were used as a reference for calculation of RMSD values. The upper RMSD limit which still fulfils criteria of acceptance was set to 2.00 Å. A visual comparison of crystal structure ligand pose and the docked poses was done after every successfully completed docking run. Afterwards, RMSD values for the top ten ranked poses were calculated and compared.

Results

The aim was to set up the docking protocols within all numbered programs in such a way that the top pose of docking will also have an RMSD less than 2.00 Å or, in the best possible situation, that the top pose will also have the lowest RMSD value. The calculated RMSD values for the top ranked ligand pose for all three crystal structures and all stated docking programs are listed in Table 2.

FRED gave the lowest RMSD values for the top ranked poses of all three ligands that were re-docked within the RMSD cut-off value of 2.00 Å (Figure 6). Furthermore, FRED performed the best among

Table 2. RMSD values obtained for re-docked ligands of different DC-SIGN CRD-ligand complexes (Jug et al., 2015)

	FRED	AutoDock	FlexX	HYDE	GOLD	CDOCKER
RMSD (Å)	**DC-SIGN 2IT6**					
	0.81 (Figure 6a)	3.50	0.61	0.79	1.68	0.97
	DC-SIGN 2XR5					
	1.32 (Figure 6b)	4.21	7.26	2.01	4.31	1.22
	DC-SIGN 2XR6					
	1.68 (Figure 6c)	5.48	7.44	6.49	6.82	2.34

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chosen docking tools when considering overall RMSD values - taking into account RMSD values of the top ten ranked poses for all three ligands. Promising results were also achieved using CDOCKER, which did not perform well only for the most complex ligand (2XR6). FlexX, which was used in previous study (Obermajer et al., 2011) showed exceptional performance with the first ligand (2IT6), but intriguingly failed later with the other two ligands. However, when FlexX docking poses were re-scored by HYDE, significant improvements of RMSD values were observed in these cases. GOLD and AutoDock performed exceptionally well in docking studies of targets with constrained binding pockets, but failed to perform satisfactorily even at re-docking of the least complex disaccharide ligand (2IT6). Authors presume that the major reason for different performance of the selected tools is the difference between their search algorithms. Majority of them were designed for non-carbohydrate ligands and druggable binding sites, where a small difference in the ligand conformation might result in a substantial difference in the score due to the steric clash and/or repulsive electrostatic interactions, but when faced with the large, flat and shallow binding sites, these algorithms presumably tend to overestimate the influence of small changes in the ligand conformation. For example, AutoDock and GOLD use a genetic algorithm (stochastic) that requires a large number of ligand conformations to explore all the conformational space available for the ligand. In this manner, a vast number of conformations combined with poorly defined (large, flat and shallow) binding site results in a combinatorial explosion that inevitably leads to a higher number of wrongly predicted docking poses. The results also suggest that the incremental construction algorithm used by FlexX should not be considered as the first choice for docking studies involving DC-SIGN and possibly to other non-druggable binding sites. On the other hand, FRED uses a systematic docking algorithm based on Gaussian function and a multi-conformer structure database pre-generated by OMEGA (Hawkins & Nicholls, 2012) that is limited to a much smaller number of ligand conformations. As a consequence, FRED's scoring function is faced with an easier task to distinguish between rather different conformations, so fewer errors are made. Furthermore, observed differences in docking tool performance might be a consequence of inadequate estimation of desolvation penalties and/or complex bonds. One rather simple and expected general rule could be extracted from the results; re-docking of all the ligands with all five docking programs showed that the RMSD increases with greater complexity of the ligand. However, relative differences between all 3 ligands do not correlate among docking tools as these are smaller when using FRED and CDOCKER compared to other docking tools, so again it is presumed that their search algorithms perform better when faced with non-druggable targets and/or large ligands.

The second aim of the study was to check how well the selected docking programs are able to reproduce the pose of the core mannose in comparison with its pose in the crystal structure. The core mannose pose is presumed to be even more important than the overall position of the ligand, as is strongly related to establishment of the coordination bond with Ca^{2+} and the hydrogen bonds with the amino acid residues in the active site. The RMSD values for core mannose for all of the three crystal structures are presented in the Table 3.

Most programs successfully predicted core mannose pose (when considering RMSD for the mannose only), but not when RMSD values for the whole ligands were considered. Once again FRED performed well (Figure 6), with an average RMSD for the core mannose of 0.79 Å. CDOCKER outperformed FRED with an average RMSD for the core mannose of 0.62 Å, which might imply that CDOCKER calculates complex interactions better than FRED. Taking into account the positioning of core mannose only, the fair performance of FlexX should be noted. Authors presume that FlexX possibility of defining Ca^{2+} ion as a "pharmacophore" allows better estimation of complex bonding and thus correct prediction of the core mannose pose. GOLD also showed promising results for the pose prediction, while AutoDock was

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Figure 6. Re-docked a) dimannoside (PDB entry: 2IT6, RMSD = 0.81 Å), b) pseudo-1,2-mannobioside (PDB entry: 2XR5, RMSD = 1.32 Å) and c) pseudo-trimannoside mimic (PDB entry: 2XR6, RMSD = 1.68 Å) using FRED docking protocol to DC-SIGN CRD (left). Zoomed view of the docking and X-ray determined pose of the core mannose residue. The position of the ligand/core mannose from X-ray determination is coloured green and the docked structure is coloured grey (right)

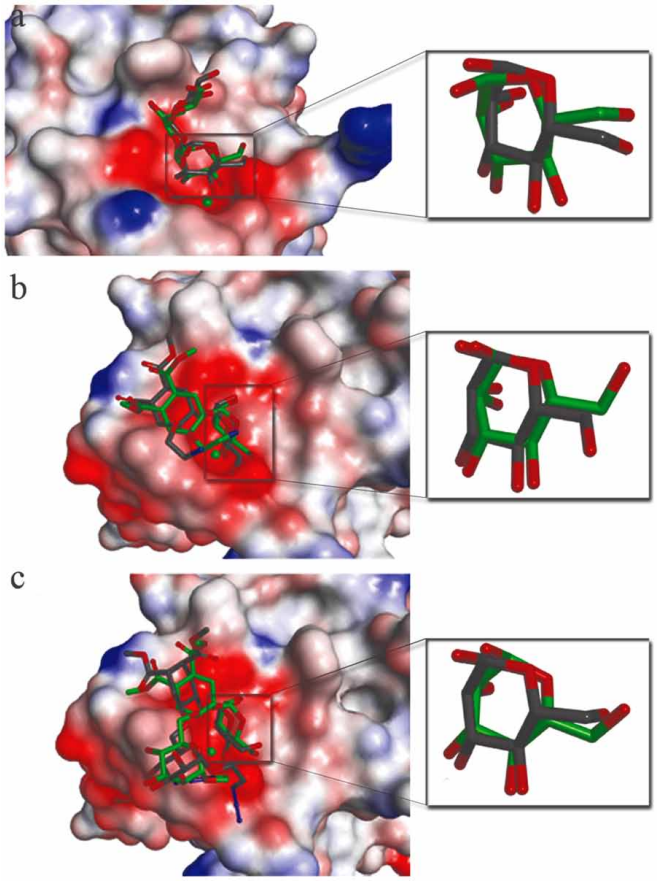


Table 3. RMSD values obtained for re-docked core mannose

	FRED	AutoDock	FlexX	HYDE	GOLD	CDOCKER
RMSD (Å)	**DC-SIGN 2IT6**					
	0.65 (Figure 6a)	3.40	0.79	0.55	1.04	0.73
	DC-SIGN 2XR5					
	0.97 (Figure 6b)	1.36	0.79	0.92	0.80	0.34
	DC-SIGN 2XR6					
	0.76 (Figure 6c)	2.35	2.96	3.11	8.71	0.78

(Jug et al., 2015).

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not satisfactory. HYDE re-scoring improved the RMSD in the case of the least complex ligand (2IT6), while in other two cases RMSD surprisingly slightly increased. The graphic presentations of the docking results can be obtained in the original publication (Jug et al., 2015).

To conclude, results in this study show that even widely used docking programs have certain limitations when faced with a rather shallow and flat (i.e. non-druggable) binding site, as is the case of DC-SIGN. FRED and CDOCKER were found to be appropriate for docking studies on DC-SIGN, as evaluated by the calculated RMSD values for each of the re-docked ligands and for the core mannose only, while other programs showed more or less weaknesses. Among these two, FRED was found as the best tool for docking ligands to DC-SIGN and therefore authors suggest it as a suitable docking program for docking studies on DC-SIGN and similar targets with non-druggable binding sites.

CASE STUDY: COVALENT DOCKING OF CATHEPSIN K

Covalent Docking

There are several drugs on the market that act by covalent inhibition (e.g. acetylsalicylic acid, amoxicillin, omeprazole, rivastigmine). Their mechanism of action is connected to their electrophilic "warheads", which target nucleophilic protein residues such as serine and cysteine, and they can form reversible or irreversible covalent linkage (London et al., 2014; Weerapana et al., 2010).

The main aim of designing covalent inhibitors is to increase selectivity and potency, particularity by targeting non-conservative protein residues and shallow binding pockets. However, covalent inhibitors could also possess promiscuous behavior causing off target effects and can be toxic due to prolonged duration off biological effects in case of irreversible binding mode. Therefore, specific noncovalent interactions are crucial for warhead orientation relative to protein nucleophile, stabilizing ligand-target complex and increasing selectivity in order to achieve desired selectivity and potency. Thus the main goal of covalent docking is to identify scaffolds that optimally orientate electrophilic warhead, and as in the case of noncovalent docking, to minimize the number of compounds that have to be prepared and biochemically/biologically evaluated (Kumalo, Bhakat, & Soliman, 2015; London et al., 2014).

In covalent docking studies, the reaction between ligand and receptor has to be taken into account. That could be achieved by "link atom" approach, which utilizes predefined link atom for both, the ligand and the target. It mimics the covalent binding event by forcing ligand link atom to occupy the same steric volume as the target link atoms (GOLD docking software) (Verdonk et al., 2003). On the other hand, "grid based method" and "modification of the flexible side chain" methods are utilized in covalent docking approach of Autodock software (Morris et al., 2009). Another covalent docking approach is based on treating covalent bond as a noncovalent interaction with the sum of additional energy contribution from the covalent bond formation - program CovalentDock (Ouyang et al., 2013).

It has to be mentioned that covalent docking has also its disadvantages; similar to noncovalent docking, poor scoring functions, limited protein flexibility, speed and accuracy are the main issues. Besides that, lack off automation and ligand preparation steps are time consuming (Kumalo et al., 2015).

There are several software packages and webservers suitable for covalent docking:

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- **Software:** GOLD (Verdonk et al., 2003), CovalentDock (Ouyang et al., 2013), Autodock (Cosconati et al., 2010), Glide (Friesner et al., 2006), CovDock (Zhu et al., 2014), CovDock-VS (Warshaviak, Golan, Borrelli, Zhu, & Kalid, 2014), etc.
- **Webservers:** CovalentDock Cloud (Ouyang, Zhou, Ge, Li, & Kwoh, 2013), Dockovalent (Irwin et al., 2009), etc.

Covalent Docking of Potential Cathepsin K Inhibitors by GOLD Docking Software

The covalent molecular docking studies were performed on 254-membered benzamide-containing aminonitriles dataset by GOLD Suite v5.1 docking package (Verdonk et al., 2003). Crystal structure of cathepsin K (Cat K) was derived from PDB with entry code 4DMX (Dossetter et al., 2012). Binding site was defined around experimental coordinates of the 0LB301 (Dossetter et al., 2012) bound ligand in radius of 6Å. In order to perform covalent docking, dummy sulfur linking atom was attached to each studied ligand. Subsequently, ligands were renumbered in a way that previously added sulfur's atom number was set to 1. Cat K sulfur atom on Cys25 was then matched with dummy sulfur atom of ligand. Preparing ligands in this way was necessary for GOLD to recognize and set the correct covalent bond between predefined ligands and proteins linking atoms (Schröder et al., 2013).

Re-docking procedure was performed by docking reference ligand 0LB301 into the binding site with early termination allowed if the top three solutions were within 1.5 Å of the root-mean-squared-deviation value in order to define optimal parameter and scoring function (Kirchmair, Markt, Distinto, Wolber, & Langer, 2008). Finally, each ligand was docked into the binding site by the selected scoring function and applying the following parameters of the GOLD genetic algorithm: population size = 100, selection pressure = 1.1, no. of operations = 100,000, no. of islands = 5, Niche Size = 2, migrate = 10, mutate = 95, crossover = 95 (Jones et al., 1997).

Results

Co-crystallized Cat K inhibitor 0LB301 was used to access visual fitness and orientation of docked compounds. Re-docking ligand validation gave satisfactory results as GOLD managed to reproduce accurate binding poses of experimentally bounded ligand 0LB301 with good accuracy. Scoring function ChemScore (CSF) yielded the lowest RMSD value (0.19 Å) for re-docking procedure and was chosen for the assessment of the binding affinities.

Post docking analyses were divided into two parts. In the first part, docking dataset was visually examined as visual fitness and orientation were compared to experimental pose of the 0LB301 ligand. If visual fitness and orientation of a studied compound were correct, the second level post docking analyses were performed by measuring interatomic distances between docked compound features and key amino acid residues of 4DMX.Visual inspection yielded 113/254 compounds from docking dataset with orientation and fitness in concordance with experimentally bound ligand.

In the second part of post-docking analyses the key distances between ligands features and binding site of protein residues 4DMX were measured. According to previously published data (Palmer et al., 2005), three H-bond interactions between ligand and protein were used: (R- N**H**-CH_3 - CN)-(Asn161-C**O**) [$d_{4DMX\text{-}1}$ = 3.28 Å], (R-C**O**-NH-CH_3-CN)-(Gly66-N**H**) [$d_{4DMX\text{-}2}$ = 3.22 Å], (R-Ph-CO-N**H**-R`)-(Gly66-C**O**) [$d_{4DMX\text{-}3}$ = 2.9 Å]. Because of dynamic nature of biological systems, the tolerance of ±1Å for each

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interaction distance is used: (R- N**H**-CH_3 - CN)-(Asn161-C**O**) [d_{4DMX-1} = 2.28 - 4.28 Å], (R-C**O**-NH-CH_3-CN)-(Gly66-N**H**) [d_{4DMX-2} = 2.22 – 4.22 Å], (R-Ph-CO-N**H**-R`)-(Gly66-C**O**) [d_{4DMX-3} = 1.9 - 3.9 Å]. Interatomic distance measurement yielded 12/254 correct compounds (Figure 7).

Additionally, QSAR model for prediction of biological activities were applied (Borišek, Drgan, Minovski, & Novič, 2014) which resulted in 4/254 finally valid compounds (Table 4).

Resulted compounds from the docking study were thereafter synthesized and proved to be reversible and selective inhibitors of Cat K in a nanomolar range (Borišek et al., 2015).

Figure 7. Close view of the 12 GOLD-calculated covalently bound combinatorial Cat K ligands; the key attachment point for covalent bond formation is Cys25 and the amino acid residues Gly66 and Asn161 (depicted in cyan) are important for the stabilization of the ligand through H-bonding.

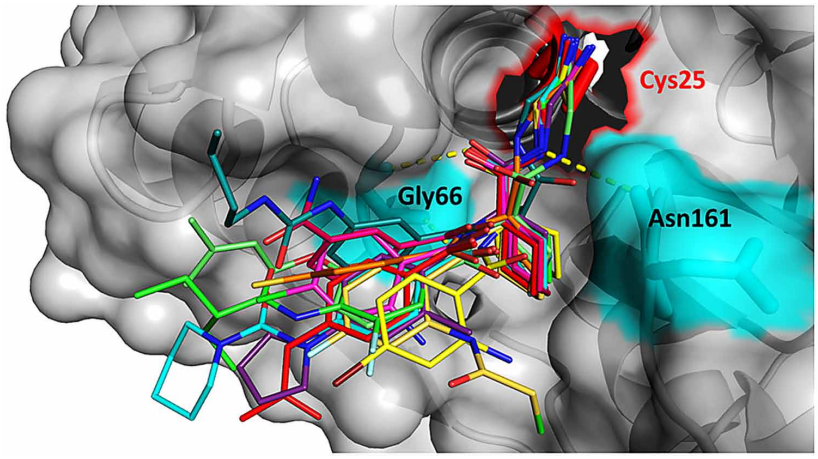
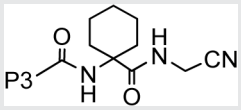
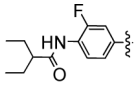
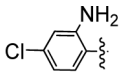
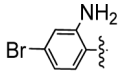
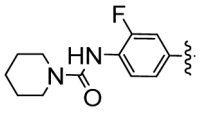


Table 4. Resulted 4/254 compounds after combining molecular docking and QSAR modeling results



ID	P3	CSF	d_{4DMX-1}	d_{4DMX-2}	d_{4DMX-3}	K_i (nM)
1		35.0	3.25	2.91	2.80	70
2		32.4	3.23	2.75	2.98	16
3		33.6	3.29	2.83	2.92	10
4		33.3	3.25	2.76	2.95	55

Borišek et al., 2015.

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Authors decided to employ GOLD docking software for covalent docking since the familiarness with this program and positive experience relating to noncovalent docking in GOLD. There is also easy to follow step by step description of covalent docking procedure in GOLD tutorial. It has to be stressed that ligand preparation for covalent docking was not automatized and was more time consuming compared to noncovalent docking. Additional studies are planned to compare several docking programs and webservers for covalent docking in order to find most suitable one for particular ligand-target system.

CONCLUSION

This chapter provides overview of computational approaches for molecular docking, considering several of the most commonly used program tools (AutoDock, FRED, CDOCKER, FlexX and GOLD). The application of these algorithms authors present in the comparative study for docking of small ligands to specific protein, lectin Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) and covalent docking of cathepsin K with GOLD. As explained in the chapter, five docking tools have been validated in the case study based on the in-house availability, but above all because of the intrinsic difference among them. They differ in the search algorithms and scoring functions, and those with most different algorithms and scoring function were selected. Of course, the selection of various other docking software would be more appropriate (like ADAM, DARWIN, DOCK, eHiTS, FLIPDock, Glide, YUCCA, etc.), but it is practically impossible to evaluate all of them. It was presumed that the mentioned intrinsic differences would most probably result in significant differences in performance of various software, which indeed turned out to be true.

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Chapter 33
Application of Docking Methodologies in QSAR-Based Studies

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ABSTRACT

The computational strategies permeate all aspects of drug discovery such as virtual screening techniques. Virtual screening can be classified into ligand based and structure based methods. The ligand based method such as Quantitative Structure Activity Relationship (QSAR) is used when a set of active ligand compounds is recognized and slight or no structural information is available for the receptors. In structure based drug design, the most widespread method is molecular docking. It is widely accepted that drug activity is obtained through the molecular binding of one ligand to receptor. In their binding conformations, the molecules exhibit geometric and chemical complementarity, both of which are essential for successful drug activity. The molecular docking approach can be used to model the interaction between a small drug molecule and a protein, which allow us to characterize the performance of small molecules in the binding site of target proteins as well as to clarify fundamental biochemical processes.

INTRODUCTION

The Molecular modeling expression is used to describe the use of computers to build compounds and carry out a variety of calculations on these compounds in order to predict their chemical characteristics and behavior. The computational strategies also permeate all aspects of drug discovery such as virtual screening techniques in comparison to the process of trial and error that was used in the search for novel drugs.

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The most important challenges that medicinal chemists face today is the design of new drugs with improved potency and less side-effects for treating human diseases such as AIDS and others. Medicinal chemists begin the process by taking a lead structure and then finding analogs exhibiting the preferred biological activities. Next, they use their experience and chemical insight to eventually choose a candidate analog for further development. This process was not only difficult but also expensive and time consuming. The conventional methods of drug discovery are now being supplemented by shorter approaches made possible by the accepting of the molecular processes involved in the original disease. In this view, the preliminary point in drug design is the molecular target, which is a receptor or enzyme in the body as an option of the existence of the known lead structure.

The effective design of chemical structures with the desirable therapeutic properties is directed towards Computer Aided-Drug Design (CADD) a well-established area of Computer Aided-Molecular Design (CAMD). The main applications of CAMD are the clarification of the basic requirements for a compound to obtain a determined activity, the simulation of the binding between a ligand and the receptor, the discovery of new active compounds and the prediction of activities for non-synthesized analogues. Two major modeling strategies right now are used in the designing of new drugs. In the first strategy, the design is based on the comparative analysis of the structural features of known active and inactive molecules that are interpreted in terms of their complementarily with a supposed receptor site model. This strategy is called *ligand based design* and one of its approaches is quantitative structure activity relationships (QSAR). This discipline was promoted by Hansch and his group (Fujita, 1990). In the second strategy, the three-dimensional features of a known receptor site are directly considered and this strategy is called *structure based design* method. In structure based drug design, the most widespread method is molecular docking (Mahajan A., Gill N.S & Arora R., 2014; Xuan-Yu Meng, Hong-Xing Zhang, Mihaly Mezei & Meng Cui, 2011) The molecular docking approach which will be discussed in details in the coming section can be used to model the interaction between a small drug molecule and a protein, which allow us to characterize the performance of small molecules in the binding site of target proteins as well as to clarify fundamental biochemical processes.

This chapter deals with the two strategies that are used in designing new drugs. At first, focus will be on the ligand based design or QSAR approach. The QSAR section starts with history and the earliest efforts made in this field, types of descriptors and statistical analysis methods. Moreover, validation of QSAR models, the internal as well as the external validations. Second, the focus will be on the structure based design or molecular docking approach. The different types of docking methods will be discussed including the different software used. At the end, applications or case studies done by the authors and others will be discussed also. For example, Exploration of human serum albumin binding sites by docking and molecular dynamics, Docking study related to non-peptide HIV-1 protease inhibitors, Homology modeling, molecular dynamics and docking simulations of rat A2A receptor, Exploring the ligand recognition properties of the human vasopressin V1a receptor using QSAR and molecular modeling studies and others. These studies will introduce the reader to the field of molecular docking and its use in structure based drug design. Finally, more attention must be dedicated to the combination of the two approaches in designing new drugs.

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BACKGROUND

The approach of designing new drugs can be classified into ligand based and structure based methods. The ligand based method such as quantitative structure activity relationship (QSAR) is used when a set of active ligand compounds is recognized and slight or no structural information is available for the receptors. In structure based drug design, the most widespread method is molecular docking. The molecular docking approach can be used to model the interaction between a small drug molecule and a protein, which allow us to characterize the performance of small molecules in the binding site of target proteins as well as to clarify fundamental biochemical processes.

LIGAND BASED DESIGN METHOD

Quantitative Structure Activity Relationships (QSAR)

QSAR is a way of finding and modeling a simple mathematical equation between molecular descriptors calculated from structures of a set of molecules with their reactivity in order to be able to predict the reactivity of unknown compounds.

Applications of QSAR can be extended to any molecular design purpose, including prediction of different kinds of biological activities, lead compound optimization and prediction of novel structural leads in drug discovery. QSAR consists of several steps which hopefully lead to the design of new compounds with the desired activity profile.

The QSAR approach follows these steps. The first step of building a QSAR model is to select a training set consisting of compounds with their experimental biological activities. The next step is to optimize the molecular structure by a proper technique. Afterwards, one should compute descriptors that contain sufficient relevant information about the biological phenomenon. Once descriptors have been calculated, it is necessary to pick which should be included in the QSAR model. Therefore, feature selection techniques are used to select the most relevant descriptors from a pool of descriptors. One way is simply to use the correlation coefficient method to select the descriptor with the highest correlation coefficient. Next step, a data analysis is needed to calculate the best mathematical expression linking together the descriptors and biological activities, In the final step, validation and predictions for non-tested compounds will take place. However, the predictive capability of the model first is verified using an external set of compounds which are not used in calibration. This is talented by biological testing of some additional compounds (test set) in the same way as the training set and then comparing the experimental finding with the values predicted by the QSAR model. If the QSAR predicts within acceptable restrictions, it may be used for a more extensive prediction of more compounds.

Hansch equation (Hansch, 1969) was developed to correlate physicochemical properties (descriptors) with biological activities and is given in a general form by the following equation (1):

$$\log \frac{1}{C} = a(\log P)^2 + b\,log\,P + cs + \cdots k \tag{1}$$

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where *C* is the molar concentration that produces the biological effect; *P* is the octanol/water partition coefficient and *s* is the electronic Hammett constant.

Descriptors

A common question in QSAR is how to describe molecules and their physicochemical properties (descriptors). The nature of the descriptors used and the extent to which they instruct the structural properties related to the biological activity is a critical step in a QSAR study It has been estimated that over a thousand of molecular descriptors can now be calculated from a given molecular structure (Devillers & Balaban, 1999; Karelson, 2000;Todeschini, Consonni & Pavan, 2001). Most of those descriptors can be calculated by using commercial software packages such as CODESSA (Katritzky, Lobanov & Karelson, 1994), DRAGON (Todeschini, Consonni & Pavan, 2001) and others. The various descriptors in use can be largely categorized as being constitutional, topological, electrostatic, geometrical, or quantum chemical.

Statistical Methods

Many different statistical methods are available in the literature and the selection of the appropriate method is critical (Xu & Zhang, 2001). However, we will try to mention some basic techniques, one linear and one non-linear, which were extensively used in QSAR/QSPR studies.

Multiple Linear Regression (MLR) (Montgomery & Peck, 1992) can be considered as an easy interpretable linear regression method. Regression analysis correlates independent X variables or descriptors with dependent Y variables (biological data). The regression model assumes a linear relationship between *m* molecular descriptors and the biological activity variable. This relationship can be expressed with the single multiple-term linear equation as follows:

$$y = b_0 + b_1 x_1 + b_2 x_2 + \cdots + b_m x_m + e \tag{2}$$

The MLR analysis calculates the regression coefficients, b_i, by minimizing the residuals, **e**, which quantify the deviations between the data (Y) and the model (Y'), as in the case of simple linear regression.

Artificial Neural Networks (ANN) method (Duprat, Huynh & Dreyfus, 1998; Tetko, Alessandro, Villa & Livingstone, 1996;) is a non-linear technique inspired in the human brain, composed of many simple processing units called neurons. This method is also recognized as learning algorithms. The aim is to simulate the various shells of the neurons, where each neuron is connected to a number of neighboring neurons with variable coefficients of connectivity that signify the strength of these associations. Several examples of this methodology are explained in Montañez-Godinez *et al* (2014).

Validation of QSAR Models

After the model equation is obtained, or it is also significant to estimate the power and the validity of the model before using it to predict the biological activity. Validity is to establish the reliability and significance of the method for a particular use. For that reason, validation of a QSAR model must be done. There are two validation methods used for a QSAR model: internal and external validation techniques to establish the confidence and strength of the model.

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Internal validation uses the data set from which the model is built and checks for internal stability. *Cross-Validation (CV) technique* is widely employed as an internal validation method of statistical models (Wold, 1991). External validation evaluates how well a built model is generalized. If a sufficiently large series of compounds with known activity are obtainable, the original data set can be divided into two subgroups, the *training set* and the *test set.* The training or calibration set is used to derive a calibration model that will be used later to predict the activities of the test or validation set compounds.

STRUCTURE BASED DESIGN METHOD

Molecular Docking

It is widely accepted that drug activity is obtained through the molecular binding of one ligand to receptor. In their binding conformations, the molecules exhibit geometric and chemical complementarity, both of which are essential for successful drug activity. The computational process of searching for a ligand that is able to fit both geometrically and energetically the binding site of a protein is called molecular docking. The docking process involves the prediction of ligand conformation and orientation within a targeted binding site. In general, there are two aims of docking studies: accurate structural modeling and correct prediction of activity.

The docking program is used to place computer-generated representations of a small molecule into the active site of an enzyme in a variety of positions, conformations and orientations. Each such docking mode is called a 'pose'. In order to identify the energetically most favorable pose each pose is evaluated or simply 'scored' based on its complementarity to the target in terms of shape and properties such as electrostatics. A good score for a given molecule indicates that it is potentially a good binder. This process is repeated for all molecules in the collection, which are subsequently rank-ordered by their scores (or their predicted affinities). Assuming that both the poses and the associated affinity scores have been predicted with reasonable accuracy, this selection will contain a relatively large proportion of active molecules

The prediction of the protein–ligand complex is usually done by searching the translational and rotational degrees of freedom of the ligand within the receptor binding site, and by searching the conformational degrees of freedom of the ligand itself. Binding conformations generated by docking programs are thus defined by both a position of the ligand on the receptor surface and a particular ligand conformer. The first docking algorithm for small molecules was developed by the Kuntz group at UCSF in 1982 (Kuntz *et al*., 1982). Before ligands can be docked against a receptor, generally the binding site has to be identified first. This is done to limit the search space on the receptor surface and thus minimize the degrees of freedom that have to be searched. The active site is often known from crystal structures of ligand-bound receptors, but it can also be predicted. The largest cavity on a protein surface is frequently the active site, but this is not always the case and different active site prediction and analysis methods have been developed. In the absence of knowledge about the binding sites, cavity detection programs such as GRID (Goodford 1985; Kastenholz, Pastor, Cruciani, Haaksma & Fox 2000), POCKET (Levitt & Banaszak 1992), SurfNet (Laskowski 1995; Glaser, Morris, Najmanovich, Laskowski & Thornton. 2006), Docking without any assumption about the binding site is called blind docking. Various excellent reviews on docking have been published in the past (Kitchen, Decornez, Furr & Bajorath 2004; Halperin, Ma, Wolfson & Nussinov 2002; Coupez & Lewis 2006; Kontoyianni, Madhav, Suchanek & Seibel 2008;

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Brooijmans & Kuntz. 2003). In the following sections, we will give more details about the docking theory including search algorithms, scoring functions, docking software's as well as docking methods.

DOCKING ALGORITHMS

Docking algorithms generally contain two search engines; one is responsible for the placement and exploration of the ligand in the binding site, while the other searches the internal degrees of freedom of the ligand. For ligands without any degrees of freedom, it is feasible to search the six translational and rotational degrees of freedom in the receptor binding site. However, as the number of rotatable bonds of the ligand increases, the number of degrees of freedom increases exponentially when finding solutions to fit into the binding site. Different algorithms are available and can be divided into three major categories. The first algorithm is a grid search that comprehensively searches the binding site by moving the rigid ligand through the available six degrees of freedom in a systematic fashion in the binding site. The original algorithm only looked for surface complementarity (Katchalski-Katzir *et al*., 1992), but later implementations also use electrostatic complementarity, as in FTDOCK (Gabb, Jackson, & Sternberg, 1997) for example. The second algorithm and significantly more efficient algorithm place ligands in the binding site by descriptor matching (Ewing & Kuntz, 1997). Descriptors to find a solution are generally points, sometimes with properties associated to them, placed in the receptor site. Ligand atoms are matched with the receptor descriptors with some tolerance. The descriptor matching algorithm orients the ligand in the binding site, which subsequently has to be optimized and scored. Usually a large number of orientations are possible, and they all have to be explored and optimized. When binding site descriptors are associated with physical– chemical properties, such as hydrophobicity and hydrogen-bonding capabilities, the ligand atoms have to match geometrically as well as chemically. DOCK (Shoichet & Kuntz, 1993; Moustakas *et al*., 2006) and FlexX (Rarey, Wefing & Lengauer, 1996) use descriptor matching to orient the ligand in the binding site. The third algorithm is an energy-based search method using molecular mechanics force fields to explore the energy surface of the ligand with molecular dynamics (MD) or energy minimization algorithms. The minima of the ligand on the surface have to be located and assessed in terms of their complementarity to the receptor. Energy minimization is a local search, and is only used to optimize binding conformations generated by other search engines. MD searches are global searches in principle, but also tend to get stuck in local minima. This search strategy generally explores all degrees of freedom simultaneously, and is therefore not strictly considered as orientation algorithms.

In summary ligand search algorithms are divided into three types of methods, namely systematic, stochastic, and deterministic. Systematic search methods are frequently used in rigid protein-ligand docking, where there are only six degrees of freedom, while genetic algorithms and Monte Carlo are stochastic search algorithms, and molecular dynamics and energy minimization are deterministic algorithms.

Scoring Functions

The search algorithm of a docking method generates a set of candidate dockings for a particular ligand, but some mechanism is needed to decide which ligand placements are better than others, and thereby rank conformations against each other. This mechanism is called a scoring function. Scoring functions can be classified into three categories: knowledge-based, empirical and force field-based. Knowledge-

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based scoring functions rely on statistical means to extract rules on preferred, and non-preferred atom pair interactions from experimentally determined protein-ligand complexes. The rules are interpreted as pair-potentials that are subsequently used to score ligand binding poses. The PMF score (Muegge & Martin 1999), for example, is a well-known knowledge-based scoring function. Empirical scoring functions sum enthalpic and entropic interactions with the relative weights of the terms based on a training set of protein-ligand complexes. The interaction terms often include Van der Waals, electrostatic interactions and hydrogen bonds. Examples of empirical scoring functions include ChemScore (Eldridge, Murray, Auton, Paolini & Mee 1997) and the FlexX (Rarey, Wefing, & Lengauer, 1996) scoring function. Force field scoring functions are similar to empirical scoring functions in that they predict the binding free energy of a protein-ligand complex by adding up individual contributions from different types of interactions. However, they differ from empirical scoring functions in that the interaction terms are derived from physical chemical phenomena as opposed to experimental affinities. Examples of force field scoring functions in docking programs include the energy score in DOCK (Shoichet and Kuntz, 1993; Moustakas *et al*., 2006), the score function used for single ligand docking in GOLD (Jones, Willett, Glen, Leach and Taylor 1997). Several reviews on the use of various scoring functions employed in docking calculations have been published (Tame, 1999; Halperin, Ma, Wolfson & Nussinov 2002; Gohlke & Klebe 2001).

Docking Software

A large number of docking programs have been published. Each program applies a certain algorithm that explores the way the ligands are treated during docking. For example, programs that follow the algorithm in which the ligand is built up incrementally, starting from a docked 'base fragment' are DOCK (Shoichet & Kuntz, 1993; Moustakas *et al*., 2006), and FlexX (Rarey, Wefing & Lengauer, 1996). In other programs, such as AutoDock (Morris *et al*., 1998; Osterberg, Morris, Sanner and Olson, 2002), Genetic Optimization for Ligand Docking (GOLD) (Jones, Willett, Glen, Leach & Taylor 1997) the ligand is treated in its entirety. Few docking programs allow protein flexibility such as AutoDock (Osterberg, Morris, Sanner & Olson, 2002), FlexE (Rarey, Wefing, & Lengauer, 1996) and QXP (McMartin and Bohacek, 1997). Another measure to classify docking programs would be according to the search strategy employed. For example, DOCK (Shoichet & Kuntz, 1993; Moustakas *et al*., 2006) and FlexX (Rarey, Wefing, & Lengauer, 1996) trying to maximize shape complementarity while AutoDock (Morris *et al*., 1998; Osterberg, Morris, Sanner & Olson, 2002), QXP (McMartin & Bohacek, 1997) and GOLD (Jones, Willett, Glen, Leach & Taylor 1997) programs incorporating an energy-driven or stochastic algorithm.

DOCKING METHODOLOGIES

Rigid Ligand and Rigid Receptor Docking

In this type of docking, the ligand has six degrees of freedom, three translational and three rotational in which the search space is limited. Only one conformation of the ligand is considered. No internal rotations about bonds are permitted in either molecule. Although no modern docking program relies exclusively on this type, they remain an integral component of many other techniques. DOCK (Shoichet & Kuntz, 1993; Moustakas *et al*., 2006) and FTDOCK (Gabb, Jackson & Sternberg, 1997) adopted this type docking. This method is divided into three types: Clique search, geometric hashing and pose clustering. All

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are based on finding complementarity between the geometry of the binding site and that of the ligand. Some early docking programs used clique detection to find docked positions of rigid ligands, and clique detection remains an element of several modern, flexible-ligand docking programs, including recent versions of DOCK. In DOCK, to place a given conformation of the ligand, the unoccupied space in the binding site of the receptor is broken down into spheres, and the docking problem is solved by matching the centers of heavy atoms in the ligand to sphere centers in the binding site using clique detection.

Flexible Ligand and Rigid Receptor Docking

Most small molecules have one or more freely rotatable bonds, allowing them to assume a variety of stable conformations, so the next logical step in the evolution of docking methods was to allow for ligand flexibility. Considering multiple ligand conformations makes it less likely that a ligand will fail to dock simply because the conformation chosen was incompatible with the receptor. Almost all the docking programs such as AutoDock (Morris *et al*., 1998) and FlexX (Rarey, Wefing, & Lengauer, 1996) have adopted this methodology of treating the ligand as flexible while the receptor is kept rigid during docking. AutoDock incorporates Monte Carlo simulated annealing, evolutionary, genetic and Lamarckian genetic algorithm methods to model the ligand flexibility while keeping the receptor rigid. The scoring function is based on the AMBER force field, including van der Waals, hydrogen bonding, electrostatic interactions, conformational entropy and desolvation terms. Each term is weighted using an empirical scaling factor obtained from experimental data. FlexX uses an incremental construction algorithm to sample ligand conformations. The base fragment is first docked into the active site by matching hydrogen bond pairs and metal and aromatic ring interactions between the ligand and receptor. Then the remaining components are incrementally built-up in accordance with a set of predefined rotatable torsion angles to account for ligand flexibility. The current version of FlexX includes terms of electrostatic interactions, directional hydrogen bonds, rotational entropy, and aromatic and lipophilic interactions. The interactions between functional groups are also taken into account through assigning the type and geometry for groups. In summary, molecular simulations such as molecular dynamics and Monte Carlo as well as ligand fragmentation will be the approach to treat flexible ligand and rigid receptor docking methodology.

Flexible Ligand and Flexible Receptor Docking

Most proteins of pharmaceutical interest are flexible objects, constantly shifting from one stable conformation to another. When they bind a ligand, many proteins undergo a conformational change in order to better accommodate the ligand and its physical properties in their binding site. This phenomenon is called ‘induced fit’. It has been demonstrated that rigid receptor methods have a high probability of failing to dock ligands to some flexible proteins when the wrong protein conformation is used (Österberg *et al.*, 2002). While some ligands readily bind to a variety of conformations, others are highly selective. Thus, in drug discovery, failure to allow induced fit and conformational change may translate into passing over viable leads. Docking methods allowing protein flexibility are therefore an active area of research (Teodoro & Kavraki, 2003; Kokh *et al.*, 2011). Incorporating the receptor flexibility is significant challenge in the field of docking. Ideally, using MD simulations could model all the degrees of freedom in the ligand-receptor complex. But MD has the problem of high computational expense, which prevents this method from being used in the screening of large chemical database. Various methods are currently available to implement the receptor flexibility. The simplest one is so-called “soft-docking” (Jiang,

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Kim, 1991) decreases the van der Waals repulsion energy term in the scoring function to allow for a degree of atom-atom overlap between the receptor and ligand. AutoDock 4 (Morris *et al*., 2009) adopts a simultaneous sample method to deal with side chain flexibility. Several side chains of the receptor can be selected by users and simultaneously sampled with a ligand using the same methods. Other portions of the receptor are treated rigidly with a grid energy map during sampling.

An extensive list of commonly used molecular docking programs, algorithms and scoring functions, can be found in Table 1 and Table 2 of the recent Review paper by Ferreira, dos Santos, Oliva & Andricopulo, (2015). Another source of the latest developments in docking programs, docking web servers, screen software, and screen webservers are listed in Table 1 of Chen's paper (2015).

APPLICATIONS OF 3D-QSAR AND DOCKING

Since (Cramer, Patterson & Bunce, 1988) introduced the 3D-QSAR concept and methodology, it has proven to be an invaluable tool in the field of drug design and activity prediction. Its impact is measured in the thousands of reports dealing with 3D-QSAR successful applications to many data sets of enzyme and receptor ligands. Excellent reviews have been written on this field (Kubinyi, Folkers & Martin, 1998, 2006; Melo-Filho, Braga and Andrade, 2014; Verma, Khedkar and Coutinho, 2010).

3D-QSAR is rooted on the hypothesis that ligands interact with their biological targets in a specific conformation, where it is assumed that identification of steric, electrostatic, and hydrophobic structural features of various drugs acting on their biological receptors can be used to explain their biological activity as well as in assisting further work on molecular design and modification.

Initially most of the research work done in this field was oriented in finding the geometric similarity among ligand and receptor. Later on, calculations of stereoelectronic properties in the binding conformation, such as comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA), along with the development of complex sophisticated docking methodologies, have allowed more accurate prediction of the biological activity of biomolecules. In the last decade there has been an explosion of reports on combining 3D-QSAR and Molecular Docking (M/D) for establishing the active conformation of a ligand in the presence of a receptor.

In 1997 a couple of articles were published on the methodology and applications of 3D-QSAR in drug design (Kubinyi, 1977a, 1977b). In 1998 (Vedani, Dobler & Zbinden) performed the first attempt to build a three dimensional receptor surface populated with atomistic properties mapped onto it. Soon after (Santos-Filho & Hopfinger, 2002) applied the 4D-QSAR idea to both receptor-independent (RI) and receptor-dependent (RD) problems. In the first method, the geometry of the receptor is not part of the data available to perform the analysis while in the second method the geometry of the receptor is available.

The first documented report of 3D-QSAR/Docking application to drug development was conducted in 2002 by (Buolamwini & Assefa) who proposed a binding mode for the cinnamoyl inhibitors at the active site of HIV-1 integrase.

In 2003 (Wu, Robertson, Books & Vieth) proposed a combined strategy involving grid docking and full force field minimization via a molecular dynamics (MD) simulated-annealing-based algorithm in order to improve docking accuracy. In their docking studies of a test set of 41 diverse protein-ligand complexes selected from the PDB, the protein was kept rigid while the ligands were treated as fully flexible and a final minimization step was used to refine the docked poses. Their results indicated that final minimization of docked structures improved the docking accuracy up to 10%.

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In 2004 (Erikson, Jalaie, Robertson, Lewis & Vieth) studied the effects of ligand and protein flexibility on M/D accuracy. To examine ligand flexibility, in a test set of 41 X-ray ligand-protein complex structures they applied several docking algorithms, finding that docking accuracy decreased substantially for ligands with eight or more rotatable bonds. To examine how protein flexibility influences docking accuracy, they applied the best docking algorithm to X-ray structures of trypsin, thrombin, and HIV-1-protease in the unbound (apo) form and also bound to several ligands. For the docking experiments they positioned each ligand to one average protein structure and to the apo form as well, and compared the results to those of docking each ligand back to its original structure. The outcome indicates that the docking accuracy falls off drastically for the average or apo structure reflecting the degree to which the protein moves upon ligand binding.

Due to the impossibility of accounting for all the research papers published in the last decade that combine M/D and 3D-QSAR to propose the best pharmacophore model, below we list a number of recent reports that we have considered most relevant in accomplishing this task. Most of the research done in this area has been focused in designing potent and effective inhibitors to HIV-1 proteins, and anti-cancer drugs. However, we must give credit to other studies in drug design such as antinociceptives, anti-tuberculosis, metabolic diseases, inflammatory and allergic processes, insecticides, osteoarthritis, antivirals, anticoagulants, anti- amyloid therapies, anti-malarial and antimicrobials.

Tubulin is an αβ heterodimer protein that forms part of microtubules essential in the cell cycle. Microtubules are involved in cell division, motility and shape and when they are compromised cell division is altered or blocked. Therefore, tubulin is the target of numerous small-molecule ligands used in cancer treatment among which is colchicine extracted from the poisonous meadow saffron *Colchicum autumnale L.* In order to construct binding models for a set of structurally diverse colchicine site inhibitors (CSIs) (Nguyen *et al*. 2005) reported docking and MD studies using the αβ-tubulin:DAMA-colchicine X-ray structure as the template. The docking studies helped in determining the binding modes of the colchicine site inhibitors. They used annealing via MD to compensate for pose inaccuracies arising from target X-ray structure resolution. They also used constrained MD simulations to undock the CSIs from the colchicine site in the binding models and to reverse the resulting trajectories to produce better models of the binding event. From these results they proposed a common structure-based pharmacophore model that accounts for the multiple structural classes of CSIs.

In order to accelerate *in silico* High Throughput Screening (Cherkasov, Ban, Li, Fallahi & Hammon, 2006) reported a progressive docking method based on "inductive" QSAR descriptors which cover a broad range of molecular properties that can be used effectively to create various binary inductive QSAR classifiers. In their approach, they used previously generated docking scores of already processed compounds in order to build predictive QSAR models that could assess hypothetical target binding affinities for yet undocked items. They tested this methodology on drug-like substances from the National Cancer Institute database that have been docked into several unrelated targets. They affirm that their approach can save hundreds of hours in terms of the docking time required to process ~90 000 potential ligands, while maintaining 80-100% hit recovery rates.

In 2007 (Markus A. Lill) questioned the usefulness of 3D-QSAR alone and use of the induced fit in determining the free energy of binding and in identifying the bioactive conformation of ligand molecules, particularly in the absence of structural information of the target protein. He reviewed the predictive power of multidimensional QSAR (mQSAR), particularly 4D-QSAR, in quantifying additional contributions to the binding energy. The concepts inherent in 4D-QSAR treat the alignment issue by incorporating molecular and spatial variety by representing each molecule in different conformations, orientations,

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tautomers, stereoisomers or protonation states. He affirmed that in classical 3D-QSAR, even those that include CoMFA or CoMSIA, one cannot sample the entire conformational space around the binding mode due to a combinatorial explosion in the number of required QSAR simulations. Since only if the 3D structure of the target protein is known, it can be used for predicting purposes using docking for example; therefore, 4D-QSAR can be interpreted as a feasible extension of 3D-QSAR to address the uncertainties during the alignment process.

In 2008 (Santos-Filho & Cherkasov) proposed a drug design approach which included docking, molecular fingerprints-based cluster analysis, and 'induced' descriptors-based receptor-dependent 3D-QSAR. They claimed that unlike other 3D-QSAR receptor-dependent, in their approach no ambiguous alignments are required for the construction of the models and that the computational cost is relatively lower. The induced descriptors presented are R_s that defines the steric influence of a group of atoms that compose a substituent G onto a single atom j, and σ^* that defines the inductive effect of G onto the reaction center j. They suggested that induced descriptors capture intermolecular interactions between ligands and receptors. This methodology was tested in eight data sets sampled from the literature and from public databases such as: human sex hormone-binding globulin, human corticosteroid-binding globulin, anthrax lethal factor, HIV-1 reverse transcriptase, neuraminidase A, thrombin, trypsin, and *Pneumocystis carinii* dihydrofolate reductase data sets. They concluded that although the presented models are interpretable, with high statistical and predictive significance, the usage of the receptor structure is not always enough for the development of significant models.

In 2009 (Pasqualoto & Ferreira) built RD 3D-QSAR models for a set of thirty seven isoniazid (INH) derivatives bound to the enoyl-acp reductase from *M. tuberculosis* (InhA). The molecular geometries of hypothesized active conformations of each ligand, resulting from a RI 4D-QSAR analysis, were used in their study. They used the 2.7 Å X-ray structure of the complex InhA-NAD-INH as starting model for the receptor geometry, and the energy-minimized structure was used as initial structure in MD calculations. Furthermore, they used the lowest energy conformation of the InhA-NAD-INH model to dock the energy-optimized structures of all ligands. They also performed MD simulations on each complex InhA-NAD-analogue. From the MD simulations these authors found that the following energy contributions were very important to the binding process: the bound ligand solvation energy, the sum of electrostatic and hydrogen bonding energies of the unbound ligand, the bending energy of the unbound ligand, the electrostatic intermolecular ligand-receptor energy, and the change in hydrogen bonding energy upon binding.

Human serum albumin (HSA), the most abundant protein in human blood plasma, binds to different types of ligands at multiple sites. Albumin is of medical importance since it transports hormones, fatty acids, metabolites, buffers pH, and maintains osmotic pressure, among other functions. To explain the high ligand promiscuity of HSA in 2010 (Deeb, Rosales-Hernández, Gómez-Castro, Garduño-Juárez, & Correa-Basurto) performed a 5 ns MD simulation on HSA. Those HSA structures sampled every 0.5 ns were subjected to a 3D-QSAR/Docking study on 94 well known HSA ligands, including warfarin and ketoprofen, known to bind HSA sites I and II. This study provided evidence that HSA binding sites I and II interact specifically with a variety of compounds through conformational adjustments of the protein structure in conjunction with ligand conformational adaptation to these sites.

In 2011 (Taha *et al*.) proposed a docking-based comparative intermolecular contacts analysis (db-CICA) as a new 3D-QSAR concept for validating docking solutions. Their approach evaluates the docking configurations based on the observations that a set of ligands inside their corresponding binding pocket interact in such a way that potent ligands contact the binding site spots in a different way than

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those ligands with low affinity. Within this framework optimal dbCICA models can be transformed into valid pharmacophore models that can be used for 3-D search of new bioactive compounds. These authors applied dbCICA methodology to search for new inhibitors of candida N-myristoyl transferase as potential antifungal agents and glycogen phosphorylase (GP) inhibitors as potential antidiabetic agents.

In in an elegant study (Deeb, da Cunha, Cormanich, Ramalho & Freitas, 2012) used a multivariate image analysis applied to QSAR (MIA-QSAR) method. MIA-QSAR is a 2D image-based approach in which images are chemical structures and, where the combination and arrangement of pixels (descriptors, binaries) provide chemical information. For this work they took 60 compounds obtained from two series of non-peptides such as HIV-1 protease inhibitors reported elsewhere. Their premise was that the sum of the substructures of two compound classes can give rise to new actives can cause synergistic effects on bioactivities and enhanced pharmacokinetic parameters. The interaction modes of the ligands with the HIV-1 protease crystal structure active site were obtained with the Molegro Virtual Docker (MVD) program using the MolDock scoring function. The MIA-QSAR models were validated through LOO-CV and Y-randomization tests. The best MIA-QSAR model was the one with the lowest RMSECV value and able to predict pIC_{50} values of new compounds that have comparable to higher HIV-1 protease inhibitory activity when compared to the existing compounds used in the study. They concluded that the MIA-QSAR method is useful in showing the synergistic effect of mixing substructures to give rise of a new compound.

In 2013 (Jimenez-Botello *et al*.) performed QSAR studies on a set of experimentally tested ligands to validate a homology built 3D model of the rat adenosine receptor (rA2AR). The rA2AR model was refined by MD, in which the initial and refined 3-D structures were used for M/D simulations. The results showed that there is a hindrance effect caused by a ribose moiety attached to agonists which plays an important role in activating the receptor via formation of several hydrogen bonds; while the lack of this moiety allows blocking of the receptor. They showed that their theoretical affinity estimation had good correlation with reported experimental data.

To improve the (Nguyen *et al*., 2005) not correctly predicted binding conformations of a few colchicine site inhibitors (CSIs), in 2013 (Da, Mooberry, Gupton & Kellogg, 2013) performed an elegant study to definitively map the αβ-Tubulin colchicine site by using 3D-QSAR, ensemble-docking and hydropathic analysis. They concluded that a combination of these techniques reveals a more detailed pharmacophore model for CSIs with a higher resolution than the ∼3.6 Å available for αβ-tubulin.

In 2013 (Ul-Haq, Usmani, Shamshad, Mahmood & Halim, 2013) performed 3D-QSAR and docking studies on Danuravir derivatives, the most potent HIV-1 protease inhibitor known so far. They applied a combined study of 3D-QSAR unto the Danuravir derivatives using ligand-based and receptor-based protocols to generate CoMFA/CoMSIA models. To align a series of 102 Darunavir derivatives in the receptor-based method, they used the bound conformation of Darunavir in the crystal structure of HIV protease as a template. To validate the 3D-QSAR results, they performed docking simulations with the most active compound and with the least active compound, and to evaluate the quality of interaction as HIV-1 protease inhibitors they were docked with the wild type and mutated proteins.

In 2013 (Contreras-Romo *et al*.) used a combined approach of protein folding, MD simulations, docking, and QSAR to elucidate the detailed interaction of the vasopressin receptor V1a (V1aR) with some of its blockers. The V1aR model was constructed by I-TASSER and refined through MD simulations. For this study 134 compounds that are structurally related to benzodiazepines were used. The energy minimized 3D chemical structures of the compounds were docked at the binding pose of conivaptan on the refined V1aR structure at the 5-ns snapshot of the MD simulations. The QSAR analysis was

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performed upon the best binding poses of the ligands, and the accepted linear models were subjected to cross-validation (CV) analysis by the leave-one-out (LOO), leave-a-group-out (LGO) procedures and Y-scrambling test. The scrambled data set was re-examined by the MLR method. The accepted MLR models were further optimized using a feed-forward neural network (NN) with back propagation of the error algorithm. Their results suggest that ligand recognition of V1aR is mediated by π-π and π-cation interactions with aromatic residues at the binding pocket of this receptor. Other nearby residues engage in hydrogen bonds and hydrophobic interactions and that a hydrogen bond interactions with the N atom of side chain of R214 is needed for a high affinity to V1aR.

It is known that protein kinase C Related Kinase 1 (PRK1) is involved in the regulation of androgen receptor signaling and has been considered as a novel potential drug target for prostate cancer therapy. Since there is little known about the PRK1 structure and its inhibitors, in 2014 (Slynko *et al*.) used the kinase domain of the protein kinase c theta (PKC-theta), with high homology to PRK1, as template in order to generate six PRK1 homology models (hm-prk1). From a group of 84 known active kinase inhibitors obtained from *in vitro* screening, they chose six compounds among which there were potent PRK1 inhibitors as well as some moderately active inhibitors. The refinement of the hm-prk1 conformations on the binding pocket and ligand was done via receptor-based docking. They observed that an inaccurate prediction of ligand binding poses occurred when using only one protein conformation and, subsequently, in low enrichment factors. To overcome this problem they docked all six chosen compounds in all six PRK1 homology models. They used MM-PB(GB)SA, QM/MMGBSA approaches to re-score the docking solutions by calculating binding free energy scores through simple QSAR models based on descriptors such as ligand charge or number of rotatable bonds. However, their results indicate that the MM-PB(GB)SA, QM/MM-GBSA rescoring approach is not accurate enough to distinguish the compounds with similar binding affinities, but it can be used to separate strong PRK1 inhibitors from moderate or weak binders. They also demonstrate that using only one snapshot derived after the minimization is sufficient to provide a reasonable prediction of the binding free energy, but only if the correct starting conformation is used.

Due to its narrow therapeutic index the CSI colchicine has limited its applications in clinical trials as antitumor agent. As a large number of small molecules possessing significant structural diversity, have been reported to bind the colchicine site in microtubules, in 2015 a hierarchical strategy was developed for structure-based virtual screening (VS) by integrating different computational methods such as M/D, 3D-QSAR, and pharmacophore model. With this procedure, based on SAR analysis, 691 novel small molecules were designed and the binding poses were predicted by a M/D into the three-dimensional X-ray structure of human tubulin complex and generated 6920 binding poses. The binding energy of the poses was calculated by MM-GBSA. Use of 3D-QSAR modeling helped in correlating correlate compound activities with interaction fields calculated based on protein crystallography or molecule superimposition. By means of the VS strategy, sixteen novel compounds with various structural features were obtained and synthetized (Li *et al*., 2015).

A CASE STUDY OF MOLECULAR DOCKING AND QSAR

In the last year, several studies have been performed using Molecular Docking-Molecular Dynamics and QSAR methodologies. For example, (Mei *et al*, 2015) have used the computational tools to study ROCK/PKA inhibitors. The 3D-QSAR results showed that the substitution containing positive charge

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attached to the phenyl ring was favored for activity and selectivity. Specially, two compounds show theses characteristics but PKA protein (PDB: 3POO) inhibitory activities were quite different. To understand this behavior, docking methodology (using Surflex-Dock module in Sybyl 2.0) were used to explain the interaction of these molecules with ROCK2 protein (PDB: 4L6Q), and finally to validate the docking results, the Molecular Dynamics (using Sybil 2.0) simulations were carried out for 5 ns.

In the same way, (Abbasi *et al*, 2015) have performed QSAR and molecular docking (AutoDock 4.2) methodology to examine 30 L-tyrosine derivatives as metalloproteinase (MMP-2, PDB: 3AYU) inhibithors. They proposed that their results could be used to design new inhibitors.

(Vats *et al*, 2015) have studied several novel flavonoid analogues as potential AChE (PDB: 4M0E) inhibitors by QSAR, Docking and MD analysis. QSAR methodology was applied by the G-QSAR module from the VLife MDS version 4.3, the docking and molecular dynamic methodologies were applied by Glide module of Schrodinger and the force field 2005 respectively, the docking was carried out only around the active site in the protein. They selected the most favorable compounds from the QSAR analysis to perform the docking and MD studied. They concluded that the stability of the complex in vivo conditions depends on the two best compounds.

(Sepehri *et al*, 2015) and (Fatemi *et al*, 2015) established a methodology, QSAR and Docking (AutoDock 4.2), to evaluate of gp41 as possible target for anti-HIV-1 (PDB: 1A1K) activity and HIV-1 protease (PDB: 4DJP) respectively. In general, docking methodology is carried out to establish the interaction between the active site and several compounds. On the other hand, (Saleh, 2015) has studied several fullerenes derivatives as HIV-1 protease (PDB: 4DJO) inhibitors. After theoretical descriptors analysis, It is concluded that the characteristics of a compound such as solubility and reactivity with the surrounding system could be sufficient to interact with the HIV-1 protease, however the docking results showed that other compounds should be more active.

(Kang *et al*, 2015) studies 36 compounds that showed inhibitory activity toward human recombinant protein of VEGFR-2 tyrosine kinase (PDB: 2IVU) by QSAR and docking (AutoDock 4.2) methodologies. Their results permitted to design seven new molecules with high predictive biological activities.

The last papers discussed before have applied the same methodology, first QSAR analysis and then molecular docking and finally, in some cases, molecular dynamic analysis. The QSAR analysis was carried out to obtain the best descriptors in the series analyzed and, in some cases, the QSAR model was used to predict new molecules with activity. The docking and molecular dynamic studies were carried out to establish the active site and the interactions between this site and the compounds.

The histone deacetylase 2 (HDAC) inhibitors are used as a new therapy for neurodegenerative diseases. Several inhibitors are in the market such as trichostatin, valproic acid, sodium butyrate, phenylbutirate, vorinostat (SAHA) (MCQouwn *et al*, 2011), however some side effects are present in its treatments. For this reason, a new compounds as HDAC2 inhibitors are required.

Our group (Martínez-Pacheco, 2012; Martínez-Pacheco, 2015; Martínez-Pacheco *et al*, 2015) has established a different methodology to find new compounds with some biological activity. First, a docking study of a series of compounds having certain biological activity took place, these compounds are exclusive of the catalytic site. In this docking analysis some new hypothetic compounds are included to identify the most probable molecules with good biological activity. After this analysis, the QSAR analysis is performed to predict the best candidate as new molecule with good biological activity.

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Results

The histone deacetylase 2 (HDAC2, PDB: 4LXZ) in complex with SAHA was used in this methodology. To prepare protein input, PyMOL Molecular Graphics System was used to remove ligands and cofactors. In this case, the HDAC2 is a trimmer and only the monomer A was used. Polar hydrogens and Kollman charges were added to monomer A. In this case, a grid box of 96Åx96Åx96Å was used as search space.

From the docking results, an occupation probability was calculated by applying the following steps:

- Selecting only the catalytic sites and the Gibbs free energy
- Consider the temperature as (298 K) and the ideal gas constant (1.98 cal/mol)
- Use the occupation probability of the results obtained in order to establish a relationship between the occupation probability and Gibbs free energy.

The following equation calculates the probability of occupation considering the Gibbs free energy:

$$p_i = \frac{e^{-\frac{\Delta G_i}{RT}}}{\sum e^{-\frac{\Delta G_i}{RT}}} \tag{3}$$

where T is the temperature at 298 K, ΔG is the difference in Gibbs free energy; R is the ideal gas constant (1.98 cal/mol).

Taking ΔE *as*:

$$\Delta E = RTln\frac{p_1}{p_2} \tag{4}$$

- Establish a p> 0.7, where $p_1 = 0.7$ and $p_2 = 0.3$

Select only those catalytic sites with higher probability of success (ex ΔG>0.499 kcal/mol).

- Analyzing the catalytic sites with a p> 0.7

Using the most stable conformation of a molecule in the catalytic site with higher probability of success and the Gibbs free energy calculated previously, it is possible to perform a QSAR study and to validate the models.

Although, many authors consider a high q^2 (for instance, $q^2>0.5$) as an indicator, or even as the ultimate proof that the model is highly predictive (Golbraikh & Tropsha, (2002)), to define the relative importance of the QSAR models, we have also performed several other tests beyond the q^2. These include the Akaike's information criterion (AIC, Akaike, (1974)), the Kubinyi fitness function (FIT, Kubiny, (1994)) and calculations of the correlation coefficient for regression through the origin (RTO, R_0^2), all of which were performed for the training and predicted activity according to (Anderson and Legendre, 1999).

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The tridimensional structure of HDAC2 used in this study was the PDB: 4LXZ, this structure correspond a trimmer, however, only a monomer was used in the molecular docking processes (Figure 1).

The monomer and Zn^{2+} ion (Figure 1) were used in the molecular docking by AutoDock 4.2 software, in a grid box of 96X96X96 Å, it is important to mention that the catalytic site is formed for the Zn2+ ion, Tyr306, His142, His143, Phe152 and Phe207

One hundred fourteen molecules were used in the docking and QSAR analysis; fifty four molecules (hydroxamic acid compounds, Figure 2) were taken as training set, fifty molecules (carboxylic acid compounds) as prediction set and ten commercial inhibitors of HDAC as validation set.

Figure 1. HDAC2 structure, only a monomer of HDAC2 and a Zn^{2+} ion (red sphere) were used in the study

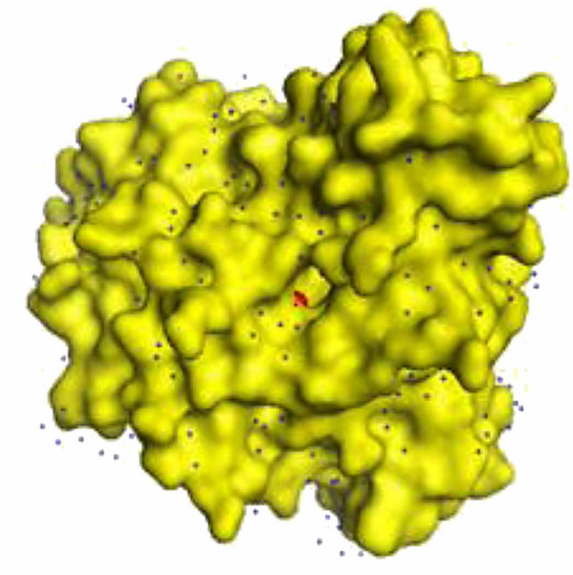
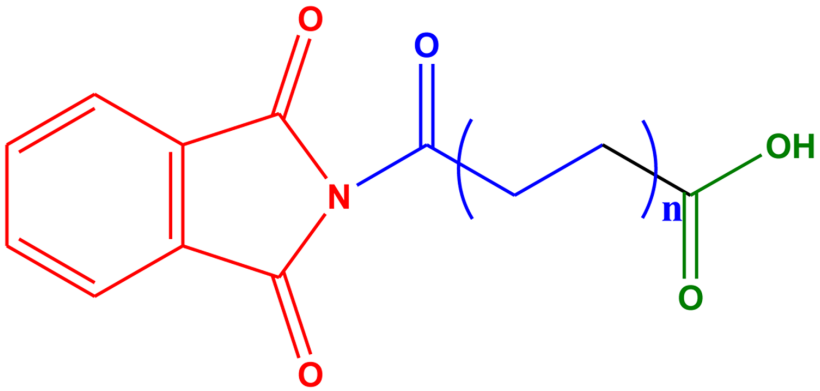


Figure 2. General structure of hydroxamic acid compounds (red color represents the lipophilic group, blue color represents the linker and the green color represents the coordination group of Zn^{2+})



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All compounds of the training set were docked into the catalytic site, and only fourteen compounds of the predicted set were docked into the catalytic site, they showed several conformations around the catalytic site. Figure 3 shows three differents conformations of a carboxyl acid derivative (14) around the catalytic site on the HDAC2 monomer.

Interactions between the compound 14 and the catalytic site were studied. The binding energy, the population distribution was calculated and only the interactions with probability higher than 0.7 were taken for QSAR analysis. The docking results showed that the active site is open when the lysine 36 is deacetiled from the histone, or close when this amino acid is acetiled. All our calculations were taken in the deacetiled system.

The QSAR model for the training set was built from 1239 molecular descriptors calculated by Dragon software. After statistical procedure, 151 molecular descriptors were used as a pool of descriptor. The best Multi-Lineal Regression (MLR) model (Figure 4A) is:

$pIC_{50}^{Exp} = -0.967\ (\pm 0.583) + 0.541\ DP06\ (\pm 0.097) + 3.317\ MATS5e\ (\pm 0.963) + 0.721\ Mor18e\ (\pm 0.224) - 1.349\ Mor13p\ (\pm 0.326) + 1.433\ GATS1e\ (\pm 0.512) + 1.015\ GATS8p\ (\pm 0.421)$

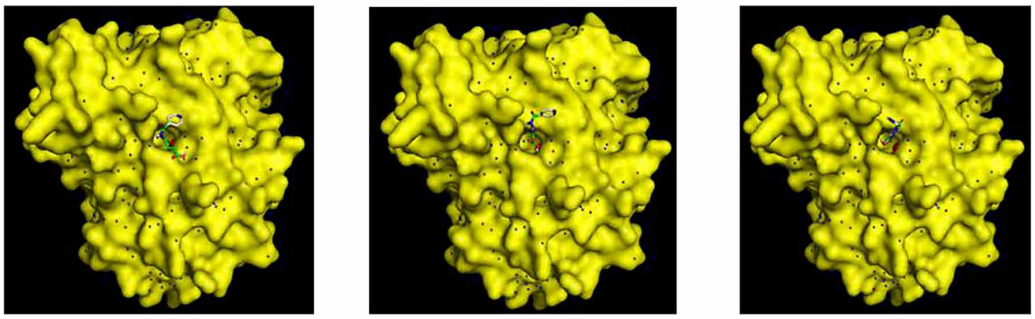
$R=0.94524$, $SD=0.23065$, $p<0.0001$, $N=52$, $SEE=2.7986$, $q^2_{LOO}=0.8498$, $q^2_{LGO}=0.7598$, $FIT=1.791$, $AIC=0.0608$, $F=60.9553$.

It is very interesting that the two commercial inhibitors as valproic acid (it does not present a bulky group) and AGK2 compounds are considered as outliers, this could be interpreted that these compounds are specific to HDAC8 and class III HDAC, respectively.

The Williams plot (Figure 4B) showed that three molecules of the prediction set are including into the region of description. These three molecules (Figure 5) were synthesized and tested as HDAC2 inhibitors (Martínez-Pacheco, (2015)), and they show potential experimental activity as theoretical prediction proposed.

Our results suggests that compoud 4 is a HDAC inhibitor with activity similar to phenylbutirate, whereas compound 14 is a HDAC inhibitor as phenylbutirate and trichostatin. Finally, compound 1 activated the HDAC2, however, theoretical results of this compound suggests the HDAC2 activation because its molecular docking is in a site different as the other two compounds.

Figure 3. Three different conformations of the carboxyl acid derivative (14), of all them are probable into the catalytic site



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Figure 4. A) MLR of 52 inhibitors of HDAC2 as training set, 14 compounds proposed from molecular docking as predicted set and 10 commercial inhibitors as validation set. There are two compounds in the validation set that are a big outliers (Valproic acid and AGK2). B) Williams plot. There are three compounds of the predicted set that are including of the description region (compounds 1, 4 and 14). These compounds are very close to the leverage limit (h).*

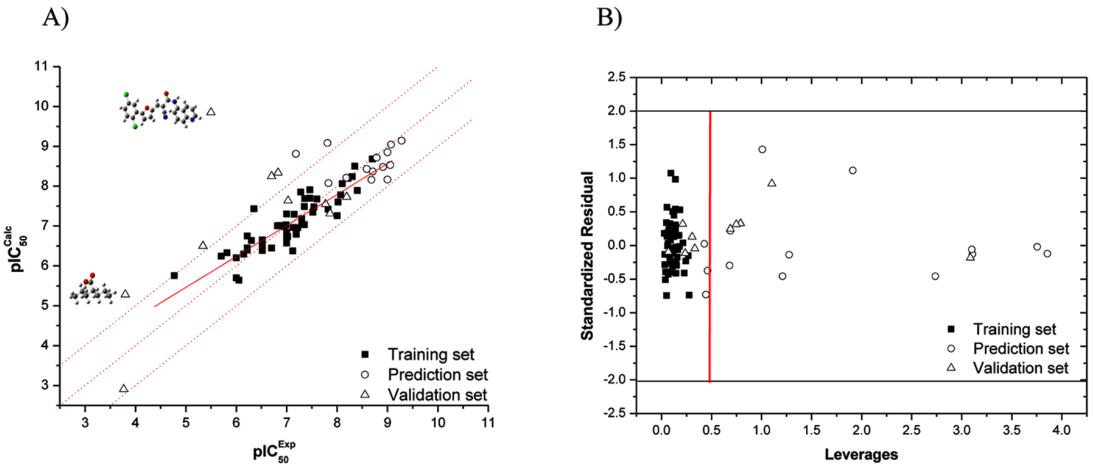
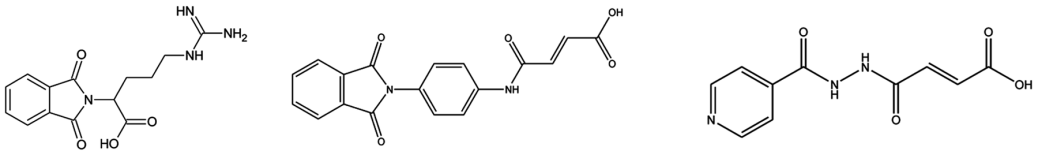


Figure 5. Compounds 1, 4 and 14. They are predicted by molecular docking and QSAR analysis as best HDAC2 inhibitors.



CHALLENGES AND FUTURE DIRECTIONS FOR CURRENT DOCKING METHODS IN DRUG DESIGN

Despite that M/D is routinely used in virtual screening or lead optimization for drug screening and design, frequently many problems arise. These issues are related to use of the wrong binding site of the target protein, use of an inappropriate small-molecule database, the method to choose the docking pose, or even a high dock score that fails in MD simulations. Sometimes docking analysis alone can generate erroneous poses of ligand binding, and occasionally the ligand will fly away during the MD simulation, in spite of having a high dock score. Such problems have been addressed by (Chen, 2015) who has given a word of caution regarding docking results.

Sampling and scoring are two of the fundamental weaknesses in simulating the binding of proteins and small ligand molecules. Protein-ligand interactions implicate a delicate balance of competing forces that occur between flexible moieties generating far too many conformations to be sampled exhaustively. These interactions are also affected by the surrounding water molecules and ions. In order to calculate the total free energy of binding, there is the need of designing accurate force-field-based approaches able

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to model the many types of interactions using physics-based parameters derived either from experiments or quantum mechanical calculations. These force fields must be able to model the entropic freedom of nearby water molecules in the vicinity of a ligand-receptor, a computationally expensive process when simulating explicit water molecules, and the water screening of the electrostatic forces between charged atoms. Such scoring function, however, will add substantial computational cost in order to estimate the favorability of a protein-ligand complex. Other qualities than one may want to score for practical reasons are toxicity and properties related to absorption, distribution, metabolism, and excretion. Since it is well-recognized that drugs may bind to different targets with significant affinities, known as drug promiscuity, another challenge is to model this tendency of ligands to bind to several sites of the protein receptor. Mainly because if a ligand binds tightly somewhere, even near the desired binding site, this does not mean that it will affect substantially the drug action, particularly important for non-traditional drug targets, such as a target found inside the interface of protein-protein interactions.

Nevertheless, some recent efforts have been oriented to help in solving the challenges mentioned above. Among these the technique known as Protein Energy Landscape Exploration (PELE) is a good example (Borrelli, Vitalis, Alcantara & Guallar, 2005) since it combines protein structure prediction algorithms and Metropolis Monte Carlo techniques in order to explore ligand diffusion on the protein energy landscape.

CONCLUSION

Computational-based rational drug design has become more common over the past decade. Most of the approaches involved in this type of design focus on ligand based drug design such as Quantitative Structure–Activity Relationship (QSAR) studies that use various molecular descriptors for chemical information training. Another branch is structure based drug design method which is molecular docking.

In this chapter, we have summarized the two methods including QSAR and Molecular Docking methodologies. Also the efforts done in 3D-QSAR and docking were presented followed by a case study.

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KEY TERMS AND DEFINITIONS

Computer Aided-Drug Design (CADD): The effective design of chemical structures with the desirable therapeutic properties, it is a well-established area of computer aided molecular design (CAMD).

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Docking Algorithm: Mechanism that two search engines; one is responsible for the placement and exploration of the ligand in the binding site, while the other searches the internal degrees of freedom of the ligand.

Molecular Docking (M/D): The computational process of searching for a ligand that is able to fit both geometrically and energetically the binding site of a protein.

Molecular Dynamics (MD): This computational method calculates the time dependent behavior of a molecular system based on Newton's second law or the equation of motion.

Quantitative Structure Activity Relationships (QSAR): A way of finding a simple equation that correlates structural molecular features (descriptors) with physicochemical properties, such as biological activities for a set of compounds by means of statistical methods.

Rigid Ligand: A drug that has no degrees of freedom to rotate around bonds.

Rigid Receptor: Protein that has only one conformation and cannot shift to another.

Scoring Function: The mechanism that is needed to decide which ligand placements (scores) are better than others, and thereby rank conformations, or candidate dockings, of a particular ligand.

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Chapter 34
Protein Structure Prediction Using Homology Modeling: Methods and Tools

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ABSTRACT

Sequence-structure deficit marks one of the critical problems in today's scenario where high-throughput sequencing has resulted in large datasets of protein sequences but their corresponding 3D structures still needs to be determined. Homology modeling, also termed as Comparative modeling refers to modeling of 3D structure of a protein by exploiting structural information from other known protein structures with good sequence similarity. Homology models contain sufficient information about the spatial arrangement of important residues in the protein and are often used in drug design for screening of large libraries by molecular docking techniques. This chapter provides a brief description about protein tertiary structure prediction and Homology modeling. The authors provide a description of the steps involved in homology modeling protocols and provide information on the various resources available for the same.

INTRODUCTION

Knowledge of the protein structure is an important step towards rational structure based drug design and virtual screening of large molecular libraries. Sequence-structure deficit marks one of the critical problems in today's scenario where high-throughput sequencing has resulted in large datasets of protein sequences, but their corresponding 3D structures still need to be determined. Currently there are about 2 million protein sequences in Swissprot and TrEMBL, among them about 30,000 proteins have had

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their structures solved experimentally. Although the rate of experimental structure determination will continue to increase, the number of newly discovered sequences grows much faster than the number of structures solved. Since experimental structures can only be determined for a small fraction of proteins, computational methods for protein structure modeling play an increasingly important role yielding models suitable for a wide spectrum of applications.

The term "homology modeling", also called comparative modeling refers to modeling of 3D structure of a protein by exploiting structural information from the known configurations of similar proteins. The method is based on the fact that structural conformation of a protein is more highly conserved than its amino acid sequence, and that small or medium changes in sequence normally result in little variation in the 3D structure. The necessary condition for successful homology modeling is a sufficient similarity (>30%) between the protein sequences. The importance and applicability of homology modeling is steadily increasing with the increase in the number of known structures in the protein structure database. Homology models contain sufficient information about the spatial arrangement of important residues in the protein, enabling us to study the binding site and design/dock drugs suitable for binding to the molecule. Stable and reliable repositories have been developed to give access to these annotated and evaluated models.

With the advent of new modeling softwares and algorithms, homology modeling is rapidly becoming the first choice for obtaining the 3D structure coordinates as well as other valuable structural and functional insights. The recent advancements in homology modeling, particularly in detecting and aligning sequences with Template structures, loop and side-chain modeling, model validation and evaluation have largely contributed to the prediction of more accurate models of protein structure, which was not possible several years ago. Improvement in sequence search/analysis, scoring systems and tertiary structure prediction methods allows development of models robust, error free models with high statistical significance. Publicly available, easy to use modeling servers and structure validation tools have further assisted the development of this technique.

Homology modeling is a multistep process, that can be summarized in following steps 1) Template identification and alignment, 2) alignment correction, 3) model generation, that includes backbone, loop and side chain generation 4) model optimization, and ; 5) validation. The first and foremost step is to fetch the Target protein sequence from various freely available databases. This is followed by Template identification, where, a protein Template whose 3D coordinates is known and that shares the maximum similarity with the Target sequence is selected. Similarity check between the Target and Template protein sequence is done by sequence alignment. The most widely used algorithm to run alignment is BLAST (Basic Local Alignment Search Tool). For a good alignment between both Target and Template sequence, the Template should not possess an E-value larger than 1. Sometimes it may be difficult to align two sequences in a region where the percentage sequence identity is very low. To resolve this issue, multiple Templates homologous to the Target protein are used, and an alignment correction is performed manually. After obtaining a good alignment, model generation starts. Quality of the model generated largely depends upon the correctness of the Template identified and accuracy of the alignment generated. If the alignment is accurate, there are less chances of the model being inaccurate. In Model generation, if the sequence alignment is good, the coordinates of those Template residues that show up in the alignment with the model sequence can be copied. If two aligned residues differ, only the backbone coordinates (N, Cα, C and O) are copied. The Target-Template alignment may contain gaps due to insertions and deletions of amino acids or bases. These loops are modeled using commercially available or manually generated loop libraries. There are generally two methods of loop modeling: 1) by

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finding similar loops in similar proteins and 2) by generating the segment *de novo*. Finding loops from similar proteins is done by taking some residues before and after the insertion as "anchor" residues and performing the loop search against databases like PDB with similar anchor residues. The best fitting loop is copied to the model. This has an advantage that all the loops are guaranteed to have reasonable internal geometries and conformations but again, they may not fit properly into the given model protein's framework. Loop selection must be done by carefully examining it for steric overlaps, and by checking loop atoms against rest of the protein's atoms. Structurally similar proteins have similar torsion angles about Cα-Cβ bond (psi angle) when comparing the side chain conformations. Therefore, in this step, the torsion angle between Cα and Cβ of the other residues can also be copied to the model because these rotamers tend to be conserved in similar proteins and many backbone configurations strongly prefer a specific rotamer. The modifications in the side chain can affect the backbone causing steric hindrance between atoms. Thus, optimization or idealization of the modeled structure using suitable a force field must be performed in order to obtain a refined model. Every homology model contains errors. Once the full scale 3D model is generated, model is validated for its authenticity, the overall correctness of the fold/structure, stereochemical parameters like bond lengths, torsion angles and bond angles, bump check etc. Moreover Ramachandran plot to visualize backbone dihedral angles (Phi)ψ against (Psi)φ of amino acid residues in protein structure have to be analyzed that whether the torsion angles fall into the allowed and the generously allowed region of the Ramachandran plot or not. Both the internal and external validation procedures need to be performed to verify that the generated model is precise and accurate. In spite of the tremendous growth in homology modeling procedures and algorithms, still there are several challenges in the technique of homology modeling which require further elucidation.

This chapter provides a brief description about the different approaches undertaken for protein tertiary structure prediction in order to lend statistical robustness to the problem of structure prediction. It provides a detailed overview of protein structure prediction, need of prediction methods and an introduction to various structure prediction approaches along with the challenges in protein structure prediction. Insights into the general features of the protein family, degree of conservation, the consensus sequence, Template identification and know-how of a correct alignment are also covered in the chapter. Recent developments in the field of protein structure prediction e.g. Comparative modeling and Threading algorithms have been discussed. Comparative modeling involves sequence-profile alignments while threading algorithms involves profile-profile alignments for identifying Templates against the query protein. Empirical techniques like *ab initio* structure prediction are also used when sufficient similarity of the Target protein to known models is not obtained. *Ab initio* strategy includes predicting the protein structure from its sequence from first principles. In different methods, this may entail calculating the physicochemical properties of proteins, evolutionary information etc. The information obtained is further used for generating spatial restraints or for identifying local structural building blocks.

Recent community-wide blind test like Critical Assessment of Protein Structure Prediction(CASP) have demonstrated significant advantages of composite (hybrid) approaches in protein structure prediction, which combine various techniques i.e Threading, *ab initio* methods, homology modeling and atomic level structure refinement. A variety of software tools, servers and meta-servers are now available for performing homology modelling. In spite of tremendous developments in structure prediction approaches, several challenges like modeling of proteins with very low similarities like GPCRs and better optimization methods still need further elucidation. The ongoing efforts in solving protein structures will continue to spur the development of novel algorithms and computational approaches to solve the problem of sequence-structure.

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BACKGROUND

Functional classification requires structural information which relies on the primary sequence of the protein. Owing to the commendable efforts in genome sequencing, there has been an overwhelming flood of sequential information. Approximately 2000 nucleotide sequences are deposited per day in publicly available databases (Kulikova et al., 2004). However, while the structural genomics efforts succeeded in determining a large number of new protein structures, there are only about 109822 protein structures deposited in the Protein Data Bank (till June 2015), the repository of protein 3D structures. The thumb rule says that more the protein structures are determined experimentally, more would be the probability to find close structure analogues in the databases of known structures for almost any protein sequence in the near future. Due to the inability of experimental procedures for determining structural coordinates for most of the proteins, a major issue of sequence-structure deficit has arisen. This huge gap between the number of available sequences and experimentally solved protein structures can only be resolved by computational prediction methods.

Structure prediction methods can be divided into two broad categories: *ab initio* method (Lesk, 1997) and homology modeling(Kolinski, Rotkiewicz, Ilkowski, & Skolnick, 1999; Rost, Fariselli, & Casadio, 1996). The first approach has prediction from physical chemistry principles while the second method predicts the three-dimensional structure of the Target sequence based on its similarity to one or more proteins of known structures. In the early 1990s, fold recognition was considered as a separate field until recently CASPs (Critical Assessment of Structure Prediction) demonstrated that Fold recognition or threading is a combination approach combining the two extremes, in which remote homologues are detected by sampling the known protein PDB conformations with scoring energies(Venclovas, Zemla, Fidelis, & Moult, 2003; Xu, Xu, & Uberbacher, 1998).

Homology modeling, also called comparative modeling or Template based modeling refers to the construction of an unknown atomic resolution model of the 'Target' protein from the amino acid sequence of the Template protein. Homology modeling approach serves well if the sequence identity between the Target and the Template sequence is sufficiently high (>30%). The quality of the homology model generated depends largely on the extent of sequence conservation between the Target and the Template sequence (Chakravarty, Wang, & Sanchez, 2005; Marti-Renom et al., 2000). This type of modeling is based on the concept that protein's structural information is more conserved than the amino acid sequence.

Besides the increasing importance of homology modeling in the recent years, there still exists a cascade of challenges in front of the scientific community. The inaccuracy of force field makes conformation sampling much more difficult, since it could guide energy minimization to the conformation of global energy minimum that is totally different from the true conformation. Homology modeling is a technique whose stringent requirement is a good and accurate force field and completeness of the conformation searching.

In an era when the rate of experimental structural determination is approximately 6000 proteins per year, homology modeling has already saved hundreds of years of human effort. Even if homology modeling produces less accurate models than experimental methods, it can be of prime importance in proposing and testing hypothesis in molecular biology, function annotation, and drug design (Francoijs, Klomp, & Knegtel, 2000; Ginalski, Rychlewski, Baker, & Grishin, 2004; Takeda-Shitaka, Takaya, Chiba, Tanaka, & Umeyama, 2004; Zhou & Johnson, 1999). It can also provide good starting models for solving structures by experimental methods (Ceulemans & Russell, 2004; Talukdar & Wilson, 1999). Structural models generated by homology modeling can be of direct medical and biological relevance. Homology-based

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structural models are widely used in predicting the effects of single nucleotide polymorphisms, *in silico* drug designing. Structural models can also be used to design mutations that lead to specific changes in the function or stability of the modeled protein in the biological system. It is approximated that within the next 10 years, structural genomics might discover all distinct protein folds in nature, making homology modeling applicable to almost any protein sequence(Vitkup, Melamud, Moult, & Sander, 2001).

MAIN FOCUS OF THE CHAPTER

After studying this chapter, you shall be able to

1. **Know:** The basics of protein structure prediction, need of prediction methods and an introduction to various structure prediction approaches along with the challenges in protein structure prediction.
2. **Learn:** The method of comparative modeling in a detailed step by step manner.
3. **Identify:** The various checkpoints in comparative modeling where more emphasis has to be laid on, along with the type of errors that can emerge in the building of the model.
4. **Evaluate:** The modeled protein for its accuracy.
5. **Analyze:** The modeled protein for its structure-function relationships and annotate it.

Issues, Controversies, Problems

The biological role of a protein is determined by its function, which is in turn is determined from its 3D fold. Thus, there is enormous benefit in knowing the three dimensional structures of all the proteins. Although more and more structures are determined experimentally at an accelerated rate, but the gap is still huge. As more and more protein sequences are determined, there is pressing need for predicting protein structures computationally.

1. The main reason for modeling of protein structure is the sequence-structure deficit. While the sequence is available for many proteins, the structure has been determined for very few. This is largely due to the fact that structure determination techniques are time and resource intensive exercise. Also, experimental difficulties in purification and crystallization of proteins make the structure determination a lengthy process.
2. Additionally, homology modeling is not possible for proteins that do not share sufficient sequence-structure similarity with any of the available proteins in the public databases like PDB.
3. Even if the structural information has been obtained by experimental techniques, there are gaps in the structure due to experimental limitations. Especially, disordered structures may be refractive to X-ray crystallographic structure determination. On the other hand, NMR techniques can not handle very large structures.
4. A third issue is that of movement leading to conformational transitions. It is well known that a protein can occur in both global and local conformations, global conformations are undoubtedly the stable conformation but it's very likely that the local conformations are biologically relevant and plays an important role in disease prognosis. Thus, it is vital to gather the structural information from the literature, mutagenesis studies and binding assays to give us more insights to elucidate a more rational model that can be taken up for further drug designing.

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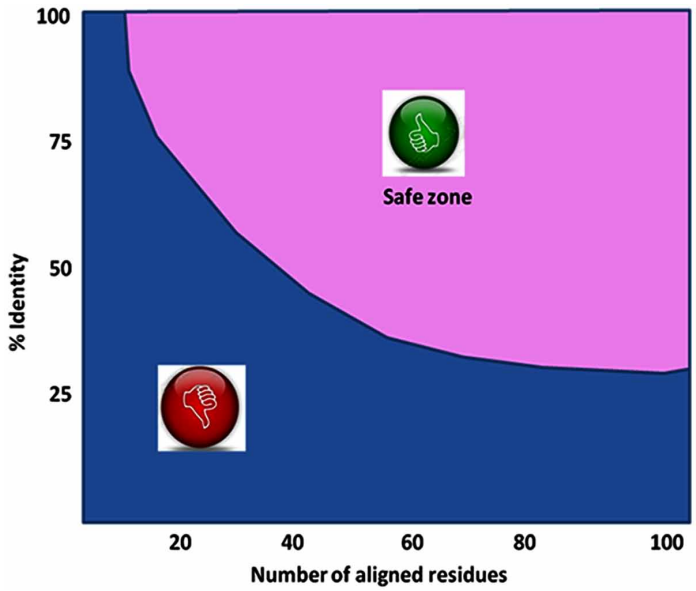
5. Accurate protein tertiary structure prediction is essential for many bioinformatics applications, including studying catalytic mechanisms of the protein, mechanism of activation, and ligand docking in drug design.
6. Protein structure prediction also assumes a critically important role for exploration of mutations and mutagenesis on the function of the molecule.

Protein Tertiary Structure Prediction

There are three methods for protein structure prediction namely 1) *ab initio* methods 2) Template based Homology modeling 3) Fold recognition or threading. As the name suggests, *ab initio* methodology calculates the protein structure from its sequence from first principles or from the beginning. A range of *ab initio* methods rely on accurate energy functions and conformational search strategies to identify the most native-like model. However, *ab initio* methods require vast computational resources and have only proved successful for small proteins. Homology Modeling and Fold recognition, on the other hand, require the identification of homologues with known structure for the protein to be modeled (Target protein).

Homology modeling is one of the easiest ways to predict the structure of a protein and is based on the observation that during evolution, the structure of a protein changes much more slowly as compared to the sequence (Chothia C, 1986; Sander & Schneider, 1991). Thus, proteins with similar sequences adopt almost identical structures while proteins with lower sequence similarity still fold into overall similar structures. It was shown that as long as the identity between two sequences and the length of the overlap fall in the "safe" region (Figure 1), two sequences will almost certainly adopt the same fold (Rost, 1999). Typically, given that sufficient number of residues between two sequences aligns, a homolog with sequence identity above 40% can be used for homology modeling of the Target protein.

Figure 1. Safe zone: schematic representation of relationship between sequence identity, overlap, and confidence in structure
Source: Authors.



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If the sequence similarity is less than 40%, the prediction can be done thorough fold recognition or threading. These methods differ from that of comparative modeling only at the level of Template selection and prediction. Here, sensitive sequence searching methods are used to align or thread the Target sequence to that of a protein with the same fold. The alignment can then be evaluated by statistical methods as well as manual inspection. If no suitable homolog is found in the database then the 3D structure is predicted through *ab initio* predictions. Typically, two or more Templates and methods of recognition are used to lend confidence to the modeling based on fold prediction.

1. **Template-Based Predictions:** Template based protein structure prediction (either Homology/ Comparative modeling OR threading based methods) uses knowledge of solved structures to model a protein sequence's native fold. First, sequence information is fetched for protein of interest (i.e. Target protein) from Expasy. BLAST (Basic Local Alignment Search Tool) search of Target protein against PDB will give us a rough idea of all the homologous protein with known 3D coordinates (Templates). Best hits may be selected from a BLAST run on the basis of low e-values (denoting statistically significant sequence similarity) or identified automatically by Homology modeling servers. Once the Template structure is found, the Target protein 3D structure is built by mapping the Target sequence onto the Template structure. This putative structure is refined using a combination of backbone moves, side-chain packing, and loop modeling. Template based protein structure prediction has always held great promise to produce atomically accurate models close to the native conformation. Comparative modeling techniques are extremely popular, reliable and fast where sequence homologues exist in the database. With increasing structural information, these techniques should prove more useful. But the major limitation with Template based selections is that it is highly dependent on the sequence identity between query and Templates. A sequence identity above 50% leads to accurate models. The Model obtained is error prone if the identity range is less than 40%. Also, at times the side chains and loops may require remodeling in order to improve the modeled protein.
2. **Fold Recognition Methods:**
    a. **Template Structure Identification Using Threading Programs:** Threading is carried out when there is not sufficient sequence similarity between the Target and Template to carry out Comparative modeling. The recognition of the Template is a problem by itself and hence it is also called fold recognition. Threading involves steps similar to Comparative modeling. It differs in the fold identification and fold fitting step. There are many approaches, but the main theme is to find "folds' from a library of folds of known protein structures (Lu, Lu, & Skolnick, 2002; Skolnick, Kihara, & Zhang, 2004). Threading algorithm for protein structure prediction uses a database of known three-dimensional structures to match sequences without known structure with protein folds. A library of different protein folds is derived from the database of protein structures. Threading methods compare a Target sequence against a library of structural Templates. The query sequence is then optimally fitted to each library fold, allowing for relative insertions and deletions in loop regions. A scoring function is assigned to assess the fit of a sequence to a given fold. These functions are database driven and generally include a pairwise atom contact and solvation terms. The 'energy' of each possible fit is calculated by summation of the proposed pairwise interactions and solvation energy. The fold library is then ranked in the order of total energy, with the lowest energy fold being taken as the most suitable match. The best score is assumed to be the one adopted by the query sequence. The

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basic assumption of the threading algorithm is that the tertiary structure is more conserved than the secondary structure and secondary structure is more conserved than the sequence. This method takes the advantage of knowledge of existing structures in the database and the physical properties they are stabilized with. Fold assignment and alignment are achieved by threading the sequence through each 3D structure in the fold library. Therefore structural modeling can be better detected through 2D or 3D structural homology instead of sequence homology. Two main kinds of Threading algorithms are available, namely:

i. **2D Threading or Prediction Based Methods:** These methods involve prediction of secondary structure (SS) or ASA (Accessible Surface Area) of the query protein. 2D Threading Algorithm also converts the coordinate information from PDB to a database containing sequence, SS and ASA information. Next, dynamic programming alignment of the query against the database is performed. These alignments are then evaluated on the basis of known SS and/or ASA matches with the Template structure. Lastly, it ranks the alignments and selects the most probable fold. Examples of 2D Threading servers are PredictProtein, 123D and GenThreader (Alexandrov & Shindyalov, 2003; Jones, 1999; Yachdav et al., 2014)

ii. **3D Profile Method:** This technique involves the alignment of the sequence to a string of descriptors that describe the 3D environment of the Target structure e.g. whether it is polar or non-polar. Other parameters could include α-helix, β-sheet or a turn conformation. Information whether a residue is fully buried, exposed or partially buried can also be considered. Here, each known fold is represented as a 2D matrix of inter-residue distance. An energy potential describes these distance dependent pair wise interactions for all possible combinations of amino acids. These are later compared with the 2D matrix of known protein structures. The trained set of contact potentials are then used to align an unknown protein sequence against a group of fold*s*. The top scoring alignments are considered as possible Templates. In order to gain confidence and to increase the accuracy of the results, it is recommended to use more than one Template. Some of programs that predict through fold recognition are Loopp (version 2), 3DPSSM, PROSPECT, ProSa etc (Kelley, MacCallum, & Sternberg, 2000; Kim, Xu, Guo, Ellrott, & Xu, 2003; Teodorescu, Galor, Pillardy, & Elber, 2004; Wiederstein & Sippl, 2007). The widely used Loopp program models the protein through a library of folds derived from CATH and SCOP. It aligns by optimizing the interaction partners for each pair of residues in contact with the structure. It uses secondary structure predictions tools like Psipred to constrain the threading. ProSa uses contact potentials while 3DPSSM is used for position specific scoring matrix to align the Target with the Template. 3DPSSM is a hybrid method that combines optimization of alignment to a family with optimization of threading energy (Hong et al., 2011; Wang et al., 2014).

3. **Ab Initio Prediction:** *Ab initio* prediction is carried out when there is no suitable homolog found in the database. It is based on Anfinsen's hypothesis that the native state of the protein represents the global free energy minimum. *Ab initio* methods try to find these global minima of the protein. Finding the correct native-like conformation requires an efficient search method to explore the conformational space in the free energy landscape to get the global minima. To simplify the computation and to reduce the search space, the following models are used:

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a. **Lattice Model:** This represents peptide chain as lattices. However, it fails to represent geometric attributes like str**a**nd twist. Also, its backbone prediction is error prone.
b. **Discrete State Off-Lattice Model:** This is an improved version of the lattice model. Off-lattice method are versatile than lattice method. It is able to represent subtle geometric considerations (strand twist, secondary structure propensities and packing) and can reproduce the backbone with greater accuracies (Park & Levitt, 1995).
c. **Using Local Structure Prediction:** Local structures can be excised from proteins that can fold independently from rest of the protein; this implies that there is a strong correlation between local structural information (biases) for short sequence segments. The strength and multiplicity of these local biases are highly sequence dependent.

The fitness of the model generated is calculated using a scoring function. There are two types of scoring functions in use, namely; knowledge based scoring function and force field based function. Former is an energy function derived from an analysis of known protein structures in the Protein Data Bank. This methodology relies on basic principle of free energy i.e. most stable conformation of a protein will be the lowest energy conformation. Force field based scoring function uses physics-based all-atom scoring function for protein structure prediction. This is analyzed with several widely used all-atom decoy sets. Currently there is not a single reliable scoring function or search method as both the scoring function are unable to explore the entire conformational space.

There is a new fully automated *ab initio* prediction method in which the Monte Carlo fragment insertion method (ROSETTA) and HMMSTR server are available, but with a limitation to model only small protein (>1000 residues) (Bystroff & Shao, 2002). The input sequence, after filtering out the low complexity region, is submitted to PSI-BLAST, and then converted to a sequence profile. The sequence profile is compared in a sliding window with each of the scoring matrices. Rosetta searches the conformational protein space using fragment insertion moves and by applying the Monte-Carlo acceptance criteria. The point where the fragment is inserted is selected at random and then a fragment of length 3 or 9 residues is selected at random from the move set. The backbone angles are changed to that of the fragment and the coordinates are then calculated. The move is accepted or rejected based on Monte Carlo criteria. The energy function is a structure based Bayesian conditional probability drawn form same PDB select database. For HMMSTR, the profile was submitted to each of the HMMSTR models namely HMMSTR-r for prediction of backbone angles, HMMSTR-d for the prediction of secondary structure and lastly HMMSTR-c for the prediction of super secondary structure.

Critical Assessment of Structural Prediction (CASP)

The biannual CASP (Moult, Fidelis, Kryshtafovych, Schwede, & Tramontano, 2014) experiments are carried out to test the different prediction methods/tools, which enables a direct comparison of a protein model to its X-ray structure. CASP is held every two years and the next one, CASP12, is to be held in mid 2016. In 2014 CASP Target list was of ~ 200 sequences with unknown 3D structures. Contestants are to predict the structure of protein using different algorithms and different methods - homology, fold recognition and *ab initio*. Subsequently, an assessment of the accuracy of the predictions is carried out. CASP concludes with a meeting to discuss the results that are published in CASP proceedings.

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Servers

There are various servers available for modeling as summarized in Table 1. This actually depends on the Templates chosen, the alignment of the query protein with the Template and also the algorithm or the method used in their prediction. It is a continuous benchmarking program and weekly evaluates the sensitivity and specificity of the different servers. Some of the servers that did well in both comparative and fold recognition modeling in CASP10 and CASP11 were 3DPSSM and Mgenthreader. Servers that outperformed in Template based modeling were I-Tasser and SwissModeller (Arnold, Bordoli, Kopp, & Schwede, 2006; Zhang, 2007).

Meta servers are servers that make their predictions based on the results of two or more different methods. Some simply look for consensus prediction between several methods while others calculate their own scores based on the results from the server. Some of the meta servers are Pcons, Pmodeller etc. The latest Live Bench experiments have demonstrated that meta servers clearly outperform the individual, autonomous servers.

Homology Modeling

Steps for Comparative Modeling

With the advent of automated protein modeling servers, submitting a sequence and obtaining its model has become a black box technique. However, all modeling protocols share certain steps, the understanding of which allows us to determine the quality of the model and the inherent errors in the system.

1. **Template Identification and Alignment:** For prediction of protein structure with reliably good accuracy, two conditions need to be fulfilled. First, the Target sequence must have significant similarity to the Template sequence. Second, computation of an accurate alignment must be feasible between the Target sequence and the Template structure. Overall model accuracy can be predicted by the degree of sequence similarity between the Target and the Template. The more the similar-

Table 1. Various modeling software tools and servers

Modeling Software	URL
SWISS Model	http://www.expasy.org/swissmod/SWISS-MODEL.html (Arnold, et al., 2006)
The CPHModels Server	http://www.cbs.dtu.dk/services/CPHmodels(Nielsen, Lundegaard, Lund, & Petersen, 2010)
EsyPred3D	http://www.fundp.ac.be/urbm/bioinfo/esypred (Lambert, Leonard, De Bolle, & Depiereux, 2002)
I-TASSER	http://zhanglab.ccmb.med.umich.edu/I-TASSER/(Yang et al., 2015; Zhang, 2008)
3D-JIGSAW	http://www.bmm.icnet.uk/~3djigsaw (Bates, Kelley, MacCallum, & Sternberg, 2001)
Modeller	http://salilab.org/modeler (Sali, 1995)
Structure Validation Resources	**URL**
WHAT IF	http://www.cmbi.kun.nl/swift/servers/ (Vriend, 1990)
PROCHECK	http://www.biochem.ucl.ac.uk (Laskowski, MacArthur, Moss, & Thornton, 1993)
VERIFY3D	http://www.doe-mbi.ucla.edu/Services(Eisenberg, Luthy, & Bowie, 1997)

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ity, more accurate is the model. However, while high accuracy models are more informative, low accuracy models may also provide coarse structural and functional annotation. Figure 2 shows a multiple sequence alignment of the Target (MoxT or the *B.anthracis* toxin) to the Template protein (*B. subtilis* YdcE) and *E.coli* Kid Toxin. The multiple sequence alignment was created using ClustalW(Thompson, Gibson, & Higgins, 2002) in Bioedit (Hall, 1999). The primary cause of errors in the final model is due to mistakes in the Target-Template alignment. Errors may also include incorrect loop building or side chain modeling. No modeling program can generate a good model from an incorrect starting alignment. Thus, good sequence-structure alignment must be ensured to improve the accuracy of the final model. Recent years mark the tremendous progress in the development of alignment methods e.g. HMMER, PSI-BLAST, HMM, SAM, HHsearch etc. Alignment can be pairwise or multiple alignments. Multiple alignments are usually heuristic, working on the principle of progressive alignment allowing large alignments of distantly related sequences eg ClustalW, ClustalX, T-Coffee. Probabilistic-based program like PROBCONS uses BAliBASE, which is a most accurate method available for multiple alignments. PROBCONS uses probabilities instead of the heuristic algorithms. HOMSTRAD is based on sequences with known 3D structures and PDB files.

2. **Alignment Correction:** An alignment is calculated by proper optimization of the scoring function. A scoring function includes gap penalty function and a substitution score matrix for matching residues in the Target and the Template sequence. Using a multiple sequence alignment program, structurally conserved region (SCRs) are searched. Insertions and deletions can be made in widely divergent parts of the molecule using multiple sequence alignment. Gaps have to be shifted to make them as small as possible. Gap is one or more empty spaces in one sequence aligned with letters in the other sequence. Gaps may or may not be treated as gap penalties. Gap penalty can be gap opening penalty or gap extension penalty. Gap penalties must be judiciously and carefully assigned as both the higher and the lower deviating from the optimum may lead to a poor alignment score. Precise importance has to be focused on residue-specific gap penalties which are responsible for

Figure 2. Multiple sequence alignment of MoxT (target) from B. anthracis with YdcE (template) from B. subtilis and Kid toxin from E. coli; the annotation of the catalytic residue E78(*) was derived by similarity from Kid.

Source: Based on Agarwal S, Mishra NK, Bhatnagar S, Bhatnagar R (2010).



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gaps in potential loop regions. Alignment score is a sum of gap penalties and the pair wise substitution scores which depend on the gap lengths and the residues matched. Despite inserting or deleting a gap and imposing gap penalties to correct the alignment, manual intervention may still be needed (Krieger, Nabuurs, & Vriend, 2003). In order to ensure a good alignment, it is recommended to choose to model only that part of the sequence which aligns without a large number of gaps in the alignment. Further, alignment errors are the main cause of deviations in comparative modeling even when the correct Template is chosen. In the alignment, gap positions should lie outside the secondary structures and in the loop areas. It is also recommended to integrate accessory information about secondary structures, conserved family residues, transmembrane helices, active site residues, etc. into the alignment in order to improve its accuracy. Last but not the least, no good alignment can be produced between sequences containing multiple repeats.

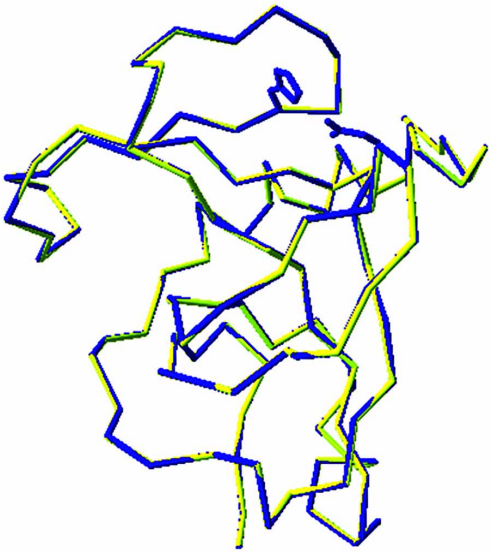
3. **Model Building:**
   a. **Backbone Generation:** When the alignment is correct, the backbone of the Target can be created. In this step, structurally conserved regions from the Template structure are used to generate the initial coordinates of the Target structure. For variable regions, the coordinates are copied from the Template structure only if the residue types are similar.
   b. **Loop Modeling:** The Target-Template alignment may contain gaps due to insertions and deletions of amino acids or bases. When the Target sequence contains a gap, the corresponding residue in the Template is deleted generating a hole. If there is an insertion in the Target, the Template will contain gap. Since, due to the gap in the Template, there will be no backbone coordinates known for these residues in the model, the Template backbone has to be cut to insert these residues. These large modifications are not feasible in secondary structural elements and therefore have to be placed in loops and strands. There are generally two methods of loop modeling: 1) by finding similar loops in similar proteins and 2) by generating the segment de novo (Li, 2013). Finding loops from similar proteins is done by taking some residues before and after the insertion as "anchor" residues and performing the loop search against databases like PDB with similar anchor residues. The best fitting loop is copied to the model. This has an advantage that all the loops are guaranteed to have reasonable internal geometries and conformations but again, they may not fit properly into the given model protein's framework. Loop selection must be done by carefully examining it for steric overlaps, and by checking loop atoms against rest of the protein's atoms.
   c. **Side Chain Modeling:** Structurally similar proteins have similar torsion angles about Cα-Cβ bond (psi angle) when comparing the side chain conformations. Therefore, in this step, the torsion angle between Cα and Cβ of the other residues can also be copied to the model because these rotamers tend to be conserved in similar proteins and many backbone configurations strongly prefer a specific rotamer. However, side chain conformations are also partially knowledge based. They are generated using rotamer libraries to select the best rotamer. To build a position-specific rotamer library, high-resolution protein structures are taken and all stretches of three to seven residues with a given amino acid at the center are collected. Prediction accuracy for side chain conformations is usually quite high for residues in the hydrophobic core (90%), while it is much lower for surface residues (50%).
   d. **Model Optimization:** The modifications in the side chain can affect the backbone causing steric hindrance between atoms. Errors in the prepared comparative model can be of different categories like: error in side chain modeling, distortions in the region correctly aligned with

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the Template structure, drift in non-aligned regions, drift in the incorrectly aligned region, and structure misfolding due to incorrect Template. For the last case, a change of Template is required. In all other errors mentioned, optimization or idealization of the modeled structure using suitable a force field must be performed in order to obtain a refined model. A forcefield is a set of energy terms used to minimize the modeled structure. It is a collection of energy terms like energies of Van der Waals and electrostatic energy between all the atoms of the structure. For this, force fields used can be either quantum-based or self-parametrizing force fields. Bond-geometry correction and removal of bad contacts can be performed by forcefields like CHARMM (Brooks et al., 1983), AMBER (Cornell et al., 1995)or GROMOS (Scott et al., 1999). Refinement can be performed by using steepest descent, conjugate gradient or other energy minimization algorithms.

4. **Model Validation:** Homology models may contain errors. The reason may either be attributed to low percentage sequence identity among Target and Template or error in the Template. Therefore, the model must be evaluated for the overall correctness of the fold/structure, stereochemical parameters like bond lengths, torsion angles and bond angles, bump check etc. other properties like the distribution of polar/ nonpolar residues, bad contacts etc can also be compared with real structures. Models are routinely compared to Template structures to show the superposition (Figure 3) and the outcome is quantitatively expressed as root mean square deviation (RMSD). RMSD values up to 2.5Å between the Target and Template are indicative of good model quality. Validation can be of two types: Internal or External Validation.
    a. **Internal Validation:** Performed for self-consistency checks. It deals with the assessment of stereochemistry of the model like bond-lengths, bond-angles, dihedral angles, etc. it can be done by programs like WHATCHECK and PROCHECK.

Figure 3. Target template superposition; the figure highlights the superposition of the modeled MoxT (blue) upon the Template YdcE (green).
Source: Based on Agarwal S, Mishra NK, Bhatnagar S, Bhatnagar R (2010).



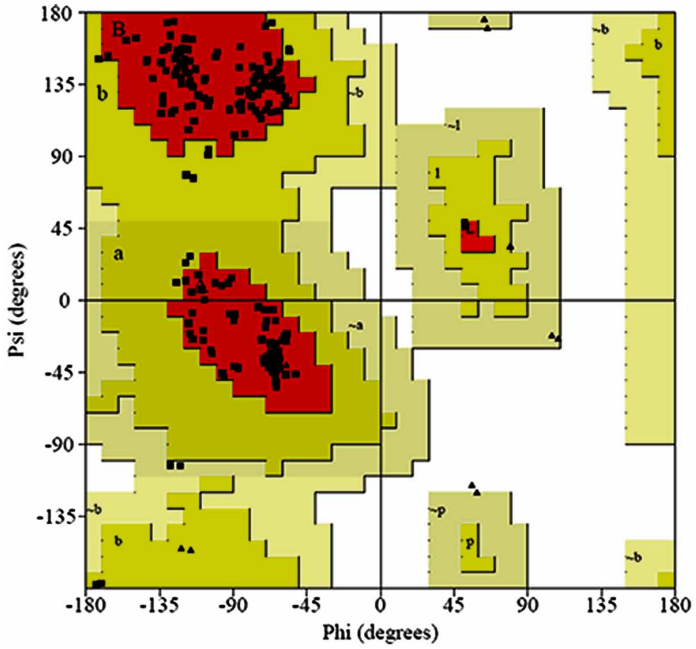
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b. **External Validation:** Based on information that was not used in calculation of the model. External validation involves the check over whether the correct Template was used (based upon identity percent). This can be predicted by comparing the Z-score of the model and the Template structure. Z-score is the measure of the compatibility between a model's sequence and its structure. Moreover Ramachandran plot to visualize backbone dihedral angles φ and ψ of amino acid residues in protein structure have to be analyzed to determine whether the torsion angles fall into the allowed and the generously allowed region of the Ramachandran plot or not (Figure 4). Thus, validation forms an indispensable part of structure modeling and must be dealt with utmost precision.

SOLUTIONS AND RECOMMENDATIONS

1. The largest improvements will come from more experimentally determined structures. This will increase the chances of modeling assignments to recognize the fold correctly, thus increasing the accuracy of the model generated.
2. Further improvement in the sequence-structure alignment can also improve the accuracy of the structure model. A better aligned profile with fewer gaps improves the accuracy of the model. One possible way of improving sequence-structure alignment might be using threading based techniques to align the sequence to structures identified in comparative modeling.
3. Protein tertiary structure prediction finds the solution to most of the issues and problems in the domain of deciphering protein structural coordinates. Multiple Template based homology modeling

Figure 4. Ramachandran plot
Source: Based on Agarwal S, Mishra NK, Bhatnagar S, Bhatnagar R (2010).



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can be applied to the low sequence similarity situations where multiple protein structural Templates can be used to model the Target protein.

4. Refinements of the modeled structure generated from homology modeling, threading, or even *ab initio* methods can be done by running molecular dynamic simulations.
5. The model is supposed to be generated on the basis of background knowledge of the protein of interest; this can be achieved by knowledge from the literature on the protein, an in-depth knowledge of the functional relevant conformation and Active site residues information. The knowledge from the literature study can help us explaining the structural and functional attributes of the protein to be modeled.

CASE STUDY

Modeling of B.anthracis Toxin MoxT: This case study is based on "Agarwal S, Mishra NK, Bhatnagar S, Bhatnagar R (2010) PemK Toxin of *B.anthracis* Is a Ribonuclease: An insight into its active site, structure, and function. JBC 285:7254–7270 (Agarwal, Mishra, Bhatnagar, & Bhatnagar, 2010). "

B.anthracis toxin-antitoxin module (MoxXT) is a TA system wherein, PemK-like toxin (MoxT) functions as a ribonuclease and thus acts as a cellular endotoxin. Under normal conditions, it exists as a dimer in complex with its cognate antitoxin and is inactive. Modeling of this toxin was used for the purpose of mutating the possible catalytic site residues.

Methodology

Homology modeling of the MoxT dimer: Homology modeling of the MoxT dimer was performed using *B. subtilis* YdcE as Template (PDB ID: 1NE8) (Gogos, Mu, Bahna, Gomez, & Shapiro, 2003) using the Swiss-Model Workspace. Figure 2 shows a multiple sequence alignment of the Target (MoxT or the *B. anthracis* toxin) to the Template protein (*B. subtilis* YdcE) and *E.coli* Kid Toxin. The multiple sequence alignment was created using ClustalW (Thompson, et al., 2002) in Bioedit (Hall, 1999) (Figure 2). Homology modeling was done using the sequence alignment with YdcE while alignment with Kid was used to infer the catalytic site residues for further mutagenesis. The modeled structure superimposed on the Template with RMSD of 0.1 Å between 232 amino acids (Figure 3). The Ramachandran plot of the predicted model revealed that 92.8% of the residues lie in the most favoured regions, and 6.2% of the residues lie in the additionally allowed regions, whereas 1.0% of the residues lie in the generously allowed region (Figure 4). The Final model was validated using Procheck (Laskowski, et al., 1993).

The residue E78 is conserved in MoxT. The other catalytic residue of Kid (R73) is replaced by a non-polar Leucine in YdcE and MoxT. However, the ribonuclease activity essentially requires the presence of a negatively charged and a positively charged (or polar group) in close proximity. In order to find the second catalytic site residue, the polar groups in the neighborhood of the conserved negatively charged E78 were identified. Interestingly, the only positively charged residue located in the close proximity of E78 was found to be H59 (Figure 5). Site directed mutagenesis of E78 and the nearby H59 resulted in abrogation of the ribonuclease activity of MoxT.

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Figure 5. Homology model of MoxT dimer; the monomer on the right shows the Cα trace of the monomer of the MoxT model (blue) superimposed upon the YdcE (black). A loop involved in binding of antitoxin is shown in orange. The catalytic base, Glu 78 and the catalytic acid, H59 are shown as magenta sticks. The monomer on the left is shown as a cartoon. The helices are shown in red, sheets in yellow, and loop regions in green. The figure rendering was accomplished using PyMOL (Delano Scientific).
Source: Delano (2002).



FUTURE RESEARCH DIRECTIONS

With the advancements in structural genomics projects, homology modeling methods is ought to play an attractive role in structure based drug discovery. Computational prediction methods provide a cost effective alternative in the absence of crystal structures. But the major drawback with prediction tools is to develop an accurate model. In this chapter, we report the current developments in this field, discuss *in silico* modeling limitations, and review the successful application of this technique to different stages of the drug discovery process. In-spite of the tremendous developments in the domain of protein tertiary structure prediction using homology modeling, there is still a requirement for better force fields and optimization functions. Better algorithmic approaches need to be developed to successfully incorporate divalent metal ions into the model

CONCLUSION

- Experimental macromolecular structure determination methods like X-ray and NMR methodology is expensive, exhaustive and cumbersome. Thus it cannot be always be undertaken.
- There are three basic approaches of structure prediction based upon the query-Template identity. If the identity is more than 30%, then the modeling lies in safe zone and comparative modeling approach is the preferred approach else threading and *ab initio* approach is used.
- If the query Template identity is less than 30%, then Threading is performed for protein structure prediction. This approach uses a database of known three-dimensional structures to match sequences without known structure with the protein folds.

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- *Ab initio* methods of protein fold prediction use force-field-based energy minimization to simulate the process of protein folding.
- Comparative modeling refers to the prediction of the Target protein from the identified Template protein structure.
- In comparative modeling, first a suitable Template is identified based upon the Target-Template alignment. After getting a proper alignment, real model building starts which encompass backbone generation, side-chain modeling and loop modeling. This is followed by model optimization and model validation.
- The accuracy of the model largely depends upon the Template identified and its alignment with the Target sequence.

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KEY TERMS AND DEFINITIONS

Alignment: The process of lining up two or more sequences to achieve maximal levels of identity for the purpose of assessing the degree of similarity and the possibility of homology.

BLAST: Basic Local Alignment Search Tool. A sequence comparison algorithm used to search sequence databases for optimal local alignment to a query.

CASP: Critical assessment of Structure Prediction is a community-wide, worldwide experiment for protein structure prediction taking place every two years since 1994.

DDBJ: DNA Databank of Japan is a biological database that collects DNA sequences.

EMBL: European molecular biology laboratory is Europe's primary collection of all publicly available nucleotide sequences.

Fold: Arrangement of secondary structural elements like helix, sheets and coils of a protein in 3D space.

Gap: A position in an alignment that represents a deletion within one sequence relative to other at that position.

Gap Penalties: Penalty applied to a similarity score for the introduction of an insertion or deletion gap, gap extension, etc. gap penalty is the deduction from the cumulative score in alignment of two sequences.

Homology: Similarity in two sequences between individuals of the same or different species.

Modeling: Process where 3D structure of the macromolecule is generated (predicted), represented and manipulated to determine their molecular properties.

PDB: Protein Databank is an archive of experimentally determined 3D structures.

RMSD: Root mean square Deviation is the measure of the average distance between the atoms of superimposed proteins.

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Chapter 35
QSAR Studies on Bacterial Efflux Pump Inhibitors

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ABSTRACT

Antimicrobial drug resistance occurs when bacteria undergo certain modifications to eliminate the effectiveness of drugs, chemicals, or other agents designed to cure infections. To date, the burden of resistance has remained one of the major clinical concerns as it renders prolonged and complicated treatments, thereby increasing the medical costs with lengthier hospital stays. Of complex causes for bacterial resistance, there has been increasing evidence that proved the significant role of efflux pumps in antibiotic resistance. Coadministration of Efflux Pump Inhibitors (EPIs) with antibiotics has been considered one of the promising ways not only to improve the efficacy but also to extend the clinical utility of existing antibiotics. This chapter begins with outlining current knowledge about bacterial efflux pumps and drug designs applied in identification of their modulating compounds. Following, the chapter addresses and provides a discussion on Quantitative Structure-Activity Relationship (QSAR) analyses in search of novel and potent efflux pump inhibitors.

INTRODUCTION

Antimicrobial drugs have been important tools of healthcare in several decades because of their effectiveness in control of bacterial infections. Unfortunately, soon after their invention it was realized that some pathogens rapidly developed resistance to antibiotics (Neu, 1992; Wood, Gold, & Moellering Jr, 1996). People infected with antimicrobial-resistant organisms are more likely to have longer, more expensive

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hospital stays, and may be more likely to die as a result of the infection. Initially, this problem was overcome by discovery of new classes of antibiotics such as aminoglycosides, macrolides and glycopeptides, but the bacteria rapidly showed an impressive array of defensive mechanisms that conferred on them, resistance to many modes of attack (Wood et al., 1996). The main mechanisms whereby the bacteria develop resistance to antimicrobial agents include enzymatic inactivation (Bush & Miller, 1998; Sabatini et al., 2012), modification of the antibiotic attack site(s) (Ruiz, 2003; Sabatini et al., 2012), and reduction of intracellular drug concentration by changes in membrane permeability (Nikaido, 2003; Sabatini et al., 2012) or by the overexpression of efflux pumps (Li & Nikaido, 2009; Sabatini et al., 2012).

One primary mechanism of antibiotic resistance is extrusion of the foreign chemical, which is termed efflux. In 1980, tetracycline was reported that it could be actively effluxed from the bacterial cell (McMurry, Petrucci, & Levy, 1980). From then on, many efflux-related mechanisms have been discovered. Overexpression of these efflux pumps may lead to antibiotic resistance. While efflux pump proteins are present in both Gram-positive and Gram-negative bacteria and also in eukaryotes, antibiotic resistance due to efflux is more of a problem in Gram-negative bacteria than in Gram-positive bacteria (Nikaido, 1996). This is because the presence of an outer membrane in Gram-negative bacteria shows comparatively lower permeability and complements the efflux activity of these pumps. Besides, studies of efflux pumps in Gram-negative bacteria have got more complicated as their double-membrane cells allow the expression of a tripartite efflux pump system such as AcrA/AcrB/TolC in Enterobacteriaceae, or MexA/MexB/OprM in *Pseudomonas aeruginosa*. One plausible practice to fight against multidrug efflux systems (MES) is the combination of conventional antimicrobial agents/antibiotics with small molecules that block MES known as multidrug efflux pump inhibitors (EPIs). An array of approaches in academic and industrial research settings, varying from high-throughput screening (HTS) ventures to bioassay guided purification and determination, have yielded a number of promising EPIs in a series of pathogenic systems (Tegos et al., 2011).

Up to now, most inhibitors of efflux pumps have been discovered through traditional random screening of synthetic compounds or natural products libraries. The assays used are very simple and easily adapted to high-throughput screening. An alternative approach is to screen libraries of known drugs. The identification of a novel mode of action in an approved drug could considerably shorten the development route and lessen the risks associated with a new chemical entity.

OVERVIEW OF BACTERIAL EFFLUX PUMPS AND ANTIBIOTIC RESISTANCE

Bacterial Efflux Pumps

Efflux pumps in Gram-positive bacteria can be categorized into four families (Handzlik, Matys, & Kieć-Kononowicz, 2013), namely ABC (ATP-binding cassette) (Higgins, 2001), MFS (major facilitator superfamily) (Saier Jr et al., 1999), SMR (small multidrug resistance) (Chung & Saier Jr, 2001) (Jack, Yang, & H Saier, 2001), and MATE (multidrug and toxic compound extrusion) (Hvorup et al., 2003).

For Gram-negative bacteria, many pump systems including *Campylobacter jejuni* (CmeABC) (Pumbwe & Piddock, 2002) (Lin, Michel, & Zhang, 2002), *Escherichia coli* (AcrAB-TolC, AcrEF-TolC, EmrB, EmrD) (Poole, 2000), *Pseudomonas aeruginosa* (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) (Poole, 2000), *Salmonella typhimurium* (AcrAB) (Nikaido, 2001) have been described. These pumps basically belong to five major families, including the MFS (major facilitator superfamily),

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MATE (multidrug and toxic extrusion), SMR (small multi-drug resistance), ABC (ATP-binding cassette) and RND (resistance-nodulation-division) families (Saier Jr, 1998). Co-expression of multiple types of efflux pumps can bring an additive or multiplicative effect on drug resistance (Lee et al., 2000).

MFS is the largest superfamily of transporters involved in symport, uniport, or antiport of various small solutes (Saier Jr et al., 1999). Examples include sugars, neurotransmitters, amino acids, Krebs cycle metabolites, and importantly, drugs. Most MFS efflux pumps have 400-600 amino acid residues and possess either 12 or 14 putative transmembrane domains. In MFS transporters, negatively charged amino acid residues located in transmembrane helices appear to play a critical role in the protonation/deprotonation step during transport. Typical MFS efflux pumps in Gram-positive bacteria include NorA, NorB, MdeA, Tet38 (*Staphylococcus aureus*), LmrB, Bmr, Bmr3, Blt (*Bacillus subtilis*), MefA (*Streptococcus pyogenes*), MefE (*Streptococcus pneumoniae*) or CmlR (*Streptococcus coelicor*) (Borges-Walmsley, McKeegan, & Walmsley, 2003; Handzlik et al., 2013; Jarmuła, Obłąk, Wawrzycka, & Gutowicz, 2010; Markham & Neyfakh, 2001; Paulsen, Brown, & Skurray, 1996; Poole, 2005).

The SMR family consists of small multidrug transporters widespread among eubacteria (Bay, Rommens, & Turner, 2008). These proteins are about 100-amino acid residues long, with four transmembrane helices. The examples of SMR efflux pumps in Gram-positive bacteria are EbrAB (*Bacillus subtilis*) or Smr, QacG, QacH (*Staphylococcus aureus*) (Jarmuła et al., 2010; Kikukawa, Nara, Araiso, Miyauchi, & Kamo, 2006; Putman, van Veen, & Konings, 2000).

All ABC transporters contain four essential modules, two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs). These four modules can be encoded by four separate genes or fused pairwise in all possible combinations (Hollenstein, Dawson, & Locher, 2007; Lubelski, Konings, & Driessen, 2007). Bacterial MultiDrug Resistance (MDR) is usually homo- or heterodimers in which one NBD is fused to one TMD. LmrA (*Lactococcus lactis*) and Rv1217c-Rv1218c (*Mycobacterium tuberculosis*) (Borges-Walmsley et al., 2003; Jarmuła et al., 2010; Wang et al., 2013) belong to this transport protein family.

The RND transporters are responsible for the high intrinsic antibiotic resistance which can be seen in Gram-negative bacteria, one component of the notorious "natural superbug" phenotype. They are also found in Gram-positive bacteria where their functions are largely unknown (Tseng et al., 1999). RND transporters are homo- or heterotrimers consisting of promoters of about 1100-amino acid residues in size. Each promoter consists of the 12 transmembrance helices and a large periplasmic domain.

Most MATE transporters consist of 400-550 amino acid residue polypeptides with 12 putative transmembrane helices which share about 40% sequence similarity. An example of MATE efflux pump in Gram-positive bacteria is MepA protein found in *Staphylococcus aureus* (Handzlik et al., 2013; Omote, Hiasa, Matsumoto, Otsuka, & Moriyama, 2006; Wasaznik, Grinholc, & Bielawski, 2008). Currently, no high-resolution structures are available for this family of transporters. Secondary structure predictions showed the symmetric repetition of conserved regions in the *N*- and *C*-terminal halves of the proteins suggesting a gene duplication event.

Bacterial Efflux Pump Inhibitors

For Gram-positive bacteria, the search for new EPIs performed in the last decade was mostly focused on the NorA of *S. aureus* (Handzlik et al., 2013). Many approaches have been made to explore new EPIs. EPI activity is often found by antibiotic–EPI combination assays using efflux-proficient and -deficient *S. aureus* strain pairs. Efflux inhibition is verified with fluorometric efflux assays using ethidium bro-

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mide, a nonspecific DNA intercalator. A number of EPIs targeting *S. aureus* NorA have been identified including current chemotherapeutic agents used as EPIs (e.g. reserpine, P-gp inhibitors, verapamil, proton pump inhibitors, phenothiazines, thioxanthenes, paroxetine and COX-2 inhibitors), naturally occuring EPIs (e.g. 4',5'-*O*-dicaffeoylquinic acid, *N-trans*-feruloyl 4'-*O*-methyldopamine, resin glycosides, pheophorbide A, diterpenes, flavonoids and capsaicin) and synthetic EPIs (e.g. piperine analogs, fluoroquinolone analogues, indole-based inhibitors, berberine-INF55 hybrids, substituted dihydronaphthalene and mesolonic compounds); of which hybrid molecules were suggested for further investigation. An EPIs must be nontoxic and highly selective in order to be a potential candidate for clinical use. In this context, reserpine cannot enter further developments due to its neurotoxicity at concentrations required to inhibit NorA (Markham & Neyfakh, 1996; Schindler, Jacinto, & Kaatz, 2013; Zhang & Ma, 2010).

For Gram-negative bacteria, promethazine, a phenothiazine, combines with penicillin G have been used to inhibit RND pumps in *E. coli* (Lehtinen & Lilius, 2007), as well as inhibit ABC pumps in yeast (Kolaczkowski, Michalak, & Motohashi, 2003). Phenothiazines are able to reverse MDR phenotypes of pathogenic bacteria like *P. aeruginosa* or *S. typhimurium* (Michalak, Wesolowska, Motohashi, Molnar, & Hendrich, 2006; Molnar et al., 1996). Paroxetine can inhibit AcrAB-TolC pump of *E. coli* (German, Kaatz, & Kerns, 2008; Kaatz, Moudgal, Seo, Hansen, & Kristiansen, 2003; Munoz-Bellido, Munoz-Criado, & Garcìa-Rodrìguez, 2000). It is pointed out that arylpiperazines reverse MDR in bacteria overexpressing AcrAB and AcrEF pumps. Moreover, NMP (1-naphthylmethylpiperazine) is the most effective inhibitor that enhances the intracellular concentration of drugs like chloramphenicol, tetracycline, linezolid, macrolides and fluoroquinolones (Kern et al., 2006). The structures with dihalogens among arylpiperidines have been shown to restore the antimicrobial activity of linezolid in *E. coli* (Thorarensen et al., 2001).

Quinolines have been shown the ability to re-establish various antibiotic activities like quinolones, cyclines and chloramphenicol. They are now applied as broad spectrum inhibitors for resistant *E. aerogenes* and *K. pneumoniae* to make them susceptible for the antimicrobials (Chevalier et al., 2004). The analogs of aminoglycoside paromomycin have been applied as inhibitors of the efflux pumps using the bacterium *H. influenzae* (Alekshun & Nelson, 2004). AcrAB efflux pump was inhibited by using antibodies in *E. coli* and this strategy has been patented (Oethinger & Levy, 2002). A recent report has introduced the use of antisense phosphorothioate oligonucleotide encapsulated in a novel anion liposome to restore the activity of fluoroquinolones in *E. coli* (Meng et al., 2011). This method could be utilized for a wide variety of pumps with known gene sequences. Using antibody or parts thereof in order to inhibit MexAB-OprM pumps in *P. aeruginosa* has been patented (Inoko & Yoshihara, 2011).

In addition, a list of about 200,000 synthetic and natural compounds was tested with the aim of finding potential compounds preventing the activity of levofloxacin against *P. aeruginosa* (Chamberland et al., 2002; Coban, Ekinci, & Durupinar, 2004; Lomovskaya et al., 2001; Lomovskaya & Watkins, 2001; Renau et al., 1999). MC-207, 110 (Phenylalanine Arginyl β - Naphthylamide/PAβN) was identified as an inhibitor of Mex pumps. The crystal structure of *E. coli* AcrB has been solved in the presence of MC-207 and its binding to the pump has been proved (Edward, Aires, McDermott, & Nikaido, 2005). This compound has been shown to decrease the frequency of the emergence of highly levofloxacin resistant *P. aeruginosa* strains and also to reduce the intrinsic resistance of the bug to levofloxacin 8-folds (Coban et al., 2004; Lomovskaya et al., 2001; Renau et al., 1999). It is a competitive inhibitor of the efflux pumps and operates by binding to the same pocket or at a site closer to the antibiotic substrate binding site (Mahamoud, Chevalier, Alibert-Franco, Kern, & Pagès, 2007; Pagès, Masi, & Barbe, 2005). The compound has not only restored the activity of levofloxacin but has also been found to potentiate the activity of other antibiotics such as oxazolidinones, chloramphenicol, rifampicin, macrolides/ ketolides

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(Zechini & Versace, 2009). Furthermore, it has these effects not only *for P. aeruginosa*, but also for *K. pneumoniae*, *C. jejuni*, *E. coli*, *S. typhimurium* and *E. aerogenes* (Malléa, Chevalier, Eyraud, & Pagès, 2002; Mazzariol, Tokue, Kanegawa, Cornaglia, & Nikaido, 2000).

Drug Design Targets Bacterial Efflux Pumps

Structure-based drug design plays an important part of the success story in the discovery of new drug leads. It is known that a thorough understanding of MDR transporter structure and function, as well as mechanism, would considerably facilitate the drug discovery. There have been, however, the scarcity of atomic structures of these membrane proteins due to difficulties related to the process of protein expression and crystallization (Chang, Ray, & Swaan, 2005; Rosenbusch, 2001). Additionally, it was suggested that there are some limitations associated with protein structures solved by crystallography that should be taken into consideration (DePristo, de Bakker, & Blundell, 2004). To date, the number of available bacterial efflux pump structures is still small compared to the number of identified bacterial transporters, which were described in previous reviews (Delmar, Su, & Yu, 2014; S. Kumar & Varela, 2012; Li & Nikaido, 2009; Van Bambeke & Lee, 2006; Zechini & Versace, 2009). This has hindered the computer-aided discovery of efflux pump chemosensitizers. The Table 1 enumerates MDR transporters with determined structures, of which recently structure-found transporters have mostly belonged to *Escherichia coli* and *Pseudomonas aeruginosa*.

Regarding MDR Gram-negative bacteria, RND transporter AcrAB-TolC and its homologues (including MexAB-OprM and MexXY-OprM) are main contributors to bacterial survival during infection. Certain studies on *Pseudomonas aeruginosa* MexAB-OprM pump, using both ligand-based and structure-based pharmacophore approaches in combination with docking method, recommended several important structural modification favoring inhibitory potentiation of known inhibitors: the incorporation of a hydrophobic group at the 2-position of the pyridopyrimidine scaffold or the inclusion of an olefin spacer between the tetrazole and pyridopyrimidine scaffold can render a boost in potency of this derivative, hydrophilic substitution was feasible on a piperidine moiety at the 2-position without adversely affecting activity, paving the way for the increase of aqueous solubility (Nakayama et al., 2004); in another study, the compound ASN05108137 exhibited maximum similarity to the pharmacophore hypothesis of MC207110, the first inhibitor against MexAB-OprM efflux system of *P.aeruginosa*, and also showed the druglikeness property satisfying the Lipinski's rule of five (Aparna, Mohanalakshmi, Dineshkumar, & Hopper, 2014).

In a recent study, the crystal structure of AcrB and MexB bound to the pyridopyrimidine derivative ABI-PP was determined. This result provides detailed insight into the mechanism by which the inhibitor ABI-PP acts on RND transporters thereby generating a structural basis for designing a universal inhibitor of multidrug efflux transporters (Nakashima et al., 2013). A pharmacophore study was aimed to circumvent the drug resistance in *P.aeruginosa* and *E.coli* by identifying natural compounds from plants having effect on MexB and its counterpart AcrB. Lanatoside C and daidzein were found to have a high possibility to counteract the MDR pumps in the resistance bacteria (Aparna, Dineshkumar, Mohanalakshmi, Velmurugan, & Hopper, 2014).

Another pharmacophore hypothesis was generated by the HipHop method to analyze the structure-activity relationships of active sites of protein-ligand interactions thereby increasing the knowledge of structure and mechanism of RND-type AdeABC EPIs in *Acinetobacter baumannii*. The achieved 3D common feature pharmacophore model revealed that two hydrogen bond acceptors (HBA) and three

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Table 1. Bacterial MDR efflux pumps with determined X-ray structures

Bacterial Strain	Protein Family	Efflux Pump	PDB Code	Resolution (Å)	Bound Ligand	Reference and Year of Release
Bacillus subtilis	MFS	BmrR	3Q5P	2.94	tetracycline	(Bachas, Eginton, Gunio, & Wade, 2011)
			3Q5R	3.05	kanamycin	(Bachas et al., 2011)
			3Q5S	3.10	acetylcholine	(Bachas et al., 2011)
Aquifex aeolicus	MFS	EmrA	4TKO	2.85	-	(Hinchliffe et al., 2014)
Neisseria gonorrhoeae	RND	MtrD	4MT1	3.54	-	(H.-T. Lei et al., 2014)
Campylobacter jejuni	RND	CmeR	3QQA	2.20	taurocholic acid	(H. T. Lei et al., 2011)
			3QPS	2.35	cholic acid	(H. T. Lei et al., 2011)
Vibrio cholerae	MATE	NorM	3MKT	3.65	-	(He et al., 2010)
			3MKU	4.20	rubidium ion	(He et al., 2010)
Escherichia coli	ABC	MacA	3FPP	2.99	-	(Yum et al., 2009)
	RND	AcrB	4CDI	3.70	-	(Du et al., 2014)
			4C48	3.30	dodecyl-β-D-maltoside and nickel (II) ion	(Du et al., 2014)
			3W9H	3.05	ABI-PP	(Nakashima et al., 2013)
			2HRT	3.00	citrate anion	(Seeger et al., 2006)
		TolC	2VDE	3.20	chloride ion	(Bavro et al., 2008)
			1EK9	2.10	-	(Koronakis, Sharff, Koronakis, Luisi, & Hughes, 2000)
Pseudomonas aeruginosa	RND	MexB	3W9I	2.71	dodecyl-β-D-maltoside	(Nakashima et al., 2013)
			3W9J	3.15	dodecyl-β-D-maltoside and ABI-PP	(Nakashima et al., 2013)
			2V50	3.00	dodecyl-β-D-maltoside	(Sennhauser, Bukowska, Briand, & Grütter, 2009)
		OprM	3D5K	2.40	chloride ion and sodium ion	(Phan et al., 2010)
			1WP1	2.56	-	(Akama et al., 2004)

(From Protein Data Bank).

hydrophobic aromatic (HpAr) were found to be significant for binding to the active site of the target protein. Three HpAr features displayed the required substitution of bulky aromatic moieties, while two HBA atoms or groups were necessary in the molecule to bind to the target protein (Yilmaz et al., 2014).

More recently, structures of interest for rational design include the advent of the pseudo-atomic structure of complete AcrAB-TolC in complex with AcrZ, a modulatory protein partner from *E.coli*. It was found that the studied structure is likely to be similar to homologous assemblies in pathogenic species that infect humans such as *Vibrio cholerae, P.aeruginosa, Neisseria gonorrhoeae* and *Salmonella enterica* (Du et al., 2014).

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QSAR APPROACHES IN SEARCH FOR EFFLUX PUMP INHIBITORS

QSAR Approaches in Search for EPIs in Gram Positive Bacteria

The methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most frequent nosocomial pathogens in developed countries. Among the Gram positive bacteria, *S.aureus* is responsible for a large number of deaths worldwide. MRSA is resistant to a variety of antibiotics including tetracyclines, aminoglycosides and fluoroquinolones. Studies found that NorA is a predominant protein efflux pump responsible for efflux mechanisms in *S.aureus* (Sabatini et al., 2012). For these reasons, NorA has been one of the most studied Gram-positive bacterial efflux pumps in the search for efflux pump inhibitors. Although other protein targets have been investigated and there are some newly found derivatives proved to have inhibitory activities towards those proteins, there is still a lack of QSAR studies focused on such inhibitors. With respect to NorA in *S.aureus*, while there have been a number of natural and synthetic product known to be inhibitors of NorA efflux pump, there is still a need to discover new and potent NorA inhibitors. However, the slow emergence of high-resolution structure of this pump has hampered the intelligent design of its modulators, necessitating a ligand-based approach. With currently available structural data regarding known NorA inhibitors, robust QSAR models were generated, paving the way for preliminary identification of compounds with NorA inhibitory activities, whereby more thorough research can be conducted in order to identify the inhibitors with high potentiality to become future "adjuvants" of antibiotics.

QSAR of Piperine Analogs for Bacterial NorA Efflux Inhibitors

Piperine, a major component of Piper nigrum, is a putative bacterial EPI (Khan, Mirza, Kumar, Verma, & Qazi, 2006). In addition, piperine has been found to be associated with several activities (Amit Nargotra et al., 2009): inhibiting several cytochrome P450-mediated pathways and phase II reactions in animal models (Atal, Zutshi, & Rao, 1981; Singh, Dubey, & Atal, 1986); inhibiting human P-glycoprotein (Bhardwaj et al., 2002) and potentially inhibiting rat hepatic microsomal constitutive and inducible cytochrome P450 activities (Koul et al., 2000).

After screening a chemical library consisting of 200 structurally diverse compounds for inhibition of the NorA efflux pump, three piperine analogues, namely SK-20, SK-56 and SK-29, were found to be the most potent inhibitors of the NorA efflux pump. The structural adjustments of piperine that can lead to bacterial efflux pump inhibitors with significantly higher activities include: (i) the introduction of an alkyl group at the C-4 position mainly contributes to potentiation, and ethyl and n-propyl show highest effect; (ii) substitution of a piperidinyl moiety by an aromatic amine such as anisidine or toluidine renders greatest potentiation, whereas other substituents such as aniline, amino esters, pyrollidine, azepine and alkylamines (except isobutyl amine) appear to be less effective; (iii) unsaturation is a critical feature for potentiation, as di- and tetra-hydro piperine show much less activity than corresponding unsaturated analogues; (iv) the omission of amide carbonyl group leads to a decrease in potentiation; and (v) the presence of a 3,4-methylenedioxyphenyl or 4-methoxyphenyl group in structure of piperine derivatives can enhance potentiation (A. Kumar et al., 2008).

In the QSAR study of piperine analogs for bacterial NorA efflux pump inhibitors (Amit Nargotra et al., 2009), researchers at Indian Institute of Integrative Medicine, India employed the Cerius2 4.10 software package (from Accelrys Inc.) to calculate 2D descriptors differentiating the molecules in terms

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of their size, degree of branching, flexibility, and overall shape. A total set of 25 compounds was divided into 20 molecules in training set and 5 molecules in test set. The general structures of the compounds are given in Figure 1.

All the molecules were energy minimized using the Cerius2 OFF module with default parameters of Smart Minimizer. The QSAR model was generated using the genetic function approximation (GFA) method and its statistical significance was validated using randomization procedure. As a result, the final QSAR equation after removal of outliers was as follows:

$$\log (PF) = 0.84 - 0.002 \times Hf + 0.01 \times Jurs_PNSA\text{-}1 - 0.01 \times Shadow_XZ$$

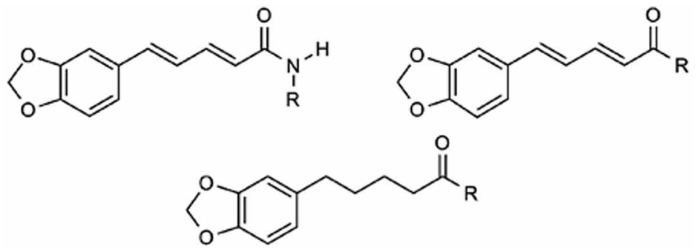
($N = 17$; LOF = 0.015; $r^2 = 0.962$; $r^2_{adj} = 0.953$; F-test = 110.08; LSE = 0.006; $r = 0.981$; $q^2 = 0.917$)

where PF (potentiation factor) of the compounds (efflux pump inhibitors – EPI) express the reduction in the MIC values of ciprofloxacin, hence represent the potentiation of activity of ciprofloxacin in the presence of piperine analogs based efflux pump inhibitors; N is number of compounds in training set, LOF is lack of fit score, r^2 is squared correlation coefficient, r^2_{adj} is square of adjusted correlation coefficient, F-test is a variance-related static which compares two models differing by one or more variables to see if the more complex model is more reliable than the less complex one, the model is supposed to be good if the F-test is above a threshold value, LSE is least-square error, r is correlation coefficient, q^2 is the square of the correlation coefficient of the cross-validation.

The QSAR model was proved robust by using the method proposed by Golbraikh *et al*., and Roy. The achieved QSAR model had the values of $R^2_{pred} = 0.78$ and $r^2_m = 0.72$, indicating the good external predictability of the QSAR model.

Jurs_PNSA-1, Shadow_XZ and Hf are important descriptors responsible for the relationship between piperine analogs' structure and activity as *S. aureus* NorA efflux pump inhibitors. Jurs_PNSA-1 is the partial negative surface area, calculated by using the sum of the solvent accessible surface area of all negatively charged atoms in the compound. Shadow_XZ is the area of the molecular shadow in the XZ plane. Hf is the heat of formation descriptor. These parameters were proved to be important contributors to the potentiating activity and must be taken into consideration while designing the efflux pump inhibitors.

Figure 1. General structures of compounds taken for QSAR study



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QSAR of Aryl Alkenyl Amides/Imines

Another QSAR study on activity of aryl alkenyl amides/imines having structural similarity to piperine (structures shown in Figure 2) in inhibiting bacterial efflux pump was also conducted by Nargotra *et al* (A Nargotra et al., 2009).

The total set of 42 compounds was divided into 34 molecules in training set and 8 molecules in test set by arranging the molecules in ascending order of their PF and selecting every fifth molecule to be included in the training set. About 200 descriptors were calculated using the Cerius2 4.10 software package. After the removal of the descriptors with zero values for all the compounds, remaining descriptors were filtered using genetic function approximation (GFA) method. The study showed that the ideal number of descriptors should be five. After analyzing the equation with five descriptors and removing outliers, the following equation was achieved:

$$\log (PF) = -0.2028 - 0.000936*Hf + 0.342484*S_dssC$$

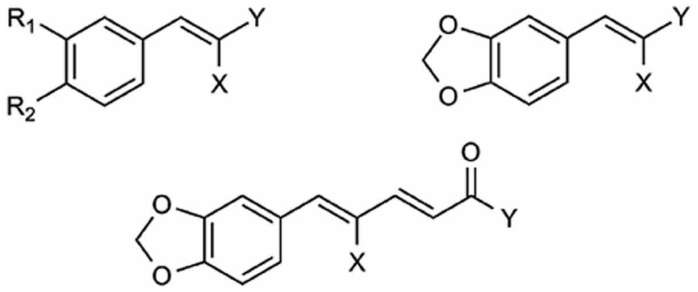
$$+ 0.441556*RadOfGyr - 0.2087*Atype\text{-}C\text{-}16$$

$$- 0.00104*Jurs\text{-}WNSA2$$

($N = 30$; LOF = 0.051; $r^2 = 0.866$; $r^2_{adj} = 0.833$; F-test = 28.875; LSE = 0.022; $r = 0.929$; $q^2 = 0.757$)

The final QSAR model had the values of $R^2_{pred} = 0.825$ and $r^2_m = 0.634$, indicating the good external predictability of the QSAR model. The QSAR study also gives us the revelation that E-state indices and A log P atom type are important descriptors responsible for describing the activity of aryl alkenyl amide/imine based efflux pump inhibitors. In addition, solvent accessible surface area, partial charged surface area of a molecule, radius of gyration and heat of formation of the molecule are also important parameters to be taken into account while designing new inhibitors belonging to the class of compounds mentioned above.

Figure 2. General structures of compounds taken for present study



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QSAR of 2-Aryl-5-Nitro-1H-Indole Derivatives

It has been found that the combinatorial administration of lead structure 5-nitro-2-phenylindole (INF55) and the antibiotic significantly increases *S. aureus* susceptibility to ciprofloxacin (Ambrus et al., 2008). Structure-activity relationship studies mostly aimed at disclosing the importance of the C5 position of INF55 (Figure 3) for inhibitory activity against the NorA pump:

1. Reduction of C5 substituent will adversely affect the activity.
2. Carbonyl based electron-withdrawing groups at C5 eliminate all activity.
3. C5 substituents such as sulfonic acids/esters/amides have no effect on potentiation.
4. INF55 derivatives bearing a nitrile group show similar potentiation activity to INF55 (compound 14) (Ambrus et al., 2008).

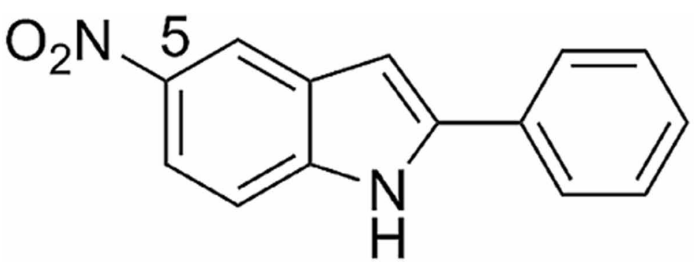
All compounds lacking 5-NO_2 groups were significantly less potent than INF55. The 3′-CO_2Me INF55 derivative showed slightly higher potency than INF55 against all three *S.aureus* strains. INF55 analogue containing a 4′-CH_2OH group was nearly equipotent with INF55 against the wild type and NorA overexpressing strain, representing a promising candidate for incorporation into dual action mutual prodrugs targeting the NorA pump (Ambrus et al., 2008).

Berberine is the active constituent of the medicinal plants echinacea and golden seal. The extrusion of berberine by bacterial efflux pumps can make this antimicrobial agent ineffective. Nevertheless, it was found that the use of berberine along with an effective MDR pump inhibitor could restore its activity. In another related study, an inhibitor of Major Facilitator MDRs, namely SS14, was made on the basis of covalent link between berberine and INF55. Compared to berberine, SS14 was 100-fold more active against *S.aureus* and 200-400 fold more active against *S.aureus* mutants overexpressing the NorA MDR (Ball et al., 2006).

In a QSAR study on MDR inhibitory activities of indole derivatives, the 3D structures of ten 2-aryl-5-nitro-1-*H*-indole derivatives (structures shown in Table 2) were optimized with the semi-empirical PM3 method and density functional theory (DFT) at b3lyp/6-31 g* level successively (Dai, Zhang, Zhang, Wang, & Lu, 2008).

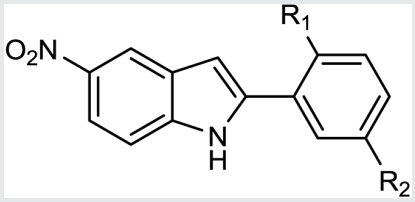
Some structural and electric parameters were subsequently obtained from single point energy and natural bond orbital analysis. These parameters included the lowest unoccupied molecular orbital energy (E_{LUMO}), the highest occupied molecular orbital energy (E_{HOMO}), the polarizability (α), the nuclear-nuclear repulsion energy (E_{rep}), the molecular total energy (E_{tot}), the dipole (μ), the molecular volume (V_{mol}), and the Mulliken atomic charges of the six carbons of the 2-aryl (Q_{C1}-Q_{C6}). A genetic algorithm-

Figure 3. Structure of INF55



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Table 2. Structures and activities of ten 2-aryl-5-nitro-1H-indole derivatives used for QSAR analysis

Compound			MIC of NorA++ K2361 Plus Berberine (30μg/mL)
	R_1	R_2	
7a	COOH	H	50
8a	CH_2OH	H	12.5
9a	CH_2N_3	H	3
7b	COOH	OCH_3	50
8b	CH_2OH	OCH_3	6.25
9b	CH_2N_3	OCH_3	1.5
8c	CH_2OH	OCH_2Ph	0.8
10a	CH_2NH_2	H	12.5
10b	CH_2NH_2	OCH_3	12.5
INF55	H	H	3

based multi-linear regression analysis (GA-MRA) was carried out to reduce the number of the structural and electric parameters originally from 13 parameters obtained from the quantum chemistry calculations, thus rendering the optimized set of descriptors for constructing the QSAR model. With each data set containing m variables, a QSAR model was built. Then the optimum combinations containing the same numbers of descriptors were screened out with GA method. For each m of 2-7, two combinations of the descriptors with the top square correlation coefficient R^2 are analyzed based on the trends of their R^2, R^2_{CV}, and F (a=0.05) values in accordance with changes of *m* values. The QSAR model was built based on partial least square (PLS) analysis.

The outcomes of genetic algorithm analysis indicated that the model containing five descriptors of μ, V_{mol}, Q_{C2}, Q_{C3} and Q_{C4} with the highest R^2_{CV} of 0.9941 and a good R^2 of 0.9982 could be considered as an ideal model for QSAR of 2-aryl-5-nitro-1-*H*-indole. These five descriptors were re-analyzed with partial least square (PLS) and the LOO cross validation. The QSAR model achieved with 5 independent variables is shown as follows:

$$-\lg \mathrm{MIC} = -0.397 \times \mu + 0.019 \times V_{mol} + 1.231 \times Q_{C2} + 9.976 \times Q_{C3} - 6.177 \times Q_{C4} - 0.198 \quad (*)$$

$$N = 10,\ R^2 = 0.998,\ R^2_{CV} = 0.996$$

The equation showed that increasing Q_{C3} and decreasing Q_{C4} could improve the activity of 2-aryl-5-nitro-1-*H*-indole derivatives as efflux pump inhibitor. With the model achieved, the electric and structural descriptors of a new compound, 2-(2-azidomethyl-5-phenoxy-phenyl)-5-nitro-1*H*-indole (8d, R_1

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= -CH_2N_3, R_2 = -OCH_2Ph) were calculated with the same quantum chemistry calculation method and they were predicted from the equation (*) to lower the MIC of berberine to 0.091 μg/ml for inhibiting K2361 of *S.aureus* with NorA efflux pump protein overexpression.

Case Study of Using QSAR Model for Virtual Screening of NorA Efflux Pump Inhibitors

In a QSAR study conducted by Thai *et al.* (Thai et al., 2015), a total of 47 compounds belonging to different structural classes in company with the efflux pump inhibitory (EPI) activity against NorA mediated ethidium bromide efflux (IC_{50}) were compiled from the literature (German et al., 2008; Kaatz, Moudgal, Seo, Hansen, et al., 2003; Kaatz, Moudgal, Seo, & Kristiansen, 2003; Michalet et al., 2007; Pieroni et al., 2010; Sabatini et al., 2012; Sabatini, Kaatz, Rossolini, Brandini, & Fravolini, 2008; Smith, Williamson, Wareham, Kaatz, & Gibbons, 2007; Stavri, Piddock, & Gibbons, 2007). In MOE, all chemical structures of the data sets were built and energy minimized before computing 184 2D molecular descriptors. Subsequently, the variables were selected using RapidMiner (RapidMiner), Weka (WEKA) and MOE (MOE) software packages respectively. This attribute selection resulted in five descriptors which were analyzed in correlation matrix with experimental pIC_{50} values. Subsequently, linear regression models ($y=a_0+a_ix_i$) were generated by using the QuaSAR-Model tool in MOE (MOE) with the method Partial Least Square (PLS) and the activity field pIC_{50}. Based on the training set, different models generated from different descriptors were internally validated by the cross-validation technique and externally validated by the test set. The descriptors used for the best one were continuously used for modeling the whole data set to get the final model which was internally validated by the cross-validation technique and externally validated by the external test set. As a result, the finally achieved model with statistical values $Q^2 = 0.8$, $\overline{r_m^2} = 0.71$ and $\Delta r_m^2 = 0.18$ met validation criteria for the data set following Roy *et al.* (Ojha, Mitra, Das, & Roy, 2011; Pratim Roy, Paul, Mitra, & Roy, 2009; Roy et al., 2011).

The yielded model was applied on drug-likeness compounds from the mixture library of 182 flavonoids (Belsare, Pal, Kazi, Kankate, & Vanjari, 2010; Farkas, Jakus, & Héberger, 2004; Park et al., 2006; Phosrithong, Samee, Nunthanavanit, & Ungwitayatorn, 2012; Seyoum, Asres, & El-Fiky, 2006; Tran, Park, Kim, Ecker, & Thai, 2009; Yokozawa et al., 1998) and the traditional Chinese medicine (C. Y. Chen, 2011) (TCM) database that met the rules of five/three to identify novel Nor-A inhibitors with $pIC_{50} \geq 4.5$. Among 182 flavonoids having the rings values ≥ 2, 27 of 169 compounds that matched the criteria of the rule of five and none of 8 compounds that matched the criteria of the rule of three were predicted as potential NorA inhibitors. Among 52957 TCM compounds with the descriptor rings ≥ 2, 2866 of 15250 compounds that matched the criteria of the rule of five and 9 of 1679 compounds that matched the criteria of the rule of three were determined as hit compounds for NorA inhibitory activity as well. Ultimately, 27 flavonoids and 2863 TCM compounds had fully satisfied at least the Lipinski's rule of five and had good predicted pIC_{50} values and thus were considered as potential drugs to reverse the efflux-mediated multidrug resistance of *Staphylococcus aureus*.

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Additional Discussion

From QSAR studies on piperine, aryl alkenyl amides/imines and 2-aryl-5-nitro-1*H*-indole derivatives as ligands of NorA efflux pump, it was observed that certain portion of any kind of ligands do involve themselves in hydrophobic interaction or electronic or in both with the receptor. The crucial binding in most of the cases is, nonetheless, hydrogen bonds. NorA is, therefore, assumed to have certain critical features capable of interacting with all kinds of structurally different ligands. These features may comprise some hydrogen-bond donor and acceptor sites, some lipophilic areas, and a few polar sites.

This view was justified by two docking studies using NorA homology models: Thai *et al*. developed homology model of NorA based on the transporter (PDB ID: 2gfp) while Kalia *et al*. used GlpT (PDB ID: 1PW4) as the protein template to create homology model. Thai *et al*. docked 6 traditional Chinese medicine (TCM) and 27 flavonoids into the central channel and the Walker B. Most molecules had better affinities to the Walker B than the central channel due to the hydrophilic property of the latter. Compared with TCM, flavonoids have higher affinities towards NorA binding site because of tight interactions (hydrogen bonding and π-π stacking) with NorA homology model made by substituents in flavonoid structures (Thai et al., 2014). Kalia *et al*. conducted docking studies of two ligands namely reserpine and capsaicin, which are two known NorA inhibitors, in the binding site of NorA. The most important finding was that a hydrophobic cleft of NorA provided stability as well as affected the orientation of the ligand within the binding pocket. Hydrogen bondings between substituents of ligands and amino acid residues of the binding site attributed to the stability of the ligand-protein complex (Kalia et al., 2012). These results consolidated the outcomes of above-presented QSAR studies that most of parameters selected for final models are associated with surface charge, polarity and molecular weight.

Compared with QSAR studies of specific derivatives, the QSAR analysis of natural products for NorA EPIs was performed with a larger and more diversified database including the compounds from different structural classes. However, this strong point became an obstacle that made the validation results of obtained models be at a disadvantage compared with those of the model generated by Koul *et al.* (A. Nargotra et al., 2009). Among three descriptors used for the model of these authors (A. Nargotra et al., 2009), this study revealed that the descriptor "partial negative surface area" might be relevant to the descriptor "total negative vdw surface area" (PEOE_VSA_NEG) that was determined as a less important variable and removed from final models.

QSAR Approaches in Search for EPIs in Gram Negative Bacteria

Gram-negative bacteria seem to be more dangerous than Gram-positive bacteria in some cases. Some can be named such as *P. aeruginosa, K. pneumoniae*, *C. jejuni*, *E. coli*, *S. typhimurium* and *E. aerogenes, etc.* Moreover, their levels of MDR have increased alarmingly. Nonetheless, no efflux pump inhibitor has been licensed for use in the treatment of bacterial infections in human or veterinary medicine. It must stimulate research that leads to the development of new EPI molecules.

With the recent advent of high-resolution 3D-structure of drug transporters, the co-crystallization of AcrB and MexB with some substrates, and the analyses of the biological effects of several mutations in the pump, it is now possible to rationally design functional targets located inside the bacterial pumps. Consequently, the synthesis of efficient molecules with strong EPI capacity will be more readily achieved. In this context, there should be more QSAR models that help to save time and money on searching "new drugs" for mediation of efflux pumps in Gram-negative bacteria. However, it is noticed with deep con-

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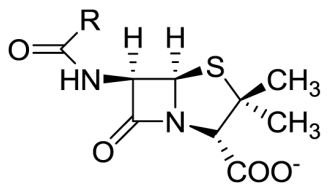
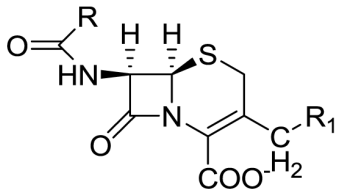
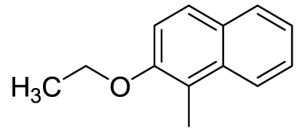
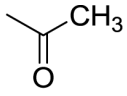
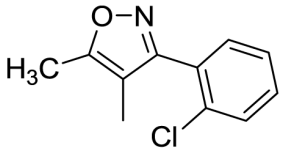
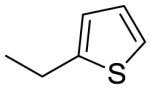
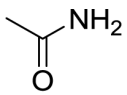
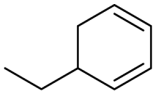
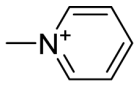
cern that scientists have not paid enough attention on this kind of QSAR studies. In comparison with a large number of other efflux pumps in Gram-negative bacteria, studies on the two kinds of efflux pumps (MATE efflux pump VmrA from *V.parahaemolyticus*, and AcrAB–TolC from *Escherichia coli*) are likely insufficient to deal with Gram-negative bacterial resistance. Therefore, it is hoped that new and solid QSAR models for MDR efflux proteins would be yielded in order to provide a more complete picture of the molecular requirements.

QSAR Study of β-Lactam Antibiotic Efflux by the Bacterial MDR Pump AcrB

A QSAR study related to Gram-negative bacterial efflux pumps was conducted by researchers Marcia Ferreira and R.Kiralj in 2004 (Ferreira & Kiralj, 2004). The target of the study was AcrAB-TolC – an efflux pump identified in several Gram-negative bacteria including *Salmonella typhymurium*. AcrAB-TolC is a tripartite efflux pump, which consists of the inner-membrane transporter AcrB (Edward, McDermott, Zgurskaya, Nikaido, & Koshland, 2003; Elkins & Nikaido, 2003; Murakami, Nakashima, Yamashita, & Yamaguchi, 2002; Pos & Diederichs, 2002), the outer-membrane channeltunnel protein (Koronakis et al., 2000) of the TolC family (Elkins & Nikaido, 2003), and the periplasmic linker lipoprotein AcrA from the membrane fusion family (Thanabalu, Koronakis, Hughes, & Koronakis, 1998).

The study was aimed at establishing multivariate quantitative lipophilicity–MIC and β-lactam structure–MIC relationships for *S. typhimurium* strains (Nikaido, Basina, Nguyen, & Rosenberg, 1998). Mass concentration MICs for 16 penicillines and cephalosporins (structures shown in Table 3) with respect to bacterial strains *S. typhimurium* SH5014 (parent strain) and its mutants low resistant SH7616 and AcrAB

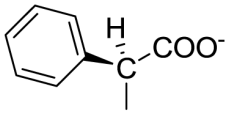
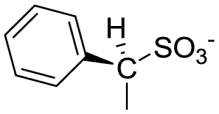
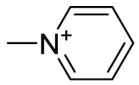
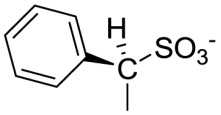
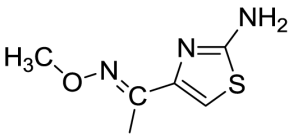
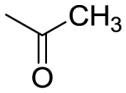
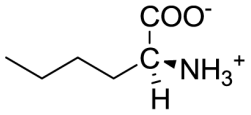
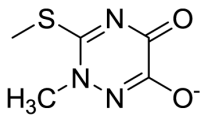
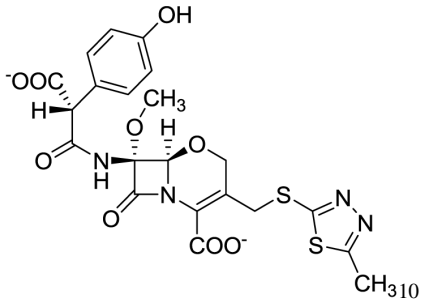
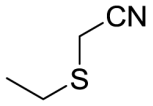
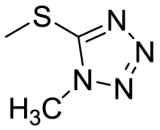
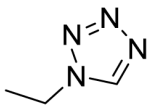
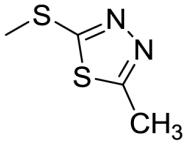
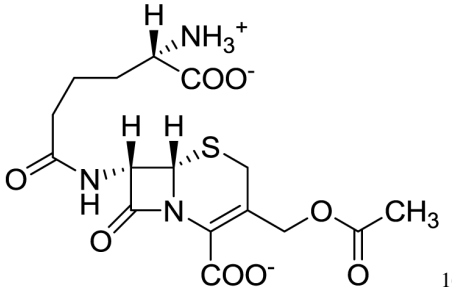
Table 3. Chemical structures of β-lactams used in QSAR study

No.	R	No.	R	R_1
				
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2		5		
3		6		

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Table 3. Continued

No.	R	No.	R	R_1
7		9		
8		11		
15		12		
		13		
		14		
				

overproducer HN891, were collected from the literature (Nikaido et al., 1998). Around 50 molecular properties of 16 β-lactam antibiotics were calculated using different methods based on their modeled molecular structures. These computed properties included lipophilicity parameters and other molecular properties which were prepared based on two-dimensional chemical formulae of studied penicillins and cephalosporins. HCA (hierarchical cluster analysis) and PCA (principal component analysis) studies indicated that the presence of charged groups and hydrophobic moieties determined the antibiotic behavior with respect to AcrAB-TolC pumps. Highly charged β-lactams with small hydrophobic fraction w_C or S_f might be poor AcrB substrates due to energetically unfavorable pump–drug interaction in molecular recognition. Nonetheless, the HCA-PCA approaches could not give answers to the question of which lipophilicity parameters were most reliable for these antibiotics, so the QSAR studies were carried out.

QSAR studies, which were performed by means of the partical least squares (PLS) and based on lipophilicity and electronic and hydrogen bonding molecular descriptors, demonstrated that

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1. Biological activities (pMICs) strongly depend both on properties of bacterial strains and drug molecules. β-lactams were classified as good, moderately good and poor AcrAB-TolC substrates;
2. Among the most important β-lactam molecular properties quantitatively related to pMICs are lipophilicity and electronic and hydrogen bonding properties; and
3. Lipophilicity parameters calculated in different ways do not necessarily present the same information about drugs, and cannot produce parsimonious and accurate regression models for MICs originated by active AcrAB-TolC pumps.

Two strains HN981 and SH5014 were found to act similarly, and the third one – SH7616 is significantly different from each of them (Table 4). This was manifested by the similarity, even with the same molecular descriptors, of the PLS models related to strains HN891 and SH5014.

Additionally, penicillins and cephalosporins which can be characterized as hydrophilic, with good hydrogen bonding properties and able to establish polar-polar interactions with bacterial pump receptors, are bad pump substrates, and so potentially good drugs. This finding has been supported by recent studies of AcrB in which most pharmacophore models generated by 49 AcrB substrates contain hydrophobic centers and hydrogen bond acceptors or donors. Molecular interactions of phytochemicals with the active site residues in the binding site of AcrB also consist of hydrogen bondings and hydrophobic contacts (Aparna, Dineshkumar, et al., 2014). Vargiu and Nikaido confirmed the similar features of AcrB binding sites. Distal pocket and proximal pocket were shown to be two main active regions of AcrB. 15 hydrophobic residues and 11 polar or charged amino acid residues in the distal binding pocket were found to contribute to the binding of all ligands examined (Vargiu & Nikaido, 2012). In another study publicized on *Nature* magazine in 2013, scientists found that AcrAB-MexAB specific inhibitor of pyridopyrimidine derivative (ABI-PP) is deeply inserted into a hydrophobic trap rich in phenylalanine residues. Based on the results of site-directed mutagenesis and molecular dynamics simulations, the trap has been considered to indirectly affect drug-binding site. Phe178 located at the edge of this trap in AcrB contributes to the tight binding of the inhibitor molecule through a π-π interaction with the pyridopyrimidine ring (Nakashima et al., 2013).

Table 4. PLS regression models for pMICs

pMIC	Parameters[a]	SEP[b]	Q[c]	R[d]	PCs (%)
HN891	w_C, S_f, GlogK_{OW}, logP_s, SlogP_s, logK_{WIN}, SlogK_{WIN}, logP_{IA}, logP_X	0.467	0.912	0.967	3 (82)
	logP_{IA}, GlogK_{OW}, SlogP_s, H_f, A_{HB}, D_y, N_{NS}	0.209	0.982	0.993	3 (85)
SH5014	w_C, S_f, GlogK_{OW}, logP_s, SlogP_s, logK_{WIN}, SlogK_{WIN}, logP_{IA}, logP_X	0.391	0.942	0.975	2 (82)
	logP_{IA}, GlogK_{OW}, SlogP_s, H_f, A_{HB}, D_y, N_{NS}	0.316	0.962	0.982	3 (85)
SH7616	w_C, S_f, GlogK_{OW}, logP_s, SlogP_s, logK_{WIN}, SlogK_{WIN}, logP_{IA}, logP_X	0.792	0.645	0.886	2 (76)
	logP_{IA}, GlogK_{OW}, SlogK_{WIN}, N_{CH}, H_f, N_{NS}	0.461	0.851	0.930	3 (83)

[a]Transformation of lipophilicity descriptors;[b] Standard error of validation; [c] Correlation coefficient from validation; [d] Correlation coefficient from prediction.

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QSAR/SAR Study of Structurally Unrelated Substrates of a MATE Efflux Pump VmrA from V.Parahaemolyticus

Multidrug transporters of MATE family have been known to expel dissimilar lipophilic and cationic drugs across cell membranes by dissipating a preexisting Na+ or H+ gradient (Lu et al., 2013). Brown *et al*. classified the MATE family into three subfamilies. Subfamily 1 includes NorM, subfamily 2 includes ERC1, and subfamily 3 includes DinF. VmrA has been considered to be a member of subfamily 3 (DinF branch). The *vmrA* gene consisted of 1,341 nucleotides, with a deduced popypeptide (VmrA) consisting of 447 amino acids residues with a calculated molecular mass of 49 kDa. VmrA was very rich in hydrophobic residues, indicating that the protein was an integral membrane protein (J. Chen et al., 2002). This characterization of MATE pump favoring hydrophobic substrates were illustrated in a QSAR study of VmrA substrates done by Rudolf Kiralj and Marcia M.C. Ferreira in 2008 (Kiralj & Ferreira, 2008).

Kiralj and Ferreira aimed at correlating the efflux activity of *V. parahaemolyticus* VmrA with molecular properties of 12 diverse agents at QSAR and SAR levels. The training set in this work consists of 12 agents (organic molecules/ions including 4',6-diamino-2-2phenylindole, tetraphenylphosphonium chloride, acriflavine chloride, ethidium bromide, chloramphenicol, norfloxacin, rhodamine 6G chloride, tetracycline, erythromycin, streptomycin, sodium deoxycholate and sodium dodecyl sulfate). The corresponding activities pMIC = -log(MIC/mol/dm^3) are pMIC(KAM) and pMIC(pVCJ6), which are efflux activity pMIC of the *E.coli* strains KAM32 and KAM32/pVCJ6, respectively. The prediction of the MDR character of VmrA with respect to 19 diverse substrates was performed at both QSAR and SAR levels.

Analysis of descriptors in correlation with the biological activities pMIC indicates that MDR of VmrA was directed against agents with shorter bonds, a few polar atoms, elevated contents of rings, planar structures, and aromatic carbon atoms. This is generally a rigid aromatic structure with exo- and/or endocyclic heteroatoms. Satisfactory prediction power statistics Q^2>0.5 and R^2>0.6 for QSAR models can be observed for all the models. The low R^2 and Q^2 values indicated that the good results in the original models were not due to a chance correlation or structural dependency of the training set.

There were also common structural features related to efflux from KAM32 and KAM32/pVCJ6 strains. In general, agent efflux was not determined by specific functional groups due to the strictly nonbonding nature of pump-mediated MDR. Exploratory analysis related to the two strains points out that good substrates of efflux systems in *E.coli* strains KAM32 and KAM32/pVCJ6 are neither linear nor extended, preferably were ring structures with some branching, rather hydrophobic, weak dipoles, difficult to polarize, possess some polar groups, and could establish hydrogen bonds with receptors. The strains differed in their preferences for good substrates: KAM32 binds rather hydrophobic species, while KAM32/pVCJ6 attracts heteroaromatics. Therefore, aromatic-aromatic and hydrophobic-hydrophobic VmrA-substrate interactions seemed to be crucial. Agent-receptor interaction statistics show that agent-receptor interaction descriptors depend on agent molecular properties, receptor molecular properties, agent position and binding mode relative to the receptor, and the presence of other molecular/ionic species. Supposing that agent properties might substantially affect agent-receptor interactions, variations in receptors and agent binding modes/positions were expected to have a secondary importance. In addition, good substrates of VmrA efflux pump from *V. parahaemolyticus* strain AQ3334 must also be rather rigid and condensed heteroaromatic systems, with very few or no flexible side-chains, with exo- or endocyclic heteroatoms, and establish aromatic-aromatic contacts with VmrA.

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This QSAR study proved that chemometric methods are very useful in practice and the agent-receptor interaction descriptors, based on PDB data, correlate reasonably with biological activities, further rationalizing the VmrA-agent interactions.

DESCRIPTORS AND METHODS

It has been recognized that most descriptors employed to build QSAR models for prediction of interactions between chemical agents and Gram-negative bacterial efflux pumps were mostly parameters of molecular field (steric, electrostatic and hydrophobic). In the study of AcrAB-TolC, critical β-lactam molecular descriptors related to pMICs were associated with lipophilicity. For the examination of VmrA substrates, VmrA in strain KAM32 extrudes agents characterized by three lipophilicity descriptors (Nh, sighyd and wh2) and two electronic descriptors (E4 and HOMO) while the regression vectors for KAM32/pVCJ6 have contributions of three electronic descriptors (FF, Np2 and Mrefn2), a steric (wr) and a hydrophobic (sighyd) descriptors accounting for agent efflux by VmrA. In studies of MDR efflux pumps of Gram-positive bacteria, in addition to molecular field descriptors as main contributors to QSAR models, other relevant descriptors relate to quantum chemical (heat of formation), constitutional (number of rings), topological and geometrical (molecular volume and radius of gyration) properties. These observations were consistent with the fact that even though the mechanisms of drug transport in bacterial efflux pumps have remained unknown and controversial, empiric observations on properties of known pump substrates and inhibitors have indicated several regular features, of which amphiphilic nature of compounds play a key role. Further discussion regarding this character of MDR pump inhibitors can be found in the review and the commentary done by Van Bambeke *et al*. in which the presence of well-defined lipophilic and hydrophilic regions of antibiotics was mentioned (Van Bambeke, Balzi, & Tulkens, 2000; Van Bambeke & Lee, 2006).

All QSAR studies concerning NorA inhibitors included in this chapter principally relied on two-dimensional (2D) descriptors to generate the models. Among the used methods, the most common was PLS, others included PCR and HCA. However, 2D descriptors could not give relevant information regarding the ligand – efflux pump protein interaction. Besides, there was only a limited number of studied transport proteins, as the researchers focused on several efflux pumps, namely NorA, AcrAB-TolC, and VmrA, out of a large number of identified antibiotic-resistant efflux pumps mentioned in previous sections of this chapter. Therefore, the need of broadening the scale of QSAR research and studies based on bacterial efflux pumps is evident.

FUTURE RESEARCH AND OUTLOOK

As far as we are concerned, since the parameters used for presented 2D-QSAR models are mostly molecular field descriptors, the COMFA/COMSIA-based 3D-QSAR might be an appropriate alternative for the exploration of relationships between certain structural properties and the binding mode in which the ligand interacts with the efflux pump at the active domains. However, this method also has several limitations. The alignment protocols used in 3D-QSAR assumes that the ligands observed in the model bind to the efflux pump at the same binding site in the same manner. As a result, researchers may have difficulties achieving 3D-QSAR models in the circumstances where the efflux pumps have either one

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large binding site or multiple binding sites which can accommodate simultaneous ligands in various ways. In such cases, VolSurf/GRIND-based descriptors should be employed to obtain 3D-QSAR models with acceptable predictive capability. These are alignment free and thus allow the analysis of structurally diverse compound sets. Although QSAR approaches do not enable one to decipher the underlying mechanism of ligand-protein interaction, they might represent versatile tools for *in silico* filtering large combinatorial libraries for compounds with bacterial efflux pump inhibitory activity.

Moreover, the studies mentioned in the chapter mainly relied on the PLS analysis to yield predictive models. This approach was limited to sets of compounds whose biological activity and molecular descriptors are in linear correlations. Thus, there should be QSAR studies using machine learning and neuron network methods which can deal with non-linear structure-activity relationships. These approaches may pave the way for the establishment of rapid *in silico* screening that are routinely applied in the early drug discovery phase. Additionally, pharmacophore modeling should also be considered in such the cases where the database includes a large number of compounds that have diverse structural properties. This method may be used alongside 3D-QSAR in order to assess the drug – transporter interactions, and also to give certain suggestions on the direction of synthesizing new chemical compounds to inhibit the studied efflux pumps. The pharmacophore models can be derived from either the binding site of the protein or a group of active compounds (Guner, 2000; Mannhold, Kubinyi, Folkers, Langer, & Hoffmann, 2006).

Recently, many researchers are working on the efflux pump namely NorA of *S. aureus*. However, no X-ray crystal structures have been found until now. Hence, physiological, pharmacological and medicinal chemistry studies on NorA pump have contributed to our understanding of the structure and mechanism of drug resistance. Further *in silico* as well as *in vitro* studies and X-ray crystal structures of certain efflux pumps like NorA have remained attractive targets to scientist working in intelligent designs of new novel NorA inhibitors as well as other EPIs.

CONCLUDING REMARKS

This review provides a summary of current knowledge about efflux-pump-mediated multidrug resistance and QSAR studies on bacterial EPIs. QSAR modeling results provide useful information for structural modifications of existing compounds in order to gain more potent compounds in use. The major benefits derived from developing efficient EPIs will be the ability to reuse various antibiotics affected by the efflux pumps as well as the control of the emergence and the dissemination of MDR efflux strains. Until now, the majority of the known inhibitors have been obtained from screening libraries of synthetic compounds, from purification of natural compounds or chemical modification of existing molecules.

As far as Gram-positive bacteria are concerned, most of the QSAR studies on bacterial MDR are dedicated to NorA in *S.aureus*. Obtained QSAR analyses, coupled with molecular docking studies, proved that hydrophobicity and hydrogen bondings are essential for NorA inhibitory activity. For Gram-negative bacterial EPIs, there has been a modest number of QSAR studies conducted. With the advent of high-resolution crystal structures of efflux pumps of *Escherichia coli* and *Pseudomonas aeruginosa*, it is hoped that consequent increase in the population of identified molecules with high EPI capacity would foster ligand-based approaches in this area. For general QSAR studies on bacterial EPIs, 2D-QSAR using partial least squares method and molecular field parameters were largely employed. Therefore, it is suggested that 3D-QSAR and machine learning methods should be taken into consideration when generating future

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QSAR models. Also, additional X-ray structures of proteins will definitely aid in the understanding of the molecular principles underlying the ligand-protein binding mode of bacterial efflux pumps.

To date, none of bacterial EPIs have entered clinical trials yet, the active compounds found with EPI-potency should still be subjects of deeper examination, including their pharmacokinetic properties and toxicity, as they give a hope for their future therapeutic usage. Moreover, the development of combination therapy poses additional complexity due to the necessity of precision tailoring of the pharmacokinetics of both agents to achieve the desired pharmacodynamic effect.

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KEY TERMS AND DEFINITIONS

Bacterial Efflux Pumps: Proteinaceous transporters located in the cytoplasmic membrane of bacteria. These transporters are responsible for moving a wide range of compounds including antibiotics out of bacterial cells, which can contribute to bacterial antibiotic resistance.

Docking Study: The method used for the simulation of optimal binding orientations of one molecule to its target protein thereby predicting the affinity and biological activity of small organic molecules. Docking may be applied in hit identification and lead optimization in the field of drug design.

Efflux Pump Inhibitors (EPIs): Compounds that can inhibit the efflux-mediated extrusion of antibiotics. The co-administrations of these compounds and antibiotics to restore antibiotic activity have been investigated, but none of the efflux inhibitors tested has been in clinical use until now.

Homology Modeling (Also Known as Comparative Modeling): The method used for construction of an atomic-resolution model of the target protein based on its amino acid sequence and an empirically determined 3D-structure of a template – the protein having the highest sequence identity with the target sequence. A homology model can be used for various purposes including molecular docking, protein-protein interaction prediction, homology model based ligand design, etc.

MultiDrug Resistance (MDR): A condition enabling pathogenic micro-organisms (including bacteria) to oppose the action of certain antibiotics, rendering the antibiotics ineffective.

Pharmacophore Hypothesis: A geometrical description of molecular features represents a binding mode of a ligand to a receptor site. Typical pharmacophore features include hydrophobic centers, aromatic rings, ionizable points, hydrogen bond donors and acceptors.

Protein Data Bank: A freely accessible source for 3D-structures of biological macromolecules such as proteins and nucleic acids. Most of these structures are determined by NMR spectroscopy and X-ray diffraction.

Protein X-Ray Crystallography: The method of characterizing the molecular structure and function of various biological molecules including proteins. In principle, when X-rays strikes a protein crystal, they are scattered into many specific directions. The patterns and relative intensities of these diffracted X-ray beams can be processed to provide information about protein structure.

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Chapter 36
QSPR/QSAR Analyses by Means of the CORAL Software:
Results, Challenges, Perspectives

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ABSTRACT

In this chapter, the methodology of building up quantitative structure—property/activity relationships (QSPRs/QSARs)—by means of the CORAL software is described. The Monte Carlo method is the basis of this approach. Simplified Molecular Input-Line Entry System (SMILES) is used as the representation of the molecular structure. The conversion of SMILES into the molecular graph is available for QSPR/QSAR analysis using the CORAL software. The model for an endpoint is a mathematical function of

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the correlation weights for various features of the molecular structure. Hybrid models that are based on features extracted from both SMILES and a graph also can be built up by the CORAL software. The conceptually new ideas collected and revealed through the CORAL software are: (1) any QSPR/QSAR model is a random event; and (2) optimal descriptor can be a translator of eclectic information into an endpoint prediction.

INTRODUCTION

Almost seventy years ago one of the areas of theoretical chemistry named the quantitative structure – property / activity relationships (QSPRs/QSARs) was established (Wiener, 1947, pp. 17-20; Wiener, 1948, pp. 425-430). However, if one considers the publication of Hammett's Free-energy relationship (Hammett, 1935, pp. 125–136) as the first exercise in the QSPR/QSAR analyses, then the introduction of this area of theoretical chemistry should be moved back by eighty years. This is obviously an evidence of the long lasting importance of the QSAR approaches. Such significance is due to their wide applications. The main target of QSPR/QSAR analyses is the prediction of the numerical data related to various endpoints (physicochemical in the case of QSPR; and biochemical, ecological, medicinal, etc., in the case of QSAR).

One can consider three periods of evolution in this field.

1. The first period can be defined as the construction of molecular descriptors and establishing of their correlation with various endpoints (Bonchev & Trinajstic, 1977; Balaban, 1982, pp. 399-404; Bonchev, 1995, pp. 137- 156; Diudea & Gutman, 1998; Ivanciuc, Ivanciuc, & Balaban, 1998; Randic, DeAlba, & Harris, 1998; Castro, Tueros, & Toropov, 2000; Randic & Basak, 2001; Nicolic, Kovacevic, Milicevic, & Trinajstic, 2003; Ren, 2003, pp. 29-39; González, Terán, Teijeira, & González-Moa, 2005; Melagraki, Afantitis, Sarimveis, Igglessi-Markopouloua, & Supuran, 2006).
2. The second period can be defined as the construction of molecular descriptors, establishing of their correlation with an endpoint for the compounds of "visible" training set with the further testing of the predictive potential of this correlation with "invisible" compounds (i.e. compounds which are not involved in building up of the model) involved in the test set (Cronin et al., 2002; Golbraikh & Tropsha, 2002; Taskinen & Yliruusi, 2003; Hemmateenejad, 2004, pp. 475–485; Duchowicz, Castro, Fernandez, & Gonzalez, 2005; Oberg, 2005, pp. 2189–2200; Afantitis et al., 2006; Coi et al., 2006; Leonard & Roy, 2006; Pan, Jiang, & Wang, 2007; Roy & Roy, 2008; Porto, Souza, Junkes, Yunes, & Heinzen, 2008; Toropova, Toropov, Benfenati, Leszczynska, & Leszczynski, 2010; Toropov et al., 2012a; Toropov, Toropova, Raska Jr, Benfenati, & Gini, 2012b; Nesmerak, Toropov, Toropova, & Kohoutova, 2013; Papa, van derWal, Arnot, & Gramatica, 2014).
3. The third period can be defined as building up of the QSPR/QSAR models according to OECD principles (Kruhlak, Contrera, Daniel Benz, & Matthews, 2007; Kar & Roy, 2010, 2012; Putz, Ionascu, Putz, & Ostafe, 2011; de Melo, 2012, pp. 213–222; Sahigara et al., 2012; Toropova & Toropov, 2014).

According to the OECD principles, the QSPR/QSAR model should be characterized by:

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1. A defined endpoint;
2. An unambiguous algorithm;
3. A defined domain of applicability;
4. Clear measures of predictive potential; and
5. A mechanistic interpretation, if possible (OECD, 2007).

It was during the second period of the methodology development, when optimal descriptors calculated with the molecular graph were suggested as a tool to solve the QSPR/QSAR tasks (Randic & Basak, 1999, 2001; Randic & Pompe, 2001; Toropov & Toropova, 2002; da Silva Junkes, Arruda, Yunes, Porto, & Heinzen, 2005). In fact, majority of molecular descriptors are not expected to correlate with a certain endpoint, but they can be involved in building up model for arbitrary endpoint. However, the optimal descriptor is aimed to correlate with a certain endpoint via a one-variable correlation.

Interestingly, simplified molecular input-line entry system (SMILES) is an attractive alternative for representation of the molecular structure by graph (Weininger, 1988, pp. 31-36; Weininger, 1990, pp. 237-243; Weininger,Weininger, & Weininger, 1989). There are databases available on the Internet where the molecular structure is represented by SMILES (Toropov, Toropova, Raska Jr, Leszczynska, & Leszczynski, 2014). Under such circumstances, the development of QSPR/QSAR approaches, where SMILES is the representation of the molecular structure becomes an attractive direction of research work in the field of the QSPR/QSAR theory and applications. The CORAL software is a tool of the QSPR/QSAR analyses with representation of the molecular structure by SMILES (Toropova, Toropov, Benfenati, Leszczynska, & Leszczynski, 2010d; Toropova et al., 2011a,b). It is to be noted that the CORAL software is able to extract from the SMILES various graph theoretical invariants such as vertex degree and extended connectivity of higher orders (Toropov & Toropova, 2002), as well as invariants for the graph of atomic orbitals (Toropov & Toropova, 2001).

The description of:

1. The organization of the CORAL software,
2. Results obtained using this software,
3. Advantages and disadvantages of the approach, as well as
4. Discussion of possible modifications of this software are the main aims of the chapter.

Background

The basic paradigm of the QSPR/QSAR prediction can be expressed as the following:

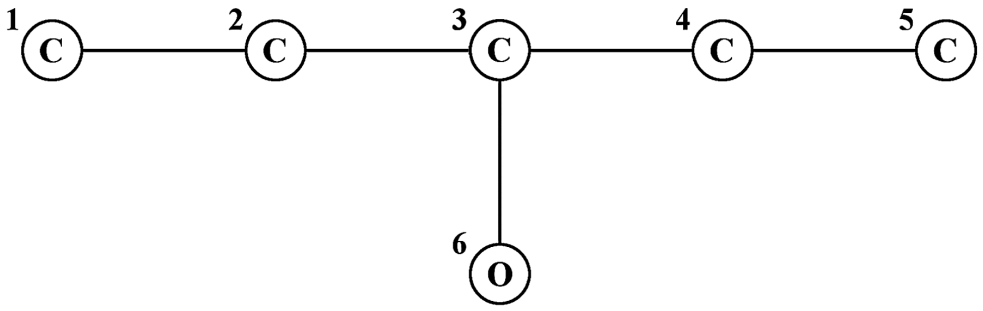
Endpoint = F(Molecular Structure).

The classic mathematical representation of the molecular structure can be done by means of the adjacency matrix A(G) (Gutman, 1988, pp. 93-94; Gutman, 1997, pp. 281-287). The hydrogen-suppressed graph (HSG) for 3-pentanol (CAS 584-02-1) can be represented as seen in Figure 1.

The hydrogen-suppressed graph is characterized by the following adjacency matrix A(HSG):

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Figure 1. The hydrogen-suppressed graph for 3-pentanol (CAS 584-02-1)



A(HSG)

	1	2	3	4	5	6	δ_k
1	0	1	0	0	0	0	1
2	1	0	1	0	0	0	2
3	0	1	0	1	0	1	3
4	0	0	1	0	1	0	2
5	0	0	0	1	0	0	1
6	0	0	1	0	0	0	1

A*(HSG)

	1	2	3	4	5	6	δ_k^*
1	x	1	0	0	0	0	$1+x$
2	1	x	1	0	0	0	$2+x$
3	0	1	x	1	0	1	$3+x$
4	0	0	1	x	1	0	$2+x$
5	0	0	0	1	x	0	$1+x$
6	0	0	1	0	0	y	$1+y$

Majority of the molecular descriptors are a mathematical function of the A(HSG), in particular the Randic index (Randic, 1975) is calculated as

$$^1X = \sum_{(i,j)edge}(\delta_i\delta_j)^{-0.5}. \tag{1}$$

The pioneer version of the optimal descriptor (Randic & Basak, 1999; Randic, 2001) is represented by the calculation of classic descriptors using the modified adjacency matrix A*(HSG):

$$^1X^* = \sum_{(i,j)edge}(\delta_i^*\delta_j^*)^{-0.5}. \tag{2}$$

The next step in the evolution of the optimal descriptors is the application instead of numerical contributions of vertex degrees (δ_k) the so-called correlation weight of the presence of a certain vertex degree, together with the correlation weight of a certain chemical element (A_k):

$$^0X_{CW} = \sum_k CW(A_k) + \sum_k CW(\delta_k). \tag{3}$$

The correlation weights are calculated by the Monte Carlo method. The numerical data on the correlation weights being used for calculation of the $^0X_{CW}$ should provide maximum of correlation coefficient

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between the descriptor and a certain endpoint for the training set of compounds. Having the numerical data one can calculate the ${}^{0}X_{CW}$ for compounds of the training set and define the model:

$$Endpoint = C_0 + C_1 \times {}^{0}X_{CW} \tag{4}$$

However, the predictive potential of the model calculated with Equation 4 should be checked up with external test set.

The representation of the molecular structure in databases by adjacency matrix is not convenient and non economical, in comparison to the representation by SMILES string. Consequently, one can define the descriptor similar to the above-mentioned ${}^{0}X_{CW}$ which can be calculated with SMILES:

$$DCW = F(SMILES) \tag{5}$$

The simplest way is to define the descriptor as a mathematical function of SMILES atoms, i.e. as a mathematical function of each character of SMILES, except situations where two symbols cannot be examined separately (e.g. 'Cl', 'Br', etc.). In the case of 3-pentanol with SMILES= "CCC(O)CC", the scheme can be illustrated as the following:

$$\begin{aligned} DCW = {} & CW('C') + CW('C') + CW('C') + CW('(') \\ & + CW('O') + CW(')') + CW('C') + CW('C') = \Sigma CW(SA_k) \end{aligned} \tag{6}$$

The scheme expressed by Equation 6, can be useful for relatively simple physicochemical properties, such as the normal boiling point of a certain class of organic compounds (alkanes, alkylbenzenes, ethers, etc.). However, in the case of mixture of various classes of organic compounds, or in the case of more complex endpoints (toxicity, carcinogenicity, etc.), the scheme gives poor statistics for both the "visible" training set and for the "invisible" test set. Fortunately, the approach can be improved if in addition to correlation weights of SMILES atoms, combinations of two and / or three SMILES-atoms would be involved in the model.

However, in the cases of two or three SMILES atoms some paradoxical situations occur. For example, SMILES fragment "CN" and "NC" (similarly, "CCN" and "NCC") will be examined as different molecular features. Fortunately, one can avoid these cases if usees normalized combines of SMILES atoms. A simple way is to select order which obey principle: the first and last characters are placed according to their ASCII codes (Toropov, Toropova, & Benfenati, 2010b). In addition, SMILES attributes which are fragments of molecular structure, the global invariants of the molecular structure are involved to build up a model. These global attributes are a mathematical function of presence (absence) of various molecular features which are represented in Table 1.

The above-mentioned Monte Carlo optimization in the case of the unlimited number of epochs will provide maximum of correlation coefficient for the training set. However (Figure 2), a maximum of correlation coefficient between optimal descriptor and endpoint for a certain number of the epochs exists for the external test set. This amount of epochs is preferable in calculations, because when the number of epochs reaches such a value the obtained model is characterized by a good predictive potential.

Vice versa, the increase of threshold is accompanied by decrease of the correlation coefficient for the training set, but again, the threshold which provide maximum correlation coefficient for the test set

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Table 1. The scheme of extraction of SMILES atoms and other SMILES attributes in order to build up a model

SMILES Attribute	Example of the Representation for the CORAL Software
S_k	SMILES-atoms, i.e. one symbol or two symbols which cannot be examined separately, e.g. 'C' and 'Cl': this information is represented by sequences of twelve symbols: C.......... Cl.........
SS_k	A combination of two SMILES-atoms 'CC' and 'CN': this information is represented by sequences of twelve symbols: C...C..... C...N.....
SSS_k	A combination of three SMILES-atoms 'CNC' and 'C#N': this information is represented by sequences of twelve symbols: C...N...C... C...#...N...
BOND	The presence / absence of double ('='), triple ('#'), and stereo chemical ('@') bonds, e.g. if SMILES = "CCC(O)CC" = # @ / 0 0 0 ⇨ BOND00000000
NOSP	Presence (absence) of nitrogen, oxygen, sulphur, and phosphorus, e.g. if SMILES="CCC(O)CC" N O S P / 0 1 0 0 ⇨ NOSP01000000
HALO	Presence (absence) of fluorine, chlorine, bromine, and iodine atoms, e.g. if SMILES='ClCC(=O)CCl F Cl Br / 0 1 0 ⇨ HALO01000000

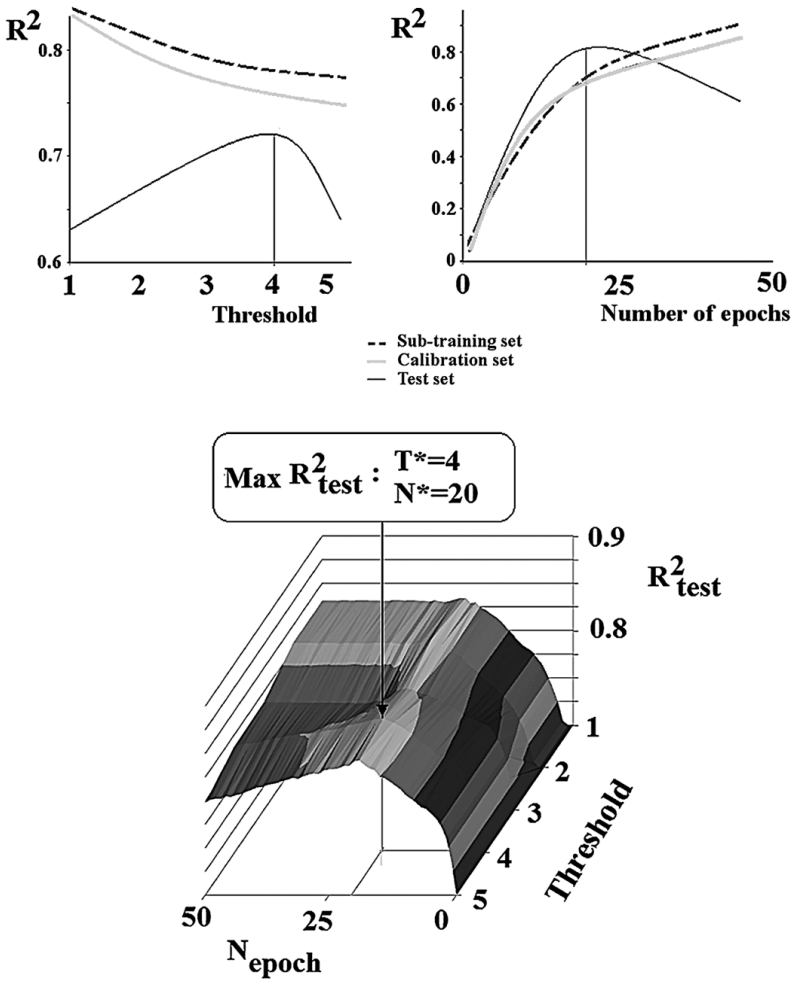
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Table 1. Continued

SMILES Attribute	Example of the Representation for the CORAL Software
PAIR	Simultaneous presence of two SMILES-atoms from the list: F, Cl, Br, I, N, O, S, P, #, =, and @; e.g. if SMILES="ClCC(=O)CCl" the following pairs will be extracted: `++++Cl..O...` i.e. 'Cl' and 'O' `++++Cl..B2..` i.e. 'Cl' and '=' `++++O...B2..` i.e. 'O' and '='

Figure 2. The generalized scheme of selection of preferable threshold and the number of epochs of the Monte Carlo optimization

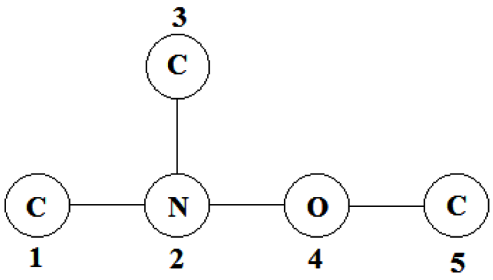
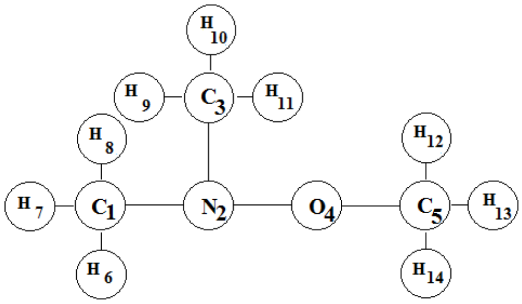
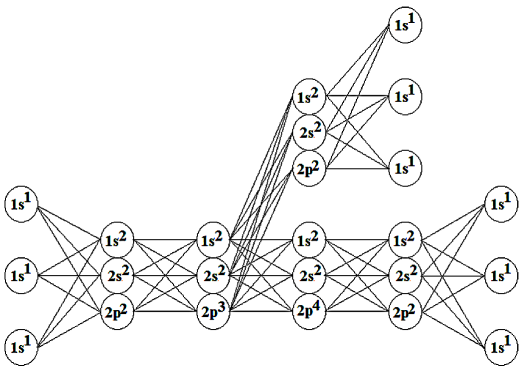


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exists. This threshold is preferable from practical point of view. Consequently, in order to prepare a good model by means of the CORAL software, one should define preferable values of the threshold and the number of epochs of the Monte Carlo optimization (T* and N*) (Toropova et al., 2011a).

Table 2 contains the list of possible representations of the molecular structure which can be used to build up a model. Thus, the CORAL software is able to provide the following types of QSPR/QSAR models:

Table 2. The representations of the molecular structure which can be used to build up a model by means of the CORAL software

The Representation of the Molecular Structure	Graphical Interpretation
SMILES	CN(C)OC
Hydrogen-Suppressed Graph, HSG	
Hydrogen-Filled Graph, HFG	
Graph of Atomic Orbitals, GAO	

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1. **SMILES Based Models:**

$$^{SMILES}DCW(Threshold, N_{epoch}) = \alpha\sum CW(S_k) + \beta\sum CW(SS_k) \\ +\gamma\sum CW(SSS_k) + x \cdot CW(NOSP) + y \cdot CW(HALO) + z \cdot CW(BOND) + t \cdot CW(PAIR) \quad (7)$$

2. **Models Based on the Representation by Molecular Graphs:** These are HSG, or HFG, or GAO (Table 2).

$$^{Graph}DCW(Threshold, N_{epoch}) = \sum CW(A_k) \\ +\alpha\sum CW(^0EC_k) + \beta\sum CW(^1EC_k) + \gamma\sum CW(^2EC_k) + \delta\sum CW(^3EC_k) \quad (8)$$

In Equations 7 and 8, coefficients α, β, γ, δ, t, x,y, and z can be 1 (yes) or 0 (no). Various combines of their values give possibility to define certain version of the optimal descriptor;

3. **The Hybrid Models which Involves Molecular Feature Extracted from Both Smiles and Graph:**

$$^{Hybrid}DCW(Threshold, N_{epoch}) = {}^{SMILES}DCW(Threshold, N_{epoch}) + {}^{Graph}DCW(Threshold, N_{epoch}) \quad (9)$$

4. Last but not least: the CORAL software is able to provide models for phenomena where eclectic information is the only basis for prediction, e.g. property and activity of proteins (Toropov, Toropova, Raska Jr, Benfenati, & Gini, 2012b) as well as endpoint related to nanomaterials (Toropova & Toropov, 2013). The generalized form of the optimal descriptor which can be used for the above-mentioned situations is the following:

$$DCW(Threshold, N_{Epoch}) = \Sigma CW(CI_k) \quad (10)$$

where CI_k represent codes of available eclectic data (e.g. concentration, size, condition of synthesis, technological details, etc.).

Selected Technical Details Related to the CORAL Software

There are some limitations of the SMILES in CORAL software. The maximal length for SMILES is 500 symbols; the maximal number of atoms (or atomic orbitals in the case of the graph of atomic orbitals) is equal to 300; the maximal number of compounds (which are involved in building up model) is equal to 50,000.

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Development of a Model by Means of the CORAL Software Is Based on the Following Principles

1. Molecular structure of each compound can be represented by molecular features which are extracted from SMILES and/or from molecular graph;
2. There are local and global molecular features which can be extracted from SMILES. The local features represent some fragments (or individual atoms). The global features are some indexes which characterized molecules as whole.
3. Building up of QSPR/QSAR model for an arbitrary split into the training and validation sets can be examined as a random event.
4. The statistical quality of each QSPR/QSAR model is a mathematical function that represents split of the data into the "visible" training set and "invisible" test set.
5. The average statistical quality of QSPR/QSAR models that is obtained for several splits into training and test sets is more robust criterion for the estimation of an approach than the statistical quality for solely one split.
6. The average statistical quality of a model for external "invisible" validation sets is more significant information related to the quality of the model than the average statistical quality for "visible" (i.e. substances involved in building up model) sub-training and calibration sets.

Every model that includes geometry-dependent molecular descriptors usually involves a relatively expensive calculation of the optimum molecular geometry, involving high computational costs and long CPU time. In this context, the conformation-independent 0D, 1D and 2D-QSPR methods emerge as an alternative approach for developing models based on constitutional and topological molecular features of compounds (Duchowich, Comelli, Ortiz, & Castro, 2012; Talevi et al., 2012). The exclusion of 3D-structural aspects also avoids problems associated with ambiguities, resulting from an incorrect geometry optimization output due to the existence of compounds in various conformational states. Such kind of problems may also lead to the vanishing of predictive capability of the QSPR/QSAR when applied for the prediction of an external test set of compounds. In the realms of the CORAL approach, the calculated flexible descriptor is a molecular descriptor which depends both on the molecular structure and the property under analysis, but does not explicitly depend on details from the 3D-molecular geometry. In previous QSPR/QSAR studies the importance of this methodology has been shown. Such approach is able to provide models having a comparable or sometimes better quality than the ones found by searching the best descriptors in a pool containing thousands of 0D-3D descriptors (Mullen, Duchowicz, & Castro, 2011; Garcia et al., 2011; Ibezim, Duchowicz, Ortiz, & Castro, 2012).

THE COLLECTION OF QSPR/QSAR MODELS

Over the years the CORAL software has been used for QSPR/QSAR analyses of a group of various endpoints. The results are listed in the following sections.

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Water Solubility of Organic Compounds

Data on water solubility of 1311 substances, i.e. their CAS number, SMILES, and the values of negative logarithm of water solubility –log S (mol / L) were taken from the web site of Virtual Computational Chemistry Laboratory (http://www.vcclab.org/lab/alogps/). These substances were distributed by means of five random splits into the sub-training set (≈55%), calibration set (≈25%), and test set (≈20%). The statistical quality of the CORAL models (Table 3) is better than the statistical quality of the models suggested earlier in the literature for the same large database (Huuskonen, 2000, pp. 773-777; Tetko, Tanchuk, Kasheva, & Villa, 2001; Yan & Gasteiger, 2003). The best prediction is characterized by n_{test}=21, r^2_{test}=0.91, s_{test}=0.63 (Huuskonen, 2000, pp. 773-777).

Solubility of [C60] and [C70] Fullerene Derivatives

The numerical data on the solubility of [C60] and [C70] fullerene derivatives in chlorobenzene in mg/mL were taken from the literature (Troshin et al., 2009). Unfortunately, since at present, the QSPR analysis of these data by other methods is not available we are not able to compare the obtained results with various approaches.

Octanol/Water Partition Coefficient of Antineoplastic Drugs

The numerical data on octanol/water partition coefficient (logP) for 55 organic compounds have been taken from the literature (Wishart at al., 2008). The CORAL model (Table 3) is better than the model described in the literature (Fouchécourt, Béliveau, & Krishnan, 2001) for the same data (n=55, r^2=0.8180).

Half-Wave Potential

The half-wave oxidation/reduction potential is very useful characteristic of electronic properties of the chemical compounds. The first SMILES-based QSPR analysis of half-wave potentials was performed for 40 benzoxazines (Toropov, Nesmerak, Raska Jr., Waisser, & Palat, 2006). The statistical quality this approach (r^2 = 0.882) is fully comparable with classical approach based on Hammett constants (r^2 = 0.897; Nesmerak, Nemec, Sticha, Waisser, & Palat, 2005). Other example of SMILES-based QSPR of half-wave potential using CORAL software is analysis of experimental data for 23 derivatives of *N*-benzylsalicylthioamides. From the statistical point of view, the model is comparable with data obtained in the experiment (Nesmerak, Toropov, Toropova, Kohoutova, & Waisser, 2013).

Rate Constants of Reactions between Organic Aromatic Pollutants and Hydroxyl Radical

The degradability of 78 compounds expressed in terms of second-order degradation rate constants, K_{OH}, between $OH^{\cdot}$- and each targeted aromatic compound have been taken from the literature (Kušić, Rasulev, Leszczynska, Leszczynski, & Koprivanac, 2009). Decimal logarithm of the K_{OH} was evaluated as the endpoint for the QSPR models. The model from the above-mentioned work (Kušić, Rasulev, Leszczynska,

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Leszczynski, & Koprivanac, 2009) is characterized by r^2_{test}=0.760. Hence the statistics of the CORAL model (Table 3) are better, because for four random splits into the training and test sets for the CORAL models (Toropov et al., 2012a) the average r^2_{test}=0.8253.

Chromatographic Retention Data

The retention factors of 41 derivatives of 1-phenyl-5-benzylsulfanyltetrazole in reverse-phase HPLC were measured for various compositions of mobile-phase. The retention characteristics of the linear solvent strength model were calculated using the linear relationship between logarithm of retention factor and volume fraction of organic modifier in mobile phase. The logarithms of the solute retention factor extrapolated to a mobile phase composition with 0% organic modifier, and constants for a given solute in a given chromatographic system (i.e. the slopes of the linear regressions) were used as endpoints. The one-variable QSPRs developed show that the CORAL software is a tool for building up a robust quantitative structure-retention relationships (QSRR) model for retention characteristics (Nesmerak, Toropov, & Toropova, 2014).

Toxicity towards *Daphnia magna*

The descriptions of toxicity of organic chemicals related to 48 h *Daphnia magna* toxicity expressed in negative decimal logarithm of the dose that kills 50% of organisms i.e. pLC50 were taken from the literature (Kar & Roy, 2010). The data set covers range of octanol/water partition coefficient from −2 to 8. According the values, it could be more possibly logarithms of octanol/water partition coefficient. The range of toxicity (daphnia) is from 0.46 to 10.09. In regard to the chemical domain, the data set includes hydrocarbons, aliphatic alcohols, phenols, ethers, and esters; anilines, amines, nitriles, nitroaromatics, amides, and carbamates; urea and thiourea derivatives; iso-thiocyanates; thiols; phosphorothionate and phosphate esters; and halogenated derivatives. The statistical quality of the model described in the original article is the following: n=222, r^2=0.695 (training set), and n=75, r^2_{pred}=0.741, R_m^2 =0.707 (test set). Thus, the statistical quality of the Coral model (Table 3) is at least comparable with the model suggested in the above-mentioned work (Kar & Roy, 2010).

Toxicity in Rats

Rat toxicity data (LD50, in mg/kg, oral exposure, n=689) was taken from the U.S. Library of Medicine (http://toxnet.nlm.nih.gov/). The $\log_{10}$[1/LD50] has been used as endpoint. Unfortunately, information on QSAR analysis of these data by other approaches is not available. However, comparison of optimal descriptors and Multiple Linear Regression (MLR) has shown (Toropov, Rasulev, & Leszczynski, 2008) that the models for rat toxicity based on optimal descriptors calculated with SMILES have better statistical characteristics than one-, two-, three-, and four-variable MLR models based on the topological and quantum mechanical descriptors.

Toxicity of Binary Mixtures

The numerical data on the toxicity of binary mixtures was taken from the literature (Zhang, Zhou, Yang, & Wang, 2007). The toxicity is expressed as pEC50 (i.e. negative decimal logarithm log[1/EC50]),

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logarithm of the inverse of the effective concentration required to bring about a 50% decrease in light emission, for *Photobacterium phosphoreum* (T3 mutation). The statistical quality of four-variables model (calculated with involvement of the quantum mechanical descriptors) suggested in the literature (Zhang, Zhou, Yang, & Wang, 2007) for the toxicity of the same 50 binary mixtures is the following: n=50, r^2=0.85, s=0.270. Consequently, the CORAL model for this endpoint provides better results (Table 3).

Carcinogenicity

Experimental values for carcinogenicity were taken from the web site of United States Environmental Protection Agency (http://www.epa.gov/ncct/dsstox/sdf_cpdbas.html). Carcinogenicity is expressed as the potency dose that induces cancer in rats (TD50, in mg/kg body weight). These values have been converted into mmol/kg body weight. The log(TD50) was selected as endpoint for modeling. The correlation coefficient of the QSAR for carcinogenicity of 45 benzene derivatives amounts to about r^2 = 0.7 (Vracko, 1997, pp.1037–1043). Another QSAR model for rodent carcinogenicity nitroso compounds obtained by multiple linear regression analysis (Helguera, Cordeiro, Pérez, Combes, & González, 2008) has the following statistical characteristics: n=48, r^2 = 0.859, s=0.361, F=42 (training set) and n=6, Q^2 = 0.71, s=0.488 (test set). The CORAL model for carcinogencity (Table 3) should be consider as comparable with the above-mentioned models, since the number of examined compounds for the CORAL model is considerable larger.

Carcinogenicity of Drugs

Numerical data on carcinogenic potentials of the selected 1464 organic compounds (chemical domain which includes hydrocarbons, aliphatic alcohols, phenols, ethers, and esters; anilines, amines, nitriles, nitroaromatics, amides, and carbamates; urea and thiourea derivatives, isothiocyanates, thiols, phosphate esters, and halogenated derivatives) are expressed by DF (Discriminate Function). The range of DF is from -9.91 to 9.86. Positive value of DF is an indicator of carcinogenic compound, negative value of DF is an indicator of non-carcinogenic compound. The predictive potentials of QSAR models for the same series of compounds suggested in the literature are characterized by n_{test}= 732, r^2_{test}=0.713 (Kar & Roy, 2011); and n_{test}= 732, r^2_{test}=0.77 (Duchowicz, Comelli, Ortiz, & Castro, 2012). Thus the statistical quality of the CORAL model is better than those available in the literature (Table 3).

Anticancer Agents (Murine P388 Leukemia)

The decimal logarithm log(1/IC50), where IC50 represents the concentration of the agent necessary to reduce cell viability by 50% against Murine P388 Leukemia (in vitro cytotoxic activity) is considered as the endpoint. Numerical data on this endpoint was taken from the literature (Atanasova, Ilieva, & Galabov, 2007). The QSAR model suggested in the published work (Atanasova et al., 2007) is characterized by n_{test} = 25; r^2_{test}=0.700. Thus, predictive potential of the CORAL model for the anticancer activity is better that the previously published results.

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Anticancer Agents (SK-MEL-5 Cell Line and UACC-62 Cell Line)

The observed cell anti-proliferative activities of the triphenylmethyl containing compounds inducing death in the human melanoma cell lines SK-MEL-5 and UACC-62 are extracted from the literature (Palchaudhuri, Nesterenko, & Hergenrother, 2008; Palchaudhuri & Hergenrother, 2008). The models calculated with MLR approch (Mullen, Duchowicz, & Castro, 2011) are characterized by n_{train}=17, r^2_{train}=0.781, s_{train}=0.16, n_{test}=6, r^2_{test}=0.243, s_{test}=0.44 (SK-MEL-5); and n_{train}=19, r^2_{train}=0.859, s_{train}=0.17, n_{test}=4, r^2_{test}=0.722, s_{test}=0.30 (UACC-62). It can be compared to the results obtained using CORAL approach, included in the Table 3.

Anti-Malaria Agents (Aryl-Piperazine Derivatives)

The experimental inhibitory concentrations (IC50) in micromolar units of aryl-piperazine derivatives against the chloquine resistant strains W2 and FCR3 and against the chloroquine-sensitive strains D10 and NF54 are extracted from the literature (Molyneaux, Krugliak, Ginsburg, & Chibale, 2005). The QSAR calculated with optimal descriptors gave possibility to suggest some perspective molecular structures of anti-malaria agents.

Anti-Malaria Agents (4(1-H)-Quinolone Derivatives)

A series of 53 endochin analogs (4(1-H)-quinolone derivatives) with anti-malarial activity against the clinically relevant multidrug resistant malarial strain TM-90-C2B has been taken from the literature (Ojha & Roy, 2011). The statistical characteristics of the model suggested in the published work (Ojha & Roy, 2011) are the following: n_{train} = 39, r^2_{train}= 0.797, s_{train} = 0.517; n_{test} = 14, r^2_{test}= 0.808. Thus, the statistical quality of the CORAL model is comparable with the above-mentioned model (Ojha & Roy, 2011).

Anti-HIV-1

The binding affinity values of fullerene derivatives pEC50 were taken from the literature (Durdagi, Mavromoustakos, & Papadopoulos, 2008). The QSAR analysis of these data has shown that even for the case of small set of data, the Monte Carlo technique gives reproducible results. In spite of relative lower statistical quality of the CORAL model for the endpoint, this model provides satisfactory prediction for a several splits of the data into the training and test sets. Thus, the optimal descriptors should be considered as reasonable alternative for the 3D QSAR analysis.

High-Affinity 5-HT1A Receptors

A dataset of 88 arylpiperazines considered as high affinity 5-HT1A receptor ligands, to which the in vitro affinity values (as measured by inhibition constants, Ki) were collected from the literature (Martinez-Esparza et al., 2001a,b) was analyzed. The CORAL software is able to be an efficient tool to build up a robust model for affinity of 5-HT1A receptor ligands of arylpiperazines. The suggested modeling process for affinity of 5-HT1A receptor ligands is based on the representation of the molecular structure by SMILES and on the application of experimental data. The predictive potential of the applied approach

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was tested with four random splits of the data into the training and test sets. In addition, the SMILES attributes, which are promoters of increase/decrease ligand binding affinity to 5- HT1A receptor, were identified. The mechanistic interpretation of these models from probabilistic point of view was suggested.

Calcium Channel-Antagonistic Effect

A dataset of 72 1,4-dihydropyridine derivatives with values for pIC50 was taken from the literature (Gecen, Saripinar, Yanmaz, & Şahin, 2012). Almost identical statistical quality of the QSAR models has been obtained for four different random splits of data. The mechanistic interpretation of these models in terms of promoter increase / decrease of the endpoint was suggested.

Anticonvulsant Agents

The experimental information on the antiepileptic activities ED50 of the molecular structures was obtained from the literature (Eddington et al., 2002, 2003; Edafiogho, Ananthalakshmi, & Kombian, 2006; Cox, Gao, Raje, Scott, & Eddington, 2001a; Cox, Scott, Gao, Raje, & Eddington, 2001b; Vamecq, Lambert, Poupaert, Masereel, & Stables, 1998). The MLR analysis gives preferable accuracy of the two ED50 models (Garro Martinez, Duchowicz, Estrada, Zamarbide, & Castro, 2011) r^2_{test}=0.856 and r^2_{test}=0.948. For this case the CORAL model provides worse results (Table 3).

Anesthetic Activity of Alkaloids

The duration of anesthesia (related to protein binding of a drug) and the onset time (determined by the pK_a) are important characteristics in assessment of local anesthetic agents. In the study (Turabekova et al., 2014) we utilized toxicity, duration, and onset of action as endpoints to construct QSAR models for the series of 34 diterpenoid alkaloids characterized by local anesthetic activity using GA-MLRA/PLS and SMILES-based optimal descriptors approach (CORAL). SMILES-based optimal descriptor approach application resulted in models of relatively improved both statistical fit of toxicity and duration of anesthesia models (r^2≈85% and r^2≈95%, respectively) and predicting ability (≈72%) for all three endpoints. This approach appeared to be more sensitive to structural peculiarities of molecules than regression methods that have to be taken into account in potential data splitting.

Membrane Damage TiO_2 Nanoparticles

Large databases related to physicochemical and biological activity of nanomaterials remain scarce. Under such circumstances, the possible way to build up a predictive model is an analysis of eclectic available data on various phenomena related to nanomaterials. As a result of exchange of classic paradigm Endpoint = F (molecular structure) by paradigm Endpoint = F(eclectic information), an analogy of classic QSPR/QSAR approach applied to nanomaterials can be obtained (Toropova & Toropov, 2013). The data obtained using this approach are provided in the Table 3.

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Table 3. Statistical quality of QSPR/QSAR models for various types of property/activity for selected sets of data calculated with the CORAL software

Endpoint	N_{train}	r^2_{train}	s_{train}	N_{test}	r^2_{test}	s_{test}	R_m^2	References
Physicochemical Parameters								
Water solubility of organic compounds	721	0.9231	0.565	261	0.9381	0.511	0.8963	Toropov et al., 2013a
Solubility of [C60] and [C70] fullerene derivatives	18	0.758	17.6	9	0.925	12.5	0.902	Toropova et al., 2011b
Octanol/water partition coefficient of antineoplastic drugs	22	0.9306	0.601	12	0.9776	0.727	-	Toropov et al., 2010a
Half-wave potential	9	0.7333	0.021	5	0.7295	0.028	0.8294	Nesmerak et al., 2013
Rate Constants of Reactions Between Organic Aromatic Pollutants and Hydroxyl Radical	38	0.8207	0.183	20	0.8033	0.145	0.6731	Toropov et al., 2012a
Biochemical Activities								
Toxicity towards *Daphnia magna*	149	0.7006	1.04	89	0.7680	0.878	0.7413	Toropova et al., 2012a
Toxicity in rats	344	0.7662	0.468	126	0.7237	0.315	0.6388	Toropova et al., 2011a
Toxicity of binary mixtures	14	0.9584	0.167	10	0.9362	0.200	0.7164	CORAL: Models of toxicity of binary mixtures. Chemometrics and Intelligent Laboratory Systems, 119
Carcinogenicity	170	0.6278	0.871	61	0.7576	0.602	-	Toropova et al., 2010b
Carcinogenicity of drugs	1088	0.8700	1.46	376	0.8520	1.53	0.835	Toropova & Toropov, 2014
Anticancer agents Murine P388 Leukemia	75	0.7688	0.48	25	0.8025	0.49	-	Toropov et al., 2010c
Anticancer agents SK-MEL-5 cell line UACC-62 cell line	19 19	0.9506 0.9409	0.14 0.13	6 6	0.752 0.760	0.36 0.40	-	Mullen et al., 2011
Anti-malaria agents aryl-piperazine derivatives	16	0.9960	0.040	6	0.8836	-	-	Ibezim et al., 2012
Anti-malaria agents 4(1-H)-quinolone derivatives	20	0.7009	0.575	10	0.6130	0.89	0.55	Toropov et al., 2013b
Anti-HIV-1	7	0.8413	0.309	5	0.8379	0.642	-	Toropov et al., 2010d
High-affinity 5-HT1A receptors	40	0.9457	0.223	18	0.9473	0.298	0.8325	Veselinovic et al., 2013a
Calcium Channel-Antagonistic Effect	50	0.8507	0.706	22	0.9269	0.521	0.8558	Veselinovic et al., 2013b
Anticonvulsant agents	46	0.7627	0.192	5	0.7001	0.313	0.6428	Garro Martinez et al., 2011
Alkaloid toxicty	26	0.83	0.19	8	0.70	0.44	-	Turabekova et al., 2014
Membrane damage TiO_2 nanoparticles	10	0.9893	0.025	5	0.9647	0.066	0.90	Toropova & Toropov, 2013

SOLUTIONS AND RECOMMENDATIONS

The variety of QSPR/QSAR models developed with the CORAL software indicates that this approach can be an efficient tool of the QSPR/QSAR analyses. However, it is to be noted, there are problems related to utilization of the CORAL software.

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Problem 1: The involvement of combinations of two and three SMILES atoms in building up a model can lead to appearance of SMILES attributes (Table 1) with unclear physical meaning, e.g.:
\...1.....
=...2.....
=...1...(...
[.../.....
[...=.....

Problem 2: The approach is able to detect three-atom fragments as basis for the mechanistic interpretation. In reality, an active fragment can involve larger number of atoms.

Problem 3: If all SMILES contain certain, an indeed active fragment, this fragment cannot be recognized by the CORAL software as an active one.

Problem 4: If an active fragment is rare, the fragment cannot be recognized by CORAL software as an active one.

However, a preliminary analysis and classification (filtration) of dataset can help to solve these problems, at least partially.

FUTURE RESEARCH DIRECTIONS

The recently published works have shown, that the CORAL software can be a tool of QSPR/QSAR analyses for other endpoints in addition to results listed in Table 3 (Singh and Gupta, 2014; Achary, 2014, pp. 73-90; Worachartcheewan, Nantasenamat, Isarankura-Na-Ayudhya, &Prachayasittikul, 2014).

QSAR Prediction Reporting Format (QPRF) (version 1.1, May 2008) available on the Internet http://ihcp.jrc.ec.europa.eu/our_labs/predictive_toxicology/qsar_tools/qrf/QPRF_version_1%201_DEREK_SS.pdf is multi-component system. Most likely, the system can be useful and attractive for the case of standardized situations. However, in order to be widely applied as a research tool, it is preferable to use OECD principles together with the following probabilistic rules:

1. A QSPR/QSAR model should be represented for a group of splits of data into the "visible" training set (compounds involved in the modeling process) and the "invisible" test set (compounds which are not involved in the modeling process);
2. The splits should be random and different (they should be far from identity). The Monte Carlo technique in general, and the CORAL software in particular, can be basis for the QSAR Prediction Reporting Format, at least for the first steps of the QSPR/QSAR analyses of ecologic, pharmacologic and other systems of endpoints which are characterized by high complexity and wide prevalence.

CONCLUSION

The CORAL software can be considered as an universal tool for the QSPR/QSAR analysis. As all software packages the CORAL has certain advantages and disadvantages. The software provides the following options:

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1. QSPR/QSAR analysis for data represented in format described at http://www.insilico.eu/coral;
2. The analyses should be carried out using a number of various data splits;
3. The software can be involved in build up models for phenomena characterized by eclectic information. Thus, the most important advantages of the approach are:
   a. The possibility of analysis of a QSPR/QSAR as a random event (not unique action); and
   b. The possibility of the translation of eclectic information into endpoint prediction.

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KEY TERMS AND DEFINITIONS

Correlation Weight: A coefficient which should be added to optimal descriptor if a given SMILES attribute or graph invariant takes place in the given SMILES or in the given molecular graph.

Global SMILES Attribute: A characteristic of molecules in whole, e.g. the presence of nitrogen together with oxygen, presence of double bonds and cycles, the presence of nitrogen and sulphur and absence of oxygen, and others (Table 1).

Graph of Atomic Orbitals (GAO): Molecular graph where vertexes are the representation of atomic orbitals (e.g. 1s2, 2p3, 3d7, etc.).

Hydrogen-Filled Graph (HFG): Molecular graph where presence of hydrogen atoms is taken into account.

Hydrogen-Suppressed Graph (HSG): Molecular graph where presence of hydrogen atoms is ignored.

Local SMILES Attribute: Fragment of SMILES line which contains one ('C', 'N', '=', etc.) or two symbols ('Cl', 'Br', '@@', etc.) which cannot be examined separately.

Monte Carlo Optimization: The calculation of the numerical data on the correlation weights which give maximal value of the correlationn coefficient between an endpoint and the optimal descriptor.

Optimal Descriptor: A descriptor which is calculated for a substances with simplified molecular input-line entry system (SMILES) or with molecular graph using correlation weights for SMILES attributes or for graph invariant (vertex degree, valence shells, etc.).

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Chapter 37
2D and 3D QSAR Studies on a Series of Antichagasic Fenarimol Derivatives

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ABSTRACT

Chagas disease is one of the most important neglected tropical diseases. Endemic in Latin America, the disease is a global public health problem, affecting several countries in North America, Europe, Asia and Oceania. The disease affects around 8-10 million people worldwide and the limited treatments available present low efficacy and severe side effects, highlighting the urgent need for new therapeutic options. In this work, the authors developed QSAR models for a series of fenarimol derivatives exhibiting anti-T. cruzi activity. The models were constructed using the Hologram QSAR (HQSAR), Comparative Molecular Field Analysis (CoMFA) and Comparative Molecular Similarity Indices Analysis (CoMSIA) methods. The QSAR models presented substantial predictive ability for a series of test set compounds (HQSAR, r2pred = 0.66; CoMFA, r2pred = 0.82; and CoMSIA, r2pred = 0.76), and were valuable to identify key structural features related to the observed trypanocidal activity. The results reported herein are useful for the design of novel derivatives having improved antichagasic properties.

INTRODUCTION

Neglected tropical diseases (NTDs) represent a group of 18 serious conditions prevalent in low-income countries with tropical and subtropical climate (Feasey, Wansbrough-Jones, Mabey, & Solomon, 2010), according to the World Health Organization (WHO). Chagas disease, one of the most important NTDs, is

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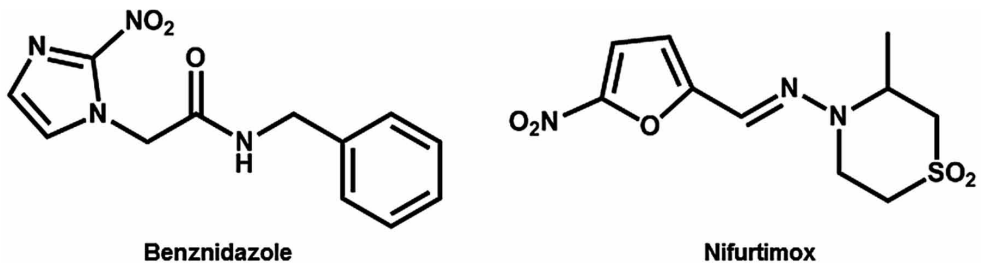
a chronic infection caused by the protozoan parasite *Trypanosoma cruzi* and affects around 8-10 million people, predominantly in Latin American countries such as Brazil, Argentina and Mexico (Viotti et al., 2014). As a result of the international population migration, the disease has reached new boundaries, primarily in North America, Europe, Asia and Oceania, thus becoming an increasing worldwide concern (Hotez et al., 2012). Chagas disease is transmitted to humans while the parasites are eliminated with the feces of triatomine hematophagous bugs during their blood meal (Rassi Jr, Rassi, & de Rezende, 2012).

The limited therapy for the disease is based only on two old drugs – benznidazole (Rochagan®, Roche) and nifurtimox (Lampit®, Bayer) (Figure 1) – which possess low efficacy and severe side effects (Castro, Montalto deMecca, & Bartel, 2006). This exceptionally unfavorable scenario supports the urgent need for the development of novel, safe and effective drugs for Chagas disease (Clayton, 2010).

The repositioning of drugs targeted to different conditions has been one of the most important strategies in NTDs, including Chagas disease (Chatelain & Ioset, 2011). Compounds possessing well-established pharmacodynamics, pharmacokinetics and toxicity profile are attractive alternatives, especially in a field that is not a priority in pharmaceutical research and development (R&D). Eflornithine, for instance, which was initially developed as an anticancer therapy, is used in human African trypanosomiasis (Brun, Blum, Chappuis, & Burri, 2010). Another example is the use of antimycotic agents, such as ketoconazole and fluconazole, in the treatment of leishmaniasis (Sundar & Chakravarty, 2013). Drug repositioning provides an excellent trade-off between risk and reward over traditional R&D strategies, and various studies on the subject have been carried out in Chagas disease drug discovery (Planer et al., 2014).

Antifungals have been investigated as trypanocidal agents since the 1980s, when the *in vitro* inhibitory activity of miconazole and econazole against *T. cruzi* was first determined (Maertens, 2004; Docampo et al., 1981). The strong interest in this class of compounds resulted in the recent approval of the fungicides posaconazole and the prodrug of ravuconazole (E1224) to undergo clinical trials for Chagas disease (de Figueiredo Diniz et al., 2013). These results have stimulated valuable repositioning studies approaching the trypanocidal activity of antifungal agents used as agrochemicals (Nosten et al., 2006). Since the approval of a given compound for use as an agrochemical requires evaluation of its toxicity profile, such molecules are a useful source for repositioning studies in the field of NTDs (Witschel, Rottmann, Kaiser, & Brun, 2012). Among these investigations, the fungicide fenarimol, broadly used as an agrochemical, was identified as a pyrimidine derivative with potent *in vitro* activity against *T. cruzi* (IC_{50} = 350 nM) (Figure 2) (Keenan et al., 2012). Recent studies have demonstrated that fenarimol analogs act by inhibiting the *T. cruzi* 14-α-sterol demethylase (CYP51), a key enzyme in the sterol biosynthesis pathway of the parasite (Keenan et al., 2013).

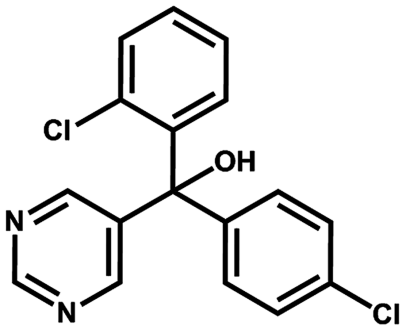
Figure 1. Currently available drugs for the treatment of Chagas disease



Benznidazole **Nifurtimox**

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Figure 2. Structure of the pyrimidine derivative fenarimol, an agrochemical and antifungal agent possessing trypanocidal properties



Fenarimol

In this work, we have developed two- and three-dimensional quantitative structure-activity relationships (2D- and 3D-QSAR) for a series of highly potent fenarimol analogs, using the Hologram QSAR (HQSAR) (Seel, Turner, & Willett, 1999), Comparative Molecular Field Analysis (CoMFA) (Cramer, Patterson, & Bunce, 1988), and Comparative Molecular Similarity Indices Analysis (CoMSIA) (Klebe, Abraham, & Mietzner, 1994) methods. These chemometric tools collect and interpret complex data from series of bioactive molecules to construct computational models that correlate chemical properties with biological activity (Vaidya, Sourabh Jain, Shweta Jain, A. Jain, & Agrawal, 2014). Through this approach, molecular features responsible for the antitrypanosomal activity of the investigated compounds were identified using the HQSAR contribution maps, and the CoMFA and CoMSIA contour plots. Furthermore, the statistical consistency of the developed models was evaluated on the basis of their correlation ability for the training set, as well as their predictive power for an external test set.

METHODOLOGY

Several QSAR methods, such as neural networks, are available for the analysis of nonlinear data (Devillers, Doucet, Doucet-Panaye, Decourtye, & Aupinel, 2012). These techniques are very useful to analyze heterogeneous compound series, and compensate for discontinuities and anomalies in the modelled data; however, such methodologies generally require large data sets to produce stable and reliable models (Eriksson et al., 2003). Given the limited number of compounds and the high degree of similarity and homogeinity of the investigated compounds, generation and analysis of linear QSAR models were carried out as previously described, using the molecular modeling platform SYBYL-X 2.0 (Tripos, St. Louis, EUA) (Ferreira & Andricopulo, 2013; Moda & Andricopulo, 2012; Guido et al., 2008). The three-dimensional structures of the data set compounds were generated by applying the default geometric and atomic parameters of SYBYL-X 2.0. Tripos force field and Powell conjugate gradient algorithm were applied to minimize (in vacuum) the 3D conformation of each compound (minimum energy change of 0.005 kcal/molÅ) (Powell, 1977; Clark, Cramer, & Van Opdenbosch, 1989). A distance-dependent dielectric function and a dielectric constant of 80 were applied to generate the Gasteiger-Hückel charges (Tsai et al. 2010). A number of methods for generating conformers have been reported in the literature

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and it is a well-known fact this is a critical aspect in the computation of molecular descriptors, which can lead to large divergences in QSAR results (Hechinger, Leonhard, & Marquardt, 2012). Given the low conformational flexibility of the investigated data set, which consists of compounds composed mainly by three aromatic rings, only the minimum energy conformers generated by the molecular mechanics-based Tripos force field were used to derive the reported QSAR models.

Data Set

The data set used to develop the QSAR models was retrieved from the literature and consists of 77 compounds inspired in the structure of the fungicide fenarimol (Table 1) (Keenan et al., 2012). The biological activity of this series was measured under the same experimental conditions and expressed as the concentration of compound required to inhibit 50% of the *in vitro* reproduction of *T. cruzi* (IC_{50}). The IC_{50} values, which vary between 1 nM and 27 μM, were transformed into - log IC_{50} (pIC_{50}), an appropriate scale for QSAR modeling purposes. This data set was select because it encloses a suitable structural diversity, along with accurate and well-distributed biological data. Besides their highly potent trypanocidal activity, these fenarimol derivatives have additionally demonstrated favorable safety profile. A recent study published by Keenan et al. (2013) confirmed that this class of compounds exhibits *in vivo* efficacy profile similar to posaconazole, and superior to that of benznidazole, in experimental models of Chagas disease. As a result, the fenarimol analogs studied in this work consist of a highly attractive chemical diversity to be explored in the development of novel drug candidates for Chagas disease.

Principal Component Analysis and Selection of Training and Test Sets

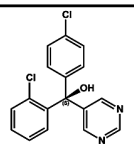
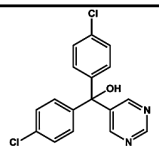
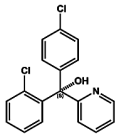
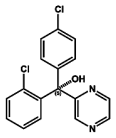
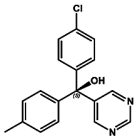
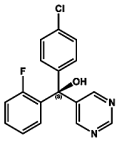
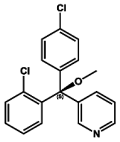
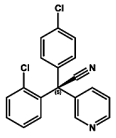
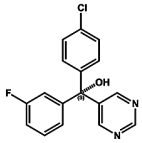
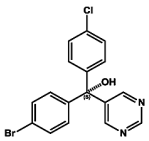
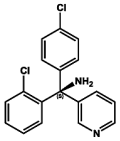
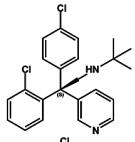
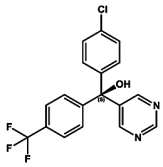
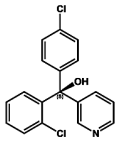
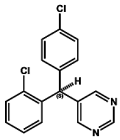
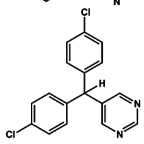
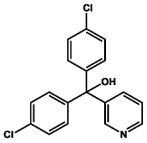
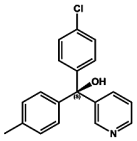
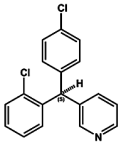
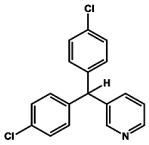
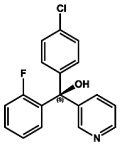
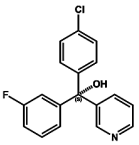
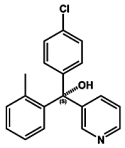
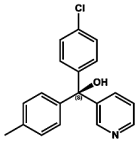
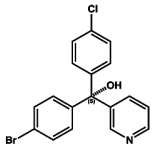
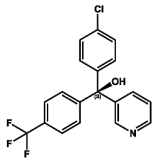
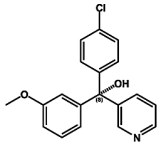
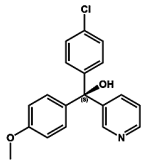
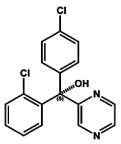
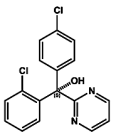
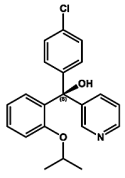
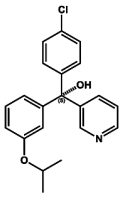
The selection of training and test sets is a critical procedure to generate reliable QSAR models (Leonard & Roy, 2006). Both sets should extend over a broad chemical and biological space across the entire data set. In this context, a principal component analysis (PCA) was performed to evaluate the structural similarity between the compounds and their distribution with respect to the biological data (Abdi & Williams, 2010). Digital fingerprints were generated for each compound using the UNITY module of SYBYL-X 2.0 and employed as molecular descriptors in the PCA (Hert et al., 2004). The output of the PCA was graphically represented as a structural similarity map, in which the distance between the points is inversely proportional to structural similarity. These points were plotted using Tanimoto similarity distances between the molecular fingerprints (Flower, 1998). In addition to the similarity analysis, a color code was employed to divide the biological data into five activity ranges. Figure 3 depicts the structural similarity map for the whole data set. Through a visual analysis of this diagram, the data set was divided into 60 training set molecules, which were employed to build the QSAR models, and 17 test set compounds used to evaluate the external predictive power of the models. The selection was carried out in such a manner as to include in both sets structurally distinct molecules covering the entire activity interval.

Structural Alignment

The structural alignment of the data set compounds in the Cartesian space is a key procedure to generate useful 3D QSAR models. Among the diverse approaches available for this purpose, the procedure adopted in this study was based on the maximum common substructure (MCS) method, as it is implemented in

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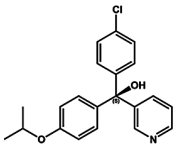
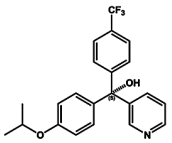
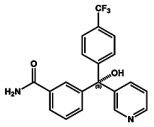
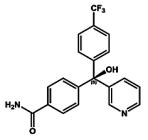
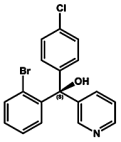
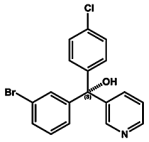
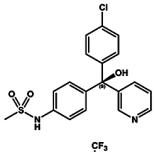
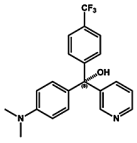
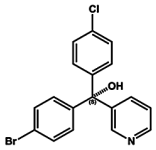
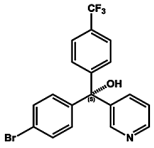
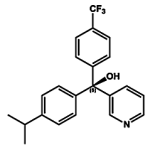
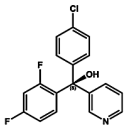
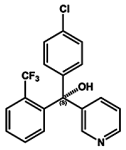
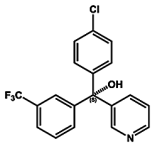
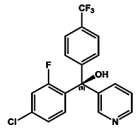
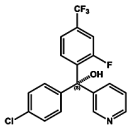
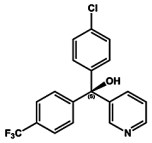
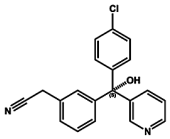
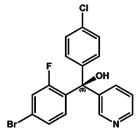
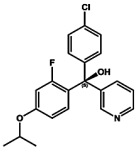
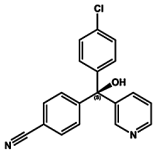
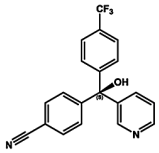
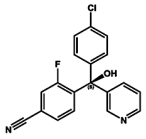
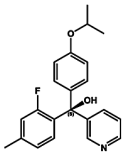
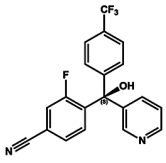
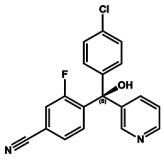
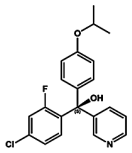
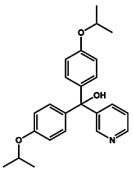
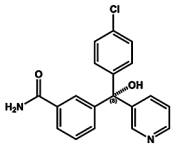
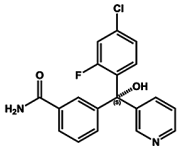
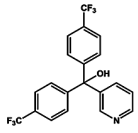
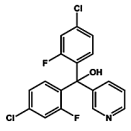
Table 1a. Structure and pIC_{50} values of the data set compounds: 1-32

Compound	Structure	pIC_{50}	Compound	Structure	pIC_{50}	Compound	Structure	pIC_{50}	Compound	Structure	pIC_{50}
1		6.46	2		6.66	17		4.57	18		5.15
3		7.62	4		6.85	19		7.05	20		7.30
5		6.08	6		6.84	21		7.05	22		6.22
7		6.71	8		6.57	23		5.97	24		6.17
9		7.02	10		7.77	25		6.64	26		7.10
11		7.46	12		7.00	27		6.43	28		7.77
13		7.51	14		8.10	29		7.22	30		7.40
15		6.36	16		4.64	31		6.35	32		7.09

the Distill Rigid module of SYBYL-X 2.0 (Patel & Ghate, 2015). First, the MCS between all compounds was identified using the default value of 5 as the minimum number of atoms enclosed in the MCS fragment. Then, the minimum energy conformation of each compound was flexibly overlaid upon the MCS. The dihedral angles of the most potent compound (molecule 61) were used as the template upon which the MCS of all the remaining compounds were superimposed. Figure 4 illustrates the complete data set aligned using the MCS approach. The searching method (i.e. the run mode) was restricted to single MCS

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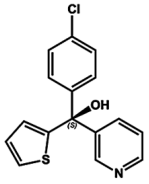
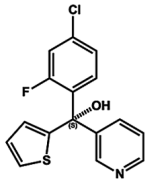
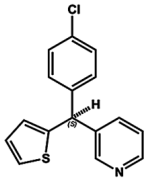
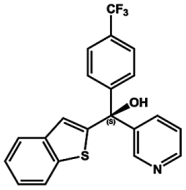
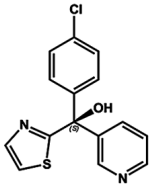
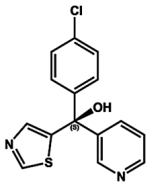
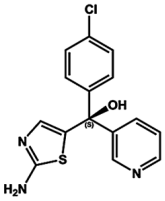
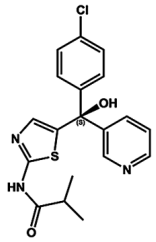
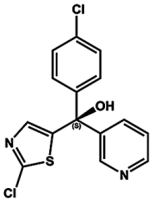
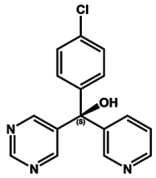
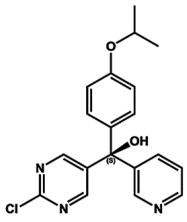
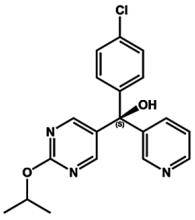
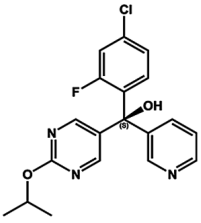
Table 1b. Structure and pIC_{50} values of the data set compounds: 33-64

Compound	Structure	pIC_{50}	Compound	Structure	pIC_{50}	Compound	Structure	pIC_{50}	Compound	Structure	pIC_{50}
33		8.70	34		8.40	49		7.15	50		6.92
35		7.15	36		7.27	51		7.52	52		8.22
37		7.51	38		7.70	53		8.70	54		7.80
39		6.92	40		7.15	55		8.70	56		7.68
41		8.10	42		6.96	57		7.59	58		7.59
43		7.15	44		7.92	59		7.59	60		8.52
45		8.70	46		8.00	61		9.00	62		7.80
47		7.52	48		7.60	63		7.82	64		7.80

fragments. The molecular alignment procedure was parameterized as follows: (1) MCS identification parameters: atom types, bond types and ring attributes on bonds; (2) target grouping size: 2; (3) ring bond weighting: 3; (4) hetero atom weighting: 4; (5) branch atom weighting: 2; and (6) score similarity: 0.5.

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Table 1c. Structure and pIC_{50} values of the data set compounds: 65-77

Compound	Structure	pIC_{50}	Compound	Structure	pIC_{50}
65		7.89	66		8.30
67		7.11	68		7.70
69		6.70	70		6.85
71		6.59	72		7.68
73		6.31	74		5.82
75		6.52	76		7.22
77		8.10			

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Figure 3. Structural similarity map and selection of training (unlabeled spheres) and test sets (highlighted spheres)

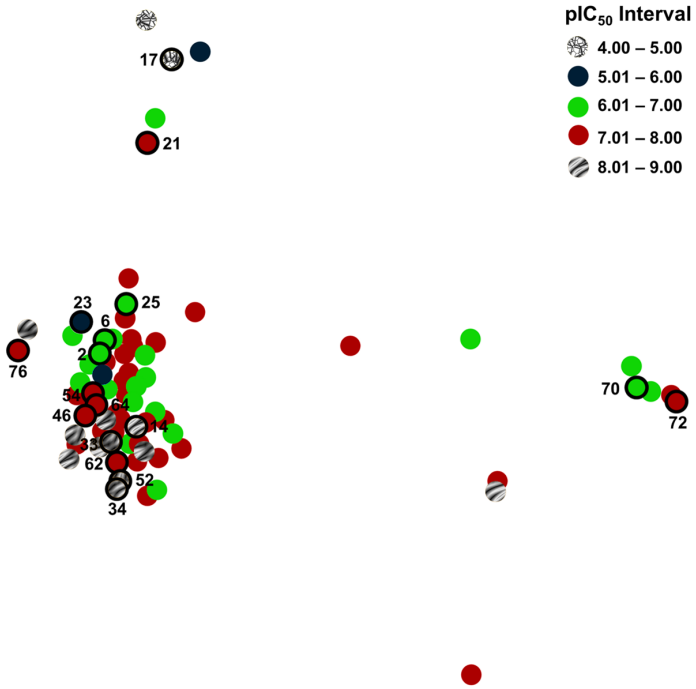
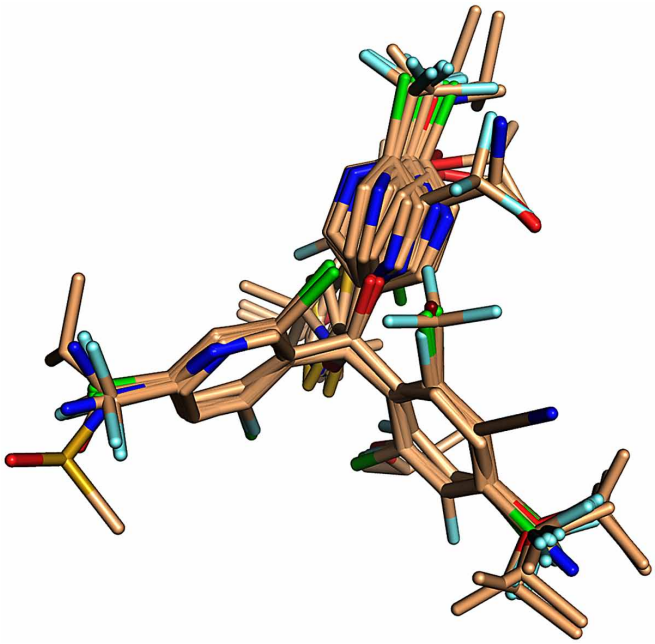


Figure 4. Molecular alignment used in the 3D QSAR modeling, generated by applying the MCS approach



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HQSAR Approach

The HQSAR method converts the two-dimensional structure of the data set molecules into a non-binary bit string called molecular hologram. Each structure is divided into all possible fragments, which are then allocated across the molecular hologram. A particular position in the bit string encodes information regarding the identity and occurrence of a given fragment (See, Turner, & Willett, 1999). The HQSAR models were generated employing the 12 default molecular hologram lengths: 53, 59, 61, 71, 83, 97, 151, 199, 257, 307, 353, and 401. The fragmentation procedure was carried out by applying several combinations of the available fragment distinction parameters: atoms (A); bonds (B); connections (C); hydrogen atoms (H); and hydrogen bond donor/acceptor (DA). Using the best fragment distinction combination, several models were further developed by varying the fragment size: 3-7, 3-9, 4-7, 4-8, 4-9 and 4-10 atoms.

CoMFA Approach

CoMFA is a 3D QSAR method based on the assumption that intermolecular interactions determine important properties, such as biological activity (Cramer, Patterson, & Bunce, 1988). The molecular descriptors employed to explain the variance in the target property consist of electrostatic and steric interaction energies calculated between an aligned set of molecules and a probe atom. According to this rationale, electrostatic and steric molecular interactions are calculated using Coulomb and Lennard-Jones potentials, respectively. This approach was used to explore the relationships between these 3D molecular descriptors and the trypanocidal activity of the investigated data set. The compounds aligned as illustrated in Figure 4 were used in the CoMFA analyses. A 3D cubic lattice with a grid spacing of 2.0 Å was created to fully enclose all molecules with an extension of 4 Å in the *x*, *y* and *z* axis. Steric and electrostatic potentials were estimated at every grid intersection with the default Tripos force field and a sp^3 carbon positively charged (+1 net charge) as the probe atom. The CoMFA standard value of 30 kcal/mol was used as the cut-off for both electrostatic and steric interaction energy. Coulombic electrostatic energy was set to vary according to a distance-dependent dielectric function (*i.e*. the energy falls as $1/r^2$). Steric and electrostatic energy values were properly scaled employing the default CoMFA scaling method. The models were refined and the resolution of the CoMFA contour maps was improved using the region focusing approach. The focusing procedure and the generation of the contour maps were carried out by weighting the product between the standard deviations (StDev) and the partial least squares (PLS) coefficients (Coeff) of each lattice point (Bush & Nachbar Jr., 1993).

CoMSIA Approach

Besides considering electrostatic and steric potentials, CoMSIA additionally estimates hydrophobic interactions and hydrogen bonds. Also, the molecular descriptors used in CoMSIA are similarity indices computed on the lattice points. These descriptors are calculated by taking into account the distance between the probe atom and the aligned compounds. The dependence of the similarity indices on the distance between the probe and the molecules is described by a Gaussian function (Klebe, Abraham, & Mietzner, 1994). The compounds aligned as illustrated in Figure 4 were used in the CoMSIA analyses. A 3D cubic lattice was built using the same methodology employed in the CoMFA proceeding. The similarity indices were calculated using hydrophobic, hydrogen bond donor and acceptor molecular in-

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teraction fields. Similar to the CoMFA modeling, the region focusing procedure was applied to improve the resolution of the CoMSIA contour maps, using the StDev*Coeff weighting method. The steepness of the Gaussian function was defined by applying the default value of the attenuation factor (0.3).

Model Validation Procedures

The statistical robustness of the generated models was assessed by calculating the non-cross-validated (r^2) and full cross-validated (q^2) correlation coefficients (Hawkins, Basak, & Mills, 2003). First, each model was subjected to a leave-one-out cross-validation analysis (LOO) to determine the optimum number of components and the q^2 value. Next, a full partial least squares (PLS) regression was performed using the optimum number of components to determine the r^2 value. Then, the stability of the best models against more significant removals of data was estimated by the leave-many-out (LMO) cross-validation procedure (Tropsha, Gramatica, & Gombar, 2003). The liability of these PLS analyses to chance correlations was determined by the progressive scrambling Y-randomization test (Clark & Fox, 2004). All of these validation strategies were executed for the training set. Finally, as an external validation protocol, the trypanocidal activity of the test set compounds was predicted. This proceeding was able to demonstrate, through the calculation of predictive r^2 values (r^2_{pred}), the power of the constructed QSAR models to estimate the activity of novel structurally related compounds.

RESULTS

HQSAR Results

HQSAR models were developed using the default hologram lengths. Initially, four models were generated employing the following combinations of fragment distinction parameters: A/B/C, A/C/DA, A/B/C/H e A/B/C/H/DA; and the default fragment size of 4-7 atoms (Table 2). The LOO validation procedure was used to determine the q^2 value and the number of components of each model. The models with the best number of components were subjected to a full PLS analysis to produce the r^2 values.

As can be observed, the best model was produced using A/B/C/H as the fragment distinction combination (q^2 = 0.60; r^2 = 0.81; and SEE = 0.37). Since fragment size interferes with the hologram

Table 2. HQSAR statistical results generated with LOO cross-validation and full non-cross-validated PLS analysis (fragment size 4-7 atoms)

Model	Fragment Distinction		Statistical Parameters				
		q^2	r^2	SEE	SEP	HL	N
1	A/B/C	0.58	0.76	0.41	0.54	97	3
2	A/C/DA	0.54	0.80	0.38	0.58	257	4
3	A/B/C/H	0.60	0.81	0.37	0.54	97	5
4	A/B/C/H/DA	0.59	0.80	0.39	0.55	97	5

A: atoms; B: bonds; C: connections; H: hydrogen atoms; DA: hydrogen bond donor/acceptor atoms. q^2: LOO cross-validated correlation coefficient; r^2: non-cross-validated correlation coefficient; SEE: standard error of estimate; SEP: standard error of prediction; HL: hologram length; N: optimum number of components.

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Table 3. HQSAR results for different fragment sizes (model 3, Table 2)

Model	Fragment Size	Statistical Parameters						
		q^2	r^2	SEE	SEP	HL	N	r^2_{pred}
5	3-7	0.59	0.86	0.32	0.55	401	6	0.64
6	3-9	0.64	0.89	0.29	0.53	401	6	0.62
7	4-7	0.60	0.81	0.37	0.54	97	5	0.61
8	4-8	0.60	0.84	0.35	0.55	53	5	0.52
9	4-9	0.63	0.89	0.29	0.53	401	6	0.62
10	4-10	0.57	0.90	0.28	0.57	257	7	0.66

q^2: LOO cross-validated correlation coefficient; r^2: non-cross-validated correlation coefficient; SEE: standard error of estimate; SEP: standard error of prediction; HL: hologram length; N: optimum number of components r^2_{pred}: predictive correlation coefficient.

generation, and therefore with the results, variation of this parameter was performed using the A/B/C/H fragment distinction. Table 3 shows the statistical indicators for the fragment sizes: 3–7, 3–9, 4–7, 4–8, 4–9 and 4–10.

The data shown in Table 3 demonstrate that the variation of fragment size was able to promote a subtle increase in r^2 and a decrease in SEE. With respect to the remaining indicators, similar results were obtained compared to the default value (fragment size of 4–7 atoms).

It is a well-known fact that internal validation procedures provide a satisfactory measure of the correlation ability of QSAR models. However, their predictive power can be assessed only by estimating the activity of compounds that were not employed in the generation of the models (Tropsha, 2010). This procedure, called external validation, employs the predictive correlation coefficient (r^2_{pred}) as the statistical indicator. Thus, the test set selected on the basis of the structural similarity map (Figure 3) was employed in the external validation process. Accordingly, the predictive strength of the models listed in Table 3 was estimated by the calculation of r^2_{pred} values. As observed, the model with fragment size of 4–10 (model 10) was the most robust in terms of predictive power for the test set compounds (r^2_{pred} = 0.66). The correlation between the experimental and predicted pIC_{50} values for this model is exhibited in Table 4 for the test set compounds, and graphically illustrated in Figure 5a for the entire data set.

Model 10 was additionally evaluated with respect to its stability against rigorous removals of data. With this purpose, the training set was randomly divided into five groups and the LMO cross-validation was applied. This process resulted in a LMO q^2 of 0.50, demonstrating the stability of model 10 when a larger number of compounds were removed for the activity prediction. This model was also evaluated for its vulnerability to produce chance correlations. The procedure was carried out using the progressive scrambling randomization method, which introduced small, random perturbations on the dependent property (pIC_{50}). After the scrambling procedure, a Q^2 of 0.36 and an effective slope (dq^2/dr^2_{yy}) of 1.01 were obtained, indicating the robustness of the original model in regard to inconsistent correlations.

HQSAR converts the chemometric analysis performed by the PLS routine into a graphical output that illustrates the contribution of each atom of a molecule to its biological activity. The method uses a color code, called contribution map, in which atoms that contribute positively are colored in yellow, green-blue and green, while those that contribute negatively are colored in red, red-orange and orange. Neutral contributions are represented as white fragments. Figure 6 depicts the contribution maps for compounds 28, 41, 45, and 53. Compound 28 is a potent chlorophenyl-pyridine (pIC_{50} = 7.77) for which the HQSAR

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Table 4. Experimental and predicted pIC_{50} values for the test set compounds (model 10, Table 3)

Compound	Experimental pIC_{50}	Predicted pIC_{50}	Residual[a]
2	6.66	6.52	0.14
6	6.84	6.64	0.20
14	8.10	7.64	0.46
17	4.57	6.00	-1.43
21	7.05	6.87	0.18
23	5.97	6.15	-0.18
25	6.64	6.48	0.16
33	8.70	7.52	1.18
34	8.40	7.94	0.46
46	8.00	7.89	0.11
52	8.22	8.13	0.09
54	7.80	7.79	0.01
62	7.80	7.27	0.53
64	7.80	7.95	-0.15
70	6.85	6.68	0.17
72	7.68	6.49	1.19
76	7.22	7.24	-0.02

[a]Difference between experimental and predicted values.

contribution map highlights the three aromatic rings as favorable to the activity of the compound (Figure 6a). As can be observed, the 4-chlorophenyl and the pyridine rings are regarded as the most important fragments compared to the *p*-tolyl moiety, since they have a higher number of atoms colored in green and yellow. Compound 41 is a highly active trifluoromethyl derivative ($pIC_{50} = 8.10$) and likewise 28 has its 4-chlorophenyl and pyridine rings labelled as key fragments (Figure 6b). Otherwise, compound 45 ($pIC_{50} = 8.70$) holds a 3-fluorobenzonitrile rather than a 4-chlorophenyl moiety, a structural modification that led to a significant increase in the trypanocidal activity of this compound compared to the derivatives 28 and 41. This aspect is graphically represented through the HQSAR map in which the 3-fluorobenzonitrile is stressed as highly favorable (Figure 6c). Finally, compound 53 differs from 45 by lacking the fluorine and carrying an isopropyl as the substituent of one of the aromatic rings. Although this strategy was not able to improve the biological activity (since both compounds are equipotent), the isopropyl-benzene fragment contributes significantly to the activity of molecule 53 (Figure 6d).

CoMFA Results

Several CoMFA models were developed aimed to investigate the 3D properties determining the activity of the studied series of fenarimol derivatives. The internal consistency of the models was primarily evaluated using LOO cross-validation to provide the q^2 values and the best number of components. Next, the best models (*i.e.* those with the highest q^2) were subjected to complete PLS analyses to produce the r^2 values. Region focusing weighted by StDev*Coeff values of 0.3; 0.6; and 0.9 was applied to improve the resolution of the CoMFA contour maps.

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Figure 5. Experimental versus predicted pIC_{50} values for the training and test set compounds. (a) HQSAR (model 10, Table 3); (b) CoMFA (model 11, Table 5); (c) CoMSIA (model 15, Table 7)

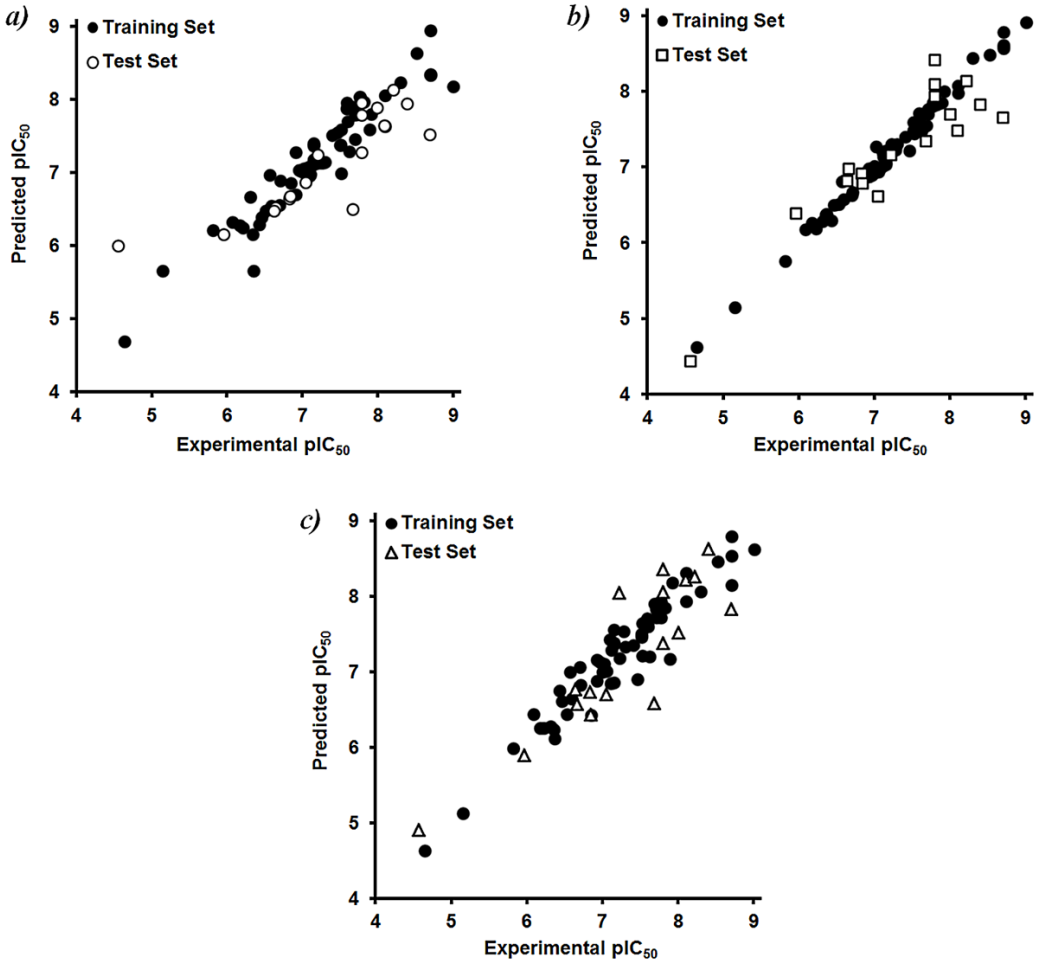
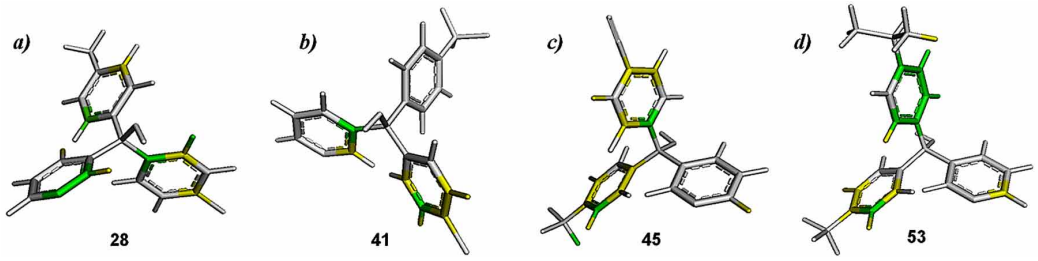


Figure 6. HQSAR contribution maps for compounds 28 (a), 41 (b), 45 (c), and 53 (d), highlighting the positive contributions of the aromatic rings and their substituents



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The same test set used in the HQSAR analysis was employed in the external validation of the CoMFA models, which had their predictive power assessed by the calculation of r^2_{pred} values. Table 5 shows the statistical indicators of the CoMFA models generated by applying the region focusing technique. As can be observed, the model weighted by a StDev*Coeff value of 0.3 (model 11) demonstrated the best external predictive ability ($r^2_{pred} = 0.82$). The correlation between the experimental and predicted pIC_{50} values for model 11 is shown in Table 6 for the test set compounds, and graphically illustrated in Figure 5b for the entire data set.

LMO cross-validation was applied to model 11, resulting in a q^2 of 0.62 for five groups. The randomization test yielded a Q^2 of 0.50 and an effective slope of 0.96, indicating the stability of the model with respect to chance correlations.

*Table 5. CoMFA models generated for different StDev*Coeff values*

Model	StDev*Coeff	q^2	SEP	r^2	SEE	r^2_{pred}	N	S	E
11	0.3	0.71	0.49	0.99	0.10	0.82	10	0.37	0.63
12	0.6	0.76	0.45	0.97	0.16	0.75	10	0.37	0.62
13	0.9	0.69	0.50	0.92	0.25	0.58	10	0.38	0.61

q^2: LOO cross-validated correlation coefficient; SEP: standard error of prediction; r^2: non-cross-validated correlation coefficient; SEE: standard error of estimate; r^2_{pred}: predictive correlation coefficient; N: optimum number of components; S: fraction of steric field; E: fraction of electrostatic field.

Table 6. Experimental and predicted pIC_{50} values for the test set compounds (model 11, Table 5)

Compound	Experimental pIC_{50}	Predicted pIC_{50}	Residual[a]
2	6.66	6.98	-0.32
6	6.84	6.91	-0.07
14	8.10	7.48	0.62
17	4.57	4.43	0.14
21	7.05	6.61	0.44
23	5.97	6.38	-0.41
25	6.64	6.82	-0.18
33	8.70	7.65	1.05
34	8.40	7.82	0.58
46	8.00	7.69	0.31
52	8.22	8.13	0.09
54	7.80	8.41	-0.61
62	7.80	8.09	-0.29
64	7.80	7.93	-0.13
70	6.85	6.78	0.07
72	7.68	7.34	0.34
76	7.22	7.16	0.06

[a]Difference between experimental and predicted values.

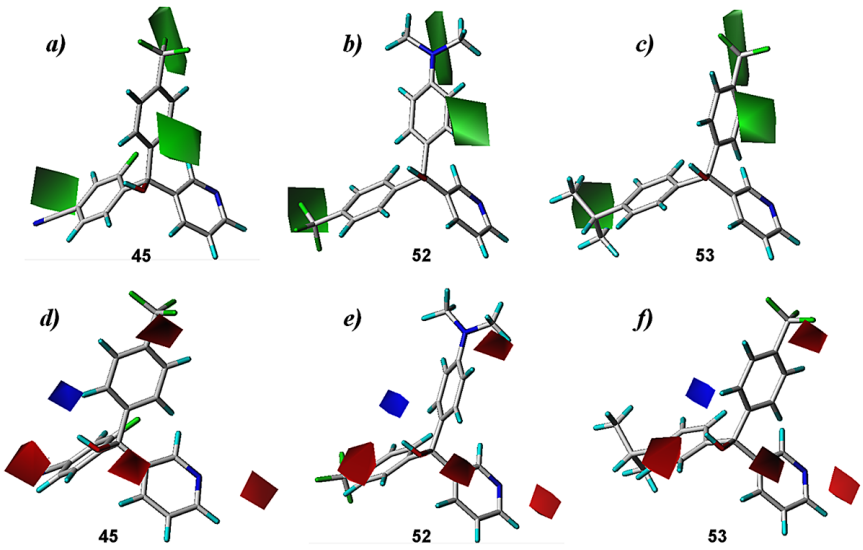
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The structure-activity relationships identified in model 11 were translated into steric and electrostatic 3D contour maps. These graphs indicate regions encompassing the van der Waals surface where variations in steric and electrostatic properties are strongly correlated with changes in biological activity. Green spots indicate areas where the presence of bulky groups is favorable to activity, whereas yellow graphs identify regions where such groups correlate with decrease in biological activity. Blue and red contours define areas where increased positive charge and electron density, respectively, contributes to improve the target property.

Figure 7 depicts StDev*Coeff steric and electrostatic contour maps for the highly active derivatives 45, 52 and 53. Compound 45 is a potent trifluoromethyl derivative ($pIC_{50} = 8.70$), for which the steric map indicates that bulky groups such as the 3-fluorobenzonitrile and the trifluoromethyl-benzene are strongly correlated with its trypanocidal activity (Figure 7a). For molecule 52 ($pIC_{50} = 8.22$), the steric map correlates the biological activity with the *p*-trifluoromethyl substituent attached to one of the benzene rings, as well as the presence of the *N,N*-dimethylaniline moiety (Figure 7b). Figure 7c shows green spots surrounding the trifluoromethyl-benzene and the isopropyl-benzene fragments of compound 53 ($pIC_{50} = 8.70$), indicating the relevance of these substituted aromatic systems for its remarkably potent activity.

Figure 7 also shows the CoMFA electrostatic contour maps generated by the region focusing procedure. As indicated by the red contours, the electron density concentrated at the pyridine nitrogen and the hydroxyl oxygen is a major contribution to the activity of the highlighted derivatives. The partial negative charge at the trifluoromethyl fluorine atoms of compounds 45 and 53 is also labelled as a favorable property (Figures 7d and 7f). Similarly, the electron density centered at the *N,N*-dimethylaniline nitrogen of compound 52 is a key element to determine the high trypanocidal activity of this analog. Although the CoMFA electrostatic maps are affected mainly by electron-withdrawing groups (which is consistent with the structural pattern of the data set), a blue spot near the hydroxyl hydrogen stresses the value of a positive dipole in this region of the compounds.

*Figure 7. CoMFA StDev*Coeff steric (a, b, and c) and electrostatic (d, e, and f) contour maps for compounds 45, 52, and 53*



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CoMSIA Results

CoMSIA models were developed based on the structural alignment illustrated in Figure 4 to investigate the relationships between the trypanocidal activity of the data set compounds and their hydrophobic and hydrogen bonding properties. The attenuation factor of the Gaussian function was maintained with the default value of 0.3 in all CoMSIA analyses. First, the q^2 values and the optimum number of components were determined by applying LOO cross-validation. Next, full PLS analyses were performed employing the best number of components. StDev*Coeff region focusing was applied to eliminate redundant descriptors, increase the weight of lattice points consistently correlated with the variation in the biological data, and refine the CoMSIA contour maps. The statistical indicators for these analyses are listed in Table 7.

The same test set used in the HQSAR and CoMFA analyses was employed in the external validation of the CoMSIA models. As observed in Table 7, the models weighted by a StDev*Coeff value of 0.6 and 0.9 exhibited the same external predictive power ($r^2_{pred} = 0.76$). Therefore the q^2 value was used as the criterion to select model 15 as the best CoMSIA model. The experimental and predicted pIC_{50} values for model 15 are shown in Table 8 for the test set, and plotted in Figure 5c for the whole data set.

As an additional validation procedure, model 15 was subjected to LMO cross-validation, which produced a q^2 of 0.62 for five randomly selected groups. The model was also evaluated using the randomization analysis, resulting in a Q^2 of 0.42 and an effective slope of 1.02.

The CoMSIA-PLS analyses were graphically interpreted through the examination of hydrophobic and hydrogen bond donor and acceptor contour maps. Figure 8 shows hydrophobic contour maps for two potent compounds. The fluorobenzonitrile derivative 46 ($pIC_{50} = 8.00$) has the chlorine atom of the chlorobenzene ring enclosed by a yellow spot, indicating the favorable character of a hydrophobic group at this position (Figure 8a). This finding also applies for the chlorofluorobenzene derivative 66 ($pIC_{50} = 8.30$). A yellow contour surrounds the thiophene ring of this compound (Figure 8b), illustrating that its trypanocidal activity is highly correlated with the presence of this hydrophobic group.

CoMSIA donor and acceptor contour maps are pictured in Figure 8 for the trifluoromethyl derivative 45. Spots colored in cyan and magenta indicate that hydrogen bond donors and acceptors, respectively, are favorable to biological activity. As displayed in Figure 8c, the cyan contour near the hydroxyl hydrogen highlights the favorable character of a hydrogen bond donor attached to the tetrahedral carbon that links the three aromatic rings. Figure 8d shows magenta spots surrounding the trifluoromethyl group, as well as the nitrile and pyridine nitrogen. In the case of the nitrile group, this finding corroborates the key role played by hydrogen bond acceptors as *para*-substituents of the phenyl ring. The CoMSIA acceptor maps also demonstrate the essential contribution of the pyridine ring for the biological activity of the investigated data set.

*Table 7. CoMSIA models weighted by different StDev*Coeff values*

Model	StDev*Coeff	q^2	SEP	r^2	SEE	r^2_{pred}	N	H	D	A
14	0.6	0.63	0.54	0.94	0.23	0.76	9	0.16	0.16	0.18
15	0.9	0.66	0.52	0.91	0.27	0.76	9	0.16	0.18	0.17
16	1.2	0.64	0.52	0.87	0.32	0.60	7	0.16	0.19	0.15

q^2: LOO cross-validated correlation coefficient; SEP: standard error of prediction; r^2: non-cross-validated correlation coefficient; SEE: standard error of estimate; r^2_{pred}: predictive correlation coefficient; N: optimum number of components; H: fraction of hydrophobic field; D: fraction of hydrogen bond donor field; A: fraction of hydrogen acceptor field.

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Table 8. Experimental and predicted pIC_{50} values for the test set compounds (model 15, Table 7)

Compound	Experimental pIC_{50}	Predicted pIC_{50}	Residual[a]
2	6.66	6.58	0.08
6	6.84	6.74	0.10
14	8.10	8.21	-0.11
17	4.57	4.90	-0.33
21	7.05	6.70	0.35
23	5.97	5.90	0.07
25	6.64	6.77	-0.13
33	8.70	7.83	0.87
34	8.40	8.63	-0.23
46	8.00	7.52	0.48
52	8.22	8.26	-0.04
54	7.80	7.38	0.42
62	7.80	8.35	-0.55
64	7.80	8.06	-0.26
70	6.85	6.43	0.42
72	7.68	6.58	1.10
76	7.22	8.04	-0.82

[a]Difference between experimental and predicted values.

Comparison between 2D and 3D Results and Design of Novel Fenarimol Derivatives

Aiming to provide a comparison amongst the final HQSAR, CoMFA and CoMSIA models, Table 9 lists the main statistical indicators for the best 2D and 3D models.

As observed, both 2D and 3D methodologies were able to produce statistically solid QSAR models. Good correlation ability was observed for the training set used to construct the models and high predictive power was achieved for the test set compounds. From the LMO and the randomization results it is possible to verify the stability of the models against rigorous removal of data and chance correlations.

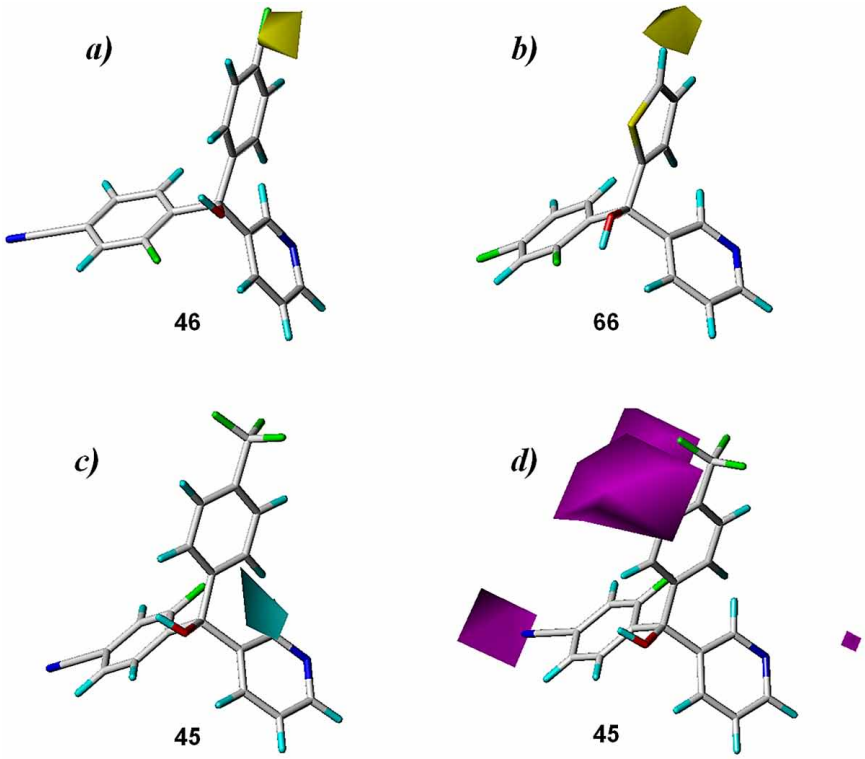
From a chemical perspective a comparison of the models can be carried out by analyzing the HQSAR contribution maps and the 3D CoMFA and CoMSIA plots. As can be seen in Figure 6 the HQSAR maps highlighted the aromatic rings as the main positive contributions for the biological activity of the

Table 9. Statistical indices for the final HQSAR, CoMFA and CoMSIA models

Model	q^2 (LOO)	r^2	r^2_{pred}	q^2 (LMO)	Q^2	dq^2/dr^2_{yy}
HQSAR (model 10)	0.57	0.90	0.66	0.50	0.36	1.01
CoMFA (model 11)	0.71	0.99	0.82	0.62	0.50	0.96
CoMSIA (model 15)	0.66	0.91	0.76	0.62	0.42	1.02

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*Figure 8. CoMSIA StDev*Coeff hydrophobic contour maps for compounds 46 and 66 (a and b, respectively). Donor and acceptor maps for compound 45 (c and d, respectively)*



fenarimol derivatives. The 4-chlorophenyl, the 3-fluorobenzonitrile and the pyridine rings were stressed as critical molecular fragments for conservation of the trypanocidal activity. Otherwise, the 3D maps identified important physical-chemical properties, which were analyzed in terms of the functional groups enclosed by such plots. As in the HQSAR analysis, the 3-fluorobenzonitrile, the 4-chlorophenyl and the pyridine rings were highlighted as important for activity; however, the 3D methods were able to detect a broader range of chemical functionalities, such as the mutual hydroxyl group linked to the tetrahedral carbon atom. As illustrated in Figures 7 and 8, the CoMFA and CoMSIA plots, differently from the HQSAR maps, concentrated mainly in the substituent groups rather than the aromatic rings. This could be explained by the structural pattern of the data set molecules, which is characterized by a conserved core of three aromatic rings substituted by a variety of functional groups.

All these findings may be used to design improved compounds. For example, the final 2D model showed that the three aromatic rings are essential for activity, therefore, this structural scaffold must be conserved in newly designed derivatives; however, replacement of the pyrimidine or pyridine with a similar system, such as an imidazole ring, could be explored as a molecular optimization strategy. As observed in the CoMFA and CoMSIA maps, introduction of steric bulk and electron-withdrawing moieties are recommended as substituents of the phenyl rings; this could be investigated by linking to these rings, bulky hydrogen-bond acceptor groups such as pyridine, pyrimidine, benzothiazole, 2-oxazolidinone and thiazole.

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CONCLUSION

Predictive QSAR models were developed for a series of highly active compounds against the parasite *Trypanosoma cruzi*, the causative agent of Chagas disease. The HQSAR, CoMFA and CoMSIA models exhibited high internal and external consistency as demonstrated by the several validation methods employed to assess their statistical quality. The external validation using a test set demonstrated the ability of the models to estimate with proper accuracy the trypanocidal activity of novel compounds structurally related to fenarimol. Moreover, the final 2D and 3D models provided a detailed description of the molecular aspects underlying the biological activity of the targeted compounds. The HQSAR contribution maps along with the CoMFA and CoMSIA contour plots were able to identify molecular fragments, functional groups and physicochemical properties strongly correlated with the biological activity of this series. The highlighted features are valuable information to delimitate the chemical space to be explored in the design of novel compounds within the investigated structural diversity.

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Chapter 38
The "ETA" Indices in QSAR/QSPR/QSTR Research

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ABSTRACT

Descriptors are one of the most essential components of predictive Quantitative Structure-Activity/Property/Toxicity Relationship (QSAR/QSPR/QSTR) modeling analysis, as they encode chemical information of molecules in the form of quantitative numbers, which are used to develop mathematical correlation models. The quality of a predictive model not only depends on good modeling statistics, but also on the extraction of chemical features. A significant amount of research since the beginning of QSAR analysis paradigm has led to the introduction of a large number of predictor variables or descriptors. The Extended Topochemical Atom (ETA) indices, developed by the authors' group, successfully address the aspects of molecular topology, electronic information, and different types of bonded interactions, and have been extensively employed for the modeling of different types of activity/property and toxicity endpoints. This chapter provides explicit information regarding the basis, algorithm, and applicability of the ETA indices for a predictive modeling paradigm.

INTRODUCTION

Chemicals are the inseparable components of human life, and thus they can affect a wide variety of industrial, agricultural as well as household processes if not monitored properly. Elucidation of the chemistry of compounds is an essential task of the designer so as to enable easy tuning of their properties. The diverse manifestation of chemicals can be systematically explored when the study of their chemistry is suitably attended with the related biology (including toxicology), mathematics and accompanying statistics. Since the past, mathematics has been considered as an effective media of natural science (Wigner, 1960) and has been employed in different branches of science to derive logical relationship employing abstract ideas. Chemistry has a very good coherence with mathematics that leads to the quantification

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of chemical information by providing suitable platform for the generation of various algorithms. How different chemicals act differently and even the same chemical elicits different responses give the hint to nurture the 'chemistry' of compounds and explore the possible quantitative phenomena involved. The attempt to quantify the chemistry of compounds has lead to the development of several independent parameters termed as predictor variables or descriptors. These descriptors are further utilized to bring under a quantitative relationship with the responses of the chemicals commonly termed as the quantitative structure-activity/property/toxicity relationship (QSAR/QSPR/QSTR) studies. Mathematics plays a great role in deriving such predictor variables as well as in the development of correlations.

Through the journey of QSAR analysis, several predictor variables have evolved by exploring the concepts of chemistry, mathematics and physics. However, the 'graph theory' has contributed much to the development of initial ideas about descriptors. The graph theory, one of the branches in mathematics, can be traced back to Euler's Königsberg bridges problem (Euler, 1736) which led to the idea of solving problems in different fields of science namely the study of electrical circuit by Kirchhoff (Kirchhoff, 1847), chemical isomer enumeration by Cayley (Cayley, 1875) etc. In a true mathematical sense, the term 'graph' also denotes cartesian plot of data, although it was Sylvester (Sylvester, 1878) who coined the term from a contemporary chemical perspective. Application of the graph theory in chemistry leads to the concept of 'chemical graph' which was supposed to have its origin in the late eighteen century holding the hand of the Scottish chemist Cullen (Crosland, 1959). William Cullen introduced the application of 'affinity diagrams', although it remains controversial with similar type of contributions by Black (Crosland, 1959). Later Higgins (Higgins, 1789) employed the use of graphical diagrams to denote the forces between atoms in a molecule. It was Dalton (Dalton, 1808) in the nineteenth century who started the concept of ball-and-stick representation of chemical models. The idea of diagrammatic presentation of chemical compounds was very primitive during that age and through various notable discoveries the chemistry got explored gradually. The famous scientist Kekulé brought the idea of three-dimensional orientation (Fischer, 1974) and later idea of chemical valence came likewise. Considering the scope of this chapter, we would like to limit this discussion at this point.

It was in the twentieth century when the chemical graphs were explored properly, and utilizing the concepts of topological distance measures the scientific fraternity started developing theoretical variables to correlate activity of chemicals with their chemistry. Although the history of QSAR starts back in the past when Cross (Cross, 1863) reported his idea of possible correlation between chemical composition and toxicity of alcohols, the concept of theoretical descriptor did not come in action properly until the year 1947 when the Wiener index (Wiener, 1947) and the Platt number (Platt, 1947) were introduced as the first two graph theoretical descriptors for modeling boiling point of hydrocarbons. Following these milestone achievements in the realm of quantification of chemistry paradigm, a new wave was hit towards the exploration of graph theoretic parameters, and in the 1960s and 1970s the pathway was systematically imputed by the following pioneering contributions namely Simon's minimal topological difference (MTD) concept (Simon, 1974), Balaban J (Balaban, 1982), Gordon and Scantlebury index (Gordon & Scantlebury, 1964), Schultz molecular topological index (MTI) (Schultz, 1989), Hosoya Z (Hosoya, 1971), Randić's branching parameter (Randić, 1975), Kier and Hall's molecular connectivity, Kappa shape, sub-graph count, flexibility and electrotopological state (E-state) atom indices (Kier & Hall, 1986), Zagreb index by Trinajstić and co-workers (Gutman & Trinajstić, 1972), Harary index (Plavšić et al., 1993), Verloop's STERIMOL parameter (Verloop, 1987), etc. Graph theoretic descriptors are purely based on a two dimensional basis, whereas concepts of three dimension was also under parallel research and various predictor variables were also derived employing three dimensional basis.

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A torrential amount of research on the descriptors has led to the development of a wide variety of predictor variables till today. The present chapter gives an account of such a group of two-dimensional novel descriptors named extended topochemical atom (ETA) indices which have been developed in the twenty first century from the modification of another set of topologically arrived unique (TAU) scheme descriptors of the late eighties. The ETA indices are now available under two generations and have been used in various modeling studies to predict property, activity as well as toxicity endpoints.

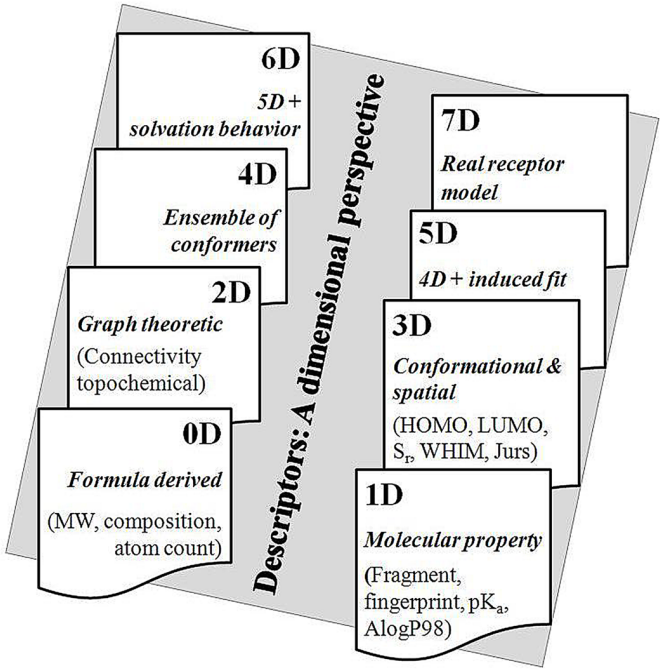
Descriptors in QSAR Studies: The Impact and Required Quality

Scientific deductions are based on logical components that aids in deriving conclusion from an observation. The chemoinformatics study also bears such inevitable components that ultimately help in developing predictive mathematical models and also leave a plausible explanation for the reasons involved. The molecular descriptors in QSAR studies can be viewed as the quantitative presentation of chemical structures obtained by processing the molecule using a specified mathematical algorithm. Todeschini and Consonni (Todeschini & Consonni, 2000) have ascribed descriptors as the useful numbers or the standardized experimental results obtained as the final result of a logic and mathematical procedure that leads to the transformation of chemical information which is encoded within symbolic representation of molecules. So, the mathematics is here utilized to unwrap the chemical information of compounds in the form of numerical entities. Descriptors are the algorithmic mathematical entities that strongly adhere to the chemistry of compounds and thereby form a core to the QSAR analysis. Since, the attributes of chemical compounds are depicted using different levels and dimensions of theory of chemistry and physics, descriptors are also defined on the basis of such theory coupled with a definite algorithm of mathematics. A good number of descriptors are available now-a-days corresponding to different algorithms which can be conveniently categorized from the dimensional perspective. Figure 1 shows a representative dimensional classification of various descriptors for QSAR analysis.

Descriptors can be defined for a whole molecule or they may also refer to specific fragments of the molecule. Again from the basis of computation, descriptors can be theoretical or experimental. Experimental descriptors correspond to various derived physicochemical properties namely partition coefficient, molar refractivity, optical rotation, pK_a values, reaction rate, parachor etc. and they usually depict characteristics of the entire molecule while theoretical descriptors are usually computationally derived mathematical entities and can define specific molecular fragment as well as the whole molecule. An interesting fact about descriptors is that they evolved from lower to higher dimensions as the practice of QSAR made its progress and so the theory. Looking back at the history of QSAR, it can be observed that Cross (Cross, 1863) was the first who observed the toxicity of primary aliphatic alcohols to be possibly correlated with their water solubility. Crum-Brown and Fraser (Crum-Brown & Fraser, 1868) are inexorably believed to be first to report the physiological action as the mathematical function of chemical constituents during an investigation of muscle paralysis activity of strychnine derivatives. Richardson (Richardson, 1869) observed a proportional relationship of narcotic effect of primary alcohols with their molecular weight and almost after two decades Richet (Richet, 1893) found the cytotoxicity of ethers, alcohols and ketones to be inversely related with their aqueous solubility measure. Then Meyer (Meyer, 1899) and Overton (Overton, 1901) came with their decisive work by correlating narcotic potency with lipid/water partition coefficient. After that, at the beginning of the twentieth century Trabe (Trabe, 1904) and Seidell (Seidell, 1912) came with their correlation analysis employing surface tension and solubility and partition coefficient respectively. Then came the pioneering discovery by Ham-

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Figure 1. Dimensional perspective of descriptors used in predictive modeling (QSAR) analysis



mett (Hammett, 1935) who initially showed relationship between reaction rates with acid dissociation constant values (pK_a) and the parameter σ (sigma) which afterwards led to a "sigma-rho" culture in the field of predictive modeling analysis. Some more notable research activities were performed during this time namely correlation of narcotic activity with partition coefficient and exploring thermodynamics of chemicals by Ferguson (Ferguson, 1939), study on the involvement of ionization and shape factors towards the bacteriostatic action of aminoacridines by Albert (Albert et al., 1941) and correlation studies on antibacterial action (*E. coli*) of sulfonamides with their ionization and shape parameters by Bell and Roblin (Bell & Roblin, 1942). In this journey, it may be observed that QSAR started with the concept of correlating toxicity of chemicals with aqueous solubility by Cross whereas the term 'chemical constituents' in Crum-Brown and Fraser's hypothesis can be connected to the elemental composition of the compounds. Richardson and Richet found toxicity to be expressible in terms of molecular weight and aqueous solubility respectively. Hence, we can see that at the dawn of QSAR, scientists used different physicochemical properties, *i.e.*, experimental descriptors as variables and at the same time the concept of zero dimensional parameter like elemental composition, molecular weight were also used. The practice of using physicochemical properties like partition coefficient, solubility etc. as descriptors was also done by other scientists namely Trabe and Seidell. The concept of extracting chemical information was getting boosted during this time and Hammett led to the discovery of a theoretical parameter σ (sigma) in connection with modeling reaction kinetics. The contributions by Albert et al. and Bell & Roblin are also notable since they were the first to focus on the ionization factor and shape factors as independent parameters to derive correlation. It was in 1947, Wiener (Wiener, 1947) and Platt (Platt, 1947) came up with some more light to the path with their first topologically derived graph theoretic descriptors namely Wiener index and Platt index respectively by developing QSPR models for the boiling points of

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paraffin hydrocarbons. The inception of graph theoretic parameters as chemical descriptors opened a big doorway in the field of theoretical descriptors, and since then a significant amount of research has taken place towards the development of more informative parameters. The discovery of Hammett (Hammett, 1935) was forwarded by Taft who introduced a steric parameter E_s (Taft, 1952) that distinguishes polar, steric and resonating effect exerted by chemicals and reported the first multiple regression method based linear free energy relationship (LFER) equation. In the early 1960s, several newer developments were made in the field of 2D-descriptors. In 1962 Hansch (Hansch et al., 1962) introduced a substituent hydrophobicity parameter π and later in 1964 Hansch and Fujita (Hansch & Fujita, 1964) extended the analysis by combining parameters of Hammett and Taft and developed LFER models comprising of σ, E_s and π. The analysis by Fujita and Hansch has undergone further parabolic extension and later it was also modified by Kubinyi (Kubinyi, 1976). Another concept of presenting molecular descriptors as the contribution of the parent moiety and substituents was depicted by Free-Wilson (Free & Wilson, 1964) which gave rise to 'indicator variables'. The Free-Wilson analysis was later modified by Fujita-Ban (Fujita & Ban, 1971) by incorporating logarithmic terminology.

It is to be noted that from the bases of the two-dimensional chemistry, concepts of three- dimensional (3D) molecular structure representation, electronic information and the descriptors thereof were also in the developing stage starting in 1930s. Some of the notable contributions can be cited namely the chemical bonding theory by Pauling (Pauling, 1939) and Coulson (Coulson, 1939), Sanderson's (Sanderson, 1952) electronegativity parameters, electronic distribution theory by Fukui et al. (Fukui et al., 1954) and Mulliken (Mulliken, 1955), charged partial surface area measures of Jurs & Stanton (Stanton & Jurs, 1990), shadow indices (Rohrbaugh & Jurs, 1987), WHIM (Todeschini & Lasagni, 1994) & GETAWAY descriptors by Todeschini and co-workers (Consonni et al., 2002), 3D-MoRSE descriptors (Schuur et al., 1996), EEVA descriptors, CoMFA and CoMSIA analysis by Cramer et al. (Cramer et al., 1988) and the ligand-receptor based analyses including multidimensional (4D, 5D, 6D and so on) features in the modern century (Lill, 2007). However, considering the scope of this article we would like to focus our topic on two-dimensional topological structure representation only.

Hence it is observed that the molecular descriptors of chemical compounds are pivotal as they play a very crucial role in predictive modeling analysis. Through the journey of QSAR paradigm, the attempt to generate quantitative numbers using the chemistry of compounds has yielded various mathematical descriptors. Now, the obvious question arises regarding the required quality of such essential component of QSAR analysis. It is apparently true that quantitative predictor variables were introduced to establish mathematical relationships so that predictions can be made when changes (*e.g.*, new substituents) are incorporated into structures. Since a good amount of mathematics is involved in this aspect, care should be taken such that the descriptors do not become just random numbers for developing correlation equations, rather they should encode proper chemical information. The experimental properties which are used as descriptors usually do not suffer from such problem, since they are the true reflection of the physicochemical behavior of the chemicals. However, there is possibility of incomplete information in theoretically derived parameters if they are not judged properly. Proper care is required while dealing with the mathematics since it only acts as a tool to define the final index employing several algebraic and arithmetic operations while the diagnosis of chemical information entirely belongs to the part of chemistry. Hence, two required criteria of molecular descriptors can be identified namely predictive ability and interpretability. From practical examples, in many cases it has been observed that descriptors giving high correlation against an endpoint of interest suffer from the lack of chemical diagnosis potential and vice-versa. A proper balance between this two makes a descriptor important for the QSAR

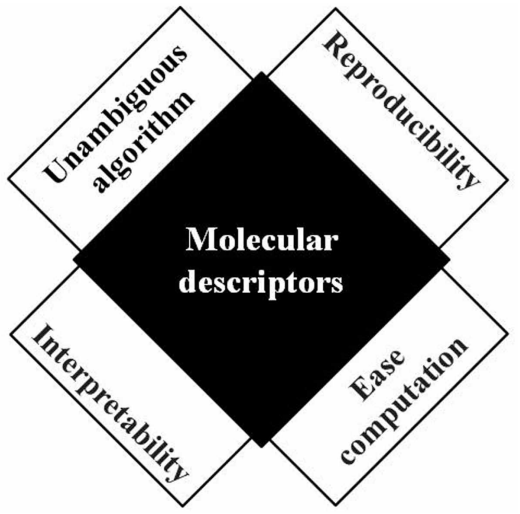
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analysis. This feature has also been indicated in the definition of descriptors prescribed by Todeschini and Consonni (Todeschini & Consonni, 2000) mentioned earlier as the 'useful numbers' which states the descriptors having the insight of the molecular attributes as well as ability to take part in predictive modeling analysis.

Four of the basic features of chemical descriptors are shown in Figure 2. Here, the term 'easy computation' refers to simplicity and explicitness of the descriptor algorithm since many of the higher dimensional variants require complex pretreatment operations. The descriptor should also be easily reproducible irrespective of the computational environment employed. The predictive ability refers to the skill of the descriptor in giving good correlation with an endpoint under investigation while the interpretability focuses on the chemical attributes of the analyzed molecular structures. Once a proper basis of chemical or biological activity of the molecule is established, the tuning of its features becomes easier and technically more specific. Two- dimensional descriptors are usually simple, derived using hydrogen suppressed molecular graphs with a high degree of precision and consume less computational time than the higher order indices. In the beginning of the development of graph theoretic descriptors, much more emphasis was given on the establishment of quantitative correlation than the search for mechanistic basis. As a matter of fact, many of the formerly defined descriptors were found to be flawed when chemometric modeling studies started in a much larger scale.

This article presents a group of two-dimensional descriptors, extended topochemical atom (ETA) indices, which were developed addressing several flaws of the previously described parameters. The ETA indices comprise a few graph theoretic parameters as well as some electronic indices to describe the bonding pattern, unsaturation, electronegativity and hydrogen-bonding related information. Till date, the ETA indices have been used to model several endpoints employing different categories of chemical compounds ranging from simple alkanes to complex drug molecules and agrochemicals. An account of the definition of various ETA indices with an overview of their modeling status is presented in the next sections.

Figure 2. The required features of theoretically derived descriptors



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THE ETA INDICES

Brief Background

The ETA indices use information from hydrogen suppressed graphical presentation of molecules where atoms are denoted by vertices while edges represent covalent bonds. The ETA formalism was derived as an extension of another group of graph theoretic descriptors called topochemically arrived unique (TAU) scheme indices. Before getting into details of the ETA indices, we would like to provide the readers with some basic information and the motivation of developing TAU scheme parameters.

The branching parameter by Milan Randić (Randić, 1975) gave the measure of ramification of branching in a graph theoretical topological problem. This idea was extended by Kier and Hall (Kier & Hall, 1986), who gave a more comprehensive description on the type of branching by incorporating the concepts of path, cluster and chain. The molecular connectivity index of Kier and Hall's was observed to produce good correlational results with some physico-chemical and biological endpoints. However, in the late eighties Pal et al. (1988, 1989, 1990) observed several limitations in Kier and Hall's parameters and argued that predictive feature of this group of descriptors is biased, especially when higher order indices are used. The following drawbacks of Kier and Hall molecular connectivity or chi (χ) indices were identified by Pal and co-workers.

1. Kier and Hall's molecular connectivity indices chiefly portray additive information with no consideration of constitutive aspects.
2. It bears a good correlation with some quantum mechanical indices and mass spectroscopic fragmentation pattern and thereby possesses a similar pattern like logPo/w.
3. The molecular connectivity index formalism does not include information for steric interaction, stereoisomerism, resonance and aromaticity.
4. The vertex valency term δ^v often shows redundancy and becomes inconsistent when atoms of different quantum levels are considered.
5. Unlike vertex valency, the relative weight of the edges is not properly distinguished which leads to the fact that only ${}^1\chi$ and ${}^1\chi^v$ have graph theoretical meaning. Furthermore, higher order indices, i.e., ${}^m\chi^v$ (m>1), were found to possess good correlation when ${}^1\chi^v$ showed similar result and reflect that ${}^m\chi^v$ is strongly correlated with ${}^1\chi^v$.
6. The sigma and pi bonds in a system are equally weighted in this scheme.
7. Suitable adjustment in the molecular structure of several compounds of varied structures which do not possess optimum biological response in the dataset can yield the ideal value of ${}^m\chi^v$ obtained from the regression equation.

With the aim of circumventing such undesirable shortcomings of molecular connectivity indices, Pal et al. (1988, 1989, 1990) proposed a new set of topological descriptors namely TAU indices emphasizing the nuclear and electronic features of the atoms. In the TAU formalism, the vertex in a connected molecular graph has been considered to be composed of a core and a valence electronic environment. The valence electronic environment has been further partitioned into a localized and a mobile electron measure. Table 1 shows the formal definitions of various TAU parameters. It should be noted that other than characterizing the core and the valence electronic environment (both local and mobile), parameters defining the branching and contribution of functional groups were also defined in the TAU scheme.

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Several modeling studies were performed using TAU indices and reliable results were obtained. Later the same group of authors also attempted to develop models employing other descriptors along with TAU parameters so as to judge the relative contribution of TAU scheme indices in predictive modeling studies. Table 2 summarizes the various endpoints modeled employing TAU scheme parameters. It was shown by Pal et al. (1988, 1989, 1990) that isosteric groups or isovertices have similar θ or θ' values.

The Motivation for Developing ETA Indices

Nearly a decade after the development of TAU indices, Roy and Ghosh (2003) observed that even the TAU scheme has some drawbacks and there are opportunities of their further development into more appropriate descriptors. The present authors' group observed the following aspects of the TAU scheme to be inconsistent:

1. The core count 'λ' was observed to have a disproportionate incremental relationship with the atomic number of different vertices.
2. The VEM vertex count was not suitably distributed and a vertex forming sigma bond with another vertex of similar electronegativity and different electronegativity were given the same value.
3. Although it provides a weighting scheme for sigma and pi bonds, the TAU scheme does not differentiate the double bonds of different types *i.e.*, ordinary alkenes, ordinary alkynes, conjugated system, aromatic system, etc.
4. Furthermore, the TAU scheme considers the contribution of functional groups at the molecular level, which should rather be considered at the atomic level or fragmental basis.

These were the chief motivations to the present authors' group towards the development of a new group of predictor variables in order to achieve better diagnostic potential. In 2003, Roy and Ghosh presented a new formalism named ETA indices (Roy & Ghosh, 2003) comprising several molecular level as well as atomic level parameters. Then these indices were subjected to a thorough chemometric modeling employing different endpoints. Later in 2011, the present authors' group realized the need for some more parameters to encode the issues related to hydrogen bonding propensity of molecules, measure of polar surface area, unsaturation and electronegativity, and they came up with a second generation indices (Roy & Das, 2011). Figure 3 presents the basic features of the ETA indices from a perspective of their developmental stages.

The ETA Indices: Formalism

Presently the ETA indices comprise a set of two generation descriptors defining chemical features at the atomic as well as molecular level. Table 3 gives a generation-wise listing of the derived ETA indices. In the present article, we would present all the ETA parameters from the viewpoint of atomic and molecular basis.

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Table 1. Definition of the descriptors under the topochemically arrived unique (TAU) scheme formalism

Sl. No.	Type	Mathematical Definition		Note (If Any)
Parameter for the Vertex Core				
1	Core count	$\text{Core count} = \lambda = \frac{Z - Z^v}{Z^v}$ where Z=atomic number, Z^v=number of valence electron, *i.e.*, number of positive charges not neutralized by core electrons.		λ shows a regular change with Pauling's electronegativity (ε^0) across the period and along the group in an opposite manner. $1/\lambda$ gives a rough measure of the strength of the positive field of the atomic core.
Parameters for the Localized and Mobile Valence Electron Environment				
		Valence Electron Local (VEL)	**Valence Electron Mobile (VEM)**	
2	Valence electron count	$\theta' = 2h + 1.5\nu + 2l$ h is the number of bonded H-atoms, ν is the number of sigma bonds other than with hydrogen and l is the lone pairs of electrons.	$\theta = \left(8 - \theta'\right) = 8 - (2h + 1.5\nu + 2l)$	θ' gives a count of the relatively localized valence electron that the positive field of the atomic core enjoys while θ represents the count of relatively mobile electrons enjoyed by the field of the core. The number '8' in the definition of VEM denotes the number of electrons in the valence octet of a bonded atom. It was assumed that an atom sigma bonded to another non-hydrogen atom shares 50% of the other electron through the sigma bond apart from its own electrons. For a covalently linked organic atom, $h + l + \pi + \nu = 4$, π being the number of pi-bonds. So, $\theta' = 8 - \left(0.5\nu + 2\pi\right)$ and $\theta = 0.5\nu + 2\pi$. Isosteric group or isovertices are characterized by similar θ or θ' values.
3	Vertex valency	$V_i' = \frac{\lambda_i}{\theta_i'}$	$V_i = \frac{\lambda_i}{\theta_i}$	
4	Edge weight	$E_{ij}' = E_{ji}' = \left(V_i' \times V_j'\right)^{\frac{1}{2}}$ when $i \neq j$ and i, j connected $= 0$ otherwise	$E_{ij} = E_{ji} = \left(V_i \times V_j\right)^{\frac{1}{2}}$ when $i \neq j$ and i, j connected $= 0$ otherwise	
5	Molecular index (1st order)	$T' = 0.5\sum E_{ij}'$	$T = 0.5\Sigma E_{ij}$	
6	Molecular index (Zero order)	${}^0T' = 0.5\sum V_i'$	${}^0T = 0.5\Sigma V_i$	
7	The composite index	$T = \sum_{i<j} E_{ij} = \sum_{i<j}\left(V_i \times V_j\right)^{0.5}$		
Parameter for Branching and Functional Group				
8	Functionality parameter	$F = T_R - T$ T_R being the first order VEM molecular index of the corresponding reference alkane.		A reference alkane of a molecule corresponds to a structure where all heteroatoms are replaced with carbon atom and multiple bond (covalent) with single bond. The branching parameter and the functionality parameter can be correlated as: $T = T_R - F = T_N - B - F$
9	Branching parameter	$B = T_N - T_R$ T_N being the first order VEM molecular index of the normal alkane.		

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Table 2. A representative list of endpoints modeled using TAU scheme indices

Endpoints Addressed	Type of Chemicals Used	Ref.
Narcosis of barnacle larvae in tomato plant and red spider	Vapor toxicity of alkanols	Pal et al., 1988
Inhibitory activity of *Mycobacterium tuberculosis*	Substituted bromophenols	Pal et al., 1989
Amoebicidal action against *Entamoeba histolytica*	Polymethylene primary diamines	Pal et al., 1990
Fuel property in terms of research octane number (RON) and performance number (PN)	Acyclic alkane.	Pal et al., 1992
Narcosis to tadpoles	Diverse functional acyclic compounds	Roy et al., 1999
General anesthetic activity	Aliphatic hydrocarbons, halocarbons and ethers	Roy et al., 2001
Heat of formation and heat of atomization	Diverse functional acyclic compounds	Roy & Saha, 2003a
Lipid–Water Partition Coefficient	Diverse functional acyclic compounds	Roy & Saha, 2003b
Aqueous solubility	Diverse functional acyclic compounds	Roy & Saha, 2003c
Molar refractivity	Diverse functional acyclic compounds	Roy & Saha, 2005

Figure 3. The principle features of ETA indices along with the path of their journey

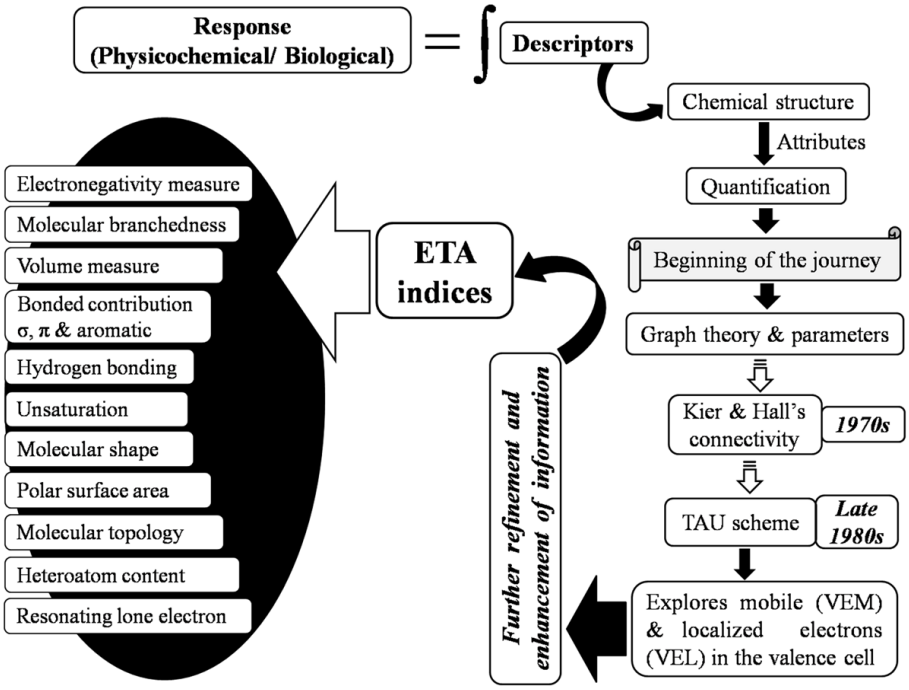


Table 3. Generation-wise presentation of the ETA indices

Generation	Indices
First generation	$\sum\alpha$, $\sum\alpha/N_v$, $(\sum\alpha)_p/\sum\alpha$, $(\sum\alpha)_v/\sum\alpha$, $(\sum\alpha)_x/\sum\alpha$, $\sum\varepsilon$, $\sum\varepsilon/N$, $\sum\beta$, $\sum\beta'$, $\sum\beta_s$, $\sum\beta'_s$, $\sum\beta_{ns}$, $\sum\beta'_{ns}$, η, η_R, η_F, η'_F, η^{local}, η_R^{local}, η_F^{local}, η'^{local}_F, η_B, η'_B
Second generation	$\Delta\alpha_A$, $\Delta\alpha_B$, ε_2, ε_3, ε_4, ε_5, $\Delta\varepsilon_A$, $\Delta\varepsilon_B$, $\Delta\varepsilon_C$, $\Delta\varepsilon_D$, ψ_1, $\Delta\psi_A$, $\Delta\psi_B$, $\Delta\beta$, $\Delta\beta'$, $\sum\beta_{ns(\delta)}$, $\sum\beta'_{ns(\delta)}$

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Definition of the Atomic Level ETA Indices

The Core Count (α)

The ETA core count is formally defined as:

$$\alpha = \frac{Z - Z^v}{Z^v} \cdot \frac{1}{PN - 1}. \quad (1)$$

Here *PN* stands for period number, while Z and Z^v represent atomic number and valence electron number respectively. Hydrogen atom being considered as the reference, α for hydrogen is taken to be zero. It was observed that α values of different atoms in common organic compounds show a high correlation (R=0.946) (Roy & Ghosh, 2003) with uncorrected van der Waals volume (Moriguchi et al., 1976).

Hence, a summed value of the parameter, i.e., $\Sigma\alpha$ for all non-hydrogen atoms in a molecule gives a measure of molecular bulk. In order to incorporate relativeness to this parameter with respect to the number of vertices, another term $\Sigma\alpha/N_v$ is commonly used where N_v is the number of non-hydrogen atoms, *i.e.*, vertices in the molecule.

The Electronegativity Count (ε)

The ETA electronegativity count can be defined as follows:

$$\varepsilon = -\alpha + 0.3 \times Z^V \quad (2)$$

The term ε is characterized by a significant correlation (R=0.937) with Pauling's electronegativity scale (EN) (Roy & Ghosh, 2003).

The Hydrogen-Bonding Propensity (ψ)

The ETA hydrogen bonding propensity measure can be defines as follows:

$$\psi = \frac{\alpha}{\varepsilon} \quad (3)$$

Based on the values of α and ε, this parameter (ψ) was defined which performs a discrimination among the atoms involved and not involved in hydrogen bonding. Table 4 gives the α, ε, and ψ values of some atoms commonly found in organic molecules.

The VEM Count (β)

In the ETA scheme, a new formalism has been incorporated to describe the VEM vertex measure. The VEM count β for a non-hydrogen vertex can be defined as:

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Table 4. α, ε, and ψ values of the commonly occurring atoms

Sl. No.	Atoms	α	ε	ψ
1	H	0.000	0.300	0.000
2	C	0.500	0.700	0.714
3	N	0.400	1.100	0.364
4	O	0.333	1.467	0.227
5	F	0.286	1.814	0.158
6	P	1.000	0.500	2.000
7	S	0.833	0.967	0.861
8	Cl	0.714	1.386	0.515
9	Br	1.333	0.767	1.738
10	I	1.643	0.457	3.595

$$\beta = \Sigma x\sigma + \Sigma y\pi + \delta \tag{4}$$

where the parameters σ and π correspond to the number of sigma and pi bonds, respectively, in the connected hydrogen suppressed molecular graph and δ represents a correction factor having a value of 0.5 per atom with loan pair of electrons capable of making resonance with an aromatic ring (e.g. nitrogen of aniline, oxygen of phenol etc.). Here x and y are the respective contributions of sigma (σ) and pi (π) bonds. We have assigned different contribution values for the sigma as well as pi bonds in the VEM count (β) of the ETA scheme. For the computation of β, contribution of a sigma bond (x) between two atoms of similar electronegativity ($\Delta\varepsilon \leq 0.3$) is considered to be 0.5, and for a sigma bond between two atoms of different electronegativity ($\Delta\varepsilon > 0.3$), it is considered to be 0.75. Again, the contributions for π bonds (y) are assigned depending on the type of double bonds as depicted below.

1. For a π bond between two atoms of similar electronegativity ($\Delta\varepsilon \leq 0.3$), the value of y is 1.
2. For a π bond between two atoms of different electronegativity ($\Delta\varepsilon > 0.3$) or for conjugated (non-aromatic) π system, y is considered to be 1.5.
3. For an aromatic π system, the value of the contribution factor is 2.

Hence, it is observable that the TAU scheme considered a value of 2.0 for all kinds of π bonds which is not in the case of ETA formalism. Furthermore, the VEM sigma contribution for a non-hydrogen vertex can be designated as β_s and defined as:

$$\beta_s = \Sigma x\sigma \tag{4a}$$

Similarly, the VEM non-sigma contribution of a non-hydrogen vertex can be designated as β_{ns} and defined as:

$$\beta_{ns} = \Sigma y\pi + \delta \tag{4b}$$

It should be noted that,

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$$\beta = \beta_s = \beta_{ns}. \tag{4c}$$

The VEM Vertex Count (γ)

The VEM vertex count γ_i of the i^{th} vertex in a connected molecular graph can be defined as:

$$\gamma_i = \frac{\alpha_i}{\beta_i} \tag{5}$$

where α_i stands for the alpha value for the i^{th} vertex and β_i being the VEM count considering all bonds connected to the atom and lone pair of electrons (if any).

The Atom Level Composite Index ($[\eta]_i$)

The contribution of a particular vertex or position (within the common substructure in a congeneric series) to the composite index η can be determined in the following manner:

$$[\eta]_i = \sum_{j \neq i} \left[\frac{\gamma_i \gamma_j}{r_{ij}^2} \right]^{0.5} \tag{6}$$

Here, $[\eta]_i$ denotes the contribution of the i^{th} vertex to η. The term in the denominator r_{ij} corresponds to the topological distance between the i^{th} and j^{th} atoms.

Definition of the Molecular Level ETA Indices

The molecular level indices indicate computation of those descriptors which involve the entire molecular structure. There are some ETA indices which can be computed at the atomic level as well as at the whole molecular level, while there are some indices for whole molecule only. Table 5 presents a comprehensive assembly of ETA indices for the whole molecule/ molecular fragment/ substructure with their notation, mathematical definition and significance. We would just like to clarify that throughout the Table 5, N indicates total number of atoms including hydrogens while N_v corresponds to the number of vertices, *i.e.*, only non-hydrogen atoms. Again few terms are associated with a subscript 'R', e.g., N_R, $[\Sigma\alpha]_R$, η_R etc. corresponding to a reference alkane which refers to a molecular graph of the original structure where all the heteroatoms are replaced with carbon and multiple (double and triple) bonds with single bonds. Furthermore, four terms in Table 5 namely $\Delta\alpha_A$, $\Delta\alpha_B$, $\Delta\psi_A$ and $\Delta\psi_B$ are spline terms (denoted by angle bracket or chevrons, $< >$) meaning that any negative value will be considered zero. Regarding the computation of VEM β count, one more subtle point to be noted is that the index β is computed for edges taking the value of vertices. Since an edge is comprised of two vertices, the following relationships exist:

$\sum\beta_s$ = Sum of β_s values of all non-hydrogen vertices / 2

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Table 5. Definition of the ETA indices at the whole molecule/fragment/substructure level

Sl. No.	Mathematical Definition	Significance
1	$\sum\alpha$	Summed α values of all non-hydrogen vertices of a molecule (or fragment/ substructure). Gives a measure of molecular bulk (or for the fragment/substructure).
2	$\Sigma\alpha/N_v$	A relative measure of molecular bulk with respect to the number of vertices.
3	$(\Sigma\alpha)_p/\Sigma\alpha$	$(\sum\alpha)_p$ stands for summation of α values of the vertices that are joined to one other non-hydrogen vertex in the connected molecular graph. Gives a measure of molecular shape.
4	$(\Sigma\alpha)_Y/\Sigma\alpha$	$(\sum\alpha)_Y$ stands for summation of α values of the vertices that are joined to three other non-hydrogen vertices in the connected molecular graph. Gives a measure of molecular shape.
5	$(\Sigma\alpha)_X/\Sigma\alpha$	$(\sum\alpha)_X$ stands for summation of α values of the vertices that are joined to four other non-hydrogen vertices in the connected molecular graph. Gives a measure of molecular shape.
6	$\Delta\alpha_A = \left\langle \frac{\sum\alpha - \left[\sum\alpha\right]_R}{N_v} \right\rangle$	A measure of count of heteroatoms.
7	$\Delta\alpha_B = \left\langle \frac{\left[\sum\alpha\right]_R - \sum\alpha}{N_v} \right\rangle$	A measure of count of hydrogen bond acceptor atoms and/or polar surface area.
8	$\varepsilon_1 = \frac{\sum\varepsilon}{N}$	Summed epsilon (ε) value in a molecule relative to the total number of atoms including hydrogen. Gives a measure of electronegative atom count.
9	$\varepsilon_2 = \frac{\sum\varepsilon_{EH}}{N_v}$	$\Sigma\varepsilon_{EH}$ stands for summed epsilon count of a molecule excluding hydrogen atoms. Gives a measure of electronegative atom count.
10	$\varepsilon_3 = \frac{\left[\sum\varepsilon\right]_R}{N_R}$	Summation of epsilon (ε) value relative to the total number of atoms including hydrogen in the connected molecular graph of the reference alkane.
11	$\varepsilon_4 = \frac{\left[\sum\varepsilon\right]_{SS}}{N_{SS}}$	Summation of epsilon (ε) value relative to the total number of atoms including hydrogen for a saturated carbon skeleton moiety of the normal molecule *i.e.*, carbon-carbon multiple bonds considered as single bond.
12	$\varepsilon_5 = \frac{\sum\varepsilon_{EH} + \sum\varepsilon_{XH}}{N_v + N_{XH}}$	Here, $\Sigma\varepsilon_{XH}$ stands for summed epsilon value for those hydrogen atoms which are connected to a heteroatom (e.g., $-$OH, $-NH_2$, $-$SH etc.) and N_{XH} gives a count of such hydrogens.
13	$\Delta\varepsilon_A = \varepsilon_1 - \varepsilon_3$	A measure of contribution of unsaturation and electronegative atom count.
14	$\Delta\varepsilon_B = \varepsilon_1 - \varepsilon_4$	A measure of contribution of unsaturation.
15	$\Delta\varepsilon_C = \varepsilon_3 - \varepsilon_4$	A measure of contribution of electronegativity.
16	$\Delta\varepsilon_D = \varepsilon_2 - \varepsilon_5$	A measure of contribution of hydrogen bond donor atoms.
17	$\sum\beta'_s$	$\Sigma\beta'_s = \Sigma\beta_s / N_v$. Summed β values for all the sigma bonds (VEM sigma contribution) relative to the number of vertices. Gives a measure of electronegative atom count of the molecule relative to the molecular size.

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Table 5. Continued

Sl. No.	Mathematical Definition	Significance
18	$\sum\beta'_{ns}$	$\Sigma\beta'_{ns} = \Sigma\beta_{ns}/N_v$. Summed β values for all the non-sigma bonds including lone electron pairs capable of resonance if any (VEM non-sigma contribution) relative to the number of vertices. Gives a measure of electron-richness of the molecule relative to the molecular size.
19	$\sum\beta$	$\Sigma\beta = \Sigma\beta_s + \Sigma\beta_{ns}$. Summation of β values of all non-hydrogen vertices in a molecule.
20	$\sum\beta'$	$\Sigma\beta' = \Sigma\beta/N_v$. Summed β values relative to the number of vertices. Gives a measure of electronic features of the molecule relative to the molecular size.
21	$\sum\beta_{ns(\delta)}$	A measure of lone electrons entering into resonance with an aromatic system.
22	$\sum\beta'_{ns(\delta)} = \sum\beta_{ns(\delta)}/N_v$	A measure of lone electrons entering into resonance relative to the molecular size.
23	$\Delta\beta = \Sigma\beta_{ns} - \Sigma\beta_s$	A measure of relative unsaturation content.
24	$\Delta\beta' = \frac{\Delta\beta}{N_v}$	A measure of relative unsaturation content with respect to the molecular size.
25	$\psi_1 = \frac{\sum\alpha}{\left[\sum\varepsilon\right]_{EH}} = \frac{\sum\alpha/N_v}{\varepsilon_2}$	A measure of hydrogen bonding propensity of the molecules and/or polar surface area.
26	$\Delta\psi_A = \left\langle 0.714 - \psi_1 \right\rangle$	A measure of hydrogen bonding propensity.
27	$\Delta\psi_B = \left\langle \psi_1 - 0.714 \right\rangle$	A measure of hydrogen bonding propensity.
28	$\eta = \sum_{i<j}\left[\frac{\gamma_i\gamma_j}{r_{ij}^2}\right]^{0.5}$	The composite index (η). Here r_{ij} stands for the topological distance between i^{th} atom and j^{th} atom. Accounts for the global topology of the molecule/fragment/substructure under investigation.
29	η'	$\eta' = \eta/N_v$. The composite index relative to molecular size.
30	$\eta_R = \left\{\sum_{i<j}\left[\frac{\gamma_i\gamma_j}{r_{ij}^2}\right]^{0.5}\right\}_R$	The composite index for reference alkane (η_R).
31	$\eta_F = \eta_R - \eta$	The functionality index (η_F). Gives a measure of the heteroatoms and multiple bonds.
32	η'_F	$\eta'_F = \eta_F/N_v$ Functionality index relative to the molecular size.
33	$\eta^{local} = \sum_{i<j, r_{ij}=1}(\gamma_i\gamma_j)^{0.5}$	The local composite index (η^{local}). Reflects the local topology of the analyzed structure since only bonded interactions are considered (r_{ij}=1).
34	$\eta_R^{local} = \left\{\sum_{i<j, r_{ij}=1}(\gamma_i\gamma_j)^{0.5}\right\}_R$	The local composite index for the corresponding reference alkane.

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Table 5. Continued

Sl. No.	Mathematical Definition	Significance
	$\eta_F^{local} = \eta_R^{local} - \eta^{local}$	Local functionality contribution.
35	$\eta_F'^{local}$	$\eta_F'^{local} = \eta_F^{local} / N_v$ Local functionality contribution relative to molecular size.
36	$\eta_B = \eta_N^{local} - \eta_R^{local} + 0.086 \times N_R$	ETA branching index (η_B), where N_R stands for the number of rings in the connected molecular graph of the reference alkane structure and η_N^{local} can be defined as: $\eta_N^{local} = 1.414 + (N_v - 3) \times 0.5$ when N_v>3, otherwise 0.
37	η'_B	$\eta'_B = \eta_B / N_v$. It is a measure of branching relative to molecular size.

$\sum\beta_{ns}$ = (Sum of β_{ns} values of all non-hydrogen vertices – Sum of all $\beta_{ns(\delta)}$ values of all vertices) / 2 + Sum of all $\beta_{ns(\delta)}$ values of all vertices

$\sum\beta_{ns(\delta)}$ = Sum of all $\beta_{ns(\delta)}$ values of all vertices

SAMPLE CALCULATION

The ETA indices are easily computed from the information of molecular composition and two dimensional representations. The topological aspects of the ETA formalism is enlightened using the hydrogen suppressed molecular graph based distance matrix, while parameters like shape, size, electronegativity, electron-richness, heteroatom content, unsaturation, hydrogen bonding propensity etc. involve chemical attributes of atoms and bonds. Since, such calculation does not require any spatial information or energy minimization operation, these indices for a large number of compounds can readily be computed. Table 6 shows sample calculations of two compounds namely 2-cholobenzaldehyde and 3-hydroxypyridine.

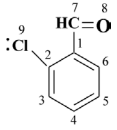
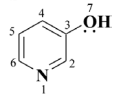
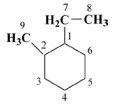
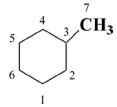
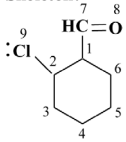
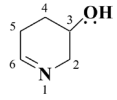
Computation of the ETA Indices

At the beginning, the ETA indices (first generations only) were computed using our in-house GW–BASIC programs namely *KRETA*1 and *KRETA*2 (GW-BASIC programs, 2003) that use distance matrix and VEM vertex count as inputs. Presently, two software platforms allow the computation of ETA indices namely Dragon (version 6) (Dragon version 6, 2010) and PaDEL-Descriptor (an open source software of the NUS, http://padel.nus.edu.sg/software/padeldescriptor/) (Yap, 2011). In Dragon (version 6), only first generation ETA indices can be computed while PaDEL-Descriptor allows the computation of all indices in this scheme. Table 7 presents the list of ETA indices corresponding to the notations used in Dragon (version 6) and PaDEL-Descriptor software.

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Table 6. Computation of ETA indices using two sample molecules 2-cholorobenzaldehyde and 3-hydroxypyridine

2-Cholorobenzaldehyde											3- Hydroxypyridine								
Structure (Arbitrary Numbering)	Atom Level Indices										Structure (Arbitrary Numbering)	Atom Level Indices							
	Index	Vertex (Arbitrary Numbering)										Index	Vertex (Arbitrary Numbering)						
		1	2	3	4	5	6	7	8	9			1	2	3	4	5	6	7
Normal Structure: 	α_i	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.333	0.714	**Normal Structure:** 	α_i	0.400	0.500	0.500	0.500	0.500	0.500	0.333
	ε_i	0.700	0.700	1.000	1.000	1.000	1.000	1.000	1.467	1.386		ε_i	1.100	1.000	0.700	1.000	1.000	1.000	1.767
	$[\beta_s]_i$	1.500	1.750	1.000	1.000	1.000	1.000	1.25	0.750	0.750		$[\beta_s]_i$	1.500	1.250	1.750	1.000	1.000	1.250	0.750
	$[\beta_{ns}]_i$	2.000	2.000	2.000	2.000	2.000	2.000	1.500	1.500	0.500		$[\beta_{ns}]_i$	2.000	2.000	2.000	2.000	2.000	2.000	0.500
	β_i	3.500	3.750	3.000	3.000	3.000	3.000	2.750	2.250	1.250		β_i	3.500	3.250	3.75	3.000	3.000	3.250	1.250
Reference Alkane: 	γ_i	0.143	0.133	0.167	0.167	0.167	0.167	0.182	0.148	0.571	**Reference Alkane:** 	γ_i	0.114	0.154	0.133	0.167	0.167	0.154	0.266
	$[\eta]_i$	0.875	0.886	0.783	0.720	0.718	0.777	0.757	0.519	1.036		$[\eta]_i$	0.500	0.587	0.665	0.627	0.594	0.548	0.574
	$[\alpha_R]_i$	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500		$[\alpha_R]_i$	0.500	0.500	0.500	0.500	0.500	0.500	0.500
	$[\varepsilon_R]_i$	1.000	1.000	1.300	1.300	1.300	1.300	1.300	1.600	1.600		$[\varepsilon_R]_i$	1.300	1.300	1.000	1.300	1.300	1.300	1.600
Saturated Carbon Skeleton: 	$[\beta_R]_i$	1.500	1.500	1.000	1.000	1.000	1.000	1.000	0.500	0.500	**Saturated Carbon Skeleton:** 	$[\beta_R]_i$	1.000	1.000	1.500	1.000	1.000	1.000	0.500
	$[\gamma_R]_i$	0.333	0.333	0.500	0.500	0.500	0.500	0.500	1.000	1.000		$[\gamma_R]_i$	0.500	0.500	0.333	0.500	0.500	0.500	1.000
	$[\eta_R]_i$	2.272	2.260	2.226	2.092	2.110	2.250	2.264	2.169	2.353		$[\eta_R]_i$	1.856	1.928	1.938	1.928	1.856	1.813	1.933
	$[\eta'_F]_i$	0.155	0.153	0.160	0.152	0.155	0.164	0.167	0.183	0.146		$[\eta'_F]_i$	0.194	0.192	0.182	0.186	0.180	0.181	0.194

Molecular Level Indices								Molecular Level Indices						
Index	Value	Index	Value		Index	Value		Index	Value	Index	Value	Index	Value	
$\sum\alpha$	4.547	η_R^{local}	4.343		$\Delta\varepsilon_C$	-0.119		$\sum\alpha$	3.233	η_R^{local}	3.394	$\Delta\varepsilon_C$	-0.115	
$\Sigma\alpha/N_v$	0.505	η_F^{local}	2.800		$\Delta\varepsilon_D$	0.000		$\Sigma\alpha/N_v$	0.462	η_F^{local}	2.321	$\Delta\varepsilon_D$	0.071	
$(\Sigma\alpha)_p/\Sigma\alpha$	0.230	η_N^{local}	4.414		$\sum\beta'_s$	0.556		$(\Sigma\alpha)_p/\Sigma\alpha$	0.103	η_N^{local}	3.414	$\sum\beta'_s$	0.607	
$(\Sigma\alpha)_\gamma/\Sigma\alpha$	0.220	η'_B	0.018		$\sum\beta'_{ns}$	0.889		$(\Sigma\alpha)_\gamma/\Sigma\alpha$	0.155	η'_B	0.015	$\sum\beta'_{ns}$	0.929	
$(\Sigma\alpha)_\chi/\Sigma\alpha$	0.000	ε_1	0.661		$\sum\beta'$	1.444		$(\Sigma\alpha)_\chi/\Sigma\alpha$	0.000	ε_1	0.631	$\sum\beta'$	1.536	
$\Delta\alpha_A$	0.005	ε_2	0.861		$\Delta\beta'$	0.333		$\Delta\alpha_A$	0.000	ε_2	0.867	$\Delta\beta'$	0.321	
$\Delta\alpha_B$	0.000	ε_3	0.433		$\sum\beta'_{ns(\delta)}$	0.056		$\Delta\alpha_B$	0.038	ε_3	0.433	$\sum\beta'_{ns(\delta)}$	0.071	
η	3.536	ε_4	0.553		ψ_1	0.586		η	2.048	ε_4	0.548	ψ_1	0.533	
η_R	9.998	ε_5	0.861		$\Delta\psi_A$	0.128		η_R	6.267	ε_5	0.796	$\Delta\psi_A$	0.181	
η'_F	0.718	$\Delta\varepsilon_A$	0.228		$\Delta\psi_B$	0.000		η'_F	0.654	$\Delta\varepsilon_A$	0.197	$\Delta\psi_B$	0.000	
η^{local}	1.543	$\Delta\varepsilon_B$	0.108					η^{local}	1.073	$\Delta\varepsilon_B$	0.083			

Table 7. List of ETA indices corresponding to the notations used in Dragon (ver. 6) and PaDEL-Descriptor software

Dragon (Version 6) Software Platform					
Sl. No.	**Notation**	**ETA Index**	**Sl. No.**	**Notation**	**ETA Index**
1	Eta_alpha	$\sum\alpha$	13	Eta_L	η^{local}
2	Eta_alpha_A	$\sum\alpha/N_v$	14	Eta_L_A	η^{local}/N_v
3	Eta_epsi	$\sum\varepsilon$	15	Eta_F	η_F
4	Eta_epsi_A	$\sum\varepsilon/N$	16	Eta_F_A	η'_F
5	Eta_betaS	$\sum\beta_s$	17	Eta_FL	η_F^{local}
6	Eta_betaS_A	$\sum\beta'_s$	18	Eta_FL_A	η'^{local}_F
7	Eta_betaP	$\sum\beta_{ns}$	19	Eta_B	η_B
8	Eta_betaP_A	$\sum\beta'_{ns}$	20	Eta_B_A	η'_B
9	Eta_beta	$\sum\beta$	21	Eta_sh_p	$(\sum\alpha)_p/\sum\alpha$
10	Eta_beta_A	$\sum\beta'$	22	Eta_sh_y	$(\sum\alpha)_Y/\sum\alpha$
11	Eta_C	η	23	Eta_sh_x	$(\sum\alpha)_X/\sum\alpha$
12	Eta_C_A	η/N_v			
PaDEL-Descriptor Software Platform					
Sl. No.	**Notation**	**ETA Index**	**Sl. No.**	**Notation**	**ETA Index**
1	ETA_Alpha	$\sum\alpha$	22	ETA_Beta_s	$\sum\beta_s$
2	ETA_AlphaP	$\sum\alpha/N_v$	23	ETA_BetaP_s	$\sum\beta'_s$
3	ETA_dAlpha_A	$\Delta\alpha_A$	24	ETA_Beta_ns	$\sum\beta_{ns}$
4	ETA_dAlpha_B	$\Delta\alpha_B$	25	ETA_BetaP_ns	$\sum\beta'_{ns}$
5	ETA_Epsilon_1	ε_1	26	ETA_dBeta	$\Delta\beta$
6	ETA_Epsilon_2	ε_2	27	ETA_dBetaP	$\Delta\beta'$
7	ETA_Epsilon_3	ε_3	28	ETA_Beta_ns_d	$\sum\beta_{ns(\delta)}$
8	ETA_Epsilon_4	ε_4	29	ETA_BetaP_ns_d	$\sum\beta'_{ns(\delta)}$
9	ETA_Epsilon_5	ε_5	30	ETA_Eta	η
10	ETA_dEpsilon_A	$\Delta\varepsilon_A$	31	ETA_EtaP	η/N_v
11	ETA_dEpsilon_B	$\Delta\varepsilon_B$	32	ETA_Eta_R	η_R
12	ETA_dEpsilon_C	$\Delta\varepsilon_C$	33	ETA_Eta_F	η_F
13	ETA_dEpsilon_D	$\Delta\varepsilon_D$	34	ETA_EtaP_F	η'_F
14	ETA_Psi_1	ψ_1	35	ETA_Eta_L	η^{local}
15	ETA_dPsi_A	$\Delta\psi_A$	36	ETA_EtaP_L	η^{local}/N_v
16	ETA_dPsi_B	$\Delta\psi_B$	37	ETA_Eta_R_L	η_R^{local}
17	ETA_Shape_P	$(\sum\alpha)_p/\sum\alpha$	38	ETA_Eta_F_L	η_F^{local}
18	ETA_Shape_Y	$(\sum\alpha)_Y/\sum\alpha$	39	ETA_EtaP_F_L	η'^{local}_F
19	ETA_Shape_X	$(\sum\alpha)_X/\sum\alpha$	40	ETA_Eta_B_RC	η_B
20	ETA_Beta	$\sum\beta$	41	ETA_EtaP_B_RC	η'_B
21	ETA_BetaP	$\sum\beta'$			

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AN OVERVIEW OF THE MODELED STUDIES

The ETA indices have been subjected to extensive chemometric modeling studies in order to judge their diagnostic potential since the inception. The initial studies reflected the predictive power of the first generation indices only, while after the development of the second generation indices, the application of both generation descriptors have been exemplified. The QSAR studies employing the ETA indices cover a wide variety of chemicals ranging from simple aliphatic hydrocarbons to complex drug molecules. Recently, the ETA indices have also been successfully employed to model relatively novel chemicals like 'ionic liquids'. Table 8 gives an overview of the *in silico* models reported so far using the ETA indices. It may be noted that in all the studies, various other topological non-ETA indices have been additionally used and the ETA indices have been found to produce models of better or comparable statistical qualities in comparison to the models from non-ETA indices. In many of the cases, the quality of the developed equations improved when ETA indices have been used in combination with other non-ETA parameters. Another observation was also very interesting during the modeling of non-specific toxicity endpoints when addition of computed lipophilicity parameter AlogP to the ETA descriptor pool gave better results. It may be noted that the non-specific toxicity of chemicals is largely encoded by the ETA bulk parameter that gives a measure of lipophilicity of the molecules. The toxicity shows either a proportionate or a parabolic relationship with molecular bulk. The toxicity is also influenced by molecular branchedness as encoded by the ETA branching and shape parameters. The ETA functionality parameters additionally show the positive effect of lipophilic heteroatoms like chlorine, bromine etc. and carbonyl oxygen group towards the toxicity. The latter factor is implicated to the Schiff base formation phenomenon with biological amines. The toxicity of chemicals is found to be negatively related to the presence of polar functional groups like hydroxy, methoxy, carboxy etc. In cases of ionic liquids, the toxicity is proportionately related to the chain length of the cationic alkyl chain. However, the same is not applicable in case of modeling AChE enzyme inhibition of eel where the head group is supposed to provide a specific spatial arrangement of the cation containing aromatic core and not for the ammonium or phosphonium core. The QSPR studies show the impact of the ETA molecular bulk parameter which relates with increased partitioning and decreased surfactancy behavior. The ETA parameters also show molecular branching and hydrogen bonding natures to be related with reduced octanol-water partitioning nature. The H-bonding phenomenon is largely attributed to the presence of polar groups like –OH, –O–, $–NH_2$, –NH– etc. However, the ETA H-bonding parameter ψ shows a negative contribution while modeling aqueous solubility of drug like compounds and agrochemicals and that has been justified by cohesive rigidity of such complex molecules due to intra- and inter-molecular H-bonding. The ETA indices for electron richness, branching and resonating lone electron pair are found to be correlated with aromatic substituent constants.

CONCLUSION AND FUTURE AVENUES

The aim of any scientific discipline lies in unraveling the reason hidden inside a system. Since, natural processes are defined by perspectives belonging to different disciplines, interdisciplinary studies are always helpful in deriving logical decisions. Predictive modeling studies aid in constituting rational basis for the physical, chemical as well as biological manifestations of chemical compounds. The chemistry suitably aided by concepts of mathematics and biology helps in unfolding the behavioral pattern of chemicals.

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*Table 8. An overview of the modeling studies performed employing ETA indices**

Sl. No.	Studied Relationship	Endpoint	Type of Chemicals	No. of Samples	Chemometric Operation	Representative Value of Metrics	Chemical Attributes Explored using ETA Indices	Ref.
1	QSTR	Toxicity towards *Tetrahymena pyriformis*	Substituted phenols	n_{total}=50	Principal component factor analysis, MLR	Q^2=0.945, R_a^2=0.950	The factors increasing the toxicity of phenols are molecular bulk, branching, η_R, η'_F, $\Sigma\beta'_{ns}$, ETA functionality of phenolic O.	Roy & Ghosh, 2003
2	QSTR	Toxicity towards fish	Substituted benzenes (phenols, anilines, other hydrocarbons)	n_{total}=92	Principal component factor analysis, MLR, all-possible-subsets regression.	R^2=0.885, R_a^2=0.876, Q^2=0.865, F (df) = 92.6 (7, 84), s=0.230	The positive contribution of molecular bulk, presence of chloro, hydroxy, methyl and nitro substituents. Presence of fluoro, ether functionality, amino or nitro functionality in an otherwise unsubstituted ring, nitro group ortho to choloro reduces toxicity.	Roy & Ghosh, 2004a
3	QSTR	Toxicity towards *Tetrahymena pyriformis*	Nitroaromatic compounds	n_{total}=42	Principal component factor analysis, MLR	R^2=0.920, R_a^2=0.910, Q^2=0.880, F (df) = 101.4 (4, 37), s=0.22	Molecular bulk (size), halogen and additional nitro group in the system increases toxicity while toxicity gets decreased by the presence of methyl and hydroxymethyl substituent.	Roy & Ghosh, 2004b
4	QSTR	Acute Toxicity towards *Vibrio fischeri*	Phenylsulfonyl carboxylates	n_{total}=56	Principal component factor analysis, MLR	R^2=0.852, R_a^2=0.837, Q^2=0.726, F (df) = 57.4 (5, 50), s=0.186	The negative impact of steric bulk, branching, chloro substituent, unsaturation, electronegative atoms towards toxicity.	Roy & Ghosh, 2004c
5	QSTR	Acute Toxicity towards *Vibrio fischeri*	Phenylsulfonyl carboxylates	n_{total}=56	GFA	R^2=0.873, R_a^2=0.861, Q^2=0.771, F (df) = 69.0 (5, 50), s=0.172	The parameters reducing toxicity are steric bulk, functionality contribution, chloro group, nitro group.	Roy & Ghosh, 2005
6	QSTR	Acute Toxicity towards *Rana japonica*	Benzene derivatives	n_{total}=51	GFA, FA, MLR, PCRA	R^2=0.915, R_a^2=0.901, Q^2=0.847, F (df) = 65.841 (7, 43), s=0.183	Toxicity is parabolically related to molecular size, increases with the presence of chloro substituent, while gets reduced due to the occurrence of groups like methoxy, hydroxy, carboxy, amino.	Roy & Ghosh, 2006a
7	QSTR	Nonspecific toxicity towards *Saccharomyces cerevisiae*	Substituted benzene derivatives	n_{total}=51	FA, PCA, stepwise MLR, PLS	R^2=0.884, R_a^2=0.874, Q^2=0.851, F (df) = 87.9 (4, 46), s=0.235	The negative contributions of amino & carboxylic acid functionalities on the benzene ring and occurrence of electronegative atom towards the toxicity while positive contributions of presence of chloro group and molecular branchedness.	Roy & Sanyal, 2006
8	QSPR	*n*-Octanol/water partition coefficient	Non-ionic organic compounds	n_{total}=122; n_{train}=92, n_{test}=30	*k*-means clustering, principal component factor analysis, MLR, PLS, stepwise MLR, PCRA	Total: R^2=0.960, Q^2=0.953 Divided: R^2=0.974, Q^2=0.970, R^2_{pred}=0.906	Partion coefficient increases in the presence of molecular bulk and degree of halogen substitution while hydrogen bonding or polar interaction reduces it.	Roy et al., 2006a
9	QSPR	Bioconcentration factors in fish	Non-ionic organic compounds	n_{total}=122	Principal component factor analysis, MLR, PLS, stepwise MLR, PCRA, *k*-means clustering	R^2=0.948, R_a^2=0.944, Q^2=0.847, F (df) = 254.9 (8, 113), s=0.362	Presence of nitro, amino, hydroxyl groups reduces BCF, while it increases by branchedness, molecular volume, and chloro substituents.	Roy et al., 2006b

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Table 8. Continued

Sl. No.	Studied Relationship	Endpoint	Type of Chemicals	No. of Samples	Chemometric Operation	Representative Value of Metrics	Chemical Attributes Explored using ETA Indices	Ref.
10	QSTR	Inhibition of seed germination rate of *Cucumis sativus*	Substituted phenols	n_{total}=41	Stepwise MLR, FA-MLR, GFA-MLR, G/PLS, FA-PLS, PCRA	R^2=0.825, R_a^2=0.811, Q^2=0.719, PRESS=2.065	Toxicity proportionately increases in presence of branching, unsaturation and groups like nitro, cholo, bromo while functionalities namely hydroxyl, carboxy, methoxy, ortho-methyl exerts negative impacts. Furthermore, toxicity was also found to possess a non-linear relationship with ETA volume parameter $\sum\alpha$.	Roy & Ghosh, 2006b
11	QSTR	Toxicity towards *Chlorella vulgaris*	Diverse functional organic compounds	n_{total}=91, n_{train}=68, n_{test}=23	*k*-means clustering, stepwise MLR, FA-MLR, PLS, PCRA	Total: R^2=0.928, R_a^2=0.925, Q^2=0.913, F (*df*) = 275.29 (4, 86) Divided: R^2=0.929, Q^2=0.900, R^2_{pred}=0.870	Molecules possessing higher amount of molecular bulk ($\sum\alpha$), electron richness ($\sum\beta'_{ns}$) and lipophilic substituents like chloro or bromo are more toxic.	Roy & Ghosh, 2007
12	QSTR	Human toxicity data (blood/serum conc.)	Organic compounds including pharmaceuticals	n_{total}=26, n_{train}=19, n_{test}=7	Stepwise MLR, *k*-means clustering, FA-MLR, GFA, PLS, G/PLS, PCRA	Total: R^2=0.903, R_a^2=0.895, Q^2=0.834, F (*df*) = 106.88 (2, 23) Divided: R^2=0.724, Q^2=0.639, R^2_{pred}=0.655	Molecular bulk, cholo group (lipophilicity), heteroatom within a chain or ring, unsaturation increase the toxicity while hydroxyl group and branching reduce it.	Roy & Ghosh, 2008
13	QSTR	Cytotoxicity in rat hepatocytes	NSAIDs	n_{total}=15	Stepwise MLR, PCRA, PLS, GFA, G/PLS	R^2=0.919, R_a^2=0.906, Q^2=0.854, F (*df*) = 67.92 (2, 12)	Toxicity increases with molecular bulk and degree of branching. Unsaturation and heteroatom content have additional impact on toxicity.	Roy & Ghosh, 2009a
14	QSTR	Toxicity towards *Tetrahymena pyriformis*	Diverse aromatic compounds	n_{total}=384, n_{train}=288, n_{test}=96	Stepwise MLR, *k*-means clustering, FA-MLR, PLS,	Divided: R^2=0.854, R_a^2=0.846, Q^2=0.821, R^2_{pred}=0.679	The parameters important for the prediction of toxicity are molecular bulk, halogen functionality representing lipophilicity and nitrogen containing functionality giving an account of polarity.	Roy & Ghosh, 2009b
15	QSTR	hERG K^+ channel blocking activity	Diverse functional drugs	n_{total}=67, n_{train}=50, n_{test}=17	Stepwise MLR, *k*-means clustering, FA-MLR, PLS	Divided: R^2=0.658, R_a^2=0.619, Q^2=0.540, R^2_{pred}=0.617	Electron-richness and volume increase hERG K^+ channel blocking activity while groups/fragments containing aliphatic nitrogen atom or –COOH reduce it. The parameter $\sum\alpha$ additionally showed a parabolic relationship with the modeled toxicity endpoint.	Roy & Ghosh, 2009c
16	QSTR	Toxicity towards *Tetrahymena pyriformis*	Aromatic aldehydes	n_{total}=77, n_{train}=50, n_{test}=17	Stepwise MLR, GFA, G/PLS	R^2=0.895, Q^2=0.870, R^2_{pred}=0.902, $r^2_{m\ (test)}$=0.896	Molecular bulk, electronegative substituents, lipophilic substituent increase the toxicity of aromatic aldehydes. The keto oxygen in aldehyde group also imparts their effect towards toxicity.	Roy & Das, 2010

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Table 8. Continued

Sl. No.	Studied Relationship	Endpoint	Type of Chemicals	No. of Samples	Chemometric Operation	Representative Value of Metrics	Chemical Attributes Explored using ETA Indices	Ref.
17	QSPR	Octanol-water partition coefficient	Diverse functional organic compounds	n_{total}=168, n_{train}=84, n_{test}=84	Stepwise MLR, PLS	Total (PLS): R^2=0.943, R_a^2=0.940, Q^2=0.933 Divided (PLS): R^2=0.922, Q^2=0.903, R^2_{pred}=0.919	Lipophilicity increases with molecular bulk while it tends to decrease in the presence of branching, relative unsaturation content and hydrogen bonding propensity measure.	Roy & Das, 2011a
	QSPR	Octanol-water partition coefficient	Aliphatic and aromatic compounds	n_{total}=139, n_{train}=70, n_{test}=69		Total (PLS): R^2=0.992, R_a^2=0.991, Q^2=0.989 Divided (PLS): R^2=0.988, Q^2=0.984, R^2_{pred}=0.972	Different electronic features, unsaturation and shape parameters were important for the partition coefficient of aliphatic and aromatic compounds.	Roy & Das, 2011a
	QSPR	Aqueous solubility	Aliphatic and aromatic compounds	n_{total}=193, n_{train}=97, n_{test}=96		Total (PLS): R^2=0.951, R_a^2=0.948, Q^2=0.937 Divided (PLS): R^2=0.918, Q^2=0.897, R^2_{pred}=0.872	Hydrogen bonding propensity and H-bond donor atoms were observed to be the important features for modeling aqueous solubility. However, relative unsaturation content and branchedness were also contributing.	Roy & Das, 2011a
	QSPR	Molar refractivity (R_m)	Aliphatic and aromatic compounds	n_{total}=166, n_{train}=83, n_{test}=83		Total (PLS): R^2=0.990, R_a^2=0.990, Q^2=0.989 Divided (PLS): R^2=0.980, Q^2=0.978, R^2_{pred}=0.993	Electronegativity, molecular bulk and hydrogen bonding propensity were contributing factors towards modeling of molar refraction of chemicals.	Roy & Das, 2011a
	QSPR	Aromatic substituent constant π	Aromatic substituents	n_{total}=98, n_{train}=50, n_{test}=48		Total (PLS): R^2=0.770, R_a^2=0.755, Q^2=0.718 Divided (PLS): R^2=0.795, Q^2=0.736, R^2_{pred}=0.540	Measure of H-bond acceptor atoms or polar surface area, H-bond donor atoms, H-bonding propensity, electron-richness, electronegativity, molecular bulk and branching were the contributing parameters for modeling π.	Roy & Das, 2011a
	QSPR	Aromatic substituent constant MR	Aromatic substituents	n_{total}=99, n_{train}=50, n_{test}=49		Total (PLS): R^2=0.986, R_a^2=0.985, Q^2=0.979 Divided (PLS): R^2=0.977, Q^2=0.967, R^2_{pred}=0.965	MR was related to the impact of molecular bulk, relative unsaturation, electron richness, electronegativity, H-bonding atom count, H-bonding propensity, resonating lone electron pair and branching of the molecules.	Roy & Das, 2011a
	QSPR	Aromatic substituent constant σ_m	Aromatic substituents	n_{total}=97, n_{train}=49, n_{test}=48		Total (PLS): R^2=0.766, R_a^2=0.758, Q^2=0.735 Divided (PLS): R^2=0.750, Q^2=0.677, R^2_{pred}=0.736	σ_m is encoded by shape parameter, electronegativity, relative unsaturation content, resonating lone electron pair, and measure of non-hydrogen bonding bulky heteroatom.	Roy & Das, 2011a

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Table 8. Continued

Sl. No.	Studied Relationship	Endpoint	Type of Chemicals	No. of Samples	Chemometric Operation	Representative Value of Metrics	Chemical Attributes Explored using ETA Indices	Ref.
	QSPR	Aromatic substituent constant σ_p	Aromatic substituents	n_{total}=99, n_{train}=50, n_{test}=49		Total (PLS): R^2=0.726, R_a^2=0.717, Q^2=0.682 Divided (PLS): R^2=0.670, Q^2=0.582, R^2_{pred}=0.585	σ_p can be described in terms of molecular branching, electronegativity, resonating lone electron pair, relative unsaturation content and molecular bulk.	Roy & Das, 2011a
18	QSTR	Toxicity towards *Pimephales promelas*	Diverse organic chemicals	n_{total}=459, n_{train}=344, n_{test}=115	*k*-means clustering, Stepwise MLR, GFA	Divided (GFA): R^2=0.763, Q^2=0.751, R^2_{pred}=0.783, $r^2_{m\ (test)}$=0.777	Apart from the positive impact of the computed lipophilicity measure, measures of molecular bulk, electron richness, relative unsaturation content, and branchedness are necessary attributes.	Roy & Das, 2012
19	QSPR	Critical micelle concentration (CMC)	Non-ionic surfactants	n_{total}=54, n_{train}=41, n_{test}=13	Stepwise MLR, GFA, PLS	Divided (PLS): R^2=0.907, Q^2=0.879, R^2_{pred}=0.923, $r^2_{m\ (test)}$=0.912	logCMC value increases with molecular shape, heteroatom content while molecular bulk and branching tends to reduce it.	Roy & Kabir, 2012a
21	QSPR	Critical micelle concentration (CMC)	Cationic surfactants	n_{total}=35, n_{train}=26, n_{test}=9	*k*-means clustering, stepwise MLR, GFA, ANN	Divided (GFA): R^2=0.949, Q^2=0.893, R^2_{pred}=0.904, $r^2_{m\ (test)}$=0.892	Hydrogen bond donor atoms, molecular size and degree of unsaturation affect the CMC of cationic surfactants.	Roy & Kabir, 2012b
20	QSPR	Critical micelle concentration (CMC)	Anionic surfactants	n_{total}=37, n_{train}=28, n_{test}=9	Stepwise MLR, GFA, ANN, PCA	Divided (GFA): R^2=0.957, Q^2=0.938, R^2_{pred}=0.923, $r^2_{m\ (test)}$=0.823	Electronegativity and topological environment impart negative contribution, while lipophilicity and branching show positive impact towards CMC of anionic surfactants.	Roy & Kabir, 2013
22	QSPR	Aqueous solubility	Drug like molecules and agrochemicals	n_{total}=565, n_{train}=282, n_{test}=283	Stepwise MLR, GFA, G/PLS	Divided (G/PLS): R^2=0.802, Q^2=0.793, R^2_{pred}=0.806, $r^2_{m\ (test)}$=0.735	Lipophilicity, unsaturation, hydrogen bonding propensity and polar surface area measure are the responsible chemical features for logS.	Das & Roy, 2013
23	QSTR	Toxicity towards rodent and interspecies toxicity correlation among daphnids, fish and algae.	Pharmaceuticals	Rodent: n_{train}=102, n_{test}=34 Daphnia-algae: n_{train}=65, n_{test}=24 Fish-algae: n_{train}=50, n_{test}=22	Stepwise MLR, PLS	**Rodent:** R^2=0.618, Q^2=0.556, R^2_{pred}=0.550 **Daphnia-algae:** R^2=0.650, Q^2=0.580, R^2_{pred}=0.604 **Fish-algae:** R^2=0.752, Q^2=0.654, R^2_{pred}=0.696	Rodent toxicity is chiefly influenced by the charge distribution and heteroatom atom count. Interspecies correlation models show the influence of different atom-type AlogP fragments, molecular shape, charge distribution and functionality measure.	Das et al., 2013

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Table 8. Continued

Sl. No.	Studied Relationship	Endpoint	Type of Chemicals	No. of Samples	Chemometric Operation	Representative Value of Metrics	Chemical Attributes Explored using ETA Indices	Ref.
24	QSTR	Ecotoxicity towards *Vibrio fischeri*	Diverse ionic liquids	LDA: n_{train}=110, n_{test}=37 MLR: n_{train}=90, n_{test}=36	*k*-means clustering, LDA, stepwise MLR, GFA	**LDA:** Wilk's λ=0.298, R_c=0.838, MCC_{trn}=0.841 **MLR:** R^2=0.694, Q^2=0.651, $\overline{r^2_{m\,(test)}}$ R^2_{pred}=0.739, =0.623, $\Delta r^2_{m\,(test)}$=0.156	Branching, molecular size and solvation entropy of cations along with a lipophilicity contribution of the anions are the contributing chemical attributes for eco-toxicity of ILs towards *V. fischeri*.	Das & Roy, 2012
25	QSTR	Toxicity towards *Daphnia magna*	Diverse ionic liquids	LDA: n_{train}=46, n_{test}=16 MLR: n_{train}=35, n_{test}=14	*k*-means clustering, LDA, stepwise MLR, PLS	**LDA:** Wilk's λ=0.257, R_c=0.862, MCC_{trn}=0.867, AUC-ROC=$0.991_{(trn)}$ & $1.0_{(test)}$ **MLR:** R^2=0.948, Q^2=0.875, $\overline{r^2_{m\,(test)}}$ R^2_{pred}=0.817, =0.802, $\Delta r^2_{m\,(test)}$=0.099	Lipophilicity, volume of heteroatoms electronegativity, presence of long cationic side chains, terminal branches and branching pattern at terminal atoms as well as at the carbon atom nearer to heteroatomic substitution are the contributing chemical features.	Roy & Das, 2013
26	QSTR	Ecotoxicity towards *Scenedesmus vacuolatus*	Diverse ionic liquids	LDA: n_{train}=42, n_{test}=18 MLR: n_{train}=35, n_{test}=14	LDA, stepwise MLR, GFA, *k*-means clustering	**LDA:** Wilk's λ=0.468, R_c=0.729, MCC_{trn}=0.802, AUC-ROC=$0.918_{(trn)}$ & $1.0_{(test)}$ **MLR:** R^2=0.883, Q^2=0.829, $Q^2_{(F1)}$=0.893, $\overline{r^2_{m\,(test)}}$ $Q^2_{(F2)}$=0.891, =0.796	Presence of bulky and multiple bonded heteroatom causing nucleophilic addition reaction is responsible for the toxicity. Long alkyl side chain on cationic N atom also increases toxicity. Toxicity can decrease by incorporation of H-bond donor group like hydroxyl (−OH).	Das & Roy, 2014a

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Table 8. Continued

Sl. No.	Studied Relationship	Endpoint	Type of Chemicals	No. of Samples	Chemometric Operation	Representative Value of Metrics	Chemical Attributes Explored using ETA Indices	Ref.
27	QSTR	Inhibition of *Electrophorus electricus* AChE enzyme	Diverse ionic liquids	LDA: n_{train}=182, n_{test}=110 MLR: n_{train}=148, n_{test}=84	*k*-means clustering, LDA, stepwise MLR, GFA, PLS, molecular docking	**LDA:** Wilk's λ=0.374, R_c=0.791, MCC_{trn}=0.802, AUC-ROC=0.959$_{(trn)}$ & 0.914$_{(test)}$ **MLR:** R^2=0.838, Q^2=0.808, $Q^2_{(F1)}$=0.822, $RMSE_p$=0.248, $\overline{r^2_{m\,(test)}}$=0.762	Increased toxicity is caused by longer alkyl chain length on N atom providing proper spatial conformation, presence of quaternary N atom in ring allowing π-cationic interaction, and distributed electron density in cations due to resonating electrons. Lipophilicity of anion additionally account for the toxicity, *i.e.*, increased enzyme inhibition. However, presence of long chains on free N^+/P^+ causes reduction of the toxicity due to free rotation of chains causing binding hindrance. Toxicity also reduces due to the presence of H-bond donors & acceptors blocking partitioning of molecule.	Das & Roy, 2014b
28	QSPR	Adsorption	Organic compounds	n_{total}=3483, n_{train}=2613, n_{test}=870	*k*-means clustering, PCA, stepwise MLR, PLS	Divided: R^2=0.815, Q^2=0.806, $\overline{r^2_{m\,(LOO)}}$=0.722, $Q^2_{(F1)}$=0.791, $\overline{r^2_{m\,(test)}}$=0.719	The adsorption behavior is characterized by molecular volume, H-bond donor groups, heteroatomic contribution, electronegativity, shape, H-bonding propensity and unsaturation.	Ray & Roy, 2013
29	QSPR	Bio-concentration factor	Diverse chemicals	n_{total}=522, n_{train}=324, n_{test}=198	*k*-means clustering, GFA, PLS	Divided: R^2=0.614, R_a^2=0.611, Q^2=0.597, R^2_{pred}=0.696, $\overline{r^2_{m\,(test)}}$=0.580	Occurrence of fused ring systems and halogen atoms exert positive contribution while electronegativity, H-bond donor groups, polarity and lowered partition coefficient have negative impact on BCF.	Pramanik & Roy, 2014
30	QSPR	Odor threshold	Aliphatic alcohols	n_{total}=53, n_{train}=42, n_{test}=11	*k*-means clustering, GFA, G/PLS	Divided: R^2=0.809, Q^2=0.778, R^2_{pred}=0.813, $\overline{r^2_{m\,(test)}}$=0.679	Odor threshold is potentiated by increased hydrophobicity and reduced electronegativity.	Pal et al., 2014

*Full form of some abbreviations used: **MLR:** Multiple linear regression; **GFA:** Genetic function approximation; **FA:** Factor analysis; **PCA:** Principal component analysis; **PCRA:** Principal-component regression analysis; **PLS:** Partial least squares; **LDA:** Linear discriminant analysis.

Mathematical descriptors are the logical basis for the exploration of the chemical fraternity. Since the introduction of predictor variables, a long way has already been traversed in depicting the chemical behavior and it has been envisaged that theoretically derived chemical descriptors are more than just simple quantification of chemical structures to derive correlations. Since, there is an amalgamation of chemical principles with mathematical algorithms, care should be taken that the encoded information is also helpful in deriving mechanistic interpretation instead of developing only correlations with the endpoint under investigation.

There is also a good amount of arguments regarding the dimensionality of analysis during the development of predictive correlation models. It is quite obvious that two-dimensional analyses are relatively easier to perform and give reproducible results. However, the molecular properties and interactions are also believed to be affected by features like shape, surface area and volume which are three dimensional. Hence, it becomes very interesting if a theoretical basis is generated to incorporate information of three-dimensional perspectives in two-dimensional indices. It may be observed that there is a good correlation and inheritance between these two and three- dimensional features since the features like size, shape, volume etc. are in turn controlled by the property of the individual atoms and bonds constituting a molecule. The three-dimensional geometry is also referred to as molecular topography (Kier & Hall, 1986) and studies have shown the occurrence of quantum chemical basis (Stankevich et al., 1995) as well as Hückel's molecular orbital (Gálvez et al., 1998) features in the topological parameters rendering them reliable predictor parameters in extracting powerful chemical information.

The ETA indices are enriched since they do not rely only on topological distance based measures of hydrogen suppressed molecular graphs. This group of parameters are advantageous as they encode suitable chemical information on different aspects of molecular chemistry, *e.g.*, volume, shape, size, branchedness, polarity, electronegative, electron-richness and hydrogen bonding property and also consider contribution of active hydrogen atoms. So, the ETA indices consider more than just graph theoretical aspects, employing detailed information on the atoms, bonds and the connectivity therein. Technically it will be difficult to assume any single descriptor to encode universal chemical features. Different aspects of chemical information are expressed in modeling different endpoints. In other words, endpoint like aqueous solubility or partition coefficient can be explained by molecular size, branchedness or hydrogen bonding aspects while electronic features and polarity information of a molecule may be of importance while developing predictive models against molar refractivity. The ETA formalism attempts to provide a platform of different chemical attributes so as to enable the designer with broader choice of predictor variables within a single category (Roy & Das, 2011b; Roy & Ghosh, 2010). The ETA parameters are also appended with dynamic chemical foundation suitable for deriving mechanistic basis. Moreover, all the studies performed till date using the ETA indices infer that they contain sufficient diagnostic potential for the development of predictive quantitative models for various classes of chemicals namely aliphatic compounds, aromatic compounds, agrochemicals, drugs and pharmaceuticals, ionic liquids etc. encompassing industrial, laboratory and house hold usage. Like every successful innovation walks through the development of new ideas and concepts, the ETA indices have followed a path of novel chemical exploration and we shall look forward to more nourishment of enriched chemical information in future.

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KEY TERMS AND DEFINITIONS

2D-Descriptor: A method where the considered chemical information corresponds to two-dimensional geometry of the molecule.

Chemical Graphs: A method of presenting chemical structures using a "hydrogen-suppressed" molecular skeleton where 'atoms' are denoted by 'vertices' and 'bonds' are represented by 'edges'.

Descriptor: A numerical quantity for the depiction of molecular structure using a suitable algorithm. Used as predictor variables in QSAR modeling analysis.

Extended Topochemical Atom (ETA) Indices: Indices where the topological information has been enriched using suitable electronic, heteroatom count & type, hydrogen-bonding and other essential chemical attributes. These are used as descriptors in QSAR modeling.

Hydrogen-Suppressed: A graph theoretical method of representing chemical structures where the hydrogen atoms attached to carbon and other atoms are not shown. Also termed as hydrogen-depleted.

QSAR Analysis: A method of developing mathematical correlation between a response and descriptors, *i.e.*, predictor variables for a series of chemical data.

Topology: A method assessing chemical structures using distance and connectivity information considering hydrogen-suppressed molecular graph. The distance corresponds to minimum path between vertices.

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Chapter 39
Importance of Applicability Domain of QSAR Models

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ABSTRACT

Quantitative Structure-Activity Relationship (QSAR) models have manifold applications in drug discovery, environmental fate modeling, risk assessment, and property prediction of chemicals and pharmaceuticals. One of the principles recommended by the Organization of Economic Co-operation and Development (OECD) for model validation requires defining the Applicability Domain (AD) for QSAR models, which allows one to estimate the uncertainty in the prediction of a compound based on how similar it is to the training compounds, which are used in the model development. The AD is a significant tool to build a reliable QSAR model, which is generally limited in use to query chemicals structurally similar to the training compounds. Thus, characterization of interpolation space is significant in defining the AD. An attempt is made in this chapter to address the important concepts and methodology of the AD as well as criteria for estimating AD through training set interpolation in the descriptor space.

INTRODUCTION

Quantitative structure-activity relationship (QSAR) modelling has become an important tool in the new drug candidate design, environmental fate modeling, toxicity and property prediction of chemicals and pharmaceuticals since they offer an economical and time-effective alternative to the medium throughput *in vitro* and low throughput *in vivo* assays (Perkins *et al*., 2003; Selassie, 2003; Walker et al., 2003). A QSAR model is a simple mathematical equation that is evaluated from a set of molecules with known activities/properties/toxicities using computational approaches. Thereafter, the developed predictive QSAR models are also applied by regulatory agencies to evaluate physical, chemical, and biological properties of individual chemical entities using applications specific for decision-making frameworks in risk and safety assessments (Kar & Roy, 2010). The QSAR modeling hypothesis also supports the 3Rs

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(replacement, refinement and reduction in animals in research) paradigm due to an increased pressure from social and economic background to trim down the use of animal testing as an important alternative method for future prediction of untested chemical entities (Benigni & Giuliani, 2003).

Robust validation of QSAR models plays a key step for the selection of a predictive model that may be considered for future prediction of new molecules. As numerous numbers of researches have been directed to the design of new molecules with the utilization of QSAR technique, validation of a QSAR model has been certified as the most considerable stride (Carlsen *et al*., 2009) for assessing the quality of data, applicability and mechanistic interpretability of the developed model. Thus, a huge number of investigations are currently directed towards the introduction of more appropriate validation approaches for more accurate and predictive QSAR model development. One important objective of QSAR modeling is to predict activity/property/toxicity of new chemical entities falling within the applicability domain of the developed models. The reliability of any QSAR model depends on the confident predictions of these new compounds based on applicability domain (AD) of the modeled compounds and therein arises the importance of the AD study (Ferreira, 2001).

In order to establish the scientific validity of a QSAR model and to facilitate its acceptance for regulatory purposes, the Organization for Economic Cooperation and Development (OECD) in its joint meeting (OECD, 2007) has agreed to five principles that should be followed during the construction of QSAR models. The OECD Principle 3 which defines the need of an AD expresses the fact that QSARs are unavoidably associated with restrictions in terms of the types of chemical structures, physicochemical properties and mechanisms of action for which the models can generate reliable predictions. The applicability domain of a QSAR model has been defined as the response and chemical structure space, characterized by the properties of the molecules in the training set. The developed QSAR model can predict a new compound truly only when it falls within the applicability domain of the developed model (Netzeva *et al*., 2005). Thus, to identify the interpolation (true prediction) or extrapolation (less reliable prediction) of query compounds is an important task for a QSAR model developer using the information of applicability domain (Netzeva *et al*., 2005).

Viewing the importance of AD in QSAR model validation, we herein focus to get an overview of different traditional as well as relatively new AD approaches used to judge the quality of the QSAR models. This book chapter will be helpful for the QSAR learners in order to have a clear idea on the principles of available AD approaches useful for judging the predictive quality of QSAR models.

BACKGROUND

A QSAR model is essentially valued in terms of its predictability, indicating how well it is able to predict the endpoint values of the compounds which are not used to develop the correlation. The models that have been suitably validated internally and externally can be considered reliable for both scientific and regulatory purposes (Golbraikh & Tropsha, 2002; Tong *et al*., 2004]. QSAR models should be validated according to the OECD principles for reliable prediction. A meeting of QSAR experts held in Setúbal, Portugal in March 2002 formulated guidelines for the validation of QSAR models, in particular for regulatory purposes (Jaworska *et al*., 2003). These principles were agreed by OECD member countries, QSAR and regulatory communities at the 37th Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology in November 2004. These principles are best pos-

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sible summary of the most important points that are required to be addressed to find consistent, reliable and reproducible QSAR models (OECD, 2004). Five OECD principles to develop reliable models are:

Principle 1: A defined endpoint;
Principle 2: An unambiguous algorithm;
Principle 3: A defined domain of applicability;
Principle 4: Appropriate measures of goodness-of fit, robustness and predictivity;
Principle 5: A mechanistic interpretation, if possible (OECD, 2004).

In order to provide guidance on the interpretation of the mentioned principles, the OECD has also provided a checklist (OECD, 2004). Thus, the current challenge in the process of development of a QSAR model is no longer in developing a model that is statistically sound to predict the activity within the training set, but in developing a model with the capability to predict accurately the activity of untested chemicals. In spite of high fitted accuracy and apparent mechanistic appeal, some published QSAR models fail rigorous validation tests, and, thus, may lack practical utility as reliable screening tools (Tropsha *et al*., 2003]. In summary, predictive power is one of the most important validation features of QSAR models. It can be defined as the ability of a model to predict accurately the target activity/property/toxicity of compounds that are not used for model development.

In this context, QSAR model predictions are most reliable if they come from the model's applicability domain (AD) which is broadly defined under OECD principle 3. The OECD includes AD assessment as one of the QSAR acceptance criteria for regulatory purposes (OECD, 2004; OECD, 2007). The Setubal Workshop report (Jaworska et al., 2003) presented the following regulation for AD assessment: "The applicability domain of a (Q)SAR is the physico-chemical, structural, or biological space, knowledge or information on which the training set of the model has been developed, and for which it is applicable to make predictions for new compounds. The applicability domain of a (Q)SAR should be described in terms of the most relevant parameters, i.e., usually those that are descriptors of the model. Ideally the (Q)SAR should only be used to make predictions within that domain by interpolation not extrapolation. This depiction is useful for explaining the instinctive meaning of the "applicability domain" approach.

Two general principles to AD estimation have been proposed till date (Nikolova-Jeliazkova & Jaworska, 2005). The first one estimates the interpolation region based on the training set in the model's descriptor space. The second approach relies on similarity analysis according to the premise that a QSAR prediction is reliable if the compound is "similar" to the compounds in the training set. It is interesting to point out that "similarity" is a relative concept as different concepts of similarity are relevant to different endpoints. The similarity approach to AD assessment should be based on measurable key features relevant to the endpoint modelled precisely (Bender & Glen, 2004; Nikolova & Jaworska, 2004).

APPLICABILITY DOMAIN (AD)

The AD (Eriksson *et al*., 2003; Gramatica, 2007; Netzeva *et al*., 2005, Tetko *et al*., 2006) is a theoretical region in chemical space encompassing both the model descriptors and modeled response. In the development of a QSAR model, the domain of applicability of molecules plays a crucial role for estimating the uncertainty in the prediction of a particular compound based on how similar it is to the compounds

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used to build the model. Thus, the prediction of a modeled response using QSAR is valid only if the compound being predicted falls within the domain of applicability of the model since it is impossible to predict an entire universe of chemicals using a single QSAR model. Again, AD can be described as the physico-chemical, structural or biological space, knowledge or information based on which the training set of the model is developed, and the model is applicable to make predictions for new compounds within the specific domain (Weaver & Paul Gleeson, 2008).

The selection method of the training and test sets has a significant effect on the applicability domain of constructed QSAR model. Thus, while splitting a dataset for external validation, the training set molecules should be selected in such a way that they span the entire chemical space for all the dataset molecules. In order to achieve successful predictions, a QSAR model should always be used for compounds within its applicability domain. Therefore, one can arguably agree that if a new compound falls outside of the AD of the training set molecules, its prediction is not reliable.

APPLICABILTY DOMAIN APPROACHES

There are various AD approaches that have been used by different groups of QSAR researchers worldwide. The most common approaches for estimating interpolation regions in a multivariate space include the following ones (Jaworska *et al*., 2005; Netzeva *et al*., 2005):

1. Ranges in the descriptor space.
2. Geometrical methods.
3. Distance-based methods.
4. Probability density distribution.
5. Range of the response variable.

The first four approaches are based on the methodology used for interpolation space characterization in the model descriptor space. On the contrary, the last one depends solely on response space of the training set molecules. A compound can be identified as out of the domain of applicability in a simple way, if:

1. At least one descriptor is out of range for the ranges approach, and
2. The distance between the chemical and the center of the training data set exceeds the threshold for distance approaches.

The threshold for all kinds of distance methods is the largest distance between the training set data points and the center of the training data set.

Along with the common approaches, Stanforth *et al*., (2007) proposed a cluster-based approach to evaluate the AD of any QSAR model. The method applies an intelligent version of the *k*-means clustering algorithm for modeling the training set as a compilation of clusters in the descriptors space. On the other hand, the test compounds of individual clusters are assigned a fuzzy membership from which an overall distance may be calculated to identify the AD of individual test compounds. A classification-based process involves calculation of regression residuals for identification of good and bad classes suggested by Guha & Jurs (2005).

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All approaches are discussed thoroughly in this section enlightening their main theories to define the interpolation space as well as the threshold criteria for AD used in QSAR modeling. A summary of hypotheses of data distribution and interpolation space of most commonly used AD methods is discussed in Table 1.

Table 1. Summary of hypothesis of most commonly used AD methods

Method		Formula/Equation	Hypothesis on Data Distribution	Shape of the AD Space
Range based		$d(x,y) = x–y$	Uniform	Boundary created by individual descriptor of the training set compounds
Geometric (Convex hull)		-	Uniform and smallest axis-aligned convex region containing all the data points	Convex
Distance-based	Mahalanobis distance/ Hotelling T^2/ leverage	$D_M(x,\mu) = \left(x-\mu\right)^T \sum^{-1} \left(x-\mu\right)$	a) Normal, b) arbitrary variances, and c) arbitrary correlation. Mean μ, Covariance matrix Σ $p\left[x\right] = \frac{1}{\left(2\pi\right)^{N/2}\left\|\sum\right\|^{\frac{1}{2}}} \exp\left\langle -\frac{1}{2} D_M\left(x,\mu\right)\right\rangle$	Ellipses (hyper-ellipses)
	Euclidean distance	$d(x,y) = \sqrt{\sum_{i=1}^{n}(x_i - y_i)^2}$ n=total number of observations for which a pair wise comparison is performed (x_i and y_i), i is the number of descriptors	a) Normal, b) equal variances, and c) uncorrelated variables Mean μ, unit covariance matrix $p\left[x\right] = \frac{1}{\left(2\pi\right)^{N/2}} \exp\left\langle -\frac{1}{2} D_E^2\left(x,\mu\right)\right\rangle$	Spheres (hyper-spheres)
	City block	$d(x,y) = \sum_{i=1}^{n}\left\|x_i - y_i\right\|$	Multivariate uniform	Rectangle
Probability density distribution		$\varphi = \frac{1}{\sqrt{\left(2\pi\right)}s} . \exp\left[\frac{-1}{\left(2s^2\right)\left(x_i - x_j\right)^2}\right]$ where, Φ (x_i and x_j) is the potential induced on x_j by x_i and width of the curve is defined by smoothing parameter s. The cut off value associated with Gaussian potential functions, namely f_p, can be calculated by: $f_p = f_i + (q–j)(f_{j+1} – f_j)$ where, $q = p \times {}^{n}/_{100}$, p is the percentile value of probability density, n is the number of compounds in the training set and j is the nearest integer value of q.	1. Parametric methods use standard distributions such as Gaussian and Poisson distributions. 2. Non-parametric techniques permit the estimation of probability density solely from data.	Captures actual data distribution and no reference data point are considered. For each data point, the method calculates the probability of belonging to the set.

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1. RANGES IN THE DESCRIPTOR SPACE APPROACH

1.1 Fixed or Probabilistic Boundaries/Bounding Box

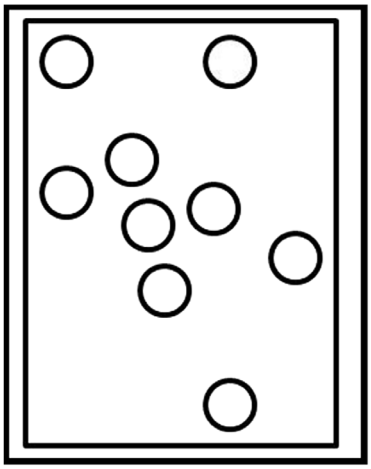
- **Theory:** The ranges of the individual descriptors are considered with a uniform distribution defining an n-dimensional hyper-rectangle developed on the basis of the maximum and minimum values of each descriptor used to build the model with sides parallel to the coordinate axes.
- **Criteria:** This method is simply based on boundary created by individual descriptor ranges of the training set compounds from which the QSAR model is developed. As mentioned earlier, this is based on the highest and lowest values of X variables (descriptors to develop the QSAR model) and Y variable (response for which the QSAR equation is created) of the training set. Any test set compounds, which are not present in any of these particular ranges, are considered out of the AD and their predictions are less reliable (Jaworska *et al*., 2005; Seber, 1984).
- **Drawbacks:**
  - For non-uniformly distributed data, the approach encloses considerable empty space,
  - Empty regions in the interpolation space cannot be recognized, as only the descriptor ranges are considered, and
  - Correlation between descriptors cannot be taken into account (Jaworska *et al*., 2005).

For the 'bounding box' method, the domain of applicability is the smallest axis-aligned rectangular box containing all the data points presented in Figure 1.

1.2 Principal Component Ranges/PCA Bounding Box

- **Theory:** Principal components convert the original data into a new orthogonal coordinate system by the rotation of axes and enable to correct for correlations among descriptors. Newly formed axes are defined as Principal Components (PCs) representing the maximum variance of the total dataset. The points between the lowest value and the highest value of each PC define an

Figure 1. Bounding box plot



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M-dimensional (*M* is the number of significant components) hyper-rectangle with sides parallel to the PCs (Nikolova-Jeliazkova & Jaworska, 2005).

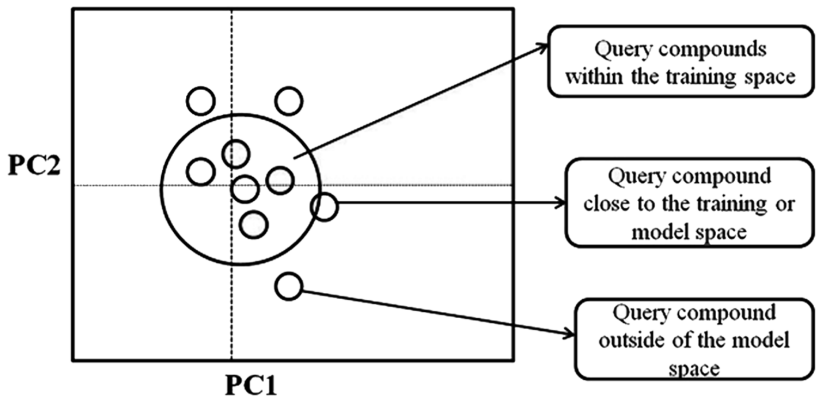
- **Criteria:** This hyper-rectangle AD also includes empty spaces depending on uniformity of data distribution (Nikolova-Jeliazkova & Jaworska, 2005). Combining the bounding box method with principal component analysis (PCA) can overcome the setback of correlation between descriptors but the issue regarding empty regions within the interpolation space still remains. It is interesting to point out that the empty space is smaller than the hyper-rectangle in the original descriptor ranges. The most important criterion of this approach remains in the selection of appropriate number of components (Jaworska *et al*., 2005, Worth *et al*., 2005). The PCA bounding box has been used to analyze the AD of the KOWWIN model.

The AD in principal component space is presented in Figure 2. The QSAR model training set is represented by the biggest circle. The predictions of query molecules within the training space are considered reliable. Query compounds which are located outside the training model space would be expected to be less reliably predicted.

1.3 TOPKAT Optimal Prediction Space

- **Theory:** Variation of PCA is implemented by the Optimum Prediction Space (OPS) from TOPKAT OPS (2000). In the PCA approach, instead of the standardized mean value, the data are centered around the mean of individual parameter range ($[x_{max}-x_{min}]/2$). Thus, it establishes a new orthogonal coordinate system which is known as OPS coordinate system. Here, the basic process is same, extracting eigenvalues and eigenvectors from the covariance matrix of the transformed data. The OPS boundary is defined by the minimum and maximum values of the generated data points on each axis of the OPS coordinate system.
- **Criteria:** The Property Sensitive object Similarity (PSS) is implemented in the TOPKAT as a heuristic solution to replicate the data set's dense and spare regions, and includes the response variable (y). Accuracy of prediction between the training set and queried points is assessed by the PSS. Similarity search method is used to evaluate the performance of TOPKAT in predicting the effects of a chemical that is structurally similar to the training structure.

Figure 2. A graphical illustration of the AD in principal component (PC) space



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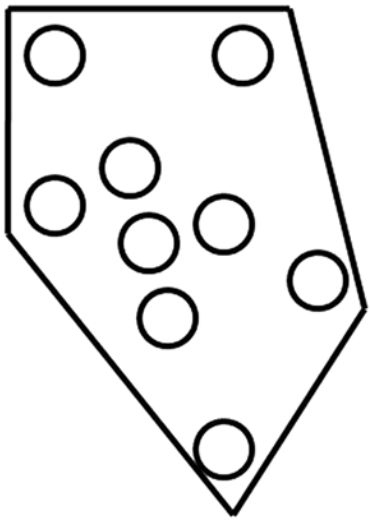
2. GEOMETRICAL METHODS

2.1 Convex Hull

- **Theory:** This approach estimates the direct coverage of an n-dimensional set using the convex hull calculation (Preparata & Shamos, 1991). Convex hull calculation is a computational geometry problem which is performed based on complex but efficient algorithms. The approach recognizes the boundary of the dataset considering the degree of data distribution.
- **Criterion:** Interpolation space is defined by the smallest convex area containing the entire training set.
- **Drawbacks:**
  - Implementing a Convex Hull can be challenging with increasing data complexity, this means that an increase in dimensions contributes to the order of complexity. Convex hull calculation is efficient for two and three dimensions. The complexity swiftly amplifies in higher dimensions. For n points and d dimensions, the complexity is of order O which can be defined as $O = [n^{[d/2]+1}]$ (Preparata & Shamos, 1991),
  - The approach only analyses the set boundaries without considering the actual data distribution, and
  - This approach cannot identify the potential internal empty regions within the interpolation space (Jaworska *et al*., 2005).

For the 'convex hull' method, the domain of applicability is the smallest axis-aligned convex region containing all the data points presented in Figure 3.

Figure 3. Convex hull plot



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3. DISTANCE-BASED METHODS

Distance-based methods are mainly founded on the 'distance-to-centroid' principle. The ellipsoidal region is centered on the dataset grand mean and has its principal axes based on the eigenvectors of the dataset variance/covariance matrix. In Figure 4, a graphical illustration of 'distance-to-centroid' is presented.

There are various types of distance-based methods which can be used for the determination of AD of QSAR models. We have tried to represent the most commonly used distance-based AD approaches in the following sections.

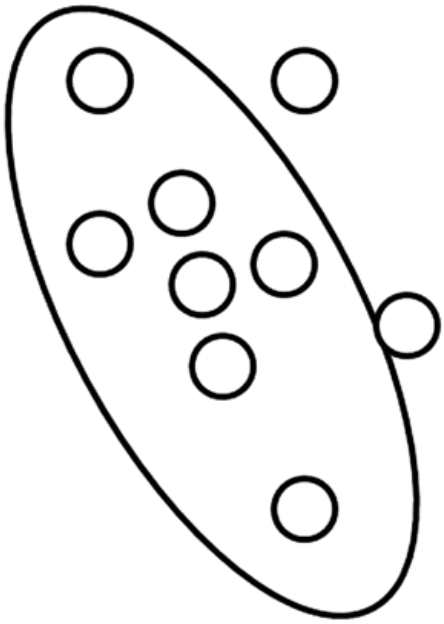
3.1 Leverage Approach

- **Theory:** The leverage (*h*) of a compound in the original variable space is calculated based on the HAT matrix as:

$$H = (X^T(X^TX)^{-1}X). \quad (1)$$

In Equation (1), H is an [n x n] matrix that orthogonally projects vectors into the space spanned by the columns of X (Eriksson *et al*., 2003; Gramatica, 2007). The AD of the model is defined as a squared area within the ±3 band for standardized residuals (σ) and the leverage threshold is defined as $h^*=3(p+1)/n$, where p is the number of variables and n is the number of compounds. The leverage values (h) are calculated for each compound and plotted vs. cross-validated standardized residuals (σ) (Y-axis) referred to as the Willam's plot.

Figure 4. Distance-to-centroid plot



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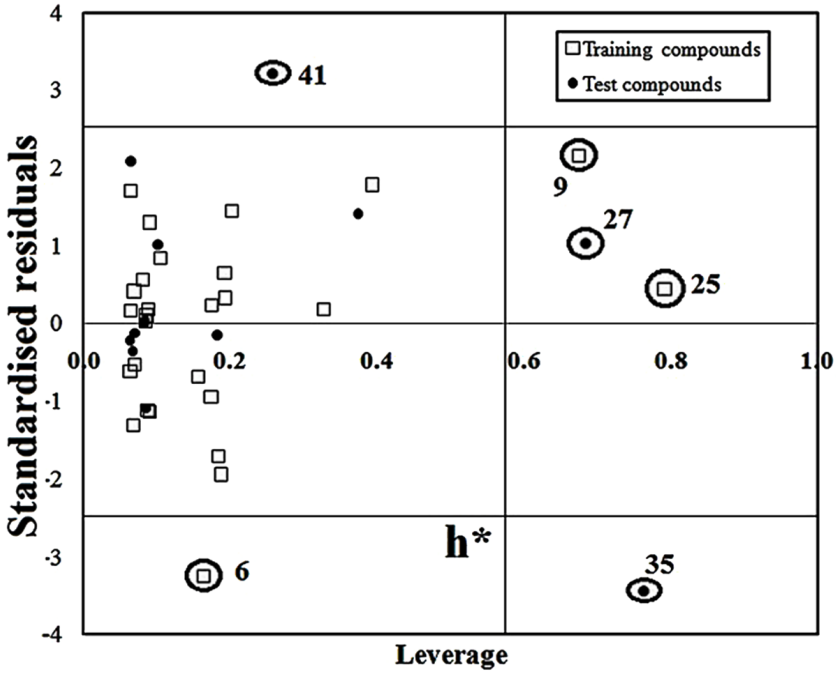
The leverage approach assumes normal data distribution. It is interesting to point out that when high leverage points fit the model well (having small residuals), they are called good high leverage points or good influence points. Those points stabilize the model and make it more accurate. On the contrary, high leverage points, which do not fit the model (having large residuals), are called bad high leverage points or bad influence points.

- **Criteria:** Graphically, the Williams plot (mostly used for the leverage approach) verifies the presence of response outliers and training set chemicals that are structurally very influential in determining model parameters. The data predicted for high leverage chemicals in the prediction set are extrapolated and could be less reliable. Here, we have tried to simplify the concept with a pictorial representation of Williams plot in Figure 5.

It is interesting to point out that this figure has been drawn based on arbitrary data to show the every possibility of AD assessment in training and test set compounds. Assessing Figure 5, one can draw the following conclusion:

1. Compound number 6 of the training set possesses standardized cross-validated residual value greater than -3σ unit, but it lies within the critical HAT (h*) value of 0.58. This compound may have flawed observed value and it can be identified as an outlier due to a wrong value of response variable. Therefore, compound number 6 is a response outlier.
2. Compounds with serial numbers 9 and 25 belonging to the training set are characterized by high leverage values, but they lie within the fixed ±3σ limit of the ordinate. The mentioned chemicals

Figure 5. Williams plot



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are not response outliers (not Y outliers), but influence the applicability domain to span over larger area and hence can be marked as influential chemicals or X outliers.

3. Compound number 35 belonging to the test set is wrongly predicted having standardized cross-validated residual value greater than -3σ unit) as well as completely outside of the AD as defined by HAT vertical line (higher leverage value than the critical value).
4. On the contrary, one of the test set compounds (41) belongs to AD as it lies within the critical HAT value but the prediction for this compound is not precise (greater than 3σ unit).
5. The test set compound with serial number 27 is within the ±3σ limit of the ordinate, but possess higher leverage value ($h > h^*$). As we can see from Figure 5, this compound is surrounded by other two influential training set chemicals (serial numbers 9 and 25), therefore its prediction can be assumed to be somewhat reliable or dependable though it lies outside the AD.

3.2 Euclidean Distance

- **Theory:** This is one of the major distance based AD assessment approaches. Like other distance-based approaches, it calculates the distance from every other point to a particular point in the data set. The distance scores are calculated by the Euclidean distance norm. A distance score, d_{ij}, for two different compounds X_i and X_j can be measured by the Euclidean distance norm. The Euclidean distance can be expressed by the following equation:

$$d_{ij} = \sqrt{\sum_{k=1}^{m} (x_{ik} - x_{jk})^2} \,. \tag{2}$$

The mean distances of one sample to the residual ones are calculated as follows:

$$\overline{d}_i = \sum_{j=1}^{n} d_{ij} \Big/ n-1, \tag{3}$$

where, i=1,2,….,n.

The mean distances are then normalized within the interval of zero to one. It is applicable only for statistically independent descriptors (Jaworska *et al*., 2004).

- **Criteria:** Compounds with distance values adequately higher than those of the most active probes are considered to be outside the domain of applicability. The mean normalized distances are measured for both training and test set compounds. The boundary region created by normalized mean distance scores of the training set are considered as the zone of applicability domain for test set compounds. If the test set compounds are inside the domain/area covered by the training set compounds, it means that these compounds are inside the applicability domain, otherwise not.

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An example of Euclidean plot is presented in Figure 6. Based on the assessment of the Euclidean plot, one can conclude that test set compounds 19 and 23 are outside of the applicability domain created by the training set compound from which the QSAR model has been developed.

3.3 Mahalanobis Distance

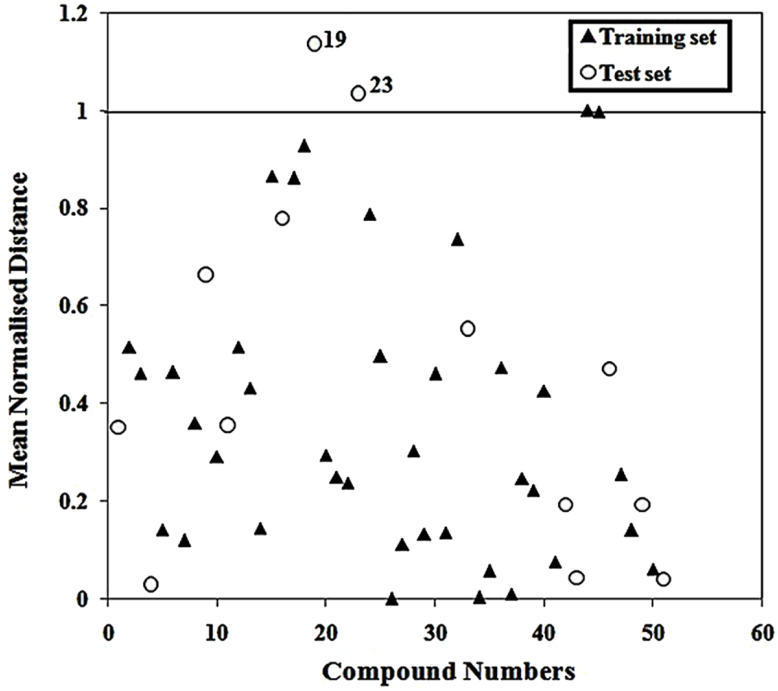
- **Theory:** The Mahalanobis distance approach considers the distance of an observation from the mean values of the independent variables but not taking the impact on the predicted value. It offers one of the unique and simple approaches for identification of outliers. Mahalanobis distance is unique because it automatically takes into account the correlation between descriptor axes (Hair *et al*., 2005).
- **Criterion:** The threshold value does not have any rule of thumb. However, observations with values much higher than those of the remaining ones may be considered to be outside the domain.

3.4 City Block Distance

City-block distance is simply the summed difference across dimensions and is calculated from the following equation:

$$d(x,y)=\sum_{i=1}^{n}\left|x_i-y_i\right|. \tag{4}$$

Figure 6. Euclidean distance plot for AD study of an arbitrary QSAR model



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It examines the absolute differences between coordinates of a pair of objects (x_i and y_i). City-block distance assumes a triangular distribution. The method is particularly useful for the discrete type of descriptors. It is used only for training sets which are uniformly distributed with respect to count-based descriptors (or fragment mapping counts) (Jaworska *et al*., 2004).

3.5 Hotelling T^2 Test

- **Theory:** The Hotelling T^2 method is a multivariate student's *t* test and proportional to leverage and Mahalanobis distance approach. It assumes a normal data distribution like the leverage approach (Hair *et al*., 2005). The method is used to evaluate the statistical impact of the difference on the means of two or more variables between two groups. Hotelling T^2 corrects for collinear descriptors through the use of the covariance matrix. Hotelling T^2 measures the distance of an observation from the center of a set of X observations. A tolerance volume is derived for Hotelling T^2.
- **Criterion:** Based on the *t* value, the significant compounds within the domain are determined.

3.6 K-Nearest Neighbors Approach

- **Theory:** The approach is based on similarity search for a new chemical entity with respect to the space created by the training set compounds. The similarity is identified by finding the distance of a query chemical from nearest training compound or its distances from *k*-nearest neighbors in the training set. Thus, similarity to the training set molecules is significant for this approach in order to associate a query chemical with reliable prediction (Sheridan *et al*., 2004). Like other methods, descriptors are used for calculate the similarity between training and test molecules.
- **Criterion:** If the calculated distance values of test set compounds or query molecules are within the user defined threshold set by the training set molecules, then the prediction of these compounds are considered to be reliable.

How One Can Find the Similarity to the Molecules of the Training Set

1. Considering all the descriptors, the mean similarity of a test set molecule to the most similar *k* members of the training set can be found. A variation of this is to calculate this similarity using only the subset of descriptors in the training set.
2. The similarity of the test set molecule to the descriptor centroid of the training set.

How to Decide the Neighboring Test or Query Compounds in the Training Set

1. The simplest method is to pick a similarity cutoff above which the test set molecule and a training set molecule can be considered "neighbors" and count the molecules that exceed that cutoff.
2. Another complicated approach is explained by Sheridan et al. (2004) which demonstrated that "A more complicated approach is to define a falloff function f(Similarity) that defines how much of a neighbor a training set molecule is as a function of similarity to the test set molecule. The number of neighbors is the sum of f (Similarity) over all members of the training set. For instance, we use a linear function where a molecule is 1.0 of a neighbor at similarity 1.0 and 0.0 of a neighbor at a similarity 0.6; anything between is linearly interpolated."

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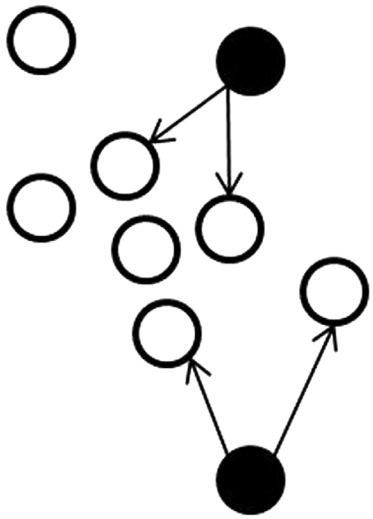
A k-nearest neighbors plot is presented in Figure 7, where the distance of a point to the domain is taken as the average distance to the k- nearest data points.

3.7 DModX (Distance to the Model in X-Space)

- **Theory:** This is another important distance based AD finding approach for QSAR models. This approach was developed by Wold *et al*., (2001) and usually applied for partial least squares (PLS) models. The basic theory lies in the residuals of Y and X which are of diagnostic value for the quality of the model. As there are number of X-residuals, one needs a summary for each observation. This is accomplished by the residual standard deviation (SD) of the X-residuals of the corresponding row of the residual matrix E. As this SD is proportional to the distance between the data point and the model plane in X-space, it is also usually called DModX (distance to the model in X-space). Here, X is the matrix of predictor variables, of size (N*K), Y is the matrix of response variables, of size (N*M) and E is the (N*K) matrix of X-residuals, N is number of objects (cases, observations), k is the index of X-variables (k = 1, 2,..., K) and m is the index of Y-variables (m = 1, 2,..., M).
- **Criteria:** A DModX value larger than around 2.5 times the overall SD of the X residuals (corresponding to an F-value of 6.25) indicates that the observation is outside the applicability domain of the model. In the DModX plot, the threshold line is attributed as D-critical line and this plot can be easily drawn in SIMCA-P software for any PLS model (SIMCA-P 10.0, 2002).

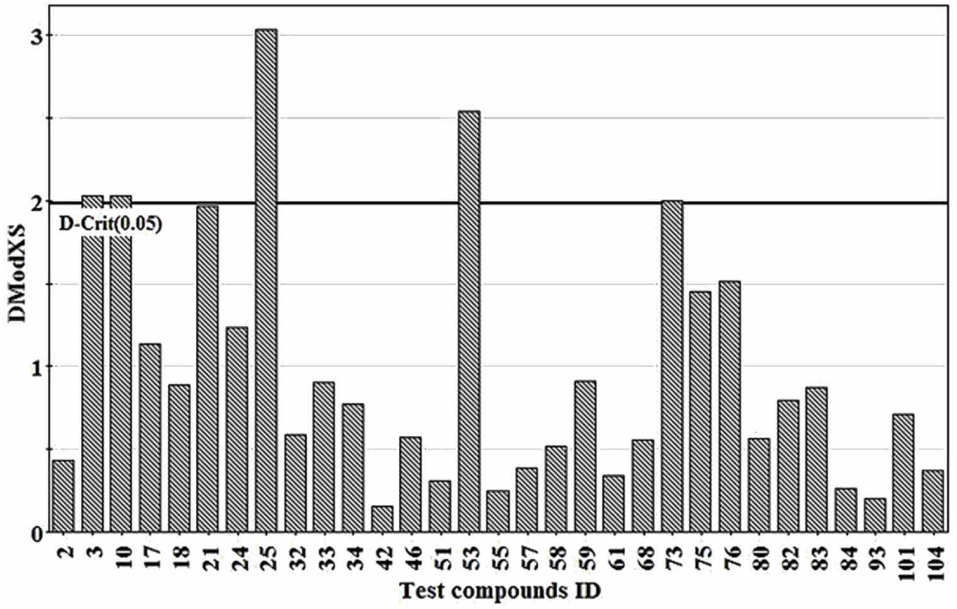
An example of DModX plot is presented in Figure 8 where the plot is constructed at 95% confidence level by SIMCA-P. The DModX values of 26 test compounds are within the critical value of 1.986. Chemical **10** along with the compound numbers **3**, **25**, **53** and **73** are outside the evaluated critical value indicating that these compounds are outside of the applicability domain of the developed model.

Figure 7. K-nearest neighbors plot



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Figure 8. DModX plot for PLS based QSAR model



3.8 Tanimoto Similarity

The Tanimoto index is a measure of similarity between two molecules based on the number of common molecular fragments (Tetko *et al*., 2008). In order to calculate the Tanimoto similarity, all unique fragments of a particular length in two compounds are calculated. The Tanimoto similarity between the compounds *J* and *I* is defined as:

$$TANIMOTO\left(J,K\right)=\frac{\sum_{i=1}^{N}\left(x_{J,i}\cdot x_{K,i}\right)}{\sum_{i=1}^{N}\left(x_{J,i}\cdot x_{J,i}\right)+\sum_{i=1}^{N}\left(x_{K,i}.x_{K,i}\right)-\sum_{i=1}^{N}\left(x_{J,i}.x_{K,i}\right)} \quad (5)$$

where, *N* is the number of unique fragments in both the compounds, $x_{J,i}$ and $x_{K,i}$ are the counts of the *i*-th fragment in the compounds *J* and *K*. Based on equation 3, the distance between two compounds *J* and *K* is "*1– TANIMOTO(J, K)*" and the distance of a compound to the model is the minimum distance between the investigated compound and compounds from the training set of the model.

3.9 Standard Deviation of the Ensemble Predictions (STD)

In order to estimate the uncertainty of the model, standard deviation of the predictions obtained from an ensemble of models can be utilized. The basic principle behind this idea is very simple and it depicts that if different models give significantly different predictions for a particular molecule, then the prediction for this compound is more likely to be unreliable. The sample standard deviation can be ideally used as an estimator of model uncertainty.

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As an example, consider that $Y(J) = \{y_i(J), i=1..N\}$ is a set of predictions for a compound J given by a set of N trained models, the corresponding distance to model STD can be defined by the following equation:

$$d_{STD}(J) = stdev\left(Y\left(J\right)\right) = \sqrt{\frac{\sum\left(y_i\left(J\right) - \bar{y}\right)^2}{N-1}} \,. \tag{6}$$

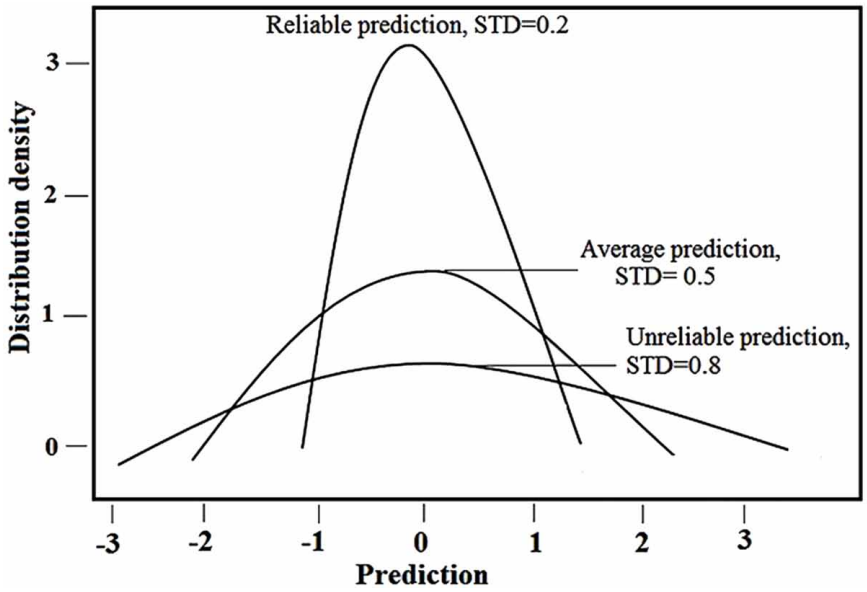
Based on the type of an ensemble used to evaluate the standard deviation, the STD approach can be classified into several subtypes. (a) Consensus STD or CONS-STD for models developed based on different machine learning techniques, (b) Associative neural networks or ASNN-STD for an ensemble of neural network models, and (c) BAGGING-STD for an ensemble of models formed utilizing the bagging technique. The distance based method STD has been verified to provide outstanding results for discrimination of highly accurate predictions for regression models (Manallack *et al*., 2003; Tetko *et al*., 2008).

A graphical illustration of the STD approach has been given in Figure 9. Here, reliable prediction has a low prediction spread and unreliable prediction has higher prediction spread.

3.10 Correlation of Prediction Vectors (CORREL)

The basic theory of this approach is based on the correlation of vectors of ensemble's predictions for the target compound and compounds from the training set (Tetko, 2008). Similar to the STD method, this measure is relevant only for ensembles of models. More specifically, CORREL measure for the target compound J is calculated according to the following expression:

Figure 9. STD graph for AD estimation



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$$d_{CORREL}\left(J\right) = 1 - \max_{i=1\ldots n}\left[corr\left(y\left(\vec{T}_i\right), y\left(\vec{J}\right)\right)\right]. \quad (7)$$

In Equation (7), $y\left(\vec{T}_i\right)$ and $y\left(\vec{J}\right)$ define the vectors of ensemble's predictions for the training set compound *Ti* and the target compound *J*, *corr* is Spearman rank correlation coefficient between the two vectors and *N* is the number of compounds in the training set. A low value of CORREL (that indicates the high Spearman correlation coefficient) specifies that for target compound *J,* there is a compound *T* from training set for which predictions of the ensemble of models are strongly correlated. If a compound *T* has the same descriptors as the target compound *J*, then predictions of models will be identical for both molecules and thus resulted *CORREL(J)* will be 0. Compounds having high correlation coefficient values are considered to be "closer to the model". The Spearman correlation outperformed several alternative measures (as e.g. Euclidean distance and Pearson correlation coefficient) due to its higher accuracy and practical requirements to store ensemble predictions for all training set molecules (Tetko & Tanchuk, 2002).

3.11 Concordance of a Classification Ensemble

The highest number or the relevant percentage of the models that produce the same prediction can be used as a measure of the concordance of an ensemble (Chen *et al*., 2005). As an example, if there are 5 models that give predictions {a; b; a; a; a}, then the concordance is 4 (or 80%). The measure that is opposite to the concordance (*i.e.* a- CONCORDANCE) can be used as a distance to model. The idea behind this method is similar to that of the standard deviation (STD) distance to model approach, but is adapted for classification models and qualitative predictions.

4. PROBABILITY DENSITY DISTRIBUTION

- **Theory:** Probability density distribution is one of the most advanced approaches for defining AD, based on estimating the probability density function for the given data. The approach is classified into two classes. The first one is a parametric method which assumes a standard distribution such as Gaussian and Poisson distributions, and the second one is a non-parametric method which does not rely on such assumptions considering the data distribution (Jaworska *et al*., 2005; Netzeva *et al*., 2005).

These methods are executed by estimating probability density of the compounds followed by identifying Highest Density Region which consists of a known fraction (as set as user input) from the total probability mass (Netzeva *et al*., 2005). A potential is formed for each compound in the training set such that it is highest for that molecule and decreases with increasing distance. Once the potential is evaluated for all the compounds, global potential is obtained by summing up the individual potentials thus indicating the probability density (Forina *et al*., 1991; Jouan-Rimbaud *et al*., 1999).

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The most important feature of these methods is their capability to identify the internal empty space. The actual data distribution can be revealed by generating concave regions around the interpolation space borders.

- **Criteria:** Here, Gaussian function is demonstrated. Given two molecules x_i and x_j, it can be determined as below:

$$\varphi = \frac{1}{\sqrt{(2\pi)}s}.\exp\left[\frac{-1}{\left(2s^2\right)\left(x_i - x_j\right)^2}\right] \tag{8}$$

where, Φ (x_i and x_j) is the potential induced on x_j by x_i and width of the curve is defined by smoothing parameter s. The cut off value associated with Gaussian potential functions, namely f_p can be calculated by methods based on sample percentile (Jouan-Rimbaud *et al*., 1999):

$$f_p = f_i + (q - j)(f_{j+1} - f_j) \tag{9}$$

with $q = p \times {}^{n}/_{100}$, where p is the percentile value of probability density, n is the number of compounds in the training set and j is the nearest integer value of q. Test compounds with potential function values lower than the set threshold are considered outside the AD.

5. RANGE OF THE RESPONSE VARIABLE

Based on the distribution plot or considering the range of the response variable, compounds can be identified as outside the AD. In a series of compounds, if the response value of a particular test compound is largely different from the mean response value of the training set compounds, then the compound can be considered as out of the AD.

6. MISCELLANEOUS APPROACHES

Along with the above-mentioned strategies to determine the AD of constructed QSAR models, there are several miscellaneous approaches introduced in the recent times. Here, we have tried to discuss these approaches briefly. The use of these approaches has been very limited compared to the classical AD methods. The below-mentioned methods are especially used for interpolation space characterization in the model descriptor space.

6.1 Stepwise Based Approach

- **Theory:** Stepwise based approach for evaluating the AD of a QSAR model is proposed by Dimitrov *et al*. (2005). This approach is useful for reflecting mechanistic rationality and transparency of the QSAR model. This approach is processed in four stages in a chronological manner. The first stage

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"General requirements domain" consists of checking of the variation of the physicochemical properties of the query chemicals in the training set compounds. The second stage "Structural domain" defines the structural similarity which is found within the chemicals that are correctly predicted by the model. The third stage "Mechanistic domain" takes into account of the mechanistic interpretation of the modeled phenomenon to define the domain of applicability. In this third step, the model domain merges the reliability of specific functional groups assumed to cause the effect and the domain of explanatory variables. Here, the latter is defined by the parameter interpolation space. In the fourth and final stage "Domain of metabolic stimulator", if stimulated metabolism of chemicals is a part of the developed QSAR model then the dependability of simulated metabolism is considered in assessing the reliability of the predictions.

- **Criterion:** An external compound is compulsory to satisfy all the conditions specified within the mentioned four stages to be considered within the AD. The approach is considered as a rigorous one as a chemical is assessed for similarity, metabolic and mechanistic check to address the reliability of predictions and permitting a better assessment of model's AD (Dimitrov *et al*., 2005).
- **Drawback:**
  - In order to determine the AD of the QSAR model, one would need a lot of information regarding the structural attributes of compounds, mechanism of action for particular response as well as their metabolic activation. In regular practice of developing QSAR models, this is lacking in major cases.
  - The method is time consuming as well as critical as a lot of variables are involved in this AD assessment approach.

6.2 Decision Trees and Decision Forests

This approach identifies the AD in terms of prediction confidence and domain extrapolation based on the consensus prediction of Decision Trees (DT) and Decision Forests (DF). The basic principle is to minimize the overfitting which can be attained by merging the DTs and keeping the differences within different DTs to maximum possible. Predictions from all the combined DTs are averaged in order to find the prediction confidence for a particular molecule, while domain extrapolation provides the prediction precision for that compound outside the training space (Netzeva et al., 2005; Tong et al., 2003, 2004).

6.3 Kernel-Based Applicability Domain

Machine learning tools are becoming an important method to evaluate the molecular activities/properties without the help of *in vitro* experiments. The major drawback of these methods is that most of them do not provide any information using the knowledge contained in the model though they are predicted adequately by the model. As a result, in machine learning based QSAR modeling, the estimation of the reliability of a model-based prediction is an important question (Fechner *et al*., 2009).

Most machine learning approaches for QSAR heavily depend on a vectorial representation of the molecules. Thus, the applicability domain is expressed as a subspace of the vector space with one dimension for individual descriptor used. However, this vectorial concept cannot be directly applied to kernel-based techniques like support vector machines. So, these methods have to rely on an implicit feature space which is only defined by the applied kernel similarity and with unknown dimensions. Therefore the domain of applicability of a kernel-based model has to be defined by means of the kernel.

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This also permits us to employ the structured similarity measures like the Optimal Assignment Kernel and its extension, instead of a numerical encoding. This method makes it possible to depict the complex chemical structure of many drugs better than the methods which are processed by using descriptors.

The concept of kernel density estimation has incorporated additional information in a trained model. The added information can be achieved by using a weighted average kernel similarity of a predicted molecule to the training data set. The weights can be attained either by utilizing the knowledge contained in the learned model or by methods that describe the feature space structure using the kernel (Fechner *et al*., 2009).

6.4 Intelligent *K*-Means Clustering

An intelligent version of the k-means clustering algorithm-based measure of distance-to-domain for QSAR models was designed by Stanforth *et al*., (2007) to obviate the difficulties of the existing methods (like convex hull, bounding box etc.) in terms of primitiveness and computational complexity. This measure combines the modeling of the training set as a collection of the intelligent k-means clusters algorithm in the descriptor space with a new interpretation of a conventional optimization criterion in fuzzy clustering which leads to a modified harmonic mean measure. A test compound is assigned fuzzy membership of each individual cluster, from which an overall distance may be calculated. Stanforth *et al*., (2007) demonstrated that this approach is more stable than existing methods and more indicative of the prediction error.

k-Means clustering is prepared based on the approximation of each point in a dataset by the centroid of that point's cluster. This estimation can be quantified by decomposing the data scatter into contributions that are explained and unexplained by the cluster model as presented in the following equation (Mirkin, 2005):

$$\sum_{i\in T} x_i \cdot x_i = \sum_{k=1}^{k} N_k c_k \cdot c_k + \sum_{k=1}^{k} \sum_{i\in C_k} \left(x_i - c_k\right)\cdot\left(x_i - c_k\right) . \tag{10}$$

In Equation (10), $\{x_i: i \in T\}$ is the training set and the k clusters C_k have sizes N_k and centroids c_k, respectively. This suggests an optimization of the so-called square-error k-means criterion

$$\sum_{k=1}^{k} \sum_{i\in C_k} \left(x_i - c_k\right)\cdot\left(x_i - c_k\right) . \tag{11}$$

Equation 11 holds for any inner product in the descriptor space.

k-Means clustering requires an initialization step to indicate the number of clusters k and the preliminary positions of their centroids. The initialization of the intelligent k-means algorithm has been achieved by the principle of Anomalous Pattern Clustering (APC) (Mirkin, 2005; Smellie, 2004). The principle can be demonstrated in the following way.

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1. Two initial centroids are specified as follows: the first one is considered as the data gravity centre where components are grand means of the corresponding components in the entire dataset, and the second is an entity which is the furthermost away from the gravity centre.
2. It is interesting to point out that the first point (grand mean) centroid never changes, so that the only changeable centroid is the second one.

The computational complexity proved to be quite acceptable for this method. Assessment of the domain of applicability using clusters affords a much faster and easier calculation than k-NN. Not only that, this one is more prudent in terms of additional stored model parameters, without negotiating the acuity of the measure.

PRACTICAL EXAMPLES OF DETERMINATION OF AD

In order to show an example of determination of the AD for a regression based QSAR model, we have shown in Table 2 a set of hypothetical X-Y data and a correspoding QSAR model developed with multiple linear regression (MLR) tool. The model consists of three descriptors. Here, we have shown the calculation and determination of parameters most commonly used in simple AD approaches like fixed or probabilistic boundaries (bounding box), range of the response variable, leverage method and Euclidean distance based method. All these methods can be used by any QSAR researchers without help of any professional software.

1. **Leverage Approach:** Based on the calculated leverage (h) and standardized residual values, we have constructed Williams plot (Figure 10). Here, the standardized residual (SR) value is calculated based on the following equation:

$$SR = \frac{\mathrm{Re}\,sidual\ \ value - Average\ \mathrm{Re}\,sidual\,value_{(Training)}}{S\tan dard\ \ deviation\,of\ \mathrm{Re}\,sidual\,value_{(Training)}}. \tag{12}$$

Then Williams plot is constructed taking standardized values in Y-axis and leverage values in X-axis. As the number of descriptors is three and number of compounds in the training set is eleven, the critical leverage (h*) value is = [3*(3+1)]/11=1.09. Taking into consideration the criteria described in section 3.1, one can firmly conclude that based on the leverage approach for the studied QSAR model, all the query compounds are within the AD.

2. **Euclidean Distance-Based Approach:** First, Euclidean distance scores are calculated (based on Equation 2) for the total set of compounds and then the mean distance scores (based on Equation 3) are calculated. The mean distances are then normalized within the interval of zero to one. From the normalized mean distances, it is clear that not a single test compound resides outside of the AD. The Euclidean plot (Figure 11) is then developed taking the mean normalized distance in Y-axis and compound numbers in X-axis.

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Table 2. A simple example demonstrating calculation of applicability domain for a regression-based QSAR model using different methods such as fixed or probabilistic boundaries (bounding box), leverage method, and Euclidean distance based approaches

Compound ID	Y* (Observed)	X_1*	X_2*	X_3*	Y (Calculated)	h[a]	Residual	Standardized Residual	Distance Score[b]	Mean Distance[c]	Normalized Mean Distance
1	3.45	1.65	1	1.65	3.46	0.41	-0.01	0.01	9.13	0.83	0.68
3	3.14	1.63	1.5	1.09	2.73	0.17	0.41	2.01	5.54	0.50	0.02
5	2.82	2.02	1.5	1.35	2.67	0.26	0.15	0.76	6.37	0.58	0.17
7	2.64	2.05	1.5	1.37	2.67	0.29	-0.03	-0.08	6.59	0.60	0.21
9	2.29	1.83	1.5	1.22	2.70	0.17	-0.41	-1.94	5.56	0.51	0.02
10	2.2	1.9	2	0.95	1.97	0.38	0.23	1.15	7.70	0.70	0.41
11	2.15	1.33	2	0.67	2.06	0.50	0.09	0.46	9.12	0.83	0.68
13	1.74	1.54	2	0.77	2.03	0.34	-0.29	-1.36	7.99	0.73	0.47
15	3.45	1.91	1	1.91	3.42	0.66	0.03	0.19	10.87	0.99	1.00
16	2.51	1.66	1.5	1.11	2.73	0.16	-0.22	-1.01	5.47	0.50	0.00
17	2.87	1.1	1.5	0.73	2.82	0.66	0.05	0.31	9.34	0.85	0.72
Test Set											
Compound ID	**Y* (Observed)**	**X_1***	**X_2***	**X_3***	**Y (Predicted)**	**h**	**Residual**	**Standardized Residual**	**Distance Score**	**Mean Distance**	**Normalized Mean Distance**
2	3.2	1.9	1	1.9	3.42	0.39	-0.22	-1.02	10.78	0.98	0.99
4	2.87	1.22	1.5	0.81	2.80	0.33	0.07	0.40	8.32	0.76	0.53
6	2.81	1.78	1.5	1.18	2.71	0.14	0.10	0.53	5.51	0.50	0.01
8	2.49	1.61	1.5	1.07	2.74	0.16	-0.25	-1.14	5.67	0.52	0.04
12	2.01	1.96	2	0.98	1.96	0.30	0.05	0.28	7.92	0.72	0.45
14	3.51	1.88	1	1.88	3.42	0.38	0.09	0.46	10.62	0.97	0.96

*Y is the response variable; X_1, X_2 and X_3 are the descriptors involved in the QSAR model, h is defined as the leverage value, [a]calculated from equation 1, [b]calculated from equation 2 and [c]calculated from equation 3.

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Figure 10. Williams plot for the arbitrarily developed QSAR model

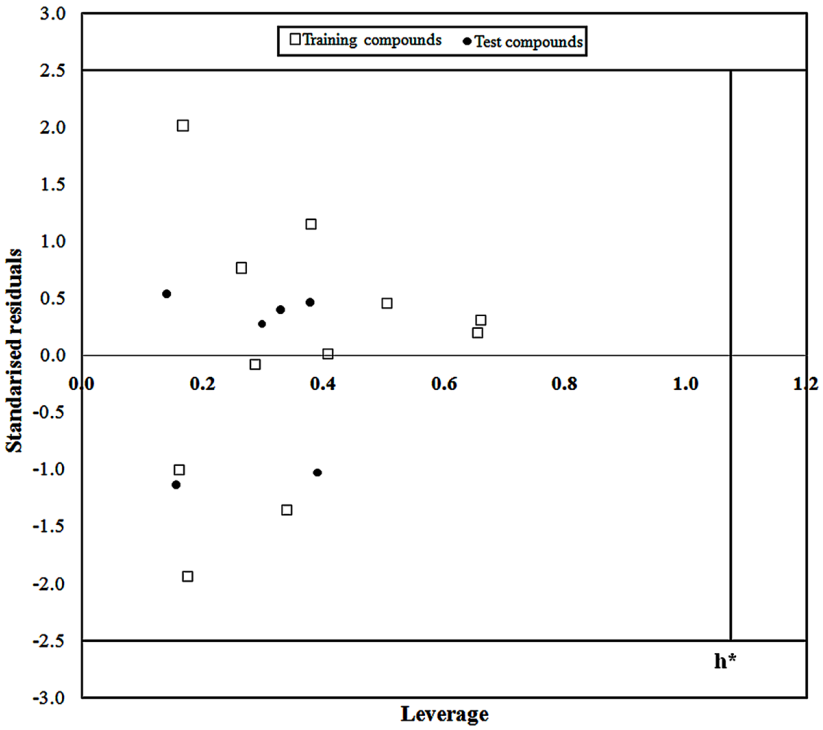
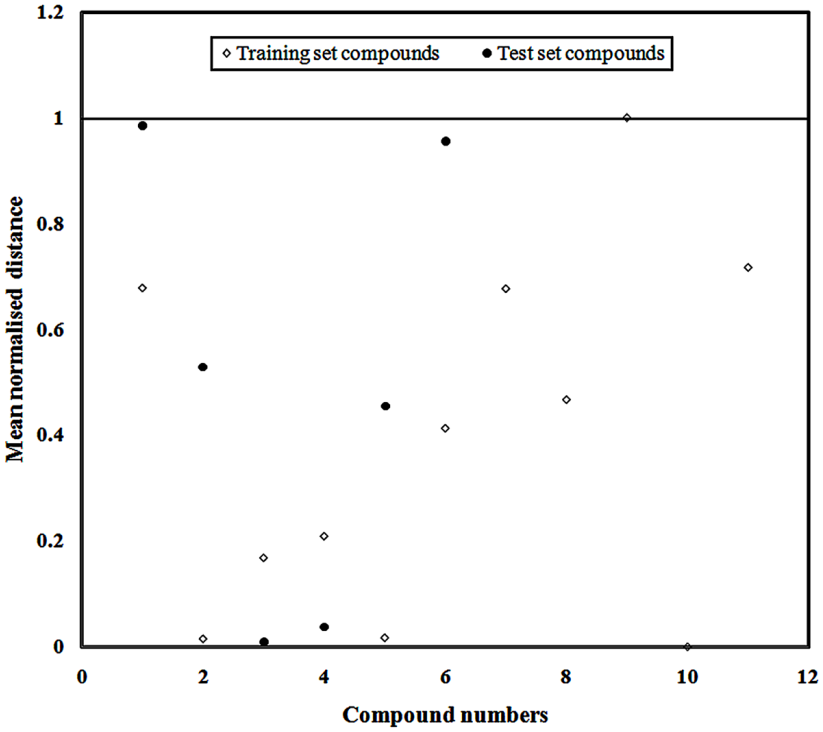


Figure 11. Euclidean distance plot for the arbitrarily developed QSAR model



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3. **Fixed or Probabilistic Boundaries (Bounding Box) and Range of the Response Variable:** Both approaches are strictly dependent on the boundary created by independent and response variables of the training set compounds. Scrutinizing the descriptor values for the training and test sets, one can easily identify that not a single test set compound falls outside the AD boundary created by the independent variables. On the contrary, only one compound (14) can be considered outside of the AD boundary when someone is taking in consideration the response variable range. Therefore, one can conclude that considering both methods, only one compound (14) resides outside the AD though it is lying very close to the boundary created by response variable.

While taking into consideration the above mentioned methods for the identification AD for a single QSAR model, one may remember that no single method of AD determination can be considered as the universally the best one. It may also be noted that the results of one specific method may not match with those from another approach as each method is based on different statistical algorithm for the determination of AD. Thus, multiple methods should be applied before coming to a final conclusion regarding AD of a QSAR model.

FUTURE RESEARCH DIRECTION

It is generally believed that no single AD approach can be relied as universally ideal one to identify the interpolation region for any QSAR model. Extensive and fruitful research is being carried out on the development of some new approaches or modification of already existing approaches to make them more acceptable and reliable ones. The following points should be considered in future research regarding AD studies.

1. A global similarity test should be developed to identify whether the structural features of a new external test compound are enclosed in the training set of compounds from which the QSAR model is developed.
2. There should be a conceptual framework for the assessment of AD for any QSAR model by the regulatory agencies and QSAR experts as no further modification has been done in the OECD guidelines after its establishment.
3. Some effort is needed to investigate the acceptable confidence limits for various AD approaches. Confidence limits could be a useful addition to the AD to predict the new set of compounds more confidently.
4. In order to make the AD estimation more user friendly, QSAR developers have to build up automated software tools where one can easily develop interpolation space as well as scatter plots for respective AD study.
5. There is a need of proper awareness of the AD concept and its implementation in the development of QSAR models. Without a proper AD study, no QSAR model should be accepted by the journals as well as regulatory agencies, as acceptability of any QSAR model is decidedly related with AD.
6. The QSAR model development and the assessment of AD (regarding algorithm and interpretation) should be transparent and it is the major responsibility of the model developer and the regulatory agency to make it clear to the readers and QSAR learners.

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CONCLUSION

Applicability domain of a QSAR model is estimated by determining interpolation regions as defined by the training data set in model descriptor space but the region varies depending on the implemented approach. It is interesting to point out that all the implemented AD approaches have their own potential and weakness. Few methods have complex algorithms behind the identification of interpolation regions, on the contrary some have strong and easy statistical background behind the AD estimation. Therefore, it is totally dependent on the model developer how he/she chooses the specific approach according to his/her need to define the applicability domain for the model more accurately and in robust way.

The basic principle of any AD approach is to find out the number of test set compounds which are falling outside of the defined domain of applicability. It is important to note that if someone uses different AD approaches for developed QSAR model from a single dataset, the result may vary in a big way and none of these established approaches can be considered universal one. Therefore, it is always recommended to assess the results from different available possible strategies before assessing a new compound set to get the result with full confidence.

Distance-based approaches are largely used over the other approaches by QSAR developers in recent times. In case of other approaches, the number of test compounds outside the AD is much higher as they are concentrated towards the training set extremities. The distance from training space is significant in defining the model's AD for test set compounds. Again, bad predictions for test compounds are also considered as outside the AD with most of the approaches. The thresholds values are also not fixed for many approaches which may lead to vary the number of compounds outside the AD. So, these issues should be considered more carefully by the QSAR fraternity and regulatory bodies to make the AD studies more transparent and robust ones.

The following points should be considered by any QSAR model developer while considering AD studies to make his/her QSAR model more robust, predictive and reproducible:

1. The utmost important aspect of any QSAR model is its prediction capability for new set of compounds. The AD study has emerged as one of the key and compulsory validation criteria considering the reliability of predictive capability of QSAR model for a new set of compounds.
2. Considering the basic principle of AD approaches, one has to rely on the AD of the QSAR before estimating activity/property/toxicity of any chemical or pharmaceuticals by the developed QSAR model.
3. The core of AD for any specific QSAR model is the training space which is constructed by the training set of compounds. Therefore, the selection of the training set is very crucial aspect as it should consist of the characteristics of the total dataset to reflect the effect of each compound in the developed model.
4. The response value or endpoint of the QSAR model is determined experimentally. So, the reliability of these values (experiments should be performed in same condition for same endpoints) should be thoroughly checked before constructing the QSAR models. As AD is also dependent on the developed QSAR model, error in response value will mislead the QSAR model which will lead to erroneous interpolation space for AD.
5. There is always a chance of uncertainty related with the assessment of AD for any QSAR model. If the constructed QSAR model is not reliable, one cannot get confidence in AD assessment. Again,

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mechanistic aspects are rarely considered in the model development by the modeler to make the fact more uncertain one.

6. Estimation of AD by various approaches is highly dependent on a number of issues like the model dimensionality, used descriptors, response value as endpoints, underlying data distribution and last but not the least algorithm of AD determination. So, there is always a chance of large number of assumptions in the AD study.

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KEY TERMS AND DEFINITIONS

3Rs: The 3Rs represent the three phrases "Reduction", "Replacement" and "Refinement". Replacement corresponds to the use of non-living materials to substitute conscious living higher animals. Reduction refers to the decrease in number of animals, and Refinement means reduction in the harshness of inhuman methodologies to be applied onto the experimental animals. *In silico* approaches like QSAR is an important alternative tool to support the 3Rs approach.

Applicability Domain: The applicability domain (AD) is a theoretical region explained by physicochemical, structural or biological space and evaluated by the model descriptors and modeled response.

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In simple explanation, AD is the knowledge or information based on which the training set of the model is constructed, and the model is applicable to make predictions for new compounds within the specific domain.

Multiple Linear Regression: Multiple linear regression (MLR) attempts to model the relationship between two or more explanatory variables and a response variable by fitting a linear equation to observed data.

Partial Least Squares: Partial Least Squares (PLS) is a generalization of regression by which strongly correlated and/or noisy or numerous X variables can be handled meticulously. The linear PLS model finds "new variables" (which can be considered as latent variables or X scores), which are linear combinations of the original ones. To avoid the problem of overfitting, a strict test for the significance of each consecutive PLS component is necessary and then stopping when the components are non-significant.

Principal Component Analysis: Principal Component Analysis (PCA) sums up all variation in X into a few more informative new variables called T scores. These new variables are linearly weighted combinations of the original X-variables. The weighting profiles are called loadings, P. For each score variable, the influence (weight) of the original variables is found by looking at its corresponding loading profile.

QSAR: Quantitative structure-activity relationship (QSAR) is a statistical modelling approach that correlates the molecular structure to a specific activity or property or toxicity derived either *in vitro* or *in vivo*. A simple mathematical relationship in any kind of QSAR method can be demonstrated as the following equation: *Biological activity = f (chemical structure or property)*. QSAR is largely used in drug discovery, environmental fate modeling, property and biological activity prediction of new, untested compounds from the molecular structures of compounds.

Randomization: Randomization is a validation technique which is performed in order to ensure the robustness of the developed QSAR model. The values of the dependent variable (Y) are randomly shuffled and new QSAR models are developed keeping the independent variable matrix unchanged. This method is of two types: process randomization and model randomization performed at varying confidence levels. The process is also known as Y-scrambling or Y-randomization technique.

Standard Deviation: Standard deviation (SD) can be defined as the measure of the deviation or dispersion of a set of data from its mean. SD can be calculated as the square root of variance.

Validation: Validation is one of the backbone steps for *in silico* models, which includes evaluation of issues such as data quality, robustness, predictability of the model and mechanistic interpretability in addition to statistical judgment. Validation strategies are largely dependent on various statistical validation metrics.

Virtual Screening: Virtual screening (VS) is a computational technique used in drug discovery to explore various existing libraries of small drug molecules in order to identify the promising structures which are most likely to bind to a drug target, especially a protein receptor or enzyme.

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Chapter 40
Molecular Dynamics Simulations for Biological Systems

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ABSTRACT

Molecular dynamics simulation is an important tool to capture the dynamicity of biological molecule and the atomistic insights. These insights are helpful to explore biological functions. Molecular dynamics simulation from femto seconds to milli seconds scale give a large ensemble of conformations that can reveal many biological mysteries. The main focus of the chapter is to throw light on theories, requirement of molecular dynamics for biological studies and application of molecular dynamics simulations. Molecular dynamics simulations are widely used to study protein-protein interaction, protein-ligand docking, effects of mutation on interactions, protein folding and flexibility of the biological molecules. This chapter also deals with various methods/algorithms of protein tertiary structure prediction, their strengths and weaknesses.

INTRODUCTION

It is difficult to perform each and every experiment practically. For example, before going for any mission astronauts are well trained and for that training they are exposed to similar kind of climatic conditions and difficulties that they expect to face on their mission. So, for these kinds of training the spatial conditions were imitated at small scale in the training centre of the space agency. Similarly, imitating conditions of real life is defined as modelling and simulation is used to visualise the operation of the model over time. These techniques give the idea of behaviour of real systems in different scenario and the way they would adapt to these conditions. Here main thrust area of the discussion is computational Molecular Dynamics (MD) Simulation of biological system where large scale computations are used to simulate the biological behaviour.

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MD Simulation is an approach that gives the opportunity to explore the assemblies of molecules at microscopic level and their kinetic behaviour with time which is not possible to decipher from conventional experiments (Alder & Wainright, 1959). Biological systems are very complex and the functions of cells mainly depend on various possible interactions that further depends on its three dimensional structure (Alberts et al., 2002). Being dynamic, the functions of bio-molecules also depend on various kinds of motions (Karplus & McCammon, 2002; Wynsberghe, Van, Chen & Cui, 2008). Biological molecules exhibit a wide range of time scales over which different specific processes occur. Among these atomic fluctuations, side chain motions and loop motions come under local motions (0.01 to 5 Å) which generally takes 10^{-15} to 10^{-1} s to finish. The processes for example, helix motions, domain motions (hinge bending), and subunit motions come under rigid body motions (1 to 10Å) that generally takes 10^{-9} to 1s. Some other processes, helix coil transitions, dissociation/association and folding/unfolding come under large-scale motions (> 5Å) that takes 10^{-7} to 10^{4} s to finish. It is very difficult to visualize most of the changes that take place in very short time span, by macroscopic experiment but by mimicking the *in vivo* conditions computationally and using MD simulation, computer can save these types of changes as different frames or snapshots that can be further visualized and analyzed. Martin Karplus, Yong Duan, Peter Kollman, Arieh Warshel, Andrew J McCammon, Wilfred Gunsteren, H J C Berendsen, Andrew Leach, Michael Levitt and C.L. Brooks are the pioneers and major contributors of molecular dynamics simulation of biological molecules. In 2013, Nobel Prize was awarded to pioneers of molecular dynamics simulations: Martin Karplus, Michael Levitt and Arieh Warshel that itself said about the recognition and application of molecular dynamics simulation. The objectives of this chapter comprise explanation of the molecular dynamics simulation theories, requirement of molecular dynamics for biological studies and application of molecular dynamics simulations. Moreover this chapter also focuses on various methods/algorithms for tertiary protein structure prediction.

BACKGROUND

Alder and Wainright are pioneers of MD simulation. They introduced these techniques in late 1950's to study the interactions of hard sphere (Alder & Wainright, 1957; Alder & Wainright, 1959). Later in 1964, Rahman carried out first simulation using realistic potential for liquid argon (Rahman, 1964). In 1970s, Rahman and Stillinger used the first realistic system (liquid water) for simulation (Rahman & Stillinger, 1971). However, the first simulation on protein was performed in 1977 (McCammon, Gelin & Karplus, 1977). In 1980s rapid calculations on biomolecules, thermodynamics analyses (free energy calculations) and protein-ligand simulations were added (Wong & McCammon, 1986). In 1990s, Duan and Kollman tried to reveal the folding mechanism of smaller villin protein using molecular dynamics simulation which is considered as land-mark achievement of this field (Duan & Kollman, 1998). The NCBI contains almost 30,000 publications with key-words molecular dynamics simulations. Continuous development of potential and sampling techniques now allow us to perform the simulation to micro-second and even milli-second scale. Here it is noteworthy that in simulation these milli-second scales are supposed to be very large that contradict the real experiment because in simulation co-ordinates are generated at femto-second level. Moving from femto seconds to milli seconds scale give a large ensemble of conformations that can reveal many biological mysteries. Recently quantum mechanics (QM) combined with molecular mechanics (MM) is also being applied rapidly for the molecular interaction. Gaussian is one of the popular software that allows electronic structure modelling of the system. The

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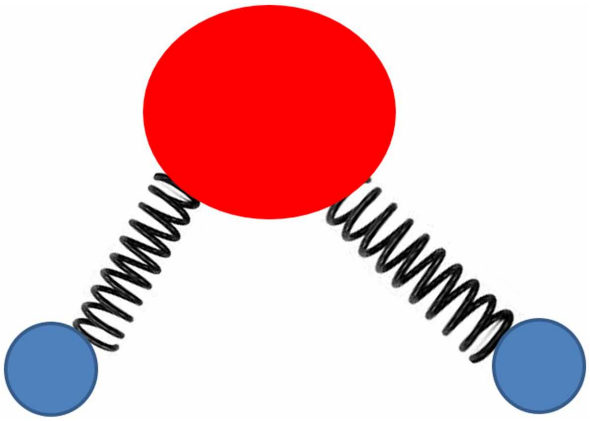
ONIOM package of Gaussian allows considering the molecules as different layers for extensive QM calculations of the area of interest and MM is applied for the rest part of system. GAMESS (Schmidt et al., 1993; Gordon, Dykstra, & Schmidt, 2005), Jaguar (Bochevarov et al., 2013), JDFTx (Sundararaman, Gunceler, Letchworth-Weaver & Arias, 2012), Quantum ESPRESSO (Giannozzi et al., 2009) etc. are other softwares used for QM calculations. A new addition in the simulation is Replica Exchange Molecular Dynamics (REMD) developed by Sugita and Okamoto that facilitates large sampling of energy landscape (Sugita & Okamoto, 1999). In this method, set of replica for the system was prepared and randomly initialized at different temperatures and then based on metropolis criteria the temperature was exchanged. This method is used to sample high dimensional rough energy landscape. Thus, molecular dynamics simulation can be a ray of hope to study the bio-molecules in detail. However, applying MD simulation to reveal the mysteries of biological world is not straight forward, since it requires optimal models that mimic the cellular environment, physical applications that can provide the motions to the model and large scale computations. But the development of more robust algorithm and theory for modeling, docking-scoring, energy-calculations etc. make the molecular dynamics simulation more robust. Here all these requisites are described in detail.

MOLECULAR MODELING

In molecular dynamics simulation the molecules are considered as "ball on spring" model that suits the dynamic behaviour. Various atoms are represented based on their atomic radii (Figure 1). Structure of bio-molecules can be generated in two different ways; either by supplying x, y and z coordinates followed by algorithms which are required to put right atoms at right place or by deducing the coordinates from the knowledge of the geometry of the molecules. If three dimensional structure of molecules are available as NMR or X-ray crystallography structure, it can act as model but when the structures are not available then one should look for the alternative option i.e. depend on the computational algorithm to find the reliable 3D structure.

There are 83955074 protein sequences in the latest release of UniProtKB/TrEMBL as of 1st oct 2014, while the Protein Data Bank (PDB) contained only 103735 protein structures till 30th Sep, 2014. Thus,

Figure 1. Ball and Spring model of water molecule



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experimental structure is not available for the vast majority of protein sequences. A list of the number of different types of molecules in PDB and their experimental methods by which the structure is determined are tabulated in Table 1. Therefore, the computational prediction of structure of proteins is must to fill the gap between the protein sequences available in public domain databases and their experimentally solved structures.

Three major methods used for the modelling of molecules are as follows:

Ab-inito Method

Ab initio structure prediction predicts the native conformation of a protein from the amino acid sequence alone. *Ab initio* prediction requires a free energy function sufficiently close to the true potential for the native state having lowest free energy minima. *Ab initio* structure prediction not only needs the free energy function but also needs the method for searching conformational space for low energy minima. Potentials can be of two types which could be used in evaluating the free energy of the peptide chain and the surrounding solvent. First one is molecular mechanics potentials which models the forces that predict the protein conformation using physically based functional forms. Examples of molecular mechanics potentials are van der Waals interactions and usually represented using a standard 6–12 potential. Another example of molecular mechanics potentials is electrostatic, which are represented by Coulomb's law with partial charges derived from QM calculations on peptide substructures. Another type of molecular mechanics potentials is called protein structure-derived potentials or scoring functions which are derived from experimental structures from the PDB (Sippl, 1995).

In *ab intio* modelling searching conformational space is also a difficult and challenging task for computational biologist. Molecular dynamics is one of the methods, generally used for searching conformational space which directly integrates Newton's equations of motion to derive the motion of a molecule in a given potential. Molecular dynamics is most often used for the study of protein folding pathways (Duan & Kollman, 1998).

A variety of methods may be used to perform broad conformational space searches through low-resolution structures; including Metropolis Monte Carlo based simulated annealing (Simons, Kooperberg, Huang & Baker, 1997), evolutionary algorithms (Bowie & Eisenberg, 1994) and genetic algorithms (Pedersen & Moult, 1997). Simulated annealing (SA) (Kirkpatrick, Gelatt & Vecchi, 1983; Simons et

Table 1. Current PDB holding as on 30th Sep, 2014

		Molecule Types				
		Proteins	**Nucleic Acids**	**Protein/NA Complexes**	**Other**	**Total**
Experimental Methods	X Ray	85854	1571	4579	5	92009
	NMR	9306	1094	220	7	10627
	Electron Microscopy	591	67	190	0	848
	Hybrid	63	3	2	1	69
	Other	159	4	6	13	182
	Total	95973	2739	4997	26	103735

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al., 1997) is probably the most popular conformational search method. Simulated annealing initially executes high temperature Monte Carlo simulation, followed by a series of simulations subject to a temperature-lowering schedule in which conformational search efficiency is not so impressive compared to other more sophisticated methods.

A single search is unlikely to find the global minimum of the free energy landscape, and may yield a structure that could be trapped in a local minimum which needs improvements in potential functions and makes identification of the most accurate models possessing the lowest free energy i.e. global local minima.

One of the best-known ideas for *ab initio* modelling is probably the generation of tertiary protein models by assembling small fragments (mainly 9-mers) taken from the PDB library (Bowie & Eisenberg, 1994). Based on the similar concept ROSETTA was developed (Simons et al. 1997), which was extremely successful and made the fragment assembly approach popular in the research area.

Ab initio modelling methods typically generate lots of structures during the simulation. How to select appropriate models structurally close to the native state is an important and challenging issue in bioinformatics/computational biology. Model Quality Assessment Programs (MQAP) was then developed in order to select the model closest to native structure (Fischer, 2006). In general, modelling selection approaches can be classified into two types, i.e. the energy based and the free-energy based. For the energy-based model selection methods, (1) physics-based energy function; (2) knowledge-based energy function; (3) scoring function describing the compatibility between the target sequence and model structures are the major contributors.

QUARK is a one of the programme for *ab initio* protein structure prediction, which aims to construct the correct protein 3D model from amino acid sequence only. QUARK models are built from small fragments (1-20 residues long) by replica-exchange Monte Carlo simulation under the guide of an atomic-level knowledge-based force field (Xu & Zhang, 2012).

TOUCHSTONE II is another new approaches to *ab initio* protein structure prediction. In this method conformational search is accelerated by newly developed parallel hyperbolic sampling algorithm with a composite movement set used in the Monte Carlo simulation processes (Zhang, Kolinski & Skolnick, 2003).

Threading

With the rapid growth in PDB and the improvement in structure prediction methods, Template-based modelling such as homology modelling and protein threading are proved powerful and key method for protein structure prediction. However, when sequence identity falls in the twilight zone (<30%), then it becomes difficult to identify good model by homology modeling. In such cases threading is popular for giving a better solution. The term "threading" was first coined in 1991 by David Jones. Threading is also called fold recognition which uses a detailed 3-D representation of protein structure. The idea behind threading was to "thread" a sequence of amino acid side chains onto a backbone structure (a fold). Thereafter the proposed 3-D structure was evaluated by using a set of pair potentials and a separate solvation potential. Threading is one of the very few methods available which can predict the fold for a protein in the absence of an evolutionary relationship. The threading or fold recognition method relies on the observation that the number of folds in nature appears to be limited and that many different remotely homologous protein sequences adopt remarkably similar structures. For a structure prediction by threading firstly, we should have a sequence of interest and a library of templates or known structures. Then query

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sequence is aligned with the various structural folds in the database, and then these subsequences are threaded and finally the best model is ranked with the help of suitable scoring function. Methods such as HHpred (Söding, Biegert & Lupas, 2005), MUSTER (Wu & Zhang, 2008)., Phyre2 (Kelley & Sternberg, 2009) I-TASSER and GenThreader are some of the popular threading based methods which combine homologous information with a variety of structure based information for remote homolog detection.

HHpred is the first fold recognition server which is based on the pairwise comparison of profile hidden Markov models (HMMs). Most conventional sequence search methods search sequence databases such as UniProt or the Non Redundant (NR) database while HHpred searches alignment databases, like Protein Data Bank (PDB), Structural Classification of Proteins (SCOP), Pfam, SMART, COGs and CDD. HHpred is very fast and performs uniformly well for single-domain as well as multi-domain query sequences (Söding et al., 2005). HHpred is also integrated with the prediction of domain boundaries.

MUSTER (MUlti-Sources ThreadER) is a new protein threading algorithm available as web-server which identifies the template structures from the PDB library. It generates sequence-template alignments by combining sequence profile-profile alignment with multiple structural informations such as secondary structure, structure fragment profiles, solvent accessibility, dihedral torsion angles and hydrophobic scoring matrix (Wu & Zhang, 2008).

The Phyre server is another popular threading programme which uses a library of known protein structures taken from the SCOP database and augmented with newer depositions in the PDB (Kelley & Sternberg, 2009). The sequence of each of these structures is scanned against a nonredundant sequence database and a profile constructed and deposited in the 'fold library'.

I-TASSER server (Zhang, 2008) is an on-line web-server for protein structure and function predictions. Here 3D models of the proteins are built based on multiple-threading alignments by LOMETS and iterative template fragment assembly simulations. In I-TASSER function predictions are achieved by matching the 3D models with BioLiP protein function database (Zhang, 2008).

GenThreader method uses a traditional sequence alignment algorithm to generate alignments which are then evaluated by a method derived from threading techniques where each threaded model is evaluated by a neural network in order to produce a single measure of confidence in the prediction (Jones, 1999). The speed of the method, sensitivity and very low false-positive rate makes it ideal and popular for automatically predicting the structure of all the proteins in a translated bacterial genome (proteome).

Homology Modeling

The first homology based model was constructed in 1969 when a wire and plastic models of bonds and atoms of α-lactalbumin was constructed by using the coordinates of a hen's egg-white lysozyme and followed by adjusting physically where two proteins exhibited 39% of sequence identity (Browne et al., 1969).

The main steps that are required to create a homology based model are summarized in following steps: (1) identification of templates structure for modelling (2) target-template sequence alignment; (3) building a model for the target based on the alignment, identity between template and target etc. (4) evaluation/validation of the model.

Some of these highly specialized, popular and frequently used homology based modeling tools/ softwares are summarized below:

3D-JIGSAW is a web-based server which generates 3D models for proteins based on homology of known structure. 3D models for proteins can be generated either in completely automatic mode by means

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of a web server or by individual modules of the program which may be executed separately followed by generation of the homology model. Templates are ranked according to the query coverage, their sequence identity and their crystallographic resolution (Bates, Kelley, MacCallum & Sternberg, 2001)

ESyPred3D is an automated homology modelling web server which implements four steps of homology modelling approach including database searching to identify structure homolog, target template alignment, model building as well as optimization and model evaluation (Lambert, Léonard, De Bolle, & Depiereux, 2002). EsyPred3D program performs better alignment by using neural networks, where alignments are obtained by combining, weighting and screening the results of several multiple alignment programs.

EasyModeller is a GUI version of MODELLER homology modeling programme in the backend. It is freely available and its usage requires pre installed MODELLER, PERL as well as PYTHON. A maximum of six templates can be used in case of multi-template based modeling in EasyModeller (Kuntal, Aparoy & Reddanna, 2010). MODELLER is one of the best method known as satisfaction of spatial restraints which is motivated by NMR spectroscopy data processing. It uses a set of geometrical criteria to produce probability density functions (pdfs) for the location of each atom in the protein used for modeling.

Geno3D is an automatic web server that uses distance geometry, simulated annealing and energy minimization algorithms to build the protein 3D homology models (Combet, Jambon, Deléage, & Geourjon, 2002). Geno3D performs homology modelling in five consecutive steps: (i) identifying homologous proteins with known 3D structures by PSI-BLAST sequence similarity programme; (ii) All potential templates are displayed with an option to choose one or more than one template structure (iii) Alignment of both target and template sequences are performed; (iv) Retrieve dihedral angles and other geometrical restraints for corresponding atoms between the target and the template; (v) 3D structure prediction of the protein by distance geometry.

CPHModel is also an automatic web server where template selection is performed on the basis of profile-profile alignment which is also guided by secondary structure and exposure predictions. In the recent version, improvement in the alignment algorithm is done by structure dependant gap penalty (Nielsen, Lundegaard, Lund & Petersen, 2010).

SWISS-MODEL is another automated web server for building protein structure homology model where template identification is carried out using various options like BLAST, PSI-BLAST and HMM-HMM-based searching (Guex & Peitsch, 1997). To estimate the quality of model(s), 'Structure Assessment' section provides stereochemistry check, global model quality and local model quality estimation.

PHYSICS BEHIND MOLECULAR DYNAMICS SIMULATION

Molecular simulation is mainly based on classical or Newtonian mechanics. However as mentioned earlier now quantum mechanics are also being incorporate in MD but still it is not possible to apply quantum mechanics to the entire bio-molecules. The difference between molecular mechanics and quantum mechanics is that molecular mechanics considered atom as smallest unit whereas in quantum mechanics explicit model of electrons are considered. Here we will discuss molecular mechanics in detail. In molecular mechanics atoms together with nucleus and electrons are considered as point charge with its associated mass. The bonded interactions are represented as Hook's law (spring connected with two atoms) and modelled as harmonic oscillator, whereas non bonded interactions are represented as Coulomb's interactions (electrostatic interaction) and Lennard-Jones potential (van der Waals interaction). Force-field

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contains the necessary building blocks for the calculation of energy and force that include bonded and non bonded interactions. Molecular mechanics force-fields are based on the four fundamental principles:

1. Born-Oppenheimer approximation: According to this approximation, speed of electrons is considered as independent from the speed of nucleus. Electrons move with much higher speed as compared to the nuclei and the nuclei are considered to be static with respect to electron.
2. Each type of bond has natural bond length and bond angle and deviation in this increases the steric energy of the system. The mathematical model of a molecule will adjust its nuclear position to yield a new geometry with lower energy according to the force-field.
3. Third principle is the set of equations that describe the potential energy of the surface of the molecule. The potential energy equations are derived from classical physics in such a way that give the best match with experimental data. After evaluating the parameters for a set they are fixed and generalized for similar compounds.
4. Fourth is the atom type that includes electronic distribution and hybridizations.
5. Generalized Force-field Equations:

$$U(\vec{R}) = \sum_{bond} K_i^{bond}(r_i - r_o)^2 + \sum_{bond} K_i^{angle}(\theta_i - \theta_o)^2 + \sum_{dihedral} K_i^{dihe}[1 + \cos(n_i\phi_i + \delta_i)] + \sum_i \sum_{j \neq i} 4\varepsilon_{ij}[(\frac{\sigma_{ij}}{r_{ij}})^{12} - (\frac{\sigma_{ij}}{r_{ij}})^6] + \sum_i \sum_{j \neq i} \frac{q_i\, q_j}{\sum r_{ij}} \quad (1)$$

In the above equations the first two terms defined the deformations in bond length and bond angle from its equilibrium position r_o and θ_o. Harmonic forms of these terms consider the correct chemical structure but it is unable to depict the chemical change e.g. bond breaking. Third term, dihedral illustrates rotation around the chemical-bond. Non-bonded interactions are described by van der Waals and electrostatic terms. Fourth term express the van der Waals' attractive and repulsive interactions in the form of Lennard Jones potential. Last term illustrates the Coulombic electrostatic potential.

Force-field has a defined set of parameters for each atom that include atomic mass, partial charge, atomic radii, equilibrium bond-length, bond angles, dihedrals, spring constant etc. Based on these parameters force-fields are able to differentiate atoms and their probable interactions. Most of the force-field e.g. AMBER (Case et al., 2014), CHARMM (Brooks et al, 1983), OPLS (Jorgensen, Maxwell & Tirado-Rives, 1996), GROMOS (Scott et al., 1999) etc. considered fixed partial charge (non-polarizable force-field) of atoms that ignores the effect of local electrostatic environment. Recently few polarizable force-fields have been developed that consider the surrounding effect of electrostatic potential either by introduction of electronic structure theory such as X-Pol (Xie & Gao, 2007) or induced dipole e.g. PIPF (Xie, Pu, Mackerell & Gao, 2007), DRF90 (Swart & Duijnen, 2006) or by considering atom as point charge carrying particle attached through spring like harmonic potential to other atoms e.g. CHARMM polarized force-field (Baker, Anisimov & Mackerell, 2012), AMBER polarized force-field (Ponder et al., 2010) etc. For, bio-molecular interactions use of polarizable force- field is still in its early stage and main reason for this is its expensive computational demand.

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Newton's Law in Molecular Dynamics Simulation

Time evolution of the system in molecular dynamics simulation is determined by the Newton's law of motion.

$$F = \frac{dP}{dt} = m\frac{d^2r}{dt^2} \tag{2}$$

Various algorithms for time integration have been developed that use Newton's law and acts as an engine for molecular dynamics simulation. Few algorithms are discussed.

Verlet Algorithm

This is the most basic and most commonly used algorithm in molecular dynamics. This is actually the two third order Taylor expansion for the position r(t), one forward and another backward in time.

$$r\left(t+\Delta t\right) = r(t) + v(t)\Delta t + \frac{1}{2}a(t)\Delta t^2 + \frac{1}{6}b(t)\Delta t^3 + O(\Delta t^4) \tag{3}$$

$$r\left(t+\Delta t\right) = r(t) - v(t)\Delta t + \frac{1}{2}a(t)\Delta t^2 - \frac{1}{6}b(t)\Delta t^3 + O(\Delta t^4) \tag{4}$$

By adding above two equations

$$F = \frac{dP}{dt} = m\frac{d^2r}{dt^2} \tag{5}$$

$$r(t + \Delta t) = 2r(t) - r(t - \Delta t) + a(t)\Delta t^2 + O(\Delta t^4) \tag{6}$$

It can be clearly observed in Equation 6 that error in verlet algorithm is in the range of Δt^4. From Newton's law acceleration is force divide by mass and force is also the function of t. So, the above equation can be written as:

$$r\left(t+\Delta t\right) \cong 2r(t) - r\left(t-\Delta t\right) + \frac{\Delta t^2}{m}f(t) \tag{7}$$

The above equation is termed as Verlet equation.

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Velocity Verlet Algorithm

In the verlet algorithm the velocity was not included; it was cancelled during the summation of Equation 3 and 4. However, it is not required during simulation but it is required to calculate kinetic energy that can be used to test the conservation of the total energy that verifies whether simulation is proceeding correctly or not. Velocity can be computed from the positions using following equation

$$v(t) = \frac{r(t+\Delta t) - r(t-\Delta t)}{2\Delta t} \tag{8}$$

Error associated with this equation is in the range of Δt^2 rather than Δt^4 in verlet algorithm.

Velocity verlet scheme shows better implementation of basic algorithm of Verlet. Here positions, velocities and accelerations are calculated at time (t+Δt) in the following ways

$$r\left(t+\Delta t\right) = r(t) + v(t)\Delta t + \frac{1}{2}a(t)\Delta t^2 \tag{9}$$

$$v\left(t+\frac{\Delta t}{2}\right) = v(t) + \frac{1}{2}a(t)\Delta t \tag{10}$$

$$a(t+\Delta t) = -\left(\frac{1}{m}\right)\nabla U(r(t+\Delta t) \tag{11}$$

Leap-Frog Algorithm

To overcome the error of velocity verlet, leap-frog algorithm was developed. In leap-frog algorithm, positions and velocities are calculated at interleaved time interval. Here position is integer time step while velocity is integer plus half time step.

$$v\left(t+\frac{\partial t}{2}\right) = v\left(t-\frac{\partial t}{2}\right) + \frac{f(t)}{m}\partial t \tag{12}$$

$$r(t+\partial t) = r(t) + v(t+\frac{\partial t}{2})\partial t \tag{13}$$

The disadvantage of leap-frog algorithm is that position and velocity are not calculated at same time so it is difficult to calculate total energy at any time point. However, it can be estimated from the following equation

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$$v(t) = \frac{v\left(t + \frac{\partial t}{2}\right) + v\left(t - \frac{\partial t}{2}\right)}{2} \tag{14}$$

Langevin Dynamics

It is based on stochastic differential equation termed as Langevin equation. In real world the molecular system is not present in the vacuum, cellular environment causes friction and the surrounding movement may perturb the system. Langevin dynamics allows incorporations of these effects in molecular dynamics simulation. It also controls the temperature of the system. So we can say that langevin dynamics mimics the viscosity of solvent but it does not include electrostatic and hydrophobic effect of the solvent. For a system of N particles having mass M and coordinates X= X (t), the langevin equation is

$$M\ddot{X} = -\Delta U(X) - YM\dot{X} + \sqrt{2YK_{B}TMR(t)} \tag{15}$$

In the above equation notations have their standard meaning.

Velocity Initialization (Maxwell-Boltzman Distributions)

To carry out molecular dynamics simulation, we need to provide initial position and velocity. Initial position is obtained from its cartesian co-ordinates of the initial geometry of the system. Initial velocity comes from Maxwell- Boltzman Distributions for specific temperature by selecting random direction.

$$E = \frac{1}{2}mv^2 = \frac{3}{2}NK_{B}T \tag{16}$$

$$v = \frac{\sqrt{3NK_{\mathrm{B}}T}}{m} \tag{17}$$

Generally, the simulation is carried out at constant temperature. To control the temperature during simulation, the equation of motion is coupled with thermostat or heat-bath. This process allows the kinetic energy of the system to be increased if the calculated instantaneous temperature is low (i.e., heat is added to the system). It also allows the kinetic energy of the system to be decreased if the calculated temperature is high (i.e., heat is removed from the system). Berendsen method is the most common approach to control the temperature of the system. In this method, the coupling between the system and the thermostat effectively leads to control temperature by scaling the velocities of the particle at regular time interval by a factor c.

$$c = \sqrt{1 - \frac{h}{\tau}\left(1 - \frac{T_{0}}{T}\right)} \tag{18}$$

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Here h is the time step, T is the current simulation temperature, T_0 is the desired temperature, τ is an empirical constant that controls the thermostat.

However, in any constant temperature simulation the temperature exhibits fluctuations since the velocity is rescaled with time. But thermostat keeps the average temperature constant.

Energy Minimization

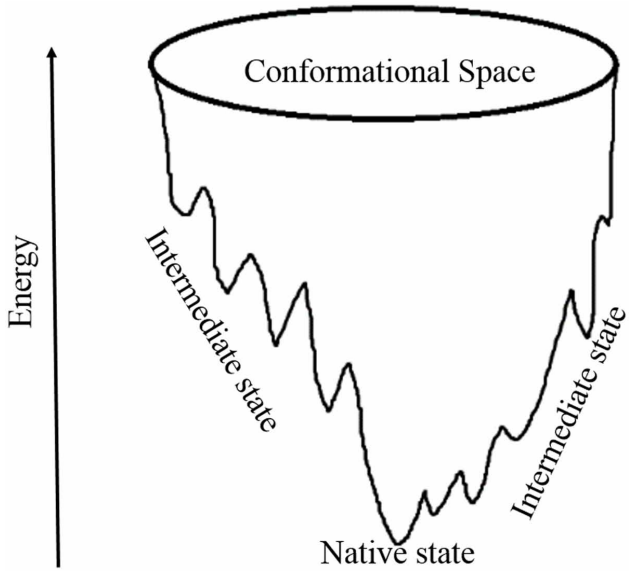
Energy minimization is also known as geometry optimization. Molecules can have multiple conformations and it always tends to be in lowest possible energy state that is the most stable state. If the entire conformational space is considered as energy well then there are large numbers of local minima (intermediate states) and one global minima (native state) (Figure 2). Energy minimization is mathematical expression that searches its nearest local minima by crossing the activation energy barrier.

The energy minimization depends on its initial conformation. Various algorithms are available for minimization e.g. Sander from AMBER, SD, Conjugant Gradient, ABNR from CHARMM etc. Before simulation it is required to optimize the geometry of the system.

Explicit and Implicit Solvation

As discussed earlier, MD simulations are performed to mimic the real environment. To study the biomolecules, it is important to consider the cellular environment. The major portion of cell (around 75%) consists of water molecules and rest of the portions consist mainly of organic compounds (carbohydrates, proteins and lipids) but to avoid the complexity, simulation is generally carried out within the water box. Size of water box should be enough to accommodate the system to be studied. Generally for smaller proteins (around 150 AA), the cubical water box includes around eight thousands of water molecules and the number of molecule will increase for the larger system. So, computationally it is not always feasible

Figure 2. Representation of conformational space of protein. Lower energy corresponds to stable structure.



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to consider all the molecules explicitly during simulation. For that, implicit solvation model is used. In implicit solvation model mathematical expressions are used that create solvent like environment. It considers solvent as a continuous medium that can miss certain important interactions mediated by the solvent molecules but nevertheless it gives very good approximation and is used in many researches. Now-a-days, well setup labs having high performance computers (HPC) even allow explicit simulation for larger system.

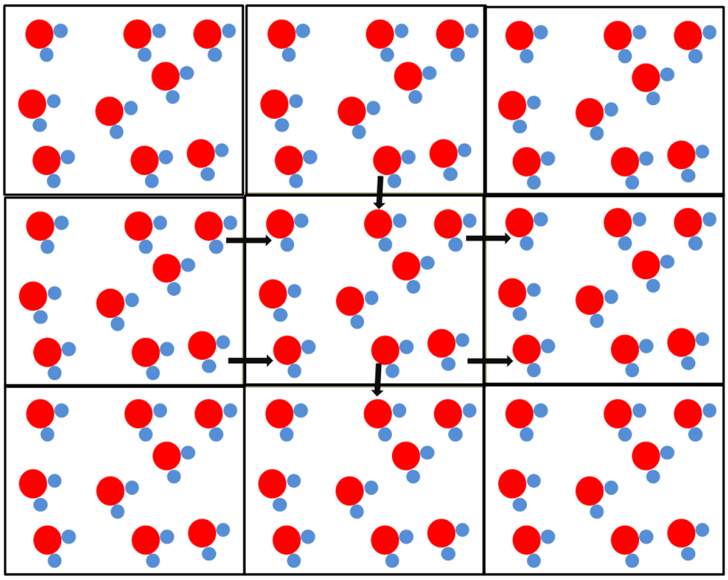
Periodic Boundary Conditions (PBC)

During the simulation of system inside a box, there is a possibility that the particles can move out of the box during simulation because of the dynamicity. To avoid this, generally a trick is applied in which replica of the box were created to cover the original box from all sides and whenever the particle moved out from the central box another will enter from the adjacent replica with the same speed (Figure 3).

Particle Mesh Ewald Method

Electrostatic potential is a long range potential. But after certain distance it becomes weaker and vanishes smoothly. So, in order to reduce the calculations, cut-off can be applied to the electrostatic potential. But cut-off does not allow the smooth fall of potential because it will become zero after certain assigned distance leading to artifacts in calculation. For, that Particle Mesh Ewald method (Darden, Perera, Li, & Pedersen, 1999) was used that divide the long range electrostatic interactions into two parts: one as short range contributions and another is long range contribution. Short range contribution is calculated in real space while long range contribution is calculated using Fourier transformations. It also allows rapid convergence of energy.

Figure 3. Representation of Periodic Boundary Condition: Removal of atoms or molecules is supposed to be filled from opposite direction during Simulation.



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Generalized steps involved in Molecular Dynamics Simulation are as follows:

1. Protein Structure File (PSF) generation
2. Energy calculation and Minimization
3. Preparatory stage involve heating and equilibration
4. Production run
5. Analysis

Protein Structure file contain all the information that are required to apply the force-field. The information that is required to generate the PSF files (atomic connectivity and partial charge, atom type etc.) come from topology and parameter file. These topology and parameters files are specific for a force-field. For standard molecule such as protein, DNA and RNA standard topology and parameters files are available. But for small molecule that act as ligand these topology and parameters files are not available rather various servers are available e.g. CHARMM-GUI (Jo, Kim, Iyer & Im, 2008), PARAM-CHEM (Vanommeslaeghe, Raman & MacKerell, 2012) etc. that can produce these files for ligand according to their atomic arrangement. After PSF generation the geometry of the structure is optimized (remove steric clash if any) by minimization (already mentioned above). During heating the temperature of the system is linearly increased from 0 to 300 K. At each integration step the velocity is re-assigned from Maxwell Boltzmann's distribution that increases the temperature. After heating to 300 K the system was equilibrated to ensure the stability. After equilibration the production run is invoked and the actual analyses are performed on the trajectory for production run.

Analysis

The major information that can be retrieved from molecular dynamics simulation are changes of atomic level interactions that may or may not lead to change in the secondary structure, changes in binding affinity in terms of binding free energy and its components, transportation and folding of molecules etc. which further gives the clue for the cellular functions.

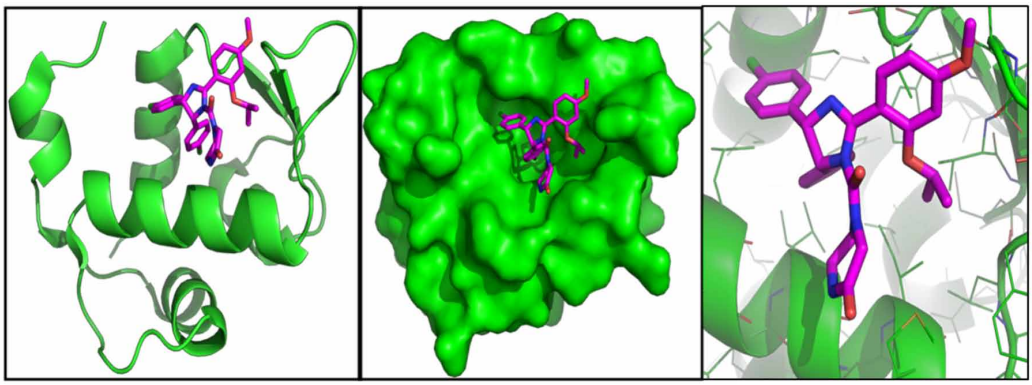
STRUCTURAL VISUALIZATION

Various visualization tools are available that gives the pictorial representation of atomic level interactions and how it varies with time over the trajectory of Molecular Dynamics Simulation. VMD (Visualization Molecular Dynamics) (Humphrey, Dalke & Schulten, 1996), Discovery Studio, Chimera (Pettersen et al., 2004), PyMoL (DeLano, 2002), Arguslab, Swiss-PDB-Viewer (Guex & Peitsch, 1997) etc. are example of visualization tools. The structural view gives the microscopic (atomic level) detail of the interactions among protein-protein, protein-ligand, protein-DNA etc. (Figure 4). This information also tells about the type of interaction and based on that it can further be improved or even diminished by applying proper mutation in the molecule.

This type of study has now-a-days become very popular in therapeutics. Apart from that, visualization software also tells about the secondary structure of protein that depends on the interactions. It is similar to the curling of hair: disulfide bonds formation in keratin allows curling of hair and when the interac-

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Figure 4. Different representations (cartoon, surface and lines) of protein (green colour) with ligand (magenta colour), prepared by PyMoL (visualization software). Co-ordinates were retrieved from Protein Data Bank (PDB ID: 4HG7).



tion is disrupted hair becomes straight. In many cases it was reported that the formation or disruption of certain interactions effect the secondary structure. To quantify the structural changes various kinds of time series calculations are performed that are briefly mentioned below.

TIME SERIES CALCULATION

These includes generally root-mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (RGYR), surface-accessibility (SA), secondary structure (phi-psi calculations) etc. These calculations tell about the atomic and molecular level changes over time period. Root mean square deviation gives the idea whether the system is stable or not over simulation whereas root mean square fluctuations give the idea about flexibility of particular residue over time period. Radius of gyration is the measure of root mean square distance of the system from its centre of mass; this is also used to get the idea of fluctuations of the system. Surface accessibility gives the idea about which portion of the system can be reachable for the solvent based on its three dimensional conformation. In biological system solvent play important role in the interactions of the molecules and binding affinity can vary with solvent accessibility.

Thermodynamics Calculations

Theory of cellular functions and drug-designing mainly depends on the selective and specific binding of molecules. These selectivity and specificity further depends on the thermodynamics and kinetics behaviour of the system. Detail knowledge of thermodynamics give better understanding of the molecular interactions by dictating almost every physical property and also allow desired modifications for improvement of molecular activities (e.g. drug-design). It can reveal many critical scientific issues such as folding, transportation and interactions of molecules which are further responsible for cellular functions. Docking-screening and virtual screening is the most significant method to study the binding affinity of the system. In 1972, Platzer et al. have used empirical energy calculations of isolated chymotrypsin with

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various amides that showed good correlation with the available experimental data (Platzer, Momany & Scherega, 1972). But it does not involve the kinetics behaviour of the system in contrast to the cellular environment, where molecule shows dynamic behaviour. To include the dynamicity, another approach is used to calculate the thermodynamics calculations over the trajectories obtained from simulation. From this approach, one can find out the dominating energy components (e.g. Bonded: covalent, H-bond etc. or Non-bonded: electrostatic, van der Waals etc.) and based on that, interactions can be modified (increased or decreased). These calculations are more accurate (ref- jmed chem) but computationally expensive. The statistical mechanical theory with molecular dynamics simulation has been combined to calculate the free energy and compare the binding affinity of various inhibitors of trypsin (Wong & McCammon, 1986). Lambda Dynamics (LD) method is another approach developed (Kong & Brooks, 1986) that use coupling parameter as a dynamics variable to increase the efficiency of free energy calculations. Linear Interaction Energy (LIE) is an empirical approach developed by Åqvist's group (Åqvist, Medina & Samuelsso, 1994) which was elaborated later (Lamb, Tirado-Rives & Jorgensen, 1999). In this method separate sampling of free and bound ligand were performed and the difference of van der Waals and electrostatic energies between the ligand and its surrounding was calculated. Chemical Monte Carlo / Molecular Dynamics (CMC/MD) developed by Ericksson et.al (Ericksson, Pitera & Kollman, 1999) also used for energy calculation. This is a stochastic free energy calculation and it allows the comparison of 5-10 similar kind of molecules at a time. In this method simulated system are divided into two different regions: monte carlo region and surrounding region. It is used to compare multiple side chain at a single position of protein and peptide (Pitera & Kollman, 1998). MM-PBSA is another robust approach developed by Srinivasan et. al. (Srinivasan, Cheatham, Cieplak, Kollman, & Case, 1998) that is based on the concept of LIE but here energy calculations can be performed by the sampling of only complex system. This method is widely used for the relative prediction of binding affinities (Kuhn & Kollman, 2000; Kuhn, Gerber, Schulz-Gasch, & Stahl, 2005). But if the system has large conformational change, it will miss out those important changes. For these kinds of systems, MM-PBSA is done on separate sample of complex and uncomplex. This approach also suffers high computational demand. Molecular Mechanics with Generalized Born and Solvent Accessibility (MM-GBSA) is another method to calculate binding energy by combining molecular dynamics and free energy scoring schemes (Lyne, Lamb, & Saeh, 2006; Zoete, Irving & Michielin, 2010, Rastelli, Del Rio, Degliesposti & Sgobba, 2010; Hayes et al., 2011). Poisson Boltzmann (PB) and Generalized Born (GB) used different but similar kind of mathematical equations to roughly mimic the solvent (polar and non-polar component) without considering the solvent molecule explicitly. MM-PBSA is considered as more accurate and slow over MM-GBSA. Binding energy and change in binding energy is calculated from equation (19) and (20) respectively.

$$G = E_{\text{int}\,ernal} + E_{vdw} + E_{electrosttaic} + E_{Solvation}^{Polar} + E_{Solvation}^{Nonpolar} - TS \tag{19}$$

Here, it should be noted that salvation terms also include the entropy of solvent and TS represent the entropy of solute. Entropy calculation of solute is non-trivial thing. Normal Mode Analysis (NMA) (ref) is the widely used and most appropriate methods available till now for entropy calculations. However, NMA is highly expensive method and generally it is done on few hundred frames of the sample and average values are used.

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$$\Delta G^{\circ}_{Binding} = < \Delta G^{\circ}_{complex} > - < \Delta G^{\circ}_{receptor} > - < \Delta G^{\circ}_{ligand} > \quad (20)$$

Free energy perturbation (FEP) and Thermodynamics integration (TI) are the methods used to calculate the absolute binding free energy. These methods are however considered as more accurate than MM-PBSA and MM-GBSA but its use is limited due to high computational demand.

COMPUTATIONAL CHALLENGES

Molecular dynamics simulation requires high performance computations. To mimic biological molecules one needs to consider the cellular environment that consists of a number of molecules and solvent. But to consider exact cellular environment computationally, is not possible because it will include huge number of atoms and molecules that is computationally not feasible and even if very efficient computation is used to consider entire molecule then the process become much complex and one is unable to get insight about all the molecules together. So, generally the system of interest is selected and solvated into virtual water box and detailed interactions is studied. To achieve global minima (native state) is also a challenge for molecular dynamics simulation, generally the system stuck in local minima although many molecules have also found to be shown its function in local minima. For this, generally the work need experimental support e.g. if the structure (NMR or crystal structure) of similar interactions are available and their binding affinity is known then one can benchmark the MD protocol by reproducing the structure or similar trends of affinity and then further proceed with those trajectory to explore the interactions (e.g. mutation can be done to improve the interaction, transition state of protein folding can be explored). If the trajectory is unable to reproduce the experimental trend for particular system then sometimes it is difficult to establish any new things based on the simulation study. But it doesn't mean that the simulation is entirely wrong, it may happen that simulation is showing something that was unexplored by experiments and it can be some clue for new kind of study and experiments. To cross-check the simulation generally independent trajectory are analyzed and average data from these independent trajectories reflect the properties of the system.

APPLICATIONS OF MOLECULAR DYNAMICS SIMULATIONS

Molecular dynamics simulations have wide applications. It is regularly used in meteorology and climatology, video games, film industries as well as in scientific world. Here major focus is to know its application to study the biomolecules. Following are the applications of molecular dynamics simulation:

Atomic Level Insight into Molecular Interactions with Time

Molecular dynamics Simulation give the dynamic pictures of atomic level insight of various biomolecular interactions. This property of MD enables to predict various possible cellular behaviours. Based on this, possible modifications of existing ligand as well as new ligands are proposed that leads us to the new era of drug-designing.

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Molecular Docking

Molecular dynamics simulation is widely used to study the protein-protein, protein-ligand, DNA-protein and DNA-ligand interactions. Researchers generally want to study the effect of new kind of molecular interactions that can act as inhibitor. For this kind of study generally virtual screening or docking is performed followed by molecular dynamics simulation to know the effect of interactions with time (Alonso, Bliznyuk, & Gready, 2006). Glide (Friesner et al., 2004), Gold (Jones, Willett & Glen, 1995; Jones, Willett, Glen, Leach & Taylor, 1997), Auto-dock (Morris et al., 1998; Storiffer, Ni & McCammon, 2000), FlexX (Rarey, Kramer, Lengauer, & Klebe, 1996) etc. are well known docking softwares. Docking is a computational method to fit a molecule into other, in various possible orientation or 'pose'. The sofwares use algorithm to calculate the dock score based on the contact surface, electrostatics etc (Kroemer, 2007) and good score is supposed to have good binding affinity. In virtual screening, docking was performed for larger data and the molecule that have shown good dock score were selected for further study. It can be further studied by *in-vitro*, *in-vivo*, pre-clinical and clinical trials. As each of these processes is time taking and expensive, the docked structure can further be filtered by thermodynamics and structural analyses over the trajectories obtained from molecular dynamics simulation.

Protein Folding

Protein folding is the most mysterious aspect of biology. Anfinsen's experiment (Anfinsen, Haber, Sela & White, 1961) revealed that the three dimensional structure of protein is guided by its amino-acid sequence but the mechanism of protein-folding is still unknown. Molecular dynamics simulation was used to study the folding mechanism of small (36 amino acids) sub-domain of villin protein (Duan & Kollman, 1998). This was a landmark implementation of molecular dynamics simulation in biological field, although is computationally expensive for larger proteins but definitely a ray of hope towards the understanding of folding mechanism.

Effect of Mutation on Interactions

To know the effect of particular amino-acid in binding, the residue is mutated and comparative study of simulated trajectories (before and after mutation) is performed. From the atomic level insights into protein-ligand interactions, the modifications in the ligand can also be proposed to improve the affinity further.

Flexibility of Molecules

Flexibility of biomolecules directly regulates its intercourse, which is the key factor of cellular function. As, molecular dynamics simulation give the dynamic picture of the system so it also reflects the flexibility to some extent. There are few techniques such as Principal Component Analysis (PCA), Elastic Network Model (ENM), Anisotropic Network Model (ANM) etc. that can extrapolate the major contributing motions of the system (Bakan & Bahar, 2009).

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FUTURE RESEARCH DIRECTIONS

Molecular Dynamics Simulation has opened a wide scope of study and research on biological system (e.g. atomic level interactions, drug-designing, protein folding etc.). But the entire theory is based on the reliability of model, force-field and various analysis techniques used to perform thermodynamics calculations. Despite many established and well accepted techniques there is lots of scope for its improvement and to reduce the level of approximation. For example MM-GBSA is a widely accepted method to check the trend of the binding affinity of different ligand with proteins but the value obtained from it is some-time far from experimental data and its values are not acceptable. Development of more robust algorithms should be at the priority list in future research that can be used synergistically with other experimental data to reveal many mysterious biological happenings.

CONCLUSION

Molecular Dynamics Simulation is a technique that gives the dynamic picture of the system. Development of high performance computation (e.g. Message Passing Interface), modeling, physical applications and analyses techniques allow the molecular dynamics simulation of biological molecules such as proteins, nucleic acids, carbohydrates etc. and their interactions with each other or small molecules inhibitors. It is used to solve the mysterious biological problems that occur within femto-second to milli-second time scale (e,g. Atomic and molecular interaction, folding etc.). It also provides insights into atomic level interactions which are related to the functions of the molecule. It discloses the detail information about the contributions of various energy components in the binding of molecules that further help to improve the affinity. We can say that using appropriate model and physical applications, molecular dynamics simulation can solve the chemistry of biological event. But still lots of improvement are required to improve the algorithms which are based on various approximations. Accurate modelling is also a crucial portion of the simulation process. Various available methods for modelling were discussed. Moreover, among the tertiary structure prediction methods homology or comparative modelling is the most accurate method for structure prediction. Besides the development of algorithms and computations, the role of macroscopic (or real world) experiments could not be ignored to solve the biological mystery. Macroscopic experiment and molecular dynamics (microscopic experiment) are synergistic processes. Various macroscopic experiments (such as Ki, ΔG, IC50, EC50, energy of the system etc.) allow validating the modelled structures that can further reveal atomic level interactions, flexibility, local and global motions etc. through microscopic (molecular dynamics simulation) experiment.

Overall, the chapter explores basic concepts that can help to enhance the communications among biologist, physicist, chemist and computation experts. Thus in the present scenario the inter connection among various disciplines of science is crucial for avoiding scientific boomerang and thereby leading to sustainable development.

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KEY TERMS AND DEFINITIONS

***Ab Initio* Modelling:** A de novo method to predict the protein structure by the general laws of physics and chemistry.

Drug Design: An inventive process of finding new medications based on the knowledge of a biological target.

Homology Modeling: (**Comparative Modelling):** A method to constructing an atomic-resolution *model* of the "target" protein from its amino acid sequence and an experimental three-dimensional structure of a related *homologous* protein (the "template").

Molecular Docking: "Best-fit" orientation of a ligand that binds to a particular protein of interest.

Molecular Dynamics: (MD) is a computer simulation of physical movements of atoms and molecules in the context of N-body simulation of biological systems.

Molecular Modelling: A technique for deriving, representing and manipulating the structures of molecules, which are dependent on these three dimensional structures.

Protein Threading: Method of computational protein structure prediction which is used to model those proteins which have the same fold as proteins of known structures.

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Chapter 41
Protein Ligand Interaction Fingerprints

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ABSTRACT

A most challenging part in docking-based virtual screening is the scoring functions implemented in various docking programs in order to evaluate different poses of the ligands inside the binding cavity of the receptor. Precise and trustable measurement of ligand−protein affinity for Structure-Based Virtual Screening (SB-VS) is therefore, an outstanding problem in docking studies. Empirical post-docking filters can be helpful as a way to provide various types of structure-activity information. Different types of interaction have been presented between the ligands and the receptor so far. Based on the diversity and importance of PLIF methods, this chapter will focus on the comparison of different protocols. The advantages and disadvantages of all methods will be discussed explicitly in this chapter as well as future sights for further progress in this field. Different classifications approaches for the protein-ligand interaction fingerprints were also discussed in this chapter.

INTRODUCTION

The number of protein-ligand complex structures from both in silico and experimental methods have highly increased during the past decade. This was due to advances in structure-based drug design in pharmaceutical industry, high-throughput NMR technology, crystallography and structural genomics. In the public domain, availability of over 5000 small molecule complexes within databases is the result of many effective studies performed in this field (Deng, Chuaqui, & Singh, 2004).

Utilizing this experimental and in silico information depends highly on the ability to organize, analyze and mine the structural data, based on virtual chemical library screening (Deng et al., 2004).

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The most important merit of virtual screening as a complementary approach to the traditional high-throughput screening is to give insights into molecular recognition in biological systems as well as to facilitate the design of novel therapeutics (Kelly & Mancera, 2004). However, some pitfalls are observed in virtual screening studies, which can be summarized as bellow (Scior et al., 2012):

1. **Detecting High Affinity Molecules by Virtual Screening:** Virtual screening is not considered as a successful tool for identifying high affinity substances. Since reaching highly attracted compounds to particular target is possible during the optimization process.
2. **Certainty of Inquiries:** The setting chosen to select potent materials from a large number of substances are very arbitrary and may depend on the performance of screening.
3. **An Expectation the False Binding Site:** Possibility of binding to exactly correct poses is low in fact. Actually, a few studies revealed exactly true prediction of binding modes.
4. **Predicting Water Mediated Interactions:** Possibly there is many water mediated hydrogen bonds between ligand and receptor. Prediction of count, situation and orientation of these interactions is very ambitious.
5. **Allosteric or Multiple Binding Sites vs. Single Ones:** Many receptors possess allosteric or multiple binding sites, but VS approaches are unable to predict the affinity to an allosteric binding site.

Retrieving useful and crucial information for rational drug discovery is not facile due to an overwhelming number of 3D X-ray and NMR structures. In order to perform virtual screening, these prospective ligands need to be filtered in order to reduce their number for more precise and intensive studies. (Kelly & Mancera, 2004).

Up to now, a variety of approaches have been developed to capture structural knowledge ranging from 2D ligand filters and pharmacophores to 3D interaction constraints and pharmacophoric features (Nandigam, Kim, Singh, & Chuaqui, 2009). All these methods can be used to limit poses generated from ligand receptor docking. Although analyzing a few structures by in silico methods is now considered as a usual practice, mining and making comparisons between a huge number of protein-ligand complexes needs special care such as simplification of the 3D information. Among the most useful simplification processes for analyzing protein-ligand interactions is the conversion of atomic coordinates into simpler 1D or 2D fingerprints (Desaphy, Raimbaud, Ducrot, & Rognan, 2013). Moreover, because binding information is encoded as 1D fingerprint strings, pattern identification of the fingerprints can be done using machine learning and clustering methods. This approach is beneficial in enhancing the ability to make useful implications that are not apparent by looking at individual structures (Nandigam et al., 2009).

BACKGROUND

Fingerprints are easy to generate and can manipulate, compare and, therefore, enable a systematic analysis of large data sets. They are largely used to describe and compare molecular objects (small molecular weight ligands, pharmacophores, proteins and protein-ligand binding sites) and can be used as descriptors. This feature of fingerprints makes it possible to use them by drug design programs as well as in silico screening tools. These fingerprints are frequently manipulated in either ligand based, structure

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based and combined approaches of drug design. They can solve problems related to finding conserved interaction patterns, explain interaction features qualitatively and perform efficient post docking processing. (Desaphy et al., 2013)

Implementations of fingerprinting methods assist scoring functions to rank the predicted ligand-protein interactions in order to select those candidates that have a greater chance of success in vitro. It also permits us to perform further analyses and similarity comparisons between these structures.(Kelly & Mancera, 2004)

Different protein ligand interaction fingerprints have been developed so far. Based on this approach, the residues involved in the interaction must be determined. In the first step, it is of great importance to consider different interactions of the ligand poses within the target for generation of a suitable algorithm. (Deng et al., 2004; Singh, Deng, Narale, & Chuaqui, 2006) Different types of interaction have been presented between the ligands and the receptor. (Chourasia, Sastry, & Sastry, 2011; Crowley & Golovin, 2005; Dalkas, Teheux, Kwasigroch, & Rooman, 2014) A common interaction observed between the ligand and protein is called cation-π interactions.(Crowley & Golovin, 2005) This interaction is made between a positively charge group such as quaternary nitrogen inside ligands with aromatic side chains of the protein such as PHE or TYR. (Crowley & Golovin, 2005). In addition, the positive charge amino acids such as LYS or ARG can form this type of interaction with aromatic fragments of the ligands. Cation-pi interaction refers to a cation fragment on the axis perpendicular to aromatic ring. (Crowley & Golovin, 2005; Dalkas et al., 2014) The hydrogen bonding interaction can be formed between a hydrogen donor and an acceptor. Two distance and angle terms normally are being considered in calculation of this interaction. π-π interaction is another interaction between the aromatic fragment of the ligand and aromatic side chain. (Dalkas et al., 2014) This interaction is among the most discussed interactions in the literature. Two different types of face to face and edge to face π- π can be investigated in docking studies. (Dalkas et al., 2014) The distance between two aromatic planes centers is normally defined as 4 Å in both types. Ionic interaction is an interaction between two groups of different charges. This interaction as well as hydrophobic and metal complex interactions have only a distance criterion. (Marcou & Rognan, 2007)

The next step for generating the bit strings is highly dependent to interactions criteria. As depicted in Figure 1, each residue is represented by a seven long bit string based on the following criteria. (Singh et al., 2006)

1. Is it in contact with the ligand or not?
2. Are any main chain atoms in contact with the ligand?
3. Are any side chain atoms involved in the binding?
4. Are there any polar interactions?
5. Are there any non-polar interactions?
6. Is the residue providing hydrogen bond acceptor?
7. Is the residue providing hydrogen bond donor?

And for nine-bit strings described as 1-any contact 2-backbone 3-side chain 4-polar 5-hydrophobic 6-H-donor 7- H-acceptor 8-aromatic 9-charged interactions (Figure 2).(Mordalski, Kosciolek, Kristiansen, Sylte, & Bojarski, 2011)

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Figure 1. A seven bit string generated on interaction criteria

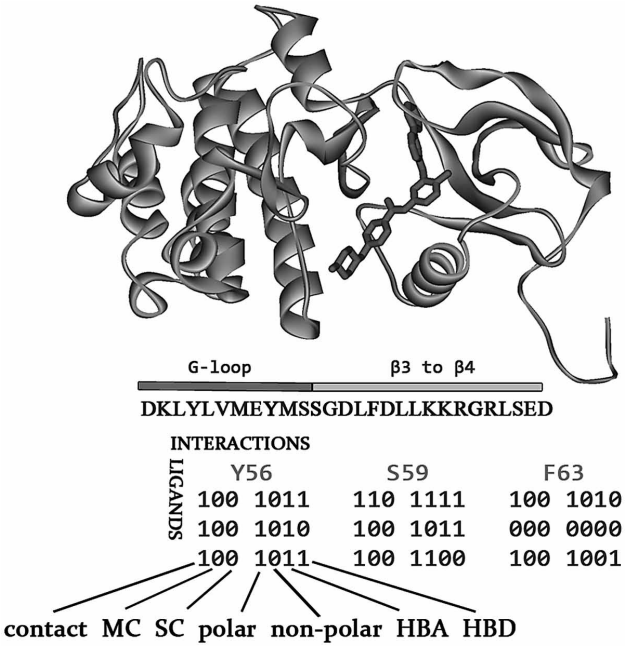
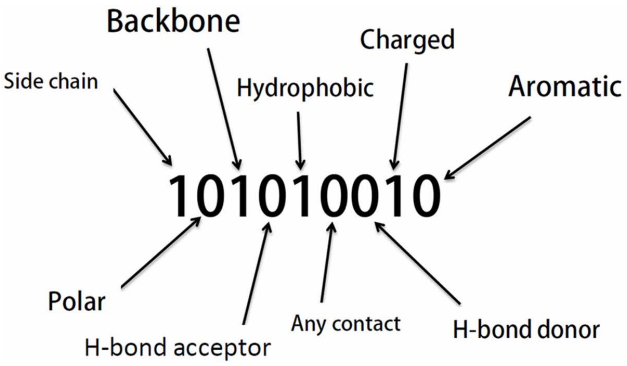


Figure 2. An example of a nine bit string



PROTEIN-LIGAND INTERACTION DESCRIPTORS

The primary methods for presenting molecular fingerprints were based upon chemical composition and structures of the molecules, First approach in ligand-protein interaction fingerprints was explained by Bajorath et al (Bajorath, 2008). The idea behind this project was that all atoms in a ligand do not take a similar role for the activity (Tan, Batista, & Bajorath, 2010). As a powerful method, interaction annotated fingerprint was more successful than the previous 2D or 3D descriptors since descriptors related to protein cavity, and those related to ligands were merged as a single fingerprint profile (Crisman, Sisay, & Bajorath, 2008). Different machine learning methods were able discriminate between true and false complex of ligand and protein (Wang et al., 2011). As an instance, this method was used in identifica-

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tion of novel ligands for oxytocin receptor and showed to be more useful than ligand based 2D and 3D descriptors (Weill et al., 2011).

Further studies by Rognan and Deng introduced two novel topological scoring methods based on molecular interaction fingerprints (IFPs). This was a novel approach to represent the properties of a ligand and a better descriptor to quantify docking success. (Deng et al., 2004; Desaphy et al., 2013)

In contrast with traditional methods based on calculating the properties of the ligands from their chemical and structural components, the structural interaction fingerprints (SIFTs) represent a ligand by its interaction in the binding site of the protein. This requires knowledge for the binding mode of each ligand inside its target protein. Basic implementation of this method has revealed effectiveness in the post processing of docking studies and as a filter during a virtual chemical library screening process. (Mordalski et al., 2011)Standard descriptors for protein cavities and their cognate ligands can also be concatenated into a single fingerprint and then used as input to train machine learning algorithms. The results can be used to discriminate true from false complexes. The structural interaction fingerprints show several promising features (Desaphy et al., 2013):

1. Enhancing the quality of pose prediction in docking experiments.
2. Clustering protein-ligand interactions for a panel of related structures according to the diversity of their interactions with a target subfamily.
   3 Assisting target-based library design.

In fact, IFPs are simple bit strings that convert 3D information of protein-ligand interactions into 1D bit vector representations that can be quickly compared with each other.

In this regard, the first step includes identification of all the residues involved in protein-ligand inter-molecular interactions. Next step is dedicated to classify the calculated interactions. Each bit can denote either the presence (1) or absence (0) of a particular discrete property such as hydrogen bond, hydrophobic or van der Waals contact. The obtained data might be utilized for ligand analysis as well describing protein binding sites and classification approaches (Nandigam et al., 2009).

Since these bit strings represent interactions between the ligand and the receptor, they can be divided as data pieces containing the contact information for all individual amino acids. In some works, seven-bit strings were used to describe those associations, while nine-bits are used in others (Desaphy et al., 2013). The fingerprint approaches are different in terms of their bit string definition and the type of interaction considered in each implementation (Desaphy et al., 2013). The initial fingerprinting approach at the residue level considered only hydrophobic and hydrogen bond contacts. This concept which was based on residues has later evolved to a new one based on the role of atoms for hydrogen-bond sites. A new concept of weighting for showing the importance of detected interactions was introduced by Kelly and Mancera (Kelly & Mancera, 2004).

The idea of this approach was to use a ligand from a co-crystal structure as a reference to score docking poses of other ligands by fingerprint similarity. The SIFT approach was shown to outperform traditional scoring functions in virtual screening and was applied successfully to cluster docking poses of individual ligands. Several extensions of the algorithms were implemented up to now, extending the accuracy of the definition of the interactions used for the fingerprint, evaluating the approach for virtual screening, scaffold docking, or the use of profile SIFTs for virtual screening (Deng et al., 2004). The largest obstacle for this approach is that a reference ligand is mandatory (Renner, Derksen, Radestock,

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& Mörchen, 2008).Docking based screening were capable of generating impressive results and many novel hits were introduced by the time being. The performance of these scoring functions can nonetheless be improved through the inclusion of tools such as clustering methods based on structural similarity.

DIFFERENT IMPLEMENTATIONS, DIFFERENT SOLUTIONS

A virtual screening can cover the ligand protein interaction for more than thousands of small molecules in a data set. The key point in performing a successful virtual screening is to comprehend the correct binding sites of the receptor as well as the most favorable binding mode for the ligand (Lyne, 2002). As described the most common method to visualize the 2D ligand-protein interaction was using softwares such as LIGPLOT (Wallace, Laskowski, & Thornton, 1995). As the number of docking poses are increasing in virtual screening studies, using other automated methods for analysis of ligand protein interactions becomes crucial (Stierand, Maaß, & Rarey, 2006). The methods must be smart enough to compare the interaction patterns of each ligand and distinguish those poses with more interactions in the active site.

Structural Interaction Fingerprints (SIFT)

This is a fingerprinting approach for mining, analyzing, organizing as well as visualizing huge number of structural binding data. The basic algorithm for generating this fingerprint is to convert structural data of ligand protein interactions in to a vector which represents every bonding as well as its nature (Deng et al., 2004). Since the binding information for each ligand is encoded as 1D string, advanced filters such as machine learning clustering methods can be implemented for further analysis of the bit strings. The statistical analysis used in any fingerprinting approach can be readily used in other methods for more comparison (Learning, 2010). In SIFT method, at first key residues that are involved in all ligand protein complexes of that target are identified. The next step includes representing all key residues by means of bit strings based on their interactions with the ligands. The first bit is the master bit which determines whether any interactions exist. The second and the third bits are related to the interactions with the main chain and the side chain, respectively. The next four bits are identifying the type of the interactions. The fourth and the fifth bits are "on" or "off" according to polar and non-polar type of the residues involved in the binding. "On" values at positions 6 and 7 of the bits are determined based on the presence of donor or acceptor functional groups in the residues (Chuaqui, Deng, & Singh, 2005). The combination of all bit strings generates a unique pattern for each complex of the ligand-protein.

In order to validate this method, docking simulations were performed by four popular docking programs (FlexX, Glide, Gold, and Surflex) for generation of poses in case of three data sets. A set of 40 compounds with low-molecular-weight from the Protein Data Bank, a collection of 40 scaffolds from protein ligands, and a database of 19 scaffolds extracted from true cdk2 inhibitors.(Deng et al., 2004)

Drugscore

Drugscore-FP is also a ligand-protein interaction fingerprint that is generated by atom score partitioning of docked inhibitors as 1D vector. Similar to the previous methods, simple distance metrics allow measuring the similarities between the fingerprints of docked structures from co-crystal structures. Furthermore, drugscore makes it possible to produce family based fingerprint profiles similar to SIFT method. The

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profiles of interaction by drugscore identify similarities and dissimilarities related to each target. The most advantage of Drugscore-FP is that near-native-docking-poses are better identified compared to SIFT and Drugscore-CSD methods. This finding was discovered using a customized dataset by Wang et al. Finally, it was seen that drugscore can be considered as a powerful filter to recognize similar binding profiles and its efficiency was verified using cross-validation studies (Pfeffer, Neudert, & Klebe, 2008).

Based on this fingerprint many x-ray structures were studied to find out their interaction patterns, which are effective for designing inhibitors with potency and selectivity. As described, the primary fingerprinting methods presented a simple overview for the protein ligand interactions, wherein all interactions are presumed to have an equal impact. But in fact, the roles of different residues are not similar in the interactions. For example, in kinase targets the interactions at hinge regions are more important for the activity compared to those in other parts of the enzyme. This was therefore a great pitfall for fingerprinting approaches and a motivating factor for the researchers to invent new algorithms for interaction fingerprinting. To overcome this limitation, Nandigan et al presented weighted structure interaction fingerprint (w-SIFT) (Nandigam et al., 2009).

Weighted Structure Interaction Fingerprint (w-SIFT)

Based on this method the ligand-protein interactions are generated as a weighted profile in such a way that the weight of each interaction determines its importance. For example, hydrogen bonding might be different weighted from polar or non-polar interactions. Therefore, by considering the interaction data distinct from each other and determining the importance of each interaction a more realistic fingerprint can be obtained. It was also possible to consider the weight of each interaction so that a reasonable correlation between experimental activity and docking results would be obtained. These equations can be used for further analysis of drug design. To obtain the profile, the weight values must be calculated in a training set based on the correlation between experimental activity and interaction fingerprints. The weighted profile includes non-negative values within the range 0 to 1. The value, 0 belong to positions which do not have any 1 values in any of its SIFTs while 1 goes to position with at least one 1 value in that position. This type of weighting is so that those interactions which are frequently observed in high potent compounds are taking more weights. On the other hand, the interactions which are frequently observed in less active compounds are taking fewer weights in the w-SIFT profile. w-SIFTS with more weights are therefore denoting the more potent inhibitors of protein. In this method tanimoto distances are finally used to compare between different w-SIFTs (Nandigam et al., 2009).

As seen in Table 1, another version of SIFT (R-SIFT) is a fragment based method for screening combinatorial libraries.

Structural Protein-Ligand Interaction Fingerprints (SPLIF)

Another algorithm is based on calculating structural protein-ligand interaction fingerprints (SPLIF) for each docking pose and comparing it with the reference ligand-protein complex (Da & Kireev, 2014).

SPLIF presents quantitative measurement of whether a docking pose interacts with the protein target in a similar way as its known ligand and can diminish the false negative active compounds with poor initial docking scores.(Da & Kireev, 2014) A trial benchmark study of 10 diverse data sets extracted from the DUD-E database has been carried out to evaluate the ability and efficiency of this protocol. SPLIF can be considered as an interaction fingerprint with the ability to explicitly encode structural

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information as well as capturing all types of ligand−protein interactions (e.g., stacking, polarization, cation-π, CH-π). (Da & Kireev, 2014)

In this regard, if the distances between two atoms are less than the predefined threshold, they are considered as being in contact with each other. Afterwards, for each protein-ligand pair, the protein and the ligand atoms are expanded to circular fragments. The third step is retrieving the 3D coordinates for the ligand-protein complex and assigning "on" and "off" bit for the contacts. A privilege for this type of fingerprinting compared to that discussed before is that in the previous algorithm the interactions types need to be encoded by empirical rules at each step. For example, in case of two parallel aromatic fragments a pi-pi interaction is anticipated.(Crowley & Golovin, 2005; Dalkas et al., 2014)

These types of interactions are merely considered if the specific criteria for them are accepted by the application. On the other hand, SPLIF is calculating any contacts between the receptor and the ligand. The ability to generate 3D fingerprints is nowadays implemented in many softwares (Vilar, Cozza, & Moro, 2008). These 3D fingerprints are utilized to express the binding mode of the ligand in the binding cavity of the receptor. These 3D fingerprints are useful in studying small sets of ligands. In case of virtual screening or studying the binding mode for a large data set, 1D bit strings must be taken.

In addition, due to analysis efficiency of IFs, they are considered as an important toolbox in post processing of docking results. Many softwares such as Schrodinger and MOE have implemented these criteria for analysis of docking results (Vilar et al., 2008).

Protein Ligand Interaction Fingerprint (PLIF)

This is a residue based interaction fingerprint developed by chemical computing group implemented in MOE software (Clark & Labute, 2007). The interaction fingerprint is generated by a presence or absence of 6 types of intermolecular interactions including hydrogen bond with donor side chain, hydrogen bond with side chain acceptor, hydrogen bond with backbone donor, and hydrogen bond with backbone acceptor, ionic attraction and surface contact. Each type of interaction can be defined as either weak or strong. Accordingly, for each residue a 12 bit string is encoded based on interaction type and strength. As described one of the most important applications of PLIF studies is their ability to be used in virtual screening or binding mode detection. Different interaction fingerprints have been developed so far. The efficiency and applicability of a PLIF algorithm are dependent upon the bit string definition implemented in the software (Clark & Labute, 2007).

A pitfall of many fingerprint methods is that a bit is related to each active site residue atom. This limits the analysis to highly homologue active sites. To overcome this problem cavity independent fingerprinting methods have emerged (Pérez-Nueno, Rabal, Borrell, & Teixidó, 2009).

Atom Pairs Based Interaction Fingerprint (APIF)

A novel fingerprinting approach called APIF (Atom Pairs based Interaction Fingerprint) which is based on atom pairs contacts for encoding protein-ligand interaction fingerprints is an example of cavity independent fingerprinting approach (Clark & Labute, 2007).

APIF has been validated by means of enrichment factor wherein poses with a similar pattern of contact towards crystallographic data were ranked as top poses. Its application as a post processing strategy using four targets HIV protease, Carboxypeptidase, trypsin and estrogen receptor α has been revealed.

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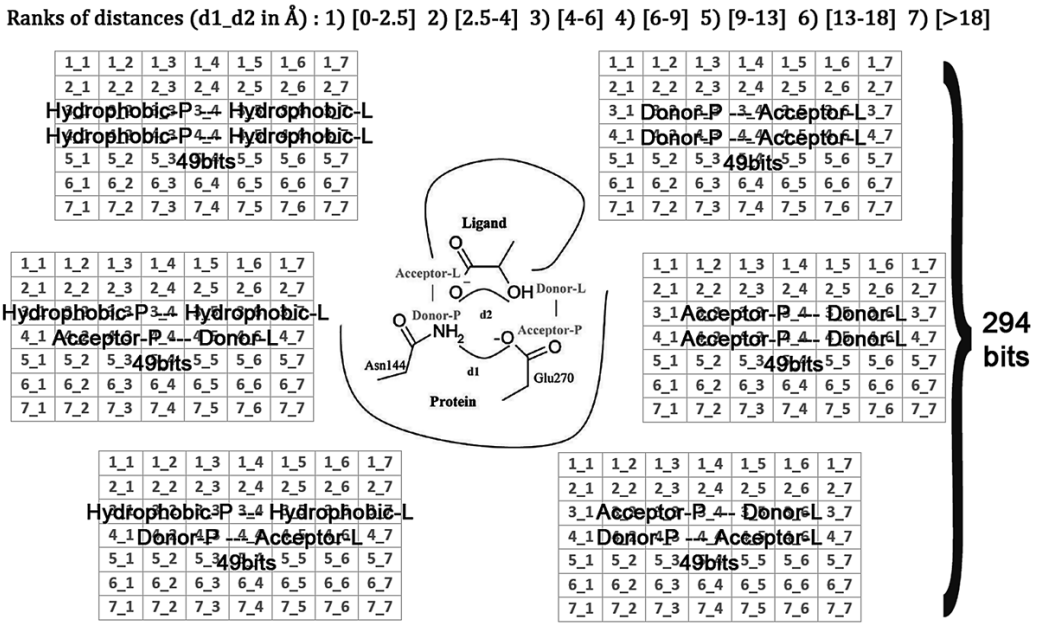
Comparison of GOLD score and APIF by means of enrichment factor has demonstrated its applicability in docking studies (Clark & Labute, 2007).

APIF algorithm was developed in SVL language as implemented in MOE package.(Pérez-Nueno et al., 2009) To generate the 1D fingerprint, first the center site is defined based on a 10 Å radius. In the next step the interactions between the protein and the ligands are calculated. Hydrogen bond acceptor, hydrogen bond donor and hydrophobic contact features of the ligand or receptor atoms can produce 6 combination types (Figure 3). Different distance zones are also expressed in the next step. Accordingly, based on different zone clusters and contact maps a 7*7*6=294 bit string is generated for each pose of the ligand (Figure 3) (Clark & Labute, 2007).

Fingerprints for Ligand and Protein (FLAP)

FLAP is a fast and efficient algorithm for describing small molecules in the active site. It includes using a reference frame work of 4 point pharamcophores for the ligand conformations which represent the shape of the molecule in the active site. In the first step, GRID force field is used for calculation of interaction fields for identification of target location for small molecules with favorable energy values. The second step includes generation of a 4 point pharmacophore based on molecular interaction fields (MIFs). This technique can be used for comparison of protein families without aligning them or further knowledge. It can be also used for descriptor generation in chemometrics analysis as well as docking procedure. On average the optimum number of the interactions between the receptor and ligand is considered to be at least 4. The first example of using FLAP in structure based virtual screening (SBVS) was in designing ligands for xa factor, where its efficiency and speed were shown to be more than the scoring functions implemented in GOLD and GLIDE (Baroni, Cruciani, Sciabola, Perruccio, & Mason, 2007).

Figure 3. An illustration of how APIF produces a 294 bit string for each pose of the ligand



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Pharmacophore Interaction Fingerprint (Pharm-IF)

Pharm-IF is another method which calculates ligand protein interaction in three steps. In the first step, the interactions are identified in the complex of ligand and receptor. Then the interaction pairs are extracted based on pharmacophore features for each ligand. The most important interactions types in this method are hydrogen bond with ligand acceptor, hydrogen bond with ligand donor, ligand anion with ionic interaction, ligand cation, with ionic interaction and hydrophobic interactions. Similar to MOE in PLIF method it is also possible to consider the weak interactions.

Pharm-if is different from the algorithm implemented in MOE in terms of the two following criteria:

1. Hydrophobic interactions are recognized when two atoms have an overlap greater than 1% of their total surface.
2. Since it is possible that the hydroxyl groups inside either ligand or receptor take role in formation of hydrogen bonds, the role of each atom is ignored. It is believed that the superior results of this method are caused by these reasons.

Pharm-if can be considered as an extended method based on 3D geometry of ligand pharmacophores for docking poses. As a perspective, the area of research in the field of protein ligand interaction fingerprints is focused on the bellow subjects:

- The orientation of interactions (for example, hydrogen bonding from ligand or protein)
- Interaction strength
- Considering atom sites for generation of bit strings rather than residues (Sato, Honma, & Yokoyama, 2009).

As summarized in Table 1, different binding site dependent and independent methods have been developed for ligand-protein interaction fingerprints. While binding site dependent methods are very sensitive and effective, cavity independent methods can be efficient in developing structure activity relationship and virtual screening.

ANALYSIS OF THE FINGERPRINTS

After construction of the bit strings using any fingerprinting approaches for every docking poses, some methods should be recruited to measure their similarities for further analysis. Traditionally, four kinds of similarity coefficient have been used to evaluate equivalence of bit stings: 1) Euclidean distance 2) Manhattan distance 3) Tanimoto coefficient 4) Simple matching coefficient. A and B are numbers of bit sets in the two interaction finger prints and C is the number of common bits (Pérez-Nueno et al., 2009). Equation of Euclidean distance is:

$$\sqrt{A + B - 2C} \tag{1}$$

Equation of Manhattan distance is:

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$$A + B - 2C \tag{2}$$

Simple matching coefficient, as called, simply shows the number of common bits in two bit strings so simple matching coefficient is C.(Pérez-Nueno et al., 2009)

Tanimoto coefficient (Tc) is the most regularly executed method to obtain similarity of bit stings among aforesaid approaches. Tc for evaluation of similarity between two sets of bits is:

$$Tc(A,B) = \quad A \cap B \; / \quad A\,UB \tag{3}$$

which $\left|\, A \cap B \,\right|$ is number of common ON bits in both A and B (previously defined as C), while $\left|\, AUB \,\right|$ is the number of bits in either A or B.(Deng et al., 2004) We can rearrange (3) as below: (Kelly & Mancera, 2004)

$$\frac{C}{A+B-C} \tag{4}$$

Since this method only considers ON bits, the results can be affected by unwanted size dependent effects. Therefor to bypass this disadvantage, Tc was developed as Modified Tanimoto coefficient (MT) (Kelly & Mancera, 2004)

To assess OFF bits, Tanimoto complement coefficient (T_0) can be defined as:

$$\frac{D}{A+B-2C+D} \tag{5}$$

where A, B and C are for the Tc and D shows the number of OFF bits in both A and B

ON bits are considered by Tc and OFF bits are considered by T_0, so by combining these two equations, modified Tanimoto (MT) is achieved as below:

$$\alpha Tc + (1-\alpha)T \tag{6}$$

where α is:

$$\frac{2-P}{3} \tag{7}$$

where P is:

$$\frac{A+B}{2n} \tag{8}$$

where n shows the total number of bits in A or B.(Kelly & Mancera, 2004)For example, in case of two bit strings 1100110 and 1010110. The calculation of similarity co-efficient for these bit strings are as below:

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Euclidean distance (1) $\rightarrow \sqrt{7+7-(2\times 3)} = 2.82$

Manhattan distance (2) $\rightarrow 7 + 7 - 6 = 8$

Simple matching coefficient = C $\rightarrow = 3$

Tanimoto coefficient (3) $\rightarrow \dfrac{3}{7+7-3} = 0.27$

Modified Tanimoto (6) $\rightarrow$ T0 $= \dfrac{2}{7+7-4} = 0.2 \rightarrow$ p $= \dfrac{7+7}{14} \rightarrow \alpha = \dfrac{2-1}{3} = 0.33 \rightarrow$ Mt $= 0.224$

Some types of scoring functions as designed for GOLD software can be obtained from Tanimoto coefficient and the original GOLD score presented by the software. According to the bellow equation:

SCORE 2 – Tanimoto = SCORE 1 – Tanimoto × GOLD score function (9)

Based on this scoring system, after docking procedure, compounds that don't have any relation to expected binding mode will be allocated (Mpamhanga, Chen, McLay, & Willett, 2006).

Clustering Methods

After calculation of similarity distances for the fingerprints in case of every docking poses, they should be subjected to further analysis.(Renner et al., 2008) One way to visualize the data is to represent them as lower-dimensional models. Projection methods are hired to represent the distribution of the poses in high dimensional descriptor space into lower dimensional ones; e.g. two dimensional.(Renner et al., 2008) There are some methods called *clustering methods* for this purpose. In other words, these methods arrange data based on their similarity.

The classification methods can be categorized as two types unsupervised (clustering methods) and supervised classification methods (Höppner, 1999).

Based on the separation patterns clustering methods can be categorized as below:

- **Well-Separated:** In this type, each cluster contains objects that are similar to each other and are completely different from the objects from another cluster. Sometimes a threshold should be considered to define rate of similarity among objects. However, this type of clusters can be utilized when data has the potential of generating natural clusters with reasonable distances. In other words, similarity distance between objects of two different clusters should be more than the distances between objects of one cluster.
- **Prototype Based:** In this type, each cluster has a prototype that is representative for other objects of its cluster. In other words members of a cluster congregate around these main objects. For continuous data, prototype is generated via a *centroid* that is calculated through averaging members of a cluster. On the contrary, for categorical data where a centroid cannot be defined, a *medoid* should be hired which is the most representative member of its cluster. Prototype based cluster is also called a center-based cluster.

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- **Graph Based:** The data are presented in a graph, where the nodes of the graph are objects of each cluster and relation between these nodes are illustrated by links. In this regard, a cluster can be defined as a connected component. In fact, a cluster contains members which are well connected with each other but completely irrelevant with members of other clusters. This type of cluster is useful when clusters are irregular and intertwined. But the pitfall of this method is that two clusters can be displayed as a merged one when noise is present in the data set. In other words, noise can play the role of a bridge between two distinct clusters.
- **Density Based:** In this type, a cluster can be defined as a dense region of data that is bordered by a region of low density. This clustering approach can be applied even when outliers and noises are present since they will be vanished into a low density region so any relation cannot be recognized between two distinct clusters.

Two of the most important tools used for clustering of docking fingerprints are presented as below: SOM –HCA – PCA – K-means – DBSCAN.(Bouvier, Evrard-Todeschi, Girault, & Bertho, 2010; Singh et al., 2006; Yan, Nie, Wang, & Wang, 2013)

Self-Organizing Map (SOM)

It is a sort of artificial neural network (ANN) presented by Kohonen, trained to organized complex, high dimensional, nonlinear data into a regular, understandable, low-dimensional (usually two dimensional) display. In other hand, this tool represents some kind of abstraction which reveals data more intelligible (Kohonen, 1998).

SOM often includes a two dimensional grid of nodes. This method orders models into a graph where similar models are closer than dissimilar ones (Kohonen, 1998).

For example YAN et al. used SOM for classification of Aurora kinase inhibitors. They collected 512 compounds of Aurora-A and -B inhibitors and classified them into three classes using Self-Organizing Map (SOM). These classes were dual Aurora-A and Aurora-B inhibitors, selective inhibitors of Aurora-A and selective inhibitors of Aurora-B. The prediction accuracy of the model (based on the training/test set splitting using SOM method) for the test set was 92.2% (Yan et al., 2013).

Hierarchical Clustering Analysis (HCA)

It's a method for clustering data as a dendrogram by state of similarity (Maimon & Rokach, 2005). This method follows two general strategy to organize the data:

- **Agglomerative:** It's a bottom-up approach that begins from each cluster and then they merge as one moves up *hierarchy.*
- **Divisive:** It's a top-down one which is started in one cluster and then divide into detailed clusters. In other words, there is an evolution from less detailed data into more ones.

A measure of dissimilarity between sets is needed for ordering clusters and deciding how clusters should be merged or divided, usually a metric measure of distance is hired (Maimon & Rokach, 2005).

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For instance, Singh et al. utilized this approach in computing the similarity metric (Tanimoto coefficient) between all of the fingerprints and then used a hierarchical clustering algorithm to group similar interaction fingerprints together (Singh et al., 2006).

There are three methods for merging of clusters in HCA:

- **Single Link (Neighbor Joining):** This method as the most common strategy calculates distance between two closest points of two neighbor clusters. This metric is then considered as distance between these clusters.
- **Average Link:** This method calculates distances between the centroids of the clusters.
- **Complete Link:** It considers distance between farthest points of two neighbor cluster.

A limitation of HCA is that it is sensitive to outlier points and some pretreatment of the data set is therefore needed before analysis.

K Means

K-means is widely utilized as a suitable clustering method. In this method at first, K cluster centroids should be defined. After that, data will be clustered by means of vicinity of two clusters centroids. This procedure is iteratively repeated until best reasonable clusters are obtained based on the selected K. Selection of K values must be done with care based on validation methods such as cross-validation. The aforementioned method can be merely utilized for numerical data and the samples should be compared using metrics such as Euclidean distance or city-block.

As seem for HCA, K-means is also sensitive to outlier points. This sensitivity can lead to disorganized cluster centroids.

The results obtained by K-means method can be inserted into a diagram called Voronoi. The cells in this diagram are called voronoi cells (Mpahnga-CHAP 8- celera).

PCA (Principal Component Analysis)

It is considered as an analysis approach for detection of patterns in the sets of data and represents data in a way which makes it possible to reveal similarities and differences between the samples.

Since finding pattern in high dimensional space is difficult, PCA can be useful in analyzing data at a low dimensional space. In other words, PCA is a tool for decreasing number of numerical data without any distract cluster structures.

In this method, major component can be determined. After that, data will be decomposed and transferred from a big high dimensional space into a lower one without loss of data.

DBSCAN (Density Based Spatial Clustering of Applications with Noise)

In this method, space should be divided into low and high density region. Number of cluster will be determined automatically by means of some special algorithms. Low density spaces will be considered as noise and should be removed. Since some parts of data will be missed, this method is not considered as a complete clustering approach. (Höppner, 1999)

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APPLICATIONS OF FINGERPRINTS AND FUTURE PERSPECTIVES

Accordingly, IFs have great potentials in scaffold hopping based on the interaction pattern of the reference ligand. This feature can be used in virtual screening to find ligands with different chemotype from the reference ligands but similar interaction patterns (Venhorst, Núñez, Terpstra, & Kruse, 2008).

Two complementary tools namely Ishape and Grim have been also developed to align protein-ligand complexes based on the intermolecular interactions obtained by the described software.

While Ishape is working by the overlap of the interactions using Gaussian function, Grim is utilizing a standard detection for providing pattern graphs. The fingerprint has focused on three main areas (Desaphy et al., 2013):

- Alignment of protein-ligand complexes based on their interaction profiles.
- Making algorithms for post-processing of docking poses according to known interaction.
- Virtual screening of bioisosteric derivatives with similar interaction behaviors.

As the applicability of IF in many studies was revealed, they became popular to score and rank docking results for more accruable hit identification. Many docking programs such as DOCK are based on using energy values of electrostatic (columbic) and steric (vanderwaals) terms. Since energy values could not always explain the best pose of the ligand foot print similarity can be a useful measure for comparison between different poses. In a study performed by Balius et al. FPS (footprint similarity) was developed for DOCK software (Balius, Mukherjee, & Rizzo, 2011). Validation of FPS was done by means of pose identification, cross-docking, enrichment and virtual screening. FPS can be explained as residue based decomposition of electrostatic, steric and hydrogen bond interactions between the ligand and the protein. Generation of bit strings was designed in such a way to make a 2D pharmacophore based on contribution of each residue in the binding cavity. Calculation of FPS was according to calculation of *x*i (interaction energy between the ligand and the ith residue). Where N denotes the number of residues in the receptor ($\vec{x} = \{x1 \ldots xN\}$). Different methods such as pearson and Euclidean distance were used for comparison of the generated bit-strings (Balius et al., 2011). A feature of this method is that it is also based on energy terms compared to those methods using interactions. For example in SIFT the tanimoto comparison are being used for the bit-strings which are merely based on presence or lack of the seven type interactions for each residue. SIFT was designed to be used in library design, dataset enrichment and identification of the natural ligands.

Most scoring functions, implemented in different docking programs, are based on energy terms rather than considering the right orientation, location and geometry of the poses. It's noticeable that these scoring functions alone are not sufficient to obtain accurate energy values for ligand-protein interaction. Identification of true binding modes for the ligands is also another challenging task in docking studies. Actually, most of them are based on approximations in their calculation. Many current limitations of docking are the results of implemented solvation, entropic and electrostatic terms for different protein systems. One strategy to overcome these problems is therefore developing different algorithms for post-processing of docking simulation results.(Kelly & Mancera, 2004)

An interaction fingerprint based scoring function is AuposSOM (Mantsyzov, Bouvier, Evrard-Todeschi, & Bertho). The efficiency of AuposSOM for Surflex-Dock 2.0 from the Sybyl 1.2 package was reported. In this implementation, the contacts between the ligands and the protein include hydrophobic, hydrogen bonding and coulombic interactions. The resulted vectors of contacts will be subsequently

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analyzed using SOM (self-organizing map) as implemented in AuposSOM software (Bouvier et al., 2010; Mantsyzov et al.). Since a bit is defined for an active site/atom/residue, the previously described methods are therefore limited to analyze interactions with highly homologous active sites sharing a fixed number of cavity-lining atoms/residues. To overcome this limitation, cavity independent fingerprints do not consider absolute but only the relative positions of pairs of protein-ligand interacting atoms. Based on this method for the case of each interaction (hydrophobic, aromatic, hydrogen bond, ionic bond, metal complexation) a psuedoatom is centered either on the interacting ligand atom, the interacting protein atom, or the geometric center of both interacting atoms. (Desaphy et al., 2013) Finally, a 210 digits fingerprint is obtained by counting all possible triplets of interaction for pseudoatoms within the six distance ranges and gathering the data of the most frequent triplets. This bit string can be used to compare any pair of protein-ligand complexes. This fingerprinting approach has been tested in case of 10 000 drug like protein-ligand complexes and made it possible to compare the interaction pattern similarity and ligand or binding site pairwise similarity. Based on this approach it was verified that the interaction pattern similarity depends on binding site similarity.

CONCLUSION

Rather than the two and three-dimensional visualization of the ligand-protein interactions, generation of bit-strings are implemented in some applications. Different protocols of protein-ligand interaction fingerprints (PLIF) have been proposed so far. While some algorithms like SIFT are dependent to the binding site of the receptor, other methods such as Pharm-IF and APIF are independent from the binding cavity. Binding site dependent methods are very sensitive and can be used for detection of the correct

Table 1. Different fingerprinting methods; used algorithms and advantages

	Method	Algorithm	Features
Binding site dependent IFP	w-SIFt (weighted IFP)	• Considering bit frequency in SIFT • Weighted profiles	• Finds interactions that are critical for compound potency • Interpretable position dependent scoring function for ligand protein interactions
	r-SIFt (R-group IFP)	• Incorporating binding information based on different fragments of a combinatorial library • Bits “on” or “off” changes based on their interaction of a ligand with either a specific R group or the core fragment	• Very sensitive and effective in separating different binding modes • Made combinatorial library analysis possible
Binding site independent IFP	APIF	• Map distance ranges for quadruplet interaction based on interaction patterns • Prepare an illustration of binding site as a 294 bits string • Calculates the fingerprints based on the relative positions of the interacting protein -ligand atom pairs	• Independent on the binding site • Comparable to current scoring functions • Showed better screening efficiency performance than the residue-based IF
	Pharm-IF	• Pharm-IF is calculated from the distances of pairs of ligand • Pharmacophore features that interact with protein atoms	• Independent on the binding site • Comparable to current scoring functions • Detected patterns of ligand pharmacophores would • Facilitate an understanding of the SAR of the target protein

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binding mode of the ligands. On the other hand binding site independent methods can be utilized for developing pharmacophore models for the ligands. PLIF patterns can be applied in developing more 3D descriptors for QSAR analysis as well as designing post-processing filters for virtual ligand screening projects. For a more convenient analysis, classification methods should be taken to provide an overview of the similarities between different docking results. Validation results of these methods with respect to experimental data seem to be crucial in making decisions about the usefulness of any of these presented algorithms in drug design studies.

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Chapter 42
Protein–Protein Docking:
Are We There Yet?

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ABSTRACT

Protein-protein docking algorithms are powerful computational tools, capable of analyzing the protein-protein interactions at the atomic-level. In this chapter, we will review the theoretical concepts behind different protein-protein docking algorithms, highlighting their strengths as well as their limitations and pointing to important case studies for each method. The methods we intend to cover in this chapter include various search strategies and scoring techniques. This includes exhaustive global search, fast Fourier transform search, spherical Fourier transform-based search, direct search in Cartesian space, local shape feature matching, geometric hashing, genetic algorithm, randomized search, and Monte Carlo search. We will also discuss the different ways that have been used to incorporate protein flexibility within the docking procedure and some other future directions in this field, suggesting possible ways to improve the different methods.

INTRODUCTION

Protein-protein interactions play key roles in several biological processes. These processes involve many essential mechanisms ranging from signal transduction and cellular transport to gene expression and immune responses. All these processes are mediated by selective and potent protein-protein interactions (Waksman & Sansom, 2005). Furthermore, many diseases have been associated with either an over-activated or an under-regulated protein-protein interaction and the cure for these diseases has been focused on either inhibiting or stimulating these interactions, respectively. For example, the p53-MDM2 interaction is associated with a severe down regulation of the p53 pathway. An inhibitor for this interaction (e.g. nutlin3) can reactivate the p53 pathway, forcing cancer cells to undergo apoptosis (Barakat, Gajewski, & Tuszynski, 2012; Barakat, Issack, Stepanova, & Tuszynski, 2011; Barakat, Mane, Friesen,

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& Tuszynski, 2010; Chène, 2003; Kojima et al., 2006). The more we know about such crucial interactions, the more we can build vital protein networks and apply this knowledge to identify treatments for many diseases. Moreover, characterizing these interactions at the atomic level can help in rationally designing new therapeutic agents that can either enhance or inhibit these interactions. Constructing a three dimensional structure of such protein complexes is an essential step toward identifying their binding interface and recognizing any hot spots that can be targeted for their regulation (Elcock et al. 2001; Kann, 2007; Kortemme & Baker, 2004).

For the last few decades X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and electron microscopy have been the main source to predict such structures. Despite their accuracy, efficiency and the huge amount of details they can provide, they are expensive and very labour and skill demanding. A simple comparison between protein structure and gene sequence databases would simply reveal the great discrepancy between the two. That is, although hundreds of thousands of gene expressions have been characterized, only less than thirty thousand protein structures have been determined so far and most of these structures are either redundant or describe only apo (unbound) proteins (Villoutreix et al., 2014). Moreover, protein complexes are more difficult to crystallize than the individual proteins, consequently, they are less represented in Protein Data Bank (PDB) (Berman et al., 2000) and constitute only a small fraction of the experimentally determined structures. This huge discrepancy and lack of structural details motivated many computational groups to fill this gap and suggest a new, rapid and cheap way to predict these interactions (Barakat & Tuszynski, 2011; Barakat, Houghton, Tyrrell, & Tuszynski, 2014; Barakat, Mane, & Tuszynski, 2011; Gógl et al., 2015; Nillegoda et al., 2015; Pedotti, Simonelli, Livoti, & Varani, 2011; Taylor et al., 2015). One solution they provided which is also the focus of this chapter is protein-protein docking.

Protein-protein docking simulations date back to the early 90s (Katchalski-Katzir et al., 1992), soon after the development of many protein-ligand docking techniques. Although the concept of docking in both cases is similar, there are still key differences that prevented the direct technology transfer between the two problems. For example, since proteins are continually dynamic and usually undergo huge structural changes, their modeling is not as easy as that of a small molecule and requires more precise predictions. Furthermore, proteins are large and have complex shapes making them harder to simulate than ligand-protein interactions (Ravikant & Elber, 2011; Sheinerman et al. 2000). On the other hand, the concept of a binding site is usually inapplicable to protein-protein interactions and in many cases proteins interact through 'hot spot' areas on their surfaces. Identifying these binding locations is particularly difficult and requires highly innovative methods. In this context, the search methods in protein-protein docking simulations are somehow different from those being used in small molecule docking (Keskin, Ma, & Nussinov, 2005). However, the reader should keep in mind that although protein-protein docking is in many ways different from protein-ligand docking, some of its reflections might be mentioned in this chapter, if applicable.

In this chapter, we will first outline the concept of docking in general, with an emphasis on the different approaches. We then briefly review the theoretical concepts, principles and specific features of the search strategies and scoring functions implemented in different programs, highlighting their strengths as well as their limitations. We will also discuss the different methods of incorporating protein flexibility within the docking procedure. After these descriptive review sections, we will guide the reader through a number of case studies and recent successes that illustrate some of the significant applications of protein-protein docking algorithms. We will also discuss the existing challenges and limitations in current algorithms and highlight some potential future directions and possible improvements in this

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field. We hope that this brief, yet comprehensive review chapter provides researchers interested in this area with current state-of-the-art technologies in this field.

THE "DOCKING PROBLEM"

The so-called protein-protein "docking problem" refers to the task of docking two individual protein structures and predicting their biologically relevant complex. It can be classified into two different approaches; "bound docking" where two proteins from a co-crystallized complex are separated and then reassembled using a computational docking algorithm or "unbound docking" (Wiehe, Peterson, Pierce, Mintseris, & Weng, 2008) where individually solved structures of two proteins are docked together to predict their most probable mode of binding. The bound docking approach has no investigational significance, but it is frequently employed to test and validate the different docking algorithms while developing a new method. Unbound docking, on the other hand, is the approach employed by the current docking programs and is a valuable tool that can predict complexes from individual proteins for producing new knowledge at the atomic level. Unbound docking is far more complicated than bound docking. This is because the two proteins, which come together to form a complex, may undergo some conformational changes upon binding, which needs to be predicted correctly (Vakser, 2014; Ravikant & Elber, 2011). In this section, we will focus mainly on the unbound docking approach involving two different methods, namely rigid-body docking and flexible docking. Initially, we will have a closer look at these two approaches and then will focus more on rigid-body docking and its different aspects.

Rigid-body docking treats the two proteins as rigid entities. In this case, the side chains and backbone of the two interacting proteins are kept fixed with no bond angles or distances allowed to change upon docking. Although this method offers a rapid way to predict the formed complex, it completely ignores any possible conformational rearrangements that may occur upon complex formation. This sampling approach adopts a simplistic, yet structurally meaningful, technique. It projects the protein coordinates onto a low-resolution three-dimensional grid and then calculates the overlap between the two grids of the interacting proteins (Ehrlich & Wade, 2001). Flexible docking, on the other hand, takes into account such conformational changes by allowing minimal movements of the side chains or the backbone of the proteins at high resolution. Rigid-body docking includes six degrees of freedom; three translations and three rotational in the Cartesian coordinates, whereas flexible docking is usually preceded by transforming the Cartesian coordinates into internal coordinates, which involves more variables compared to rigid docking (Guo, Li, Ma, & Wang, 2013; Vakser, 2014).

It is noteworthy to mention that in many cases, no significant conformational changes take place upon the binding of the two proteins and a "lock-and-key" approach would be sufficient to address their interaction (Betts & Sternberg, 1999). This usually occurs when the two protein structures are not fluctuating too much, particularly at their binding interface. In this case, employing a rigid docking protocol to study these interactions would be adequate and can predict, with an acceptable degree of accuracy, their mode of binding. However, to predict higher resolution structural details at the interface some degree of flexibility is required, and the more protein dynamics incorporated during the docking simulation, the more structural details can be revealed. In this case, flexible docking comes into play and more details on that are described in the next sections.

It is important to emphasize the significance of incorporating experimental data within the docking procedure. This type of data not only helps in validating the docking outcomes, but also can help in

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identifying the potential binding interface of the two proteins, reducing the search space to a manageable region (Grinter & Zou, 2014; Wodak & Méndez, 2004). In this context, a number of docking algorithms can restrict the search space to a specific region and therefore feeding these algorithms with a minimal knowledge of the binding site residues called, 'hot spots' can significantly improve the search process (Grimm et al., 2005; Grosdidier & Fernández-Recio, 2012).

DOCKING STEPS: SAMPLING AND SCORING

Within the scope of the two classes (rigid-body docking & flexible docking) discussed above, the process of docking is implemented through two main interconnected steps: *sampling* which will search for all possible binding modes between the two proteins and *scoring* for the differentiation between correct, near-correct or incorrect poses. In the following two sections, we will describe these two processes in details, giving examples of different software packages employing the different methods. We will then describe how protein flexibility is incorporated during docking and post-docking approaches. Finally, a brief evaluation of the different docking techniques will follow.

Sampling Techniques

We will begin by briefly introducing the sampling stage. The reader is referred to the references included her and the references within for a more details on this area of research. The sampling stage of docking involves generating a large number of alternative poses for the proteins by sampling all the possible binding modes. The correct prediction of the complex is more of a near-native approximation than an accurate native (co-crystalized) complex of the two proteins, which is computationally impossible to generate (Smith & Sternberg, 2002). Standard search methods are computationally expensive and lead to a huge number of solutions; most of them are physiologically irrelevant. It is for this reason more sophisticated sampling approaches and strategies are usually incorporated to correctly predict realistic solutions at this stage. This includes exhaustive global search methods as adopted by fast Fourier transform (FFT) algorithms, local shape feature matching used in geometrical hashing and genetic algorithms, randomized search employed in Monte Carlo (MC) search or Boolean operations and effective energy gradients to determine new atomic positions (Ehrlich & Wade, 2001; Huang, 2014). Table 1 lists the most commonly used software packages used for protein-protein docking with their respective sampling techniques. Although the focus of this chapter is particularly on protein-protein docking, there are a number of small-molecule docking programs optimized to become applicable to protein-protein docking and therefore, they will be discussed in various sections to illustrate the different aspects of docking.

Some recently developed and/or optimized protein-protein docking programs are DockTrina (docking triangular protein trimmers) (Popov, Ritchie, & Grudinin, 2014), ATTRACT (Vries & Zacharias, 2013), MEGA-DOCK (Matsuzaki et al., 2013), F(2)Dock 2.0 and GB-Rerank (Chowdhury et al., 2013), Swarm-Dock (Moal, Torchala, Bates, & Fernández-Recio, 2013). In addition to the programs listed in Table 1, there are numerous docking programs, which facilitate the use of docking algorithms by availing online servers. Some of them include: ClusPro (Comeau, Gatchell, Vajda, & Camacho, 2004), GRAMM-X (Tovchigrechko & Vakser, 2006), ZDOCK SERVER (B. G. Pierce et al., 2014), 3D-GARDEN (Lesk & Sternberg, 2008), PatchDock & SymmDock (Schneidman-Duhovny, Inbar, Nussinov, & Wolfson, 2005), HADDOCK (Dominguez, Boelens, & Bonvin, 2003), RosettaDock (Gray et al., 2003), FIBERDOCK

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Table 1. The most commonly used protein-protein docking programs with their method of sampling

Name of Software	Method of Sampling	References
ZDOCK, FTDOCK, GRAMM, F2DOCK, DOT, Cell-Dock	FFT-correlation	(Chen et al., 2003; Gabb et al.,1997; Vakser, 1995; Bajaj et al., 2011; Mandell et al., 2001; Pons et al., 2012)
HEX, FRODOCK	FFT- Spherical Harmonics	(Ritchie & Kemp, 2000; Garzon et al., 2009)
PatchDock, LZerD	Geometric Hashing	(Duhovny et al., 2002; Venkatraman et al., 2009)
BIGGER, SKE-DOCK, SoftDock	Direct Search in Cartesian Space	Palma et al., 2000; Terashi et al., 2007; Jiang & Kim 1991)
DOCK	Distance Geometry Algorithm	(Kuntz et al., 1982)
DARWIN	Genetic Algorithm	(Taylor & Burnett, 2000)
RosettaDock, ATTRACT, ICM	Monte Carlo Search	(Gray et al., 2003; Zacharias, 2003; Fernandez-Recio et al., 2002)
HADDOCK	Based on both Biochemical/ Biophysical Information and MC Search	(Dominguez et al., 2003)

(Mashiach, Nussinov, & Wolfson, 2010), FIREDOCK (Mashiach, Schneidman-Duhovny, Andrusier, Nussinov, & Wolfson, 2008), pyDockWEB (Jiménez-García, Pons, & Fernández-Recio, 2013), pyDockSAXS (Jimenez-Garcia, Pons, Svergun, Bernado, & Fernandez-Recio, 2015) and ATTRACT web interface (Vries, Schindler, Chauvot de Beauchêne, & Zacharias, 2015).

Fast Fourier Transform (FFT) correlation approach is regarded as one of the most popular and widely used techniques of rigid-body search, developed in 1992 by Katchalski-Katzir and coworkers (1992). This technique allows an accelerated exhaustive global search of the binding orientations over six degrees of freedom in 3D translational plus 3D rotational space. The popularity and wide usage of this technique is attributed to its ability to rapidly perform an exhausted sampling of all the possible binding modes of the interacting proteins. In this method, the two proteins (receptor protein and ligand protein) are discretized onto two different 3-dimensional grids each with the size N * N * N (where N is the number of grid points), and each point on the grid with coordinates *l,m,n* is assigned a value based on its geometric characteristics (a $_{l,m,n}$). This includes a value of one for grid points on the surface of the protein, a value of zero for points outside of the protein surface and a value of ρ for points located within the protein core structure (where ρ usually has a negative value). The complementarity of shape function *C* (p,q,r) between the two proteins is then evaluated from the Equation 1, shown below:

$$C(p,q,r) = \sum_{l=1}^{N}\sum_{m=1}^{N}\sum_{n=1}^{N} R(l,m,n) \times L(l+p, m+q, n+r)$$

The translational space is then sampled by calculating a correlation between the two discrete grids. FFT correlation has been employed in a number of docking programs: FTDock (Gabb, Jackson, & Sternberg, 1997), GRAMM (Vakser, 1995), DOT (Mandell et al., 2001), ZDOCK (R. Chen & Weng, 2002), F2DOCK (Bajaj, Chowdhury, & Siddahanavalli, 2011) and Cell-Dock (Pons et al., 2012). The basic approach is the same for all of them and the difference lies in the way the proteins are projected onto the grids.

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A successful modification of the FFT algorithm is the spherical Fourier transform-based search, initially introduced in the program HEX (Ritchie & Kemp, 2000), which employs spherical harmonics to rapidly search the complete conformational space of the proteins. Another exhaustive sampling technique employs a direct search in Cartesian space to identify the correct binding mode between two proteins using 3D Cartesian grid space. This technique has been adopted in many docking packages including SOFTDOCK (Jiang & Kim, 1991), BIGGER (Palma et al., 2000), SKE-DOCK (Terashi et al.,2007). The program BIGGER (Palma et al., 2000) for example, employs a surface implicit method based on Boolean operations, in which the molecular shape of the two proteins is mapped onto a 3D grid in Cartesian space. Each point on the grid is assigned a value of '1' if it is on the protein core and a '0' if it is outside. Geometric fitting between the two proteins is then performed by shape matching directly in the Cartesian space. The advantage of this technique over FFT-based methods is the ability to have more control over the search process because of the direct operation in Cartesian space, which allows inclusion of protein flexibility and biological information (Huang, 2014).

Geometric hashing techniques are another type of global protein-protein docking search using a shape-explicit algorithm. The technique is based on finding a set of shape descriptors on the molecular surface of the proteins (one of them called the receptor and the other the ligand). Following that, a set of local coordinate frames is defined, for which the positions of nearby critical points are used as indices hashed into a table. The hash table stores the current coordinate frame of each protein. After superimposing the local coordinate frames of the ligand and the receptor, the positions of the critical points of the ligand in proximity of the receptor are then used to scan the hash table for the corresponding ligand reference frames (Smith & Sternberg, 2002). Several programs employ different shape descriptors. For example patch descriptors are used in PatchDock (Duhovny, Nussinov, & Wolfson, 2002) and 3D Zernike descriptors are adopted by LZerD (Venkatraman, Yang, Sael, & Kihara, 2009). High shape similarity is revealed by the frequency of correspondences to a specific frame. The method has been improved by introducing surface softness as a tolerance value of about 1.5 A in the coordinates.

Genetic algorithm is the basis for a number of other successful docking algorithms. This generally involves a real-space search method, which is based on a local shape feature matching. The DARWIN (J. S. Taylor & Burnett, 2000), for example, includes a solvent accessible area (SAA) for the proteins with normals, surface curvature and associated hydrogen bonding character. The complementarity between the curvatures and normals are scanned for the two proteins to find the most probable binding mode. The 'chromosome' of the genetic algorithm (GA) consists of the relative positions and orientations of the molecules (6D space).

Randomized search is another broad category of sampling techniques. Monte Carlo (MC) search is one of the approaches in which sampling of all possible conformations with a technique like FFT correlation is replaced with a random approach. As such, random translational and rotational orientations for the two proteins are performed and the final positions are selected based on a Metropolis criterion. This is then followed by a multistage optimization of the side-chains and rigid-body orientations based on MC simulations (Gray et al., 2003). The advantages of MC methods over FFT methods includes the allowance of more physical decoy distribution, arbitrary energy functions and structural flexibility to some additional extent (Lorenzen & Zhang, 2007). RosettaDock (Gray et al., 2003) is an efficient protein-protein docking software that is based on the MC method. Some other software based on randomized search are HADDOCK (Dominguez et al., 2003), ICM-DISCO (Fernandez-Recio, Totrov, & Abagyan, 2002) and ATTRACT (Zacharias, 2003).

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More recent practical successes in sampling techniques, include the recent work of Zhang et al. (2015), who identified a variation of MC search for the RosettaDock package to improve the sampling stage towards a more efficient identification of the near native docking geometries. This variation involves combining the well-tempered ensemble method with a 2-dimensional temperature and Hamiltonian replica exchange scheme (WTE-REMC). A comparative study of the results from different docking protocols of Rosetta, using the same set of targets has shown the enhanced performance of WTE-REMC in exploring the phase space.

HADDOCK (High Ambiguity Driven Protein-Protein Docking) software (Dominguez et al., 2003) is implemented with a different scheme for the randomized search process. It employs a data-driven approach and supports various types of biochemical and/or biophysical data sets such as chemical shift perturbation data from NMR titration experiments or mutagenesis data. HADDOCK is able to handle almost full flexibility (side-chain and backbone) and incorporate explicit water during the modeling process. In addition, the software provides a user-friendly web server (De Vries, Van Dijk, & Bonvin, 2010), which is accessible through four interfaces: Easy, Expert, Guru and parameter file upload interfaces, for different levels of expertise and type of application allowing more control and modification on the docking process. Several CAPRI experiments (Janin, 2013), have confirmed the profound ability of HADDOCK in precise predictions of protein-protein complexes based on experimental information. In addition it is stated that HADDOCK has generated about 70 protein-protein complexes in PDB (Grosdidier & Fernández-Recio, 2012).

Scoring Functions

The large numbers of possible conformations (poses) generated from the sampling stage are ranked using a scoring function that involves the assessment of the overall favorability of a protein-protein complex and/or a score between the two proteins in the bound form. The final docking results comprise of the top ranked complexes, which are in order of their binding score (Huang, 2014). These different scoring methods can be categorized into different groups based on the basis they are derived from; for example the *forcefield-based* scoring functions employ non-bonded interaction terms as the scoring method. *Empirical* scoring functions, on the other hand make use of regression methods to differentiate between the different poses. *Knowledge-based* scoring functions use statistical atom pair potentials from the structural databases as the scoring method. Yet, another category is the *heuristic* scheme of scoring, which makes use of chemical scores or geometrical scores such as contact/surface scores, or shape complementarity scores (Ehrlich & Wade, 2001; Muegge & Rarey, 2001)

Docking programs use different scoring functions that largely vary from one algorithm to the other. Usually, a docking program makes use of a composition of different scoring functions to increase the efficacy and accuracy of the ranking process. For instance, although shape complementarity between the interacting proteins is regarded as a simple, inexpensive and powerful scoring function, it does not necessarily distinguish near-native from non-native complexes and is thus combined with other functions such as electrostatic field, hydrophobic complementarity, desolvation free energy, etc. (Szilagyi et al., 2005). An example of implementing such composite scoring functions is the ZDOCK program (R. Chen & Weng, 2002). FTDOCK (Gabb et al., 1997) and DOT (Mandell et al., 2001), which combine electrostatic and geometric scores but in different ways. FTDOCK uses geometric matching in the initial stage and then filters the docked poses based on their electrostatics scores in later stages. In the DOT algorithm, two types of scoring methods are used in conjunction. The GRAMM package performs the

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scoring through a geometric surface matching approach by involving the hydrophobic contacts between the two proteins. PyDock (Cheng, Blundell, & Fernandez-Recio, 2007) uses an energy function based on ICM potentials, composed of van der Waals, Coloumbic electrostatics and Atomic Solvation Parameter (ASP)-based desolvation energy. The scoring function in ICM-DISCO (Fernandez-Recio et al., 2002) takes into consideration the intermolecular grid-based energy terms and the Accessible Surface Area (ASA)-based desolvation energy. RosettaDock program also employs a similar scoring scheme.

Incorporating Protein Flexibility

Currently, all modern docking techniques use different approximations to include protein flexibility within the docking process. This may include rotating the protein side chains and possibly involve simple dynamics of the backbone. Flexibility in general is treated in two different ways: "implicit flexibility" and "explicit flexibility". Rigid-body approaches incorporate protein flexibility in an implicit way, by either using soft docking (surface softening) or through more advanced techniques such as ensemble-based docking. Surface softening techniques include low resolution docking methods, simplified models for the side chains and surface layer thickening (Szilagyi et al., 2005). Rigid-body docking methods show successful results in many cases (Viricel, Ahmed, & Barakat, 2015; Xu et al., 2013). Nevertheless, an accurate and biologically relevant prediction of these protein-protein interactions which involve considerable conformational changes of backbone and/or side chains upon complex formation is merely not possible through rigid-body docking (Grosdidier & Fernández-Recio, 2012; Wang & Schueler-furman, 2005).

Explicit treatment of the protein flexibility is only possible in docking algorithms that involve the atomic coordinates of the different atoms, rather than those that implicitly represent the protein structure on a grid. Each approach treats flexibility to a specific extent in a different way, such as accounting for the dynamic changes of the protein domains, amino acid side-chains and less frequently the backbone of the protein. The different approaches to include protein flexibility are based on energy minimization, atomic-level simulations or optimization of the shape-based characteristics and/or complementarity. Others include considerations related to electrostatic interactions and hydrogen bonding which have been known for a long time. All the mentioned approaches are time-consuming and take several hours to days for an effective treatment in a docking process and this represents the main reason for which flexibility remains a major challenge in the field of protein-protein docking (Ehrlich & Wade, 2001). For example, FiberDock (Mashiach et al., 2010) includes protein flexibility through several energetic calculations and ranking through normal mode analysis (NMA) along with side-chain optimizations (using a rotamer library) and restrained energy minimization with limited degrees of freedom. Another randomized search algorithm, RosettaDock (Gray et al., 2003) that is based on Monte Carlo principles, employs side-chain repacking, followed by optimizing the docked poses using a MC search with rigid-body displacements. Hinge-bending treatment is used in FlexDock (Schneidman-Duhovny, Ruth, & Wolfson, 2007) and FlexDoBi (Guo et al., 2013) programs in which the hinge regions (regions of the protein causing large-scale motions) are specified on the proteins prior to the docking simulation and the docking procedure is then performed, taking these specifications into account. HADDOCK (Dominguez et al., 2003) is regarded as an experimental data-driven program which performs a semi-flexible simulated annealing. Biochemical & biophysical interaction data from NMR experiments, mutagenesis investigations or bioinformatics predictions are used to introduce ambiguous interaction restraints (AIRs). These are then used to control the docking process with allowance for both backbone and side-chain

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flexibility on the interface. Average interaction energies are then used as a basis for the clustering and ranking of top predictions.

Post Docking Refinement

Docking procedures can be followed by a second ranking/scoring stage to refine the docking results and remove any steric clashes that may emerge form the docking algorithm. This is generally required due to the lack of precision in docking scoring functions. There are many different methods that have been used in the post-docking refinement stage. This includes a biased probability side-chain optimization approach as implemented in the ICM program (Abagyan, Totrov, & Kuznetsov, 1994) or side-chain minimization as employed by Multidock (Multiple copy side-chain refinement Dock) (Jackson, Gabb, & Sternberg, 1998) algorithm. The RosettaDock program (Gray et al., 2003) uses another effective method, which involves the correction of main-chain displacements. In addition, some post-docking algorithms are specialized in refining the results to a higher extent. These include ZRANK (Pierce & Weng, 2007), EMPIRE (Liang, Liu, Zhang, & Zhou, 2007), DARS (Chuang, Kozakov, Brenke, Comeau, & Vajda, 2008), DECK (Liu & Vakser, 2011), RDOCK (Li, Chen, & Weng, 2003), pyDock (Cheng et al., 2007), Eigen-Hex (Venkatraman & Ritchie, 2012), RPScore (Moont, Gabb, & Sternberg, 1999), Multidock (Jackson et al., 1998) among others. DOCKSCORE (Huang et al., 2015) and FiltRest3D (Gajda, Tuszynska, Kaczor, Bakulina, & Bujnicki, 2010) are two of the online webservers which allow ranking of poses from protein-protein docking.

Docking Evaluation

When a new docking technique is developed and is claimed to be good at predicting the structure of a protein-protein complex, it needs to be evaluated to provide reliable data and information to scientists and research groups to obtain the highest quality results from their investigations. The Critical Assessment of Predicted Interactions (CAPRI) experiment (Janin et al., 2003) is created to compare and evaluate the performance of various docking algorithms developed by different groups. CAPRI is modeled after Critical Assessment of Structural Prediction (CASP), which started in 1994 for comparing the performance of protein-folding algorithms (Moult et al., 2014). The way CAPRI aims at evaluating the docking algorithms is through using a set of standard benchmark proteins (unbound pairs), with the correct match known (co-crystallized complex) which is kept confidential. These are assigned to the computational research groups and the results obtained are further assessed and compared. CAPRI has attracted significant attention of the scientific community and the results have led to great improvements to the docking techniques (Vakser, 2014). Furthermore, systematic studies are being constantly conducted to provide up to date insights into different aspects of docking programs (Chen, 2015; Moal et al., 2013).

The variability in the conformational sampling methods and scoring employed by the different docking algorithms, makes selecting a particular docking software a hard task. Each docking tool discussed so far has its own strengths as well as shortcomings. A fair way to compare these different tools is to test them against the same set of data using exactly the same set of parameters with identical amount of elapsed time (Cole, et al., 2005). This has been the case employed by CAPRI evaluations, which provide reliable and periodical comparison among the different groups developing PPI docking software packages and servers.

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Table 2 summarizes a comparative overview of protein-protein docking software and/ servers with an emphasis on the most recent CAPRI experimental outcomes. For the complete references for each docking tool, please refer to Table 1. The latest CAPRI experiment (5th Evaluation) which was carried out in 2013 (Bonvin, 2013), reported the ClusPro server as the best in the automatic protein docking server category. In particular, the server's performance was equivalent to the best human predictor group. HADDOCK (De Vries et al., 2010), SwarmDock (Moal et al., 2013) and PIE-DOCK (Ravikant & Elber, 2010) servers were ranked next. In the human predictor category, HADDOCK (Dominguez et al., 2003) was ranked first followed by SwarmDock (Venkatraman & Ritchie, 2012), Vakser group, Vajda/Kozakov group (Camacho & Vajda, 2002; Chuang et al., 2008) and ICM (Fernandez-Recio et al., 2002) in the 2nd to 5th position (Kozakov et al., 2013) (Lensink & Wodak, 2013).

However, it is worth noting that a single docking algorithm cannot provide the ultimate answer for a particular problem and it would be better to combine at least two algorithms for the same research question. This will ensure a high quality of the final results, which should also make use of additional computational tools along with the docking methods to fulfill their gaps and shortcomings. Likewise, other numerical measures such as root-mean-square deviation (RMSD), careful analysis of interaction-based measures and visual examinations may provide additional credibility to the results (Hernández-santoyo et al., 2013).

Furthermore, we carried out a quick citations' survey for the top ranked docking algorithms (using Google Scholar; http://scholar.google.com), which resulted in the pie chart shown in Figure 3. This chart includes the citations of only the software packages. All docking servers were excluded to avoid

Table 2. Comparative overview of different docking software and tools with respect to 2013 CAPRI experiment

Docking Software and /Server	Strength	Weakness
HADDOCK	• Semi-flexible simulated annealing permitting almost full protein flexibility • Allows for ambiguous interaction restraints • Efficient scoring performance •CAPRI 2013: software ranked 1st, server ranked 2nd	•Reduced accuracy without structural information
ClusPro	• Efficient prediction •CAPRI 2013: Server ranked 1st	•Rigid-body docking; no/less inclusion of flexibility
RosettaDock	• Flexible protein docking • Post-docking correction of main-chain displacements.	•Sχορινγ φυνχτιονσ require improvement
ZDOCK	• Efficient scoring through a combination of functions. • Allows for specifying residual restraints.	• Rigid-body docking; no/less inclusion of flexibility • Inaccurate for docking of homology models
SwarmDock	• Εφφιχιεντ πρεδιχτιον using normal modes and flexibility during docking •CAPRI 2013: Server ranked 3rd	•Inconsistency ιν σχορινγ φυνχτιονσ.
PIE-DOCK (software and server	•Εφφιχιεντ πρεδιχτιον •CAPRI 2013: server ranked 4th.	•Poor scoring as indicated by CAPRI 2013
PatchDock	•Scalable ΦΦΤ βασεδ αλγοριτημ •Efficient computer run time	•Poor scoring as indicated by CAPRI 2013 •Takes minimal flexibility into account •Poor refinement

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Figure 1. A depiction of two proteins shown individually and when docked together in complex

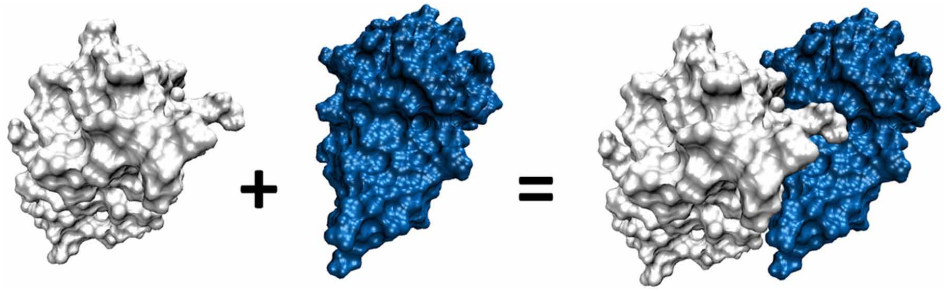
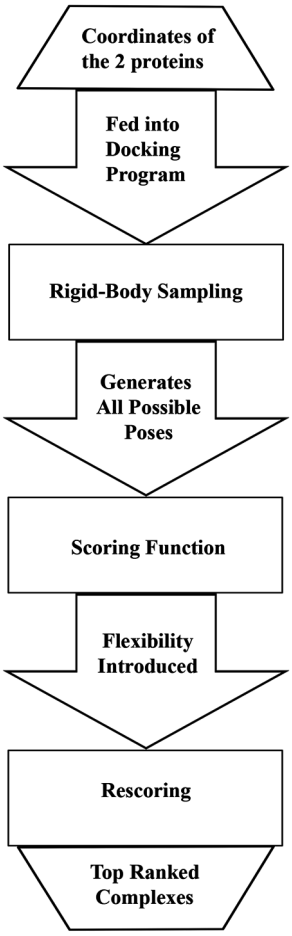
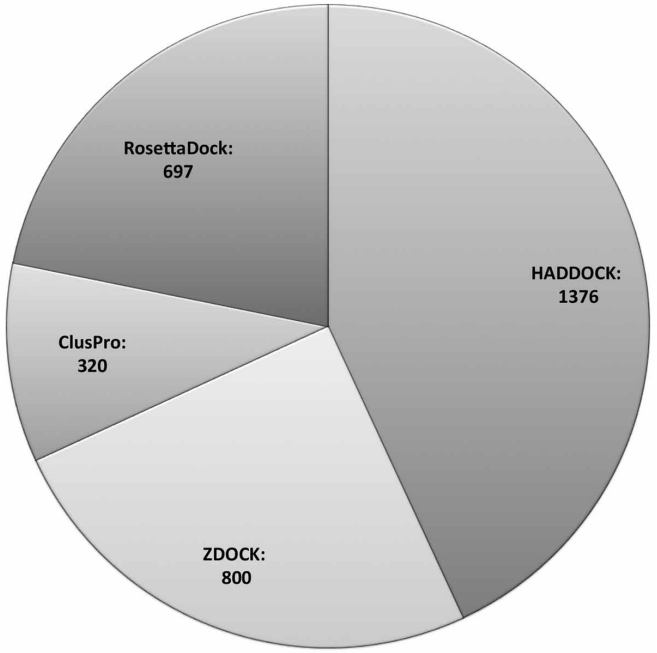


Figure 2. General scheme of docking and the different stages involved. The docking process usually starts with rigid-body docking, covering only the six dimensional space of the two proteins in 3D translational and 3D rotational. This is followed by ranking the generated complexes using a simple scoring function (e.g. shape complementarity). A more complex energy-based scoring method is next employed. Flexibility is then introduced to allow side chains or backbone dynamics. Incorporating protein flexibility usually leads to a more realistic and detailed picture of interaction. Rescoring stage is sometimes implemented for a more sophisticated ranking of poses.



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Figure 3. Pie chart presenting the relative number of citations to each docking tool; for the sake of consistency, only the docking software are mentioned and servers are excluded.



discrepancies in the presented data. Accordingly, HADDOCK software was considered to be one of the most popular protein-protein docking tools, which confirms its proven accuracy and credibility amongst scientific groups.

CASE STUDIES

In our recent work (Ahmed & Barakat, 2015; Viricel et al., 2015), we have applied ensemble-based docking technique to understand the interaction between Programmed Death- 1 (PD-1) and PD-Ligand-1 proteins using the ZDOCK software (R. Chen, Li, & Weng, 2003) and the FFT search. These proteins belong to the immune system and play important functions in the T-cell inhibition pathway, leading to immune tolerance and inactivity against cancer and infectious cells. The novel results produced in this study predicted how these proteins interact in human. This interesting modeling approach, which combined molecular dynamics simulations, docking and free energy calculations, provided a good example for how protein-protein docking techniques can help understand complex protein interactions at the atomic level. In another interesting study (Zhang et al., 2005), the ZDOCK server (Pierce et al., 2014) was used for finding the possible protein-protein complex formed between the severe acute respiratory syndrome coronavirus (SARS-CoV) S1 protein and its receptor, human angiotensin-converting enzyme 2 (ACE2). This study illustrates successful identification of the interfacial key residues involved in the process of complex formation and has provided valuable information for further study of the mentioned system.

Protein-protein docking tools are frequently employed for investigating the structural details of various types of ion channels. These multimeric biological complexes are difficult to crystallize and the

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available literature indicates wide use of computational techniques for their study. A good example of such successful attempts is the work by Smith et al. (2007), who employed Rosetta-based docking for modeling the transmembrane domain of KCNQ1/KCNE1 cardiac ion channel. The docking approach in this study has produced one of the most accurate starting models and has provided significant information on the structural basis for this ion channel.

The HADDOCK software (Dominguez et al., 2003) has been quite successful in resolving a large number of accurate models for protein-protein complexes. One good example is the study of the complex formed between plectasin, a member of the innate immune system and the bacterial cell-wall precursor Lipid II (Schneider et al., 2010). The bacterial cell wall biosynthesis is targeted by plectasin, through complex formation with Lipid II. The study has clearly identified the residues involved at the binding site between the two proteins by using the HADDOCK protein-protein docking tool, providing valuable information for the design of novel antibiotics.

CHALLENGES AND FUTURE DIRECTIONS

Despite the outstanding success of many protein-protein docking algorithms and their application throughout the last decade, there are still a number of challenges that need to be addressed within the next few years to advance the field for more realistic predictions. The initial sampling stage of most of docking algorithms is generally rapid and efficient, however there are certain shortcomings of protein-protein docking approaches, which fall into different categories mainly, related to scoring and flexibility issues.

The scoring functions are regarded as one of the major challenges of docking algorithms and frequently fail to accurately rank the resultant complexes. Even with the considerable improvements so far, detection of correct conformations is still considered to be beyond the scope of current docking algorithms. Using post-docking filtering techniques usually conceals this drawback, although it complicates the overall docking procedure. Accordingly, it is crucial to reach a point where the docking algorithms themselves gain the ability of completely refining the complexes correctly. The scoring functions also need to focus on solvation, entropy and the effects of protonation states of the charged residues. Development of new implicit and explicit models for further investigation of the role of water in the proteins interfaces during protein-protein docking, requires more effort. (Moal et al., 2013)

The treatment of proteins' structural flexibility remains an active area of research and is still lagging behind. The problem is even more puzzling when dealing with the proteins' backbone flexibility and large structural changes as seen in antigen-antibody complexes (Chen & Tou, 2013).

Furthermore, advancements in the structural genomics studies are leading to more number of therapeutically significant 3D structures being determined and thus, opening doors for docking studies to explore such interactions at the atomic level. However, it is predicted that there would be more number of 3D structures obtained by homology modeling rather than experimental techniques. This would produce more controversies and errors in the resulting complexes, due to uncertainties and limitations of homology modeling in correct prediction of 3D structures (Wass, Fuentes, Pons, Pazos, & Valencia, 2011).

The success of the current docking algorithms is largely dependent on the availability of experimental data (such as binding sites, interaction partners and conformational changes), without which there are high chances of obtaining incorrect conformations. This reliance on experimental data would always remain a challenge for many docking studies (Smith & Sternberg, 2002).

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Improvements to the docking techniques in general, may include the optimization of existing algorithms or the development of new ones to overcome these limitations. With the aid of community efforts such as CAPRI, docking algorithms are being successfully evaluated to identify the potential limitations and the benchmark testing has led to overcoming a large number of them. The remaining challenges such as flexibility incorporation are still under investigation and with the accelerated pace of research in this area, the problem of flexibility will be soon tackled. Other tricky challenges like the prediction of multi-protein complex structures could be addressed and investigated more in the future (Grimm et al., 2005; Wodak & Méndez, 2004).

CONCLUSION

Understanding protein-protein interactions is an essential step to comprehend many important biological processes and to identify new treatments for many diseases. The huge discrepancy between the amount of genomic data deposited in the literature and the experimentally determined protein structures place a huge pressure on the computational groups to predict the structure of many proteins and understand how they interact. Amongst the current computational tools in use is protein-protein docking. This technique involves several algorithms that have been developed to analyze the protein-protein interactions at the atomic-level. They predict the mode of binding between two interacting proteins and involve two interconnected stages, namely sampling of protein conformations and scoring the docked poses. More advanced algorithms include the proper incorporation of protein flexibility and sophisticated scoring methods, referred to as post-docking refinement.

This chapter briefly introduced the concepts behind protein-protein docking, with an emphasis on the different approaches. It also reviewed the theoretical concepts, principles and specific features of the search strategies and scoring functions implemented in different programs, highlighting their strengths as well as their limitations. The main sampling techniques reviewed here include FFT correlation, direct search in Cartesian space, randomized search and MC algorithm. Scoring of the protein-protein docking solutions span different methods including force field-based methods, empirical scoring, knowledge-based and heuristic scoring functions.

Despite the great efforts of many research groups in this exciting area of research, there are still many challenges that need to be addressed to improve the method. This includes mainly developing more accurate scoring methods and the proper incorporation of protein flexibility. Although many algorithms are on the edge of overcoming these problems, more work need to be done to fully solve these issues. With the accelerated pace of improvements in the field of computational approaches and their successful applications in understanding many biological systems, we believe that the field of protein-protein docking will evolve rapidly towards a more accurate method to predict protein-protein interactions. We hope this chapter provides a brief, yet comprehensive, overview to researchers working in this area.

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KEY TERMS AND DEFINITIONS

Docking: The computational task of predicting a biologically relevant and stable complex formed by two individual molecules.

Hot Spot Regions: The critical residues at the interface regions of proteins, where the binding takes place. These are the main components responsible for protein interactions and contribute the most to the binding free energy in bound forms of proteins.

Molecular Dynamic Simulations: Computer simulation techniques for prediction of the dynamic behaviour of molecules based on integration of Newton's equations of motion.

Post-Docking Refinement: A number of methods employed for more accurate ranking of the conformational poses from the docking process. Due to lack of precision in docking scoring functions, this stage is implemented to ensure the correctness of predictions.

Protein Flexibility: Proteins are dynamic entities and continuously undergo deviations from their equilibrium state. This is caused either due to the motion of the protein side-chains or sometimes the backbone structure. The issue of flexibility is often considered a challenge in docking studies and requires special treatments.

Sampling: A set of techniques in docking process for generating a large number of alternative poses for the two molecules (receptor and ligand) by sampling all the possible binding modes.

Scoring: Mathematical methods to rank the poses from docking with different types of scoring functions based on the binding free energy (non-bonded interactions) between the two docked molecules.

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Chapter 43
Protein–Protein Interactions (PPIs) as an Alternative to Targeting the ATP Binding Site of Kinase:
In Silico Approach to Identify PPI Inhibitors

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ABSTRACT

Most of the developed kinase inhibitor drugs are ATP competitive and suffer from drawbacks such as off-target kinase activity, development of resistance due to mutation in the ATP binding pocket and unfavorable intellectual property situations. Besides the ATP binding pocket, protein kinases have binding sites that are involved in Protein-Protein Interactions (PPIs); these PPIs directly or indirectly regulate the protein kinase activity. Of recent, small molecule inhibitors of PPIs are emerging as an alternative to ATP competitive agents. Rational design of inhibitors for kinase PPIs could be carried out using molecular modeling techniques. In silico tools available for the prediction of hot spot residues and cavities at the PPI sites and the means to utilize this information for the identification of inhibitors are discussed. Moreover, in silico studies to target the Aurora B-INCENP PPI sites are discussed in context. Overall, this chapter provides detailed in silico strategies that are available to the researchers for carrying out structure-based drug design of PPI inhibitors.

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INTRODUCTION

Protein kinase enzymes, also known as phosphotransferases, are the most extensively pursued class of drug targets in current pharmaceutical research. Several kinase inhibitors are registered for pre-clinical & clinical trial evaluation for different ailments, including cancer (O'Brien & Fallah Moghaddam, 2013; Rask-Andersen, Zhang, Fabbro, & Schioth, 2014; J. Zhang, Yang, & Gray, 2009). These enzymes transfers ATP terminal phosphate group to the substrate protein, and thereby regulate various activities including cell proliferation, differentiation, survival, transcription, apoptosis, metabolism, and a wide array of other signal transduction process (Adams, 2001; P. Wu, Nielsen, & Clausen, 2015). The involvement of kinases in various pathological conditions, makes them an attractive drug target for therapeutic intervention in diseases such as cancer (Fabbro, Cowan-Jacob, Mobitz, & Martiny-Baron, 2012), vascular (Abeyrathna & Su, 2015; Kikuchi et al., 2014) & central nervous system (CNS) disorders (Chico, Van Eldik, & Watterson, 2009), inflammatory disease conditions (Barnes, 2013; Rommel, 2010), and diabetes (Banks et al., 2015; Y. Wu & Chakrabarti, 2015). Consequently, for the past one and half decades, pharmaceutical companies and academic researchers are mounting intense efforts to develop small molecule kinase inhibitors. As a result of this, the first successful kinase inhibitor imatinib was approved in 2001 by FDA for the treatment of chronic myeloid leukemia (Gambacorti-Passerini & Piazza, 2015; Wisniewski et al., 2002). Since then, understanding of kinase structural and functional mechanism and their involvement in pathological conditions has significantly improved by the advancement of cell and molecular biology, structural biology, genetics, and associated fields. Concomitantly, the kinase inhibitors approval has also increased, and presently around 28 small molecule kinase inhibitors are approved by the US FDA for therapeutic usage in various cancers (Fabbro, 2015; P. Wu et al., 2015; Z. Zhao et al., 2014). The structures of approved kinase inhibitor drugs are represented in Figure 1.

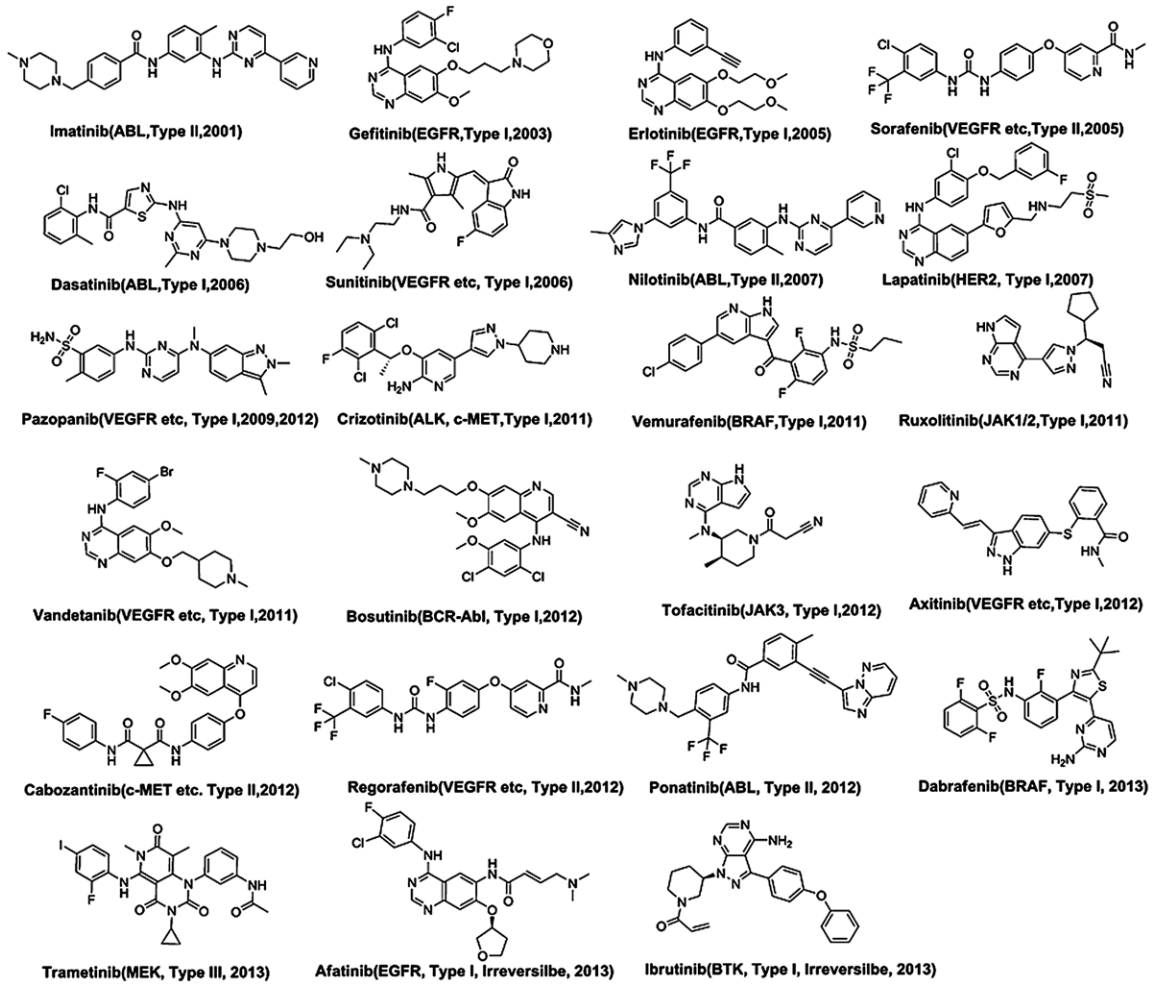
The entire human genome encodes approximately 518 protein kinases (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002), and have conserved kinase domain fold. The 3D structure of kinase contains a helix-dominated conserved C-terminal region, and a β sheet-dominated N-terminal region, which varies in sequence length and amino acid composition. A flexible hinge region connects the N- and C-terminal lobes, and in between these two lobes there exist a conserved ATP binding cleft. Glycine rich loop contains a conserved GXGXXG motif, and acts like a clamp to stabilize the binding of ATP at this cleft. Binding of ATP to the active site is controlled by the activation loop N-terminal starting residues Asp-Phe-Gly, also called DFG motif which adapts "DFG-in" and "DFG-out" conformation according to protein active and inactive state, respectively. The DFG motif residue Asp together with metal ion Mg^{2+} regulates kinase transactivation mechanism during phosphorylation process (Eswaran & Knapp, 2010; Thaimattam, Banerjee, Miglani, & Iqbal, 2007).

KINASE INHIBITORS, THEIR BINDING MECHANISM AND DRAWBACKS

The small molecule kinase inhibitors are useful as therapeutic agents, as well as to understand various cellular functions in normal and disease conditions. To date, most of the discovered kinases inhibitors are ATP competitive in nature; the heterocyclic core group in the inhibitor forms one to three hydrogen bond interactions with the conserved hinge region residues of the kinase (Garuti, Roberti, & Bottegoni, 2010; J. Zhang et al., 2009). Thus, they mimic the typical hydrogen bond interactions of adenine ring of ATP with the kinase. Based on the inhibition mechanism, kinase inhibitors are classified as either reversible

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Figure 1. FDA-approved kinase inhibitors with binding modes.
Reproduced from reference Z. Zhao et al. (2014).



or irreversible (P. Wu et al., 2015). The reversible inhibitors are further categorized as Type-I, Type-II, Type-III and Type-IV inhibitors, based on their binding mechanism as well as DFG motif conformation (Z. Zhao et al., 2014). The type-I inhibitors binds to active state of the protein where the DFG motif adapts into "DFG-in" conformation; in this state, the DFG motif residues Asp and Phe are placed inside and outside of the ATP binding pocket, respectively. The type-II inhibitors bind to the inactive state of the protein, where the DFG motif adapts into "DFG-out" conformation; in this conformation, the DFG motif residues Asp and Phe are placed outside of the ATP binding (under αC-helix) pocket. Moreover, in this conformation, the gate keeper residue permits the inhibitors to access the back pocket region. The type-III inhibitors also called allosteric inhibitors which binds exclusively to the ATP adjacent pocket (back pocket), and modulates the ATP binding by allosteric mechanism. While, type-IV inhibitors binds to a distant place from ATP binding site. Both type-III and type-IV inhibitors show highest degree of kinase selectivity because of their allosteric binding mode and regulatory mechanism that are unique to particular kinase enzymes (P. Wu et al., 2015). The irreversible inhibitors of kinases have tendency

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to bind covalently with ATP binding site residue, most frequently by interacting with nucleophilic Cys residue, which results in irreversible inhibition (Garuti, Roberti, & Bottegoni, 2011).

So far, most of the developed kinase inhibitors are ATP competitive in nature, and have serious drawbacks because of the shared conserved ATP binding site among the human kinome. The primary concerns regarding ATP competitive inhibitors are: off- and on-target toxicity issues (Burtness, 2014; Ho, Yeo, Kang, & Chua, 2014; Liu & Kurzrock, 2014), development of drug resistance (Barouch-Bentov & Sauer, 2011; Murray & Miller, 2015) and unfavorable intellectual property situation. Because of the conserved ATP binding site, development of selective kinase inhibitors is very challenging, and most of the approved and developed kinase inhibitors are also having reasonable multi-kinase activity which ultimately causes serious on- and off-target toxicity. The second major concern about kinase inhibitors is the development of drug resistance because of mutations in the gate keeper residue of the ATP binding site, which eventually causes loss of drug binding and drug efficacy to a particular kinase target. Because, at this particular position (gate keeper residue), the size of amino acid side chain regulates the inhibitors accessibility to the back pocket region or ATP adjacent pocket. As the kinase inhibitors make crucial hydrophobic interactions at the ATP adjacent pocket, loss of these interactions decrease the binding affinity of the inhibitors to the target kinase (J. Zhang et al., 2009).

The most frequently reported gate keeper residue mutations are observed in different kinases, including BCR-ABL1 (T315I), EGFR (T790M), FLT3 (G697R), KIT (T670I), PDGFR (T674I) and BTK (C481S) which results in drug resistance (J. Zhang et al., 2009). For example, gate keeper residue mutation (T315I) of BCR-ABL1 confers drug resistance to imatinib (Gleevec) in chronic myeloid leukemia patients (O'Hare, Eide, & Deininger, 2007). Further, mutation of EGFR gate keeper residue T790M results in drug resistance to geftinib and erlotinib by decreasing inhibitors accessibility to the ATP back pocket region (Godin-Heymann et al., 2008). Moreover, the recently approved Bruton's tyrosine kinase (BTK) inhibitor ibrutinib also showed resistance to gate keeper residue mutation (C481S) (D'Cruz & Uckun, 2013). In addition to these gate keeper mutations, other aberration such as target amplification and up-regulation of alternative kinase pathways also leads to the development of drug resistance to ATP competitive inhibitors (Barouch-Bentov & Sauer, 2011). The third concern regarding kinase inhibitors is the development of diverse set of molecules with favorable IP (intellectual property) situation. Because of the conserved ATP binding pocket, it is very difficult to develop kinase inhibitors with diverse set of functional groups which are protectable by IP laws (Backes, Zech, Felber, Klebl, & Muller, 2008). Thus, there is an urgent need to develop alternative strategies to develop small molecule inhibitors towards these indispensable drug targets.

Beside the ATP binding pocket, protein kinases have other prominent structural features including the binding sites that are involved in protein-protein interactions (PPIs) (Dickson, Wilbur, Reinke, Young, & Vojtek, 2015; Pearson et al., 2001; Rechfeld, Gruber, Hofmann, & Kirchmair, 2011; Rudolph, 2007; White, Westwell, & Brahemi, 2008). These PPIs directly or indirectly monitor the protein kinase activity, and interruption of interaction at these sites (PPI sites) have emerged as an attractive and alternative strategy to develop selective kinase inhibitors with favorable IP situation (Cierpicki & Grembecka, 2015; Jin, Wang, & Fang, 2014). As the activity of different kinases are controlled by numerous protein-protein interactions, and their binding interface significantly varies from one kinase family to other kinase family member proteins, targeting these PPI sites have advantage over targeting the conserved ATP binding site. This might allow higher level of selectivity as compared to the ATP competitive inhibitors. Moreover, development of kinase PPI inhibitors permits for combinatorial treatment with ATP competitive inhibitors which could synergistically block kinase activity as compared to ATP competitive inhibitors

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alone. However, targeting kinases PPI site is very challenging, because of their flat and featureless large hydrophobic interface region; further, these interactions are transient and are exposed to solvent regions, causing difficulties in PPI inhibitor development. Nevertheless, growing evidence indicates that targeting PPI sites of kinases are emerging as an important therapeutic strategy in drug discovery and development (Corbel et al., 2011; Dickson et al., 2015; Han, Kim, & Yang, 2012; Kaidanovich-Beilin & Eldar-Finkelman, 2006; Rechfeld et al., 2011; B. Zhang et al., 2011).

PROTEIN-PROTEIN INTERACTIONS AS THERAPEUTIC TARGETS

The fundamental functions of living cell such as cell growth, morphology, nutrient uptake, gene expression, signaling network, and apoptosis are accomplished by macromolecular interactions; among them, protein-protein interactions (PPIs) have critical role in various biological and pathological conditions (Ryan & Matthews, 2005). It is reported that a single cell of a human can have around 130,000 to 650,000 PPIs (Stumpf et al., 2008; Venkatesan et al., 2009). Thus, understanding the importance of PPIs in various pathological conditions has wide application in rational drug design and protein engineering. However, previously it was assumed that PPI sites are "undruggable (or) intractable", because of their flat and featureless large hydrophobic region that couldn't accommodate a small molecule inhibitor (Wells & McClendon, 2007). This misconception was assumed due to lack of understanding of these sites, and also, there weren't many known small molecule inhibitors that could bind to PPI sites as compared to the traditional drug targets such as enzymes, receptors and ion channels. Further, lack of experimental assay methods, and small molecule libraries that were suitable for identification of PPI inhibitors were not available in yesteryears. However, recent developments in molecular biology, structural biology, computational chemistry and associated fields have helped in gaining better knowledge of PPIs (Cierpicki & Grembecka, 2015; Kuenemann et al., 2015; Villoutreix et al., 2014; Watkins & Arora, 2015).

Traditional drug targets such as receptors, enzymes and ion channels have served as attractive drug targets for many of the pathological conditions, including cancer. At the same time, toxicity issues and drug resistance development was concurrently observed for many of these drug targets (Barouch-Bentov & Sauer, 2011; Liu & Kurzrock, 2014; Wallace, 2015) Consequent to this, quest for new targets for therapeutic intervention turned the focus on PPIs, because of their importance in various pathologies (Ryan & Matthews, 2005; Wells & McClendon, 2007). Thus, design of small molecule inhibitors that directly block PPIs has become an attractive and an alternative strategy to develop small molecule inhibitors for various diseases. However, structural analysis of PPIs revealed that as compared to traditional drug targets, PPI sites are complex and flexible; they lack cavities that are essential for small molecule binding (Cierpicki & Grembecka, 2015). The interfaces of PPIs are considerably flat, and contain featureless large hydrophobic surface area (around 1200 to 3000 $Å^2$) (Cierpicki & Grembecka, 2015; Lo Conte, Chothia, & Janin, 1999). Further, the intrinsic properties of PPIs such as flexibility and dynamic nature is different in solution as compared to crystal binding mode, and it is very difficult to design small molecule inhibitors for PPIs when structure based drug design methods are applied.

In spite of these challenges, the current body of literature evidences suggest that only few of the clustered residues of PPIs sites contributes to majority of the binding free energy (called as 'hot spots'), and these hot spot residues are critical for protein-protein structure stability and function (Bogan & Thorn, 1998; Clackson & Wells, 1995; Kuttner & Engel, 2012; Moreira, Fernandes, & Ramos, 2007). Thus, targeting PPIs hot spots residues provide an attractive opportunity to explore these drug targets

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for therapeutic intervention (Cukuroglu, Engin, Gursoy, & Keskin, 2014; Guo, Wisniewski, & Ji, 2014). Further, structural analysis revealed that PPIs sites could be subdivided into three to four sub-pockets (Fuller, Burgoyne, & Jackson, 2009; Jubb, Blundell, & Ascher, 2015) and the binding to these sub-pockets could be explored using natural products and peptidomimetics like compounds. Recently, few small molecule and peptide inhibitors of PPIs are discovered using the above information (Arkin, Tang, & Wells, 2014). The interface residues of PPIs are conserved and are less prone to the development of drug resistance (Kozakov et al., 2011; X. Li, Keskin, Ma, Nussinov, & Liang, 2004; Ma, Elkayam, Wolfson, & Nussinov, 2003). Moreover, growing evidences indicate that PPIs are challenging but tractable drug targets for various diseases. As a proof of concept, few small molecule inhibitors have already entered into clinical trials and a number of small molecule inhibitors are in pre-clinical development (Arkin et al., 2014; Bogoyevitch, Barr, & Ketterman, 2005; Cierpicki & Grembecka, 2015). In the following section, few important PPI drug targets (IAP family proteins, bromodomains, BCL family proteins, MDM2-p53 complex interface, and HIV integrase) and their inhibitors that have progressed towards clinical trials are described in brief. Interested readers may also refer to other review articles for additional information (Arkin et al., 2014; Ivanov, Khuri, & Fu, 2013; Jin et al., 2014; Kuenemann et al., 2015; Laraia, McKenzie, Spring, Venkitaraman, & Huggins, 2015; Sheng, Dong, Miao, Zhang, & Wang, 2015).

IAP Inhibitors

The Inhibitors of Apoptosis Proteins (IAPs), including XIAP, cIAP1, cIAP2, Survivin, and Levivin, play vital role in various biological function including cell division, duplication, cell death, DNA damage, autophagy and immunity (Budhidarmo & Day, 2015; Damgaard & Gyrd-Hansen, 2011). On the other hand, it has been demonstrated that these proteins are over expressed in many of the human solid tumors. Consequently, these proteins have become attractive drug targets for cancer therapy (Dubrez, Berthelet, & Glorian, 2013). These proteins contain at least one conserved BIR domain, and involves in PPIs. IAPs BIR domain directly binds to N-terminal tetra-peptide sequence of caspase-3, caspase-7 and caspase-9 during their proteolytic activation; as a result it inhibits their pro-apoptotic mechanism. However, an endogenous peptide inhibitor of IAPs called Smac (Second mitochondrial activators of caspases) binds to their BIR domain, and activates the apoptosis mechanism. Therefore, developing a small molecule inhibitor of IAPs that mimics the Smac is an attractive approach to inhibit the IAPs protein-protein interaction with caspases. Several Smac mimetics have been developed by many scientific groups; among them, seven compounds have reached clinical trials and five compounds including LCL-161, AT-406, TL-32711, GDC-0152, and GDC-0917 are actively pursued in clinical development for the treatment of cancer (Arkin et al., 2014).

Bromodomain (BRD) Inhibitors

The bromodomain family proteins (BET, BRD2, BRD3, BRD4 and BRDT) are ‘epigenetic readers’, which have significant role in targeting multiple enzymes, protein scaffolds and DNA binding proteins to their specific sites. Usually, these proteins recognize the acetylated lysines (Kacs) on histone tail, and thereby govern chromatin remodeling and gene transcription (Sanchez & Zhou, 2009). However, dysfunctions of these proteins have been reported in various cancers, and targeting these proteins interaction with acetylated lysine peptide has become an attractive approach for the design of small molecule inhibitors for cancer (Muller, Filippakopoulos, & Knapp, 2011). Consequently, the hydrophobic pocket

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of bromodomains that recognizes acetyl-lysine peptide has been used to design and develop the Kac mimetic inhibitors. Currently, few compounds including I-BET762, CPI-0610, Ten-010 and OTX15 are in clinical development for cancer (Arkin et al., 2014).

BCL Family Protein Inhibitors

BCL family proteins include pro- and anti-apoptotic proteins which regulates cell death mechanism in various biological events (Hardwick & Soane, 2013). The pro-apoptotic proteins BAD and BAK are tightly regulated or inhibited by anti-apoptotic proteins including BCL-2, BCL-xl and MCL1. Over expression or deregulation of anti-apoptotic proteins have been observed in various cancers (Besbes, Mirshahi, Pocard, & Billard, 2015). The anti-apoptotic proteins are helical proteins which forms an open cavity that binds to BH3 domain (single helix) of pro-apoptotic proteins. Hence, disrupting these PPIs using small molecule inhibitors has become an attractive approach for cancer therapy. In the past, several inhibitors were reported to modulate these PPIs; currently, ABT-199 and ABT-263 are in clinical development for cancer (Arkin et al., 2014).

MDM2-p53 PPI Inhibitors

p53 is the principal tumor suppressor protein, also called as "guardian of genome" because of its central role in protecting and stabilizing the human genome from various mutations. It tightly regulates cell-fate decisions including cell cycle arrest, senescence and apoptosis (Surget, Khoury, & Bourdon, 2013). Mouse Double Minute 2 homolog (MDM2), also known as E3 ubiquitin-protein ligase is one of the major regulator of p53 (Momand, Wu, & Dasgupta, 2000). MDM2, constantly ubiquinates p53 leading to degradation by nuclear and cytoplasmic proteasome enzymes, thereby limiting its tumor suppressor function in unstressed cells. However, mutations are reported in p53 and its regulator protein, which results in loss of p53 tumor suppressor function. Thus, disruption of MDM2-p53 PPIs results in p53 activation in tumor cells. The inhibitor nutlin is the first breakthrough that was discovered by large high-throughput screening for disruption of MDM2-p53 PPIs (Shangary & Wang, 2009). Subsequently, several small molecule inhibitors were discovered by many scientific groups. Currently, RG-7112 (Ro5045337), RG-7388 (Ro5503781) and MI-888 are in clinical development for the treatment of cancer (Arkin et al., 2014; Y. Zhao, Aguilar, Bernard, & Wang, 2015).

HIV Integrase Inhibitors

HIV integrase (IN) allows integration of viral genome into host genome by catalyzing 3′ processing and stand transfer. In this process, the human protein - Lens Endothelial Growth Factor (LEDGF) acts as IN cofactor to activate the integration process, and also to protect from proteolytic degradation. Thus, targeting these host-pathogen PPIs (LEDGF-IN) is an alternative and attractive approach for the design of small molecule inhibitors for treating AIDS. Recently, a second class of IN inhibitors that mimics the LEDGF peptide has entered into clinical trials (Engelman, Kessl, & Kvaratskhelia, 2013). Currently, the inhibitor *tert*–Butoxy-(4phenyl-quinolin-3-ly-acetic acid (*t*BPQA) derivative and BI-224436 are in clinical development for the treatment of AIDS (Arkin et al., 2014).

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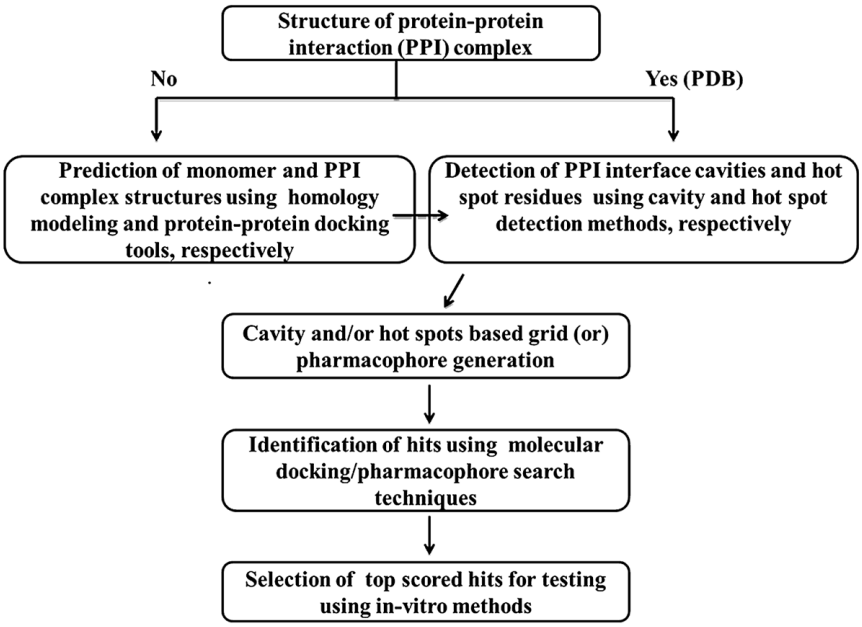
STRUCTURE-BASED DRUG DESIGN OF PPI INHIBITORS USING *IN SILICO* APPROACHES

The arena of structure based drug design (SBDD) has rapidly grown because of the advancements in genomics, proteomics, structural biology and computational chemistry. SBDD has an important role in drug discovery, and many successful lead molecules have been discovered using this approach (Anderson, 2003; Lionta, Spyrou, Vassilatis, & Cournia, 2014). Once the 3D structure of a particular disease target is resolved using experimental (X-ray or NMR) or computational methods (homology modeling and threading), then it could be used for virtual screening or for de-novo design to identify lead molecule inhibitors. In this chapter, the authors describe in a step-wise manner how SBDD methods could be applied to discover small molecule inhibitors of PPIs. Figure 2 summarizes the SBDD approach for the identification of PPIs inhibitors.

Modeling of Protein and PPI Complex Structures

For the rational design of PPI inhibitors using structure-based drug design (SBDD), it is essential to have 3D structures of PPI complexes involved in the particular disease state. However, available 3D structures of protein-protein complexes are very few in Protein Data Bank (PDB), as compared to the traditional drug targets such as enzyme and receptor structures. Resolving the 3D structures of PPIs using X-ray and NMR methods is much more challenging (Widmer & Jahnke, 2004). Due to the experimental limitations, *in silico* methods including comparative modeling (homology modeling), threading and fold recognition and Ab initio methods can be used to build the protein structure. Knowledge of resolved homologue protein structures and physical principles of protein such as that mimic the protein folding

Figure 2. Structure-based drug design (SBDD) approach for the identification of PPI inhibitors



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or stochastic (global optimization of energy function) methods are used to search the possible solutions. The *in silico* tools or modules that are available for protein 3D structure predictions are listed in Table 1. Among them, MODELLER (Eswar et al., 2006), SWISS-MODEL (Biasini et al., 2014) (for comparative modeling), I-TASSER (Yang et al., 2015) (for threading), ROBETTA server (Kim, Chivian, & Baker, 2004) (combination of comparative and Ab initio methods) are well known modules for protein 3D structure prediction.

Further, in the absence of experimental PPIs complex structures, the available monomer structures (from PDB or modeling) of two individual protein partners could be used to build the PPI complexes using protein-protein docking (Vakser, 2014). The ultimate goal of protein-protein docking is to predict the 3D structure of the macromolecular complexes of interest as they would occur in a living organism.

Table 1. List of available protein 3D structure prediction tools and web servers

Sl.No	Name of the Tool	Description and Web-Links
Homology Based or Comparative Methods		
1	Modeller	Downloadable (http://salilab.org/modeller/) and webserver (https://modbase.compbio.ucsf.edu/modweb/)
2	SWISS-MODEL	Webserver (http://swissmodel.expasy.org/)
3	PHYRE2	Webserver (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index)
4	ESyPred3D	Webserver (http://www.unamur.be/sciences/biologie/urbm/bioinfo/esypred/)
5	AS2TS system	Webserver (http://as2ts.llnl.gov/)
6	3D-JIGSAW	Webserver (http://bmm.cancerresearchuk.org/~3djigsaw/)
7	Yasara	Webserver (http://www.yasara.org/)
8	Biskit	Downloadable (http://biskit.pasteur.fr/)
9	FoldX	Downloadable (http://foldx.crg.es/)
10	Prime	Downloadable (http://www.schrodinger.com/Prime)
11	CPHModel	Webserver (http://www.cbs.dtu.dk/services/CPHmodels/)
12	HHpred	Downloadable (ftp://ftp.tuebingen.mpg.de/pub/protevo/HHsearch/) and webserver (http://toolkit.tuebingen.mpg.de/hhpred)
Threading and Fold Recognition Based Methods		
13	I-TASSER	Downloadable (http://zhanglab.ccmb.med.umich.edu/I-TASSER/download/) and webserver (http://zhanglab.ccmb.med.umich.edu/I-TASSER/)
14	RaptorX	Downloadable (http://raptorx.uchicago.edu/) and Webserver (http://raptorx.uchicago.edu/)
15	LOOPP	Webserver (http://cbsuapps.tc.cornell.edu/loopp.aspx)
16	MUSTER	Webserver (http://zhanglab.ccmb.med.umich.edu/MUSTER/)
17	SPARKS-X	http://sparks-lab.org/yueyang/server/SPARKS-X/_
Ab Initio and Combination of Threading and Ab Initio Methods		
18	ROBETTA	Webserver (http://www.robetta.org/)
19	Bhageerath	Webserver (http://www.scfbio-iitd.res.in/bhageerath/index.jsp)
20	EVfold	Webserver (http://evfold.org/evfold-web/evfold.do)
21	QUARK	Webserver (http://zhanglab.ccmb.med.umich.edu/QUARK/)
22	PEP-FOLD	Webserver (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/)

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Thus, execution of protein-protein docking of two interested proteins would produces number of plausible candidate complex structures, and these candidates must be ranked using scoring functions to identify structures that are most likely to occur in the native state. There are a number of algorithms and scoring functions developed to model PPIs complexes with reasonable accuracy (Sable & Jois, 2015). The most commonly used protein-protein docking tools and their algorithms are listed in Table 2.

Table 2. List of available protein-protein docking tools and web servers

Sl. No	Name of the Tool	Applied Methodology	Description and Web Links
1	ZODOCK	It uses Fast Fourier transformation (FFT), shape complementarity, desolvation energy and electrostatic interactions	It is available as a webserver (http://zdock.umassmed.edu/) and also as a standalone tool; implemented in Accelrys software suit (discovery studio).
2	HADDOCK	Data-driven bimolecular docking	It is an information-driven flexible docking program used to model bimolecular complexes (http://haddocking.org/)
3	GRAMM-X	Fast Fourier Transformation	It employs smoothed potentials, refinement and knowledge-based scoring function to find the possible solution. (http://vakser.compbio.ku.edu/resources/gramm/grammx/)
4	ClusPro	Rigid-body docking using the FFT correlation approach and RMSD clustering	Webserver (http://nrc.bu.edu/cluster)
5	Patch dock	Global search	It uses shape complementarity and atomic desolvation energy to search possible solution (http://bioinfo3d.cs.tau.ac.il/PatchDock/)
6	RosettaDock	Rigid body docking (energy based method)	It optimizes the rigid body docked poses and side chains to finds lowest energy conformation (http://rosettadock.graylab.jhu.edu/)
7	GPU.proton. DOCK	Fast Fourier Transform bottleneck and electrostatic fields computation	It is a state of the art server for prediction of protein–protein interactions via rigorous and ultrafast docking code (http://gpudock.orgchm.bas.bg/)
8	HexServer	Fast Fourier transformation	(http://hexserver.loria.fr/)
9	3D-Garden	Marching cubes algorithm	http://www.sbg.bio.ic.ac.uk/~3dgarden/_
10	SymmDock	Symmetry by Geometry Based Docking	http://bioinfo3d.cs.tau.ac.il/SymmDock/_
11	SwarmDock	SwarmDock algorithm	This algorithm includes docking using a hybrid particle swarm optimization / local search, and minimization, re-ranking and clustering of docked poses http://bmm.cancerresearchuk.org/~SwarmDock/
12	PIPER	FFT-based docking with pairwise potentials	It is implemented in Schrodinger software suit http://www.schrodinger.com/PIPER/
13	DOT 2.0	FFT based global rigid search	Downloadable (http://www.sdsc.edu/CCMS/DOT/)
14	ICM	Monte Carlo based global rigid search	Downloadable (http://www.molsoft.com/docking.html)
15	MolFit	FFT and shape complementarity based global search	Downloadable http://www.weizmann.ac.il/Chemical_Research_Support/molfit/
Refinement or post docking refinement modules			
16	RDOCK	CHARMM force field	It is implemented in discovery studio (Accelrys software suit). Refines the ZODOCK poses using CHARMM force field
17	FireDock	Fast interaction refinement	It is an efficient method for refinement and re-scoring of rigid-body protein-protein docking poses (http://bioinfo3d.cs.tau.ac.il/FireDock/)
18	HADDOCK-Refinement interface	MD simulated annealing refinement	Refines other protein-protein docking poses http://haddock.science.uu.nl/services/HADDOCK2.2/haddockserver-refinement.html

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Identification of Ligand Binding Cavities and Hot Spot Residues at the PPI Interface

The next step for the identification of inhibitors of PPIs is to identify the cavities and ligand binding hot spot residues in PPIs interface region. It has been demonstrated that PPIs interface are flat and contains featureless large hydrophobic surface region (Cierpicki & Grembecka, 2015). Nevertheless, the large hydrophobic surface area is separated into three to four superficial sub-pockets which could be of 50 $Å^3$ size (Fuller et al., 2009). Moreover, it is also demonstrated that only few clustered residues of each partners of PPIs contributes to the majority of binding free energy (affinity) towards the complex formation. The clustered residues which are mainly contributing to the binding energy are called 'hot spots' (Bogan & Thorn, 1998; Clackson & Wells, 1995). Further, these hot spots of PPIs are conserved throughout evolution and show high propensity for ligand binding. Thus, identification of superficial cavities and ligand binding hot spots are two primary steps that are very critical for the SBDD of PPIs inhibitors. It should also be noted that sometimes it is not possible to get both these structural features at the same site. The ligand binding cavity may exists at one site and the hot spot residues exist at other site or the hot spot residues may be projected into the ligand binding cavity. Therefore, the integrated knowledge that is derived from both the cavity and hot spots detection can be used for SBDD of PPI inhibitors (Kuenemann et al., 2015; Laraia et al., 2015; Watkins & Arora, 2015).

Ligand binding pockets or cavities on the protein surface can be identified using various *in silico* tools which are mentioned in the virtual ligand screening website (http://www.vls3d.com/links/bioinformatics/binding-pockets). Generally, these tools are classified into three categories: (1) protein geometry based methods; (2) energy based methods and (3) combination of above two methods (H. Li, Kasam, Tautermann, Seeliger, & Vaidehi, 2014; Zheng, Gan, Wang, & Wang, 2013). Most of the tools using these methods are also suitable for detecting the cavities in traditional drug targets. More specifically, few energy based methods such as FTMap server (Ngan et al., 2012) and FindBindSite (H. Li et al., 2014) were specifically developed to detect the ligand binding cavities at PPIs interface. These methods use molecular docking approach to map the whole protein surface using small probes or fragments. Consequently, mapping small molecule fragment on protein surface by docking effectively identifies the superficial cavities at PPIs.

The PPIs hot spots can be identified using either experimental or computational methods. Among these methods, experimental alanine scanning mutagenesis is well known and is widely employed in the literature to study the structural and functional aspects of the protein (Lefevre, Remy, & Masson, 1997). Moreover, it exactly identifies the hot spots by substituting the selected residue with alanine. Upon substitution (mutation) it estimates binding free energy by means of thermodynamic properties. After alanine scanning mutagenesis, if the total binding free energy of mutant protein drops more than 1-2 kcal/mol, as compared to the native protein then the mutated amino acid is considered as hot spot residue. However, identification of hot spots using experimental alanine scanning mutagenesis is very expensive and time consuming. On the other hand, various *in silico* methods such as Robetta server (Kim et al., 2004), DrugScorePPI server (Kruger & Gohlke, 2010), HotRegion database (Cukuroglu, Gursoy, & Keskin, 2012), KFC server (Darnell, LeGault, & Mitchell, 2008) and PocketQuery server (Koes & Camacho, 2012a) could be used to detect the hot spot residues in PPIs interface region. These methods use different algorithms, energy functions and parameters to optimize and to correlate with the experimental results. These methods are classified into three categories: (1) simple free energy function based methods, (2) molecular dynamics simulation based methods and (3) machine learning based

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methods. In fact, all the developed methods have their own advantages and limitation and are widely used by various scientific groups in their research. Moreover, these are very fast, reliable and also correlate with experimental results. Thus, along with the superficial cavities that are identified by cavity detection methods, hot spots knowledge could be used to define the ligand binding sites or to derive the pharmacophore features and used for screening the small molecule databases. Some of the hot spot and ligand binding cavity detection methods and tools that are specially developed for PPIs are listed in Table 3.

Virtual Screening for the Identification of PPI Inhibitors

After the identification of ligand binding sites of PPIs using the cavity detection methods and the detection of hot spots, small molecule inhibitors or initial hits for PPIs can be identified using virtual screening (VS) methods (Reddy, Pati, Kumar, Pradeep, & Sastry, 2007). It is a common practice in the drug discovery project to search the libraries of small molecules to identify molecules that are most likely to bind to a drug target. VS can be carried out using either molecular docking or pharmacophore based methods.

Molecular docking (Ferreira, Dos Santos, Oliva, & Andricopulo, 2015) is a computational technique, used in drug design projects to predict the binding orientation of small molecule inhibitors in their target proteins. Thus, the knowledge derived from binding orientation could be used to predict the binding affinity or activity of the small molecules using scoring functions. Hence, docking has become a powerful *in silico* tool in various pharmaceutical industries. Some of the well-known docking programs that are

Table 3. List of PPIs hot spots and ligand binding cavity detection tools and webservers

Sl.No	Name of the Tool	Web Links
Hot Spot Detection Server and Tools		
1	Robetta server	http://www.robetta.org/alascansubmit.jsp_
2	KFC2 server	http://kfc.mitchell-lab.org/_
3	HotRegion	http://prism.ccbb.ku.edu.tr/hotregion/_
4	DrugScorePPI	http://cpclab.uni-duesseldorf.de/dsppi/main.php_
5	HotPoint	http://prism.ccbb.ku.edu.tr/hotpoint/_
6	PredHS	http://nar.oxfordjournals.org/content/early/2014/05/22/nar.gku437_
7	HotSpot Wizard	http://loschmidt.chemi.muni.cz/hotspotwizard/_
8	PCRPi-DB	http://www.bioinsilico.org/cgi-bin/PCRPIDB/htmlPCRPI/home_
9	PCRPi	http://www.bioinsilico.org/PCRPi/_
10	FoldX	http://foldx.crg.es/_
11	Hot Spot Prediction	Standalone tool, can be downloaded from the link http://sfb.kaust.edu.sa/pages/software.aspx
Ligand Binding Cavity Detection Servers and Databases		
12	FTmap	http://ftmap.bu.edu/login.php_
13	FindBindSite	PPIs binding site prediction methods. Not yet developed as a server
14	Pocketquery	http://pocketquery.csb.pitt.edu/_
15	MAPPIS	http://bioinfo3d.cs.tau.ac.il/MAPPIS/_
16	ANCHOR	http://structure.pitt.edu/anchor/_

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commonly used by scientific communities are: AutoDock (Morris et al., 2009), GOLD (Jones, Willett, Glen, Leach, & Taylor, 1997), DOCK (Ewing, Makino, Skillman, & Kuntz, 2001), Glide-XP (Friesner et al., 2006), LibDock (Diller & Merz, 2001), CDOCKER (G. Wu, Robertson, Brooks, & Vieth, 2003), FlexX (Rarey, Kramer, Lengauer, & Klebe, 1996), LigandFit (Venkatachalam, Jiang, Oldfield, & Waldman, 2003), etc. All these programs use different algorithms and parameters, and these could be classified broadly as shape based (e.g. LibDock) and energy based (e.g. Glide XP) methods.

Virtual screening using structure based pharmacophore (SBP) is an important and an alternative method to docking (Pirhadi, Shiri, & Ghasemi, 2013). SBP method uses a set of essential features (steric and electrostatic interactions) that are necessary to maintain the optimal intermolecular interactions with a specific biological target to activate or block its biological response. Generally, pharmacophore features include hydrogen bond acceptor, hydrogen bond donor, hydrophobic, aromatic, positive and negative ionizable groups. Earlier, only ligand based pharmacophore hypothesis were usually employed to screen the small molecule databases in the absence of the protein structure. But, advancement of structural biology and computational chemistry greatly helped to derive the pharmacophore features from protein or protein-ligand complex structures. Thus, several tools are developed to derive the pharmacophore features using protein or protein-ligand structural information. Among them, Catalyst (Discovery Studio 3.1), Phase and E-pharmacophore (Schrödinger Suite 9.2 software package), Ligand and Structure-Based Query Editor (MOE), LiganScout (Wolber & Langer, 2005), and ZINCPharmer (Koes & Camacho, 2012b) are few routinely employed tools in drug discovery projects. Using these tools, the pharmacophore features could be derived from the known protein-ligand crystal structure or exclusively from the identified binding sites or from the identified hot spot residues of the given target PPIs. Moreover, the pharmacophore features can be derived from docking the fragments at the defined binding site of protein using E-pharmacophore module (Schrödinger software suit), which takes the advantages of both ligand and structure based methodologies to generate an energetically optimized pharmacophore hypothesis that can be used to screen the small molecule databases. Interested readers may refer to Clik2Drug website (http://www.click2drug.org/directory_Docking.html) for an exhaustive list of tools available for SBDD.

The PPIs inhibitors identified by VS could be further evaluated using various computational methods. One approach is, assessing the binding stability of the compound with the target protein using molecular dynamics simulation (Mortier et al., 2015). Using this, the dynamic behavior of the compounds with the target protein in the presence solvent molecules could be studied. The second approach is to assess the drug-like properties of the identified hits, using Lipinski's rule of five (Lipinski, Lombardo, Dominy, & Feeney, 2001). Based on the favorable results from the above *in silico* investigations, the identified inhibitors could be subjected to *in vitro* testing and further optimization to identify PPIs inhibitors.

An *In Silico* Approach for the Identification of PPI Inhibitors: Aurora B-INCENP PPI as a Case Study

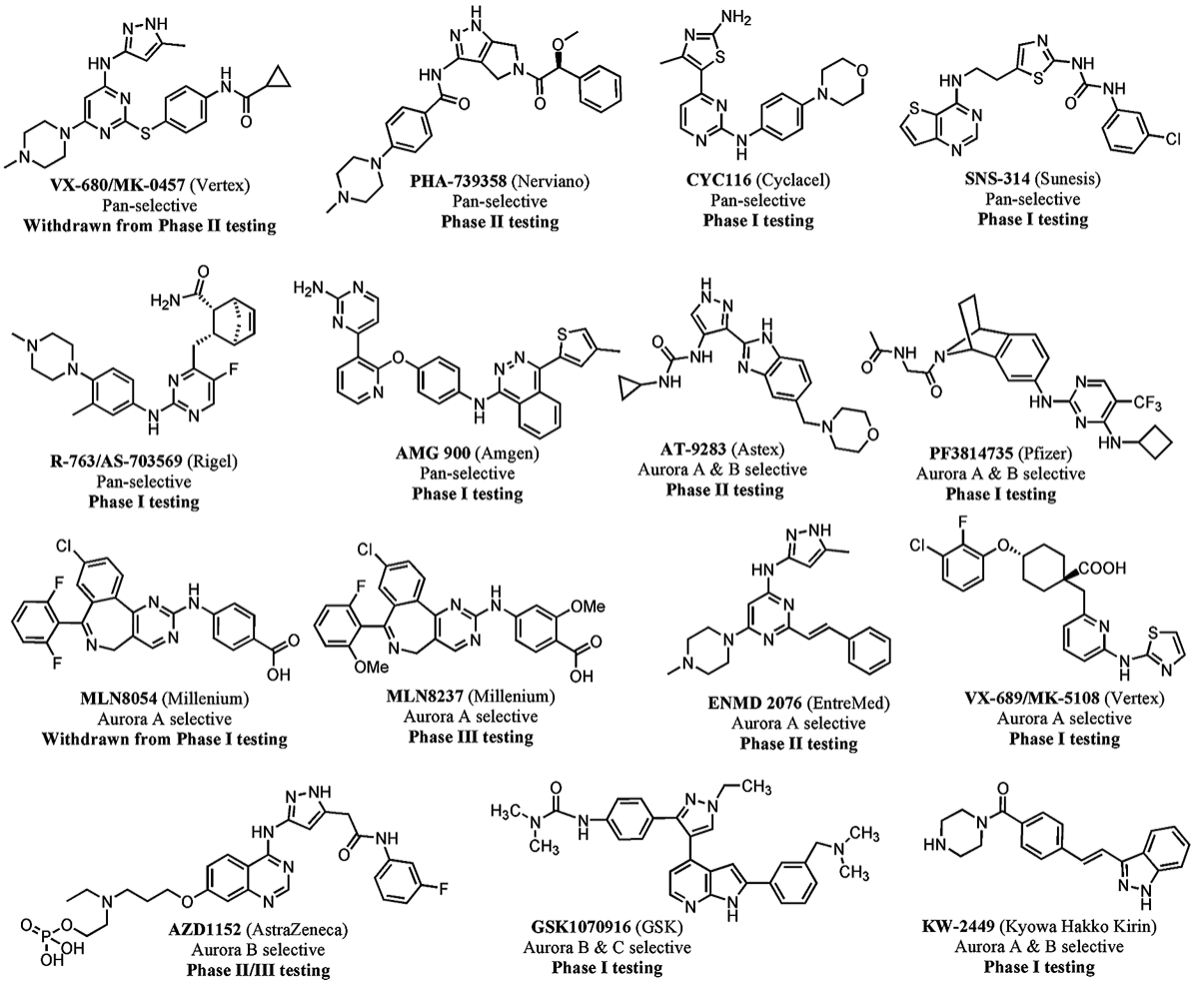
Aurora kinases are a subclass of protein kinase that plays a crucial role in mitosis through phosphorylation of serine/threonine residues of the substrate protein. There are three isoforms of Aurora kinases (Aurora-A, B and C) which share a conserved ATP binding site. Among these three isoforms, Aurora A and B are over expressed in many of the human solid tumors (Fu, Bian, Jiang, & Zhang, 2007). Consequently, scientific communities are developing Aurora kinase inhibitors for cancer therapy. Presently, over a dozen Aurora kinase inhibitors that target the ATP binding site are in various phases of clinical

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development (Figure 3) (Cheung, Coumar, Chang, & Hsieh, 2011; Cheung, Coumar, Hsieh, & Chang, 2009; Cheung, Sarvagalla, Lee, Huang, & Coumar, 2014). Most of the developed Aurora kinase inhibitors are pan-selective (Aurora A, B and C selective), and few of them are isoform selective (Aurora A or B selective); but none of them have been approved for clinical use so far. The major reasons for the failure is due to on- and off-target toxicity problems associated with the administration of non-selective Aurora kinase inhibitors (Sarvagalla & Coumar, 2015).

As Aurora kinase family shares a conserved ATP binding site, developing isoform selective inhibitors is very difficult. Hence, most of the developed Aurora kinase inhibitors are pan-selective, and evaluation of these inhibitors in the clinical trials reported on- and off-target toxicity (Kollareddy et al., 2012; Sarvagalla & Coumar, 2015). The major on-target toxicities that are reported in clinics are: neutropenia, leukopenia, myelosuppression, septicemia, pneumonia and thrombocytopenia. In addition to this, several off-target problems including mucositis, proctalgia, hypertension, somnolence, stomatitis, grade 3 increases in aspartate aminotransferase and grade 2 ventricular dysfunctions have been reported. Drug resistance, is another major problem that need to be addressed for Aurora kinase inhibitors (Girdler et al.,

Figure 3. Chemical structures of Aurora kinase inhibitors in clinical development



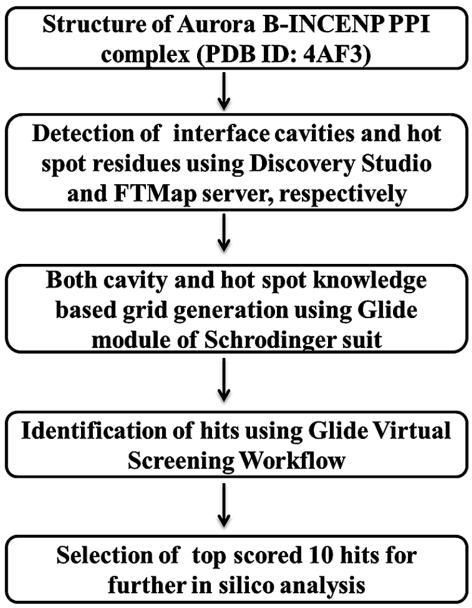
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2008). Because of the development of drug resistance, drug efficacy and affinity is greatly affected. It is also reported that various types of cancer cell lines develop resistance to many Aurora kinase inhibitors. Another drawback of Aurora kinase inhibitors is the lack of chemical diversity, because of the conserved ATP binding site, resulting in difficulties in IP protection (Backes et al., 2008).

The above reported problems could be overcome by targeting the PPI sites of Aurora kinases. Aurora kinase A interacts with TPX2 (Bayliss, Sardon, Vernos, & Conti, 2003); whereas Aurora B interacts with INCENP protein (Sessa et al., 2005). The interactions of TPX2 and INCENP with their respective Aurora kinase isoform are very specific and essential for the full activation of the kinase. Thus, inhibiting these PPIs would be very specific and serves as an alternative strategy to target the Aurora kinase for cancer treatment with reduced toxicity profile. Recent evidence also indicates that the design of isoform selective Aurora kinase inhibitor has advantage over the pan-selective Aurora inhibitors (Sarvagalla & Coumar, 2015). This could be achieved by targeting PPI sites of Aurora enzymes (Gohard, St-Cyr, Tyers, & Earnshaw, 2014). To this end, to design isoform selective small molecule inhibitors of Aurora kinase B, here the authors have applied the cavity and hot spot detection techniques discussed above to identify ligand binding PPIs sites in the interface of Aurora B-INCENP. Further, molecular docking was carried out to identify PPI inhibitors by screening approved drugs (http://www.drugbank.ca/). The methodology applied for the identification of Aurora B-INCENP PPIs inhibitors is depicted in Figure 4.

For this process, first the Aurora B-INCENP protein crystal structure (PDB ID: 4AF3) was downloaded from PDB, and was processed for docking as reported earlier (Sarvagalla et al., 2015). Then, the prepared protein was used to identify the ligand binding cavities using two different methods: The first one is shape based method (Define Site- discovery studio module) and the second one is based on the energy based method (FTMap server). These two methods identified a common ligand binding cavity at the interface of Aurora B-INCENP complex. Further, comparison of the results from both the

Figure 4. Schematic representation of the workflow used to identify PPI inhibitors of Aurora B-INCENP complex



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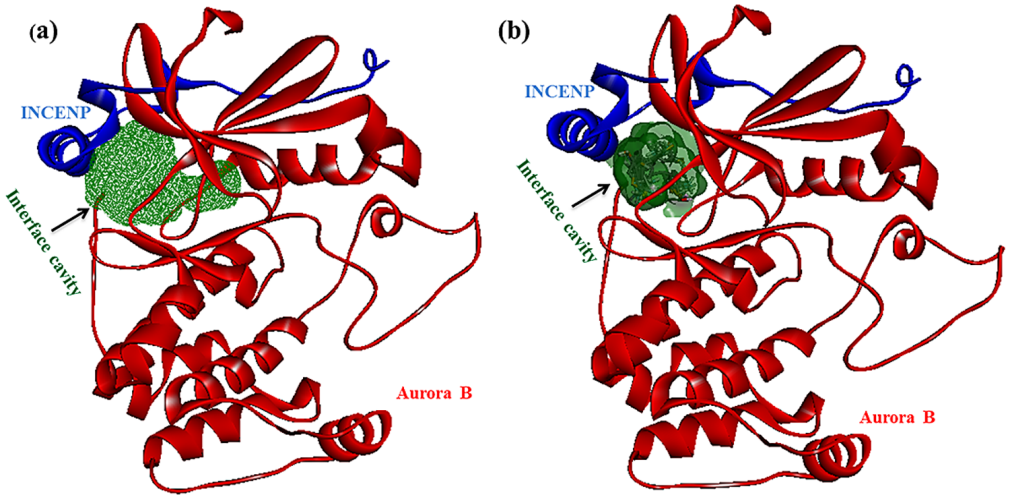
method revealed that most of the residues identified in the cavity are common. The residues involved in the cavity are shown in Table 4, and the location of the cavity that was identified by discovery studio and FTMap are shown in Figure 5.

Further, the hot spot residues of both the proteins (Aurora B and INCENP) were identified by mutating the interface residue to alanine using discovery studio module and Robetta web-server. Results from both the methods are shown in Table 5. Analysis of the results revealed that four hot spots residues (Phe101, Ile102, Tyr141 and Leu210) from Aurora B and six hot spot residues (Ile855, Gln858, Tyr859, Leu865, Leu868 and Phe869) from INCENP protein are projected into the identified cavity. Next, the integrated knowledge derived from the cavity and hot spot detection was used to define the ligand binding site using 'Receptor Grid Generation' option of glide module; this was followed by Virtual Screening of approved drug bank compounds. For VS, Glide module 'Virtual Screening Workflow' was employed; this module provides three stages of screening: first HTVS (high-throughput virtual screening) mode, then SP (standard precision) mode and the final XP (extra precision) mode. The VS workflow, enriches

Table 4. Results from cavity detection using Discovery Studio and FTMap server for Aurora B-INCENP complex

Residues Identified in the Cavity of Aurora B-INCENP Complex	
Discovery Studio Results	**FTMap Server Results**
Aurora B: Leu93, Phe101, Ile102, Val103, Ala104, Leu103, His133, His134, Pro135, Asn136, Ile137, Leu138, Agr139, Leu140, Tyr141, Ile153, Leu154, Glu155, Tyr156, Ala157, Pro158, Agr159, Leu207, Leu208, Leu210, Lys211, Glu213, Leu214, Lys215, Leu335, Pro336, Pro337 and Ser338.	**Aurora B:** Phe101, Ile102, His134, Pro135, Asn136, Ile137, Leu138, Agr139, Tyr141, Glu155, Tyr156, Ala157, Pro158, Leu207, Leu208, Gly209, Leu210, Lys211, Glu213, Leu214, Lys215, Pro336, Pro337 and Ser338.
INCENP: Ala854, Ile855, Ile856, His857, Gln858, Tyr859, Tyr860, His861, Pro862, Pro863, Leu865, Leu868, Phe869	**INCENP:** Ile855, Gln858, Tyr859, His861, Pro862, Pro863 and Leu865

Figure 5. Location of the ligand binding cavity in the Aurora B-INCENP interface region. (a) Identified by Discovery Studio and (b) identified by FTMap server.



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Table 5. List of identified hot spot residues of Aurora B- INCENP complex using Robetta server and Discovery Studio

Sl.No	Residue	Robetta Server	Discovery Studio	Sl.No	Residue	Robetta Server	Discovery Studio
Aurora B Interface Residues				**INCENP Interface Residues**			
1	Phe72	H (1.20)	H (1.26)	17	Trp845	H (4.62)	H (4.54)
2	Glu78	H (1.00)	NH (0.28)	18	Leu851	H (1.05)	H (1.26)
3	Leu93	H (1.33)	H (1.10)	19	Ile855	H (1.83)	H (1.27)
4	Arg95	H (1.33)	H (1.73)	20	Gln858	H (1.83)	H (1.36)
5	Phe101	H (2.31)	H (1.47)	21	Tyr859	H (3.58)	H (2.89)
6	Ile102	H (1.65)	H (1.25)	22	Leu865	H (1.05)	NH (0.34)
7	Lys110	H (1.46)	H (1.97)	23	Leu868	H (1.05)	H (0.96)
8	Leu122	NH (0.0)	H (0.57)	24	Phe869	H (3.65)	H (2.73)
9	Ile126	NH (0.0)	H (0.69)	25	Ile872	H (2.26)	H (2.21)
10	Tyr141	H (1.40)	H (1.72)	26	Leu875	H (2.32)	H (1.96)
11	Asn142	NH (0.0)	H (1.21)	27	Leu877	H (1.89)	H (1.91)
12	Tyr143	H (2.88)	H (2.48)	28	Ile880	H (1.35)	H (1.30)
13	Tyr145	H (1.87)	H (1.40)	28	Phe881	H (2.99)	H (2.76)
14	Ile150	NH (0.0)	H (1.03)	30	Lys882	NH (0.0)	H (0.94)
15	Tyr156	NH (0.0)	H (0.73)				
16	Leu210	H (1.89)	H (0.95)				

the data in every step by eliminating false positives by applying more extensive sampling and scoring methods. After screening, top scored 10 compounds were selected as initial hits for Aurora B-INCENP PPI inhibition. The selected 10 compounds, Glide XP docking scores and the hot spot residues involved in non-bonded interaction are listed in Table 6. Analysis of these hits in the PPI binding site shows that few hot spot residues of Aurora B (Phe101, Tyr141 and Leu210) and INCENP (Ile855, Gln858, Tys859 and Leu865) are consistently making hydrogen bonds as well as hydrophobic interactions with the identified hits. Thus, the selected hits might disrupt the PPI of Aurora B-INCENP complex by interacting with the important hot spot residues. For illustration, top scored two compounds 3D and 2D binding mode in the Aurora B-INCENP complex is shown in Figure 6. As a next step, the identified hits need to be tested in *in vitro* assay, to confirm their ability to interfere in Aurora B-INCENP PPI.

CONCLUSION AND FUTURE DIRECTIONS

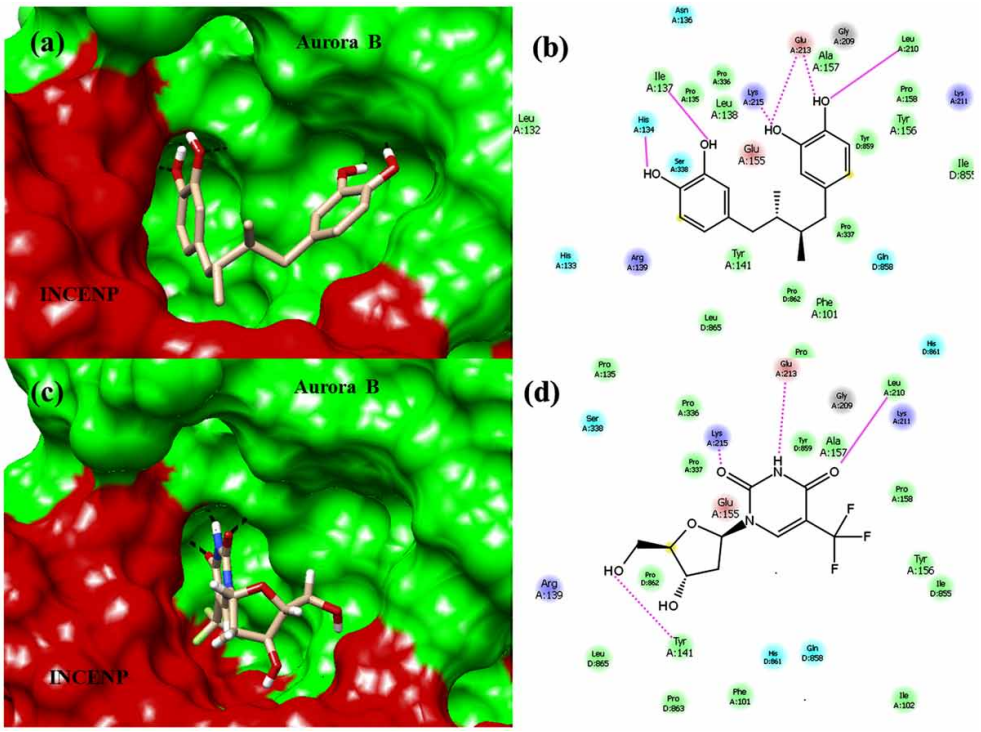
Most of the kinase inhibitors in the market or those under development act by interacting with the ATP binding site of the kinase. Due to the conserved nature of the ATP binding site of the kinase, existing kinase inhibitors are burdened with drawbacks including off- and on-target toxicity and development of drug resistance. These drawbacks could be overcome by targeting the protein-protein interactions (PPIs) of kinase, instead of targeting the ATP binding site. Though targeting the PPIs is a difficult task

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Table 6. Top 10 hits identified by virtual screening of DrugBank database as Aurora B-INCENP PPI inhibitors

SI.No	Drug Name	Glide XP Docking Score	Hot Spot Residues Involved in Non-Bonded Interaction with the Drugs	
			Aurora B	INCENP
1	Masoprocol	-10.065	Phe101, Tyr141 and Leu210	Ile855, Gln858, Tys859 and Leu865
2	Trifluridine	-9.342	Phe101, Tys141 and Leu210	Ile855, Gln858, Tys859 and Leu865
3	Fenoldopam	-8.948	Phe101 and Tys141	Tys859 and Leu865
4	Telbivudine	-8.942	Phe101, Tys141 and Leu210	Ile855, Gln858, Tys859 and Leu865
5	Dexrazoxane	-8.729	Phe101, Ile102, Tys141 and Leu210	Ile855, Gln858, Tys859 and Leu865
6	Dobutamine	-8.300	Phe101, Tys141 and Leu210	Ile855, Gln858, Tys859 and Leu865
7	Pioglitazone	-8.206	No	No
8	Miglustat	-8.116	Phe101, Tys141 and Leu210	Ile855, Gln858, Tys859 and Leu865
9	Idoxuridine	-8.079	Phe101, Tys141 and Leu210	Ile855, Gln858, Tys859 and Leu865
10	Mitoxantrone	-8.070	Leu210	Leu865

Figure 6. Binding mode of the top two hits in Aurora B (green)-INCENP (red) complex interface region. (a) 3D binding mode of Masoprocol (b) 2D interaction map of Masoprocol (c) 3D binding mode of Trifluridine and (d) 2D interaction map of Trifluridine.



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and success in the form of a marketed drug is yet to come, it is worthwhile exploring these targets, as they are very selective in nature and provides distinct advantages. Moreover, if the PPIs are considered as potential drug targets, then the number of targets would exceed the druggable human kinome; because large numbers of PPIs exist in a living cell as compared to human kinome, and many of them are involved in disease conditions.

Towards this end, here the authors have provided an *in silico* framework for the identification of kinase PPI inhibitors. By utilizing the structural knowledge (cavities and hot spots) of kinase PPI interfaces, structure-based drug design could be carried out. As a case study, Aurora B-INCENP PPI inhibitor identification using the *in silico* framework is discussed. In the future, availability of 3D protein-protein interaction complex, advancement in protein-protein docking algorithms and reliable *in silico* tools for the detection of hot spots, could greatly enhance the speed at which PPI inhibitors could be reliably identified.

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KEY TERMS AND DEFINITIONS

Aurora Kinases: Human genome encodes three members of Auora kinases: Aurora A, Aurora B, and Auora C. These members belong to serine/threonine kinase families that are essential for cell division and duplication.

Docking: Docking is a computation tool which predicts predominant binding mode of biomolecule interactions including protein-ligand, protein-protein, protein-DNA, protein-RNA and protein-carbohydrate.

Drug Target: Drug target is a biomolecule within the living organism to which an endogenous ligand or drug molecule binds to produce the therapeutic effect.

Hot Spots: In the protein-protein interaction interface region, a residue or cluster of residues that contributes to the majority of binding free energy for the complex formation is called hot spots.

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INCENP: The human gene *INCENP* encodes a protein known as - Inner Centromere Protein, which is essential for the full activation of Aurora B kinase.

Kinase: Kinase is an enzyme which catalyzes the transfer of an ATP terminal phosphate group to a specific substrate protein; the process is called phosphorylation.

Pharmacophore: Pharmacophore is a 3D hypothetical model of molecular features which are essential for the molecular recognition of a ligand by a drug target.

Protein-Protein Interactions: Protein-protein interactions (PPIs) refer to a physical contact that is formed between two or more proteins. As a result of PPIs, a wide array of biological functions including cell division, cell growth, signal transductions, metabolic reactions, and transportation of molecules from one location to another will occur within the living organism.

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Chapter 44
Rational Drug Design
Rational Drug Design: One Target, Many Paths to It

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ABSTRACT

For the past three decades rationale drug design (RDD) has been developing as an innovative, rapid and successful way to discover new drug candidates. Many strategies have been followed and several targets with diverse structures and different biological roles have been investigated. Despite the variety of computational tools available, one can broadly divide them into two major classes that can be adopted either separately or in combination. The first class involves structure-based drug design, when the target's 3-dimensional structure is available or it can be computationally generated using homology modeling. On the other hand, when only a set of active molecules is available, and the structure of the target is unknown, ligand-based drug design tools are usually used. This review describes some recent advances in rational drug design, summarizes a number of their practical applications, and discusses both the advantages and shortcomings of the various techniques used.

INTRODUCTION

Once, a US General summarized his philosophy on warfare in just four concise statements, "The art of war is simple enough. Find out where your enemy is. Get at him as soon as you can. Strike him as hard as you can, and keep moving." Although these overarching statements formed the basic premise of modern war strategies, the same concepts have been applied in designing new drugs aimed at combating a broad range of diseases. In this context, rational drug design (Hao, Yang, & Zhan, 2012; Hedvat et al., 2012; Mandal, Moudgil, & Mandal, 2009) has been established as an exciting research approach

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aimed at developing safer and more efficacious drugs using modern computational tools which are fast and inexpensive compared to traditional methods. The ultimate goal of this research is to design small organic non-peptidic compounds that bind to a specific molecular target, and result in the inhibition (or less frequently, activation) of a particular protein or enzyme involved in a given cellular pathway, i.e. a blockage of a specific protein-protein interaction. The development of such drugs has been recognized early on by the pharmaceutical industry as a principal foundation that provides it with the necessary return on investment to fuel further research and development (Szymkowski, 2005) leading to a discovery and development cycle.

Our understanding of cell mechanisms and pathways at a molecular level becomes deeper and clearer every day. This is largely due to the great efforts and hard work of genomic and proteomic research groups who add novel targets for drug intervention on a regular basis (Drews, 2000; Fishman & Porter, 2005; Hopkins & Groom, 2002). Thus far, several hundred proteins have been synthetically expressed and many of them are currently evaluated for their druggability (Hopkins & Groom, 2002). These targets involve several families comprised of G-protein coupled receptors (GPCRs), ligand-gated ion channels (LGICs), cytoskeleton proteins, phosphatases, kinases, nuclear receptors (NRs) and DNA repair proteins. The growing list of potential drug targets encourages a bold question if it is in principle possible to restore any diseased cell to a healthy state by uncovering a drug for every potential druggable target? Certainly, if this dream is ever realized, many diseases will be cured and relegated to the dustbin of history in a manner similar to the effect of the discovery of vaccines in the 19th and early 20th century. We think this is most definitely achievable as a result of rapid progress made in the computational drug discovery area.

A typical rational drug design effort involves many tools that can be used either separately or in combination depending on the available structural and kinetic data. Once the structure of a target (typically a protein) is available, docking algorithms can be used to place each ligand (i.e. a molecule or a molecular fragment included in a typical library of compounds) and predict its most probable binding mode within the binding site of the target (Abagyan & Totrov, 2001; Schneider & Bohm, 2002). Moreover, most docking programs can rank the predicted activity of each compound by analyzing the different ligand-target interactions in terms of the estimated binding affinity of the complex. In addition to docking techniques, one can define the essential interactions between the ligand and the binding site of the receptor and translate this information into the formulation of binding-site pharmacophore models (Good, Krystek, & Mason, 2000). These models can be used to search the available chemical space for compounds that can complement the physicochemical features of the receptor. As these two procedures require a comprehensive understanding of the structural arrangement of the target, they have been commonly termed as structure-based drug design (SBDD). On the other hand, and for most of the cases, the three-dimensional structure of the target, the binding site or even the target itself are not accurately known, although there may be a number of known active compounds that have been identified experimentally. In this case, data mining algorithms can be used to screen for compounds that are structurally similar to the known actives (similarity search) (Willett, 2006) or that comprise the chemical features of these compounds (pharmacophore search) (Krovat, Fruhwirth, & Langer, 2005), in what is called ligand-based drug design (LBDD). Thus, these two fundamental procedures, SBDD and LBDD, form the general layout of present-day rational drug design protocols. This review summarizes the recent advances in rational drug design, focusing on the methods and their successful applications in drug discovery. Moreover, we will summarize the shortcomings of the various techniques used, pointing out to future directions of the field.

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STRUCTURE-BASED DRUG DESIGN (SBDD)

SBDD requires the knowledge of the three-dimensional structure of the target protein (Hedvat et al., 2012; Heikamp & Bajorath, 2013; Lyne, 2002; Schneider & Bohm, 2002; Shoichet, Stroud, Santi, Kuntz, & Perry, 1993). Therefore, an essential step prior to the actual screening procedure is to generate or predict the structure of the target protein. The target structure can be obtained by experimental techniques such as NMR, X-ray crystallography, electron crystallography or it can be predicted computationally using homology modeling (Levit, Barak, Behrens, Meyerhof, & Niv, 2012). In addition to the 3D-structure, it is also important to identify the relevant binding site(s) within the protein, which is (are) deemed responsible for its biological activity. In general, the binding site is a pocket, a groove or a protrusion having an assortment of apparent hydrogen bond donors and acceptors, hydrophobic features and it can be associated with molecular adherence surfaces (Berezovsky, 2013; Nussinov & Tsai, 2013). There may be a number of metal ions or water molecules as part of the active site that are essential for the activity of the protein and they must be considered during the screening procedure in order to produce a correct result. There are two basic approaches for SBDD namely, docking (Abagyan & Totrov, 2001) and receptor-based pharmacophore modeling (Good et al., 2000).

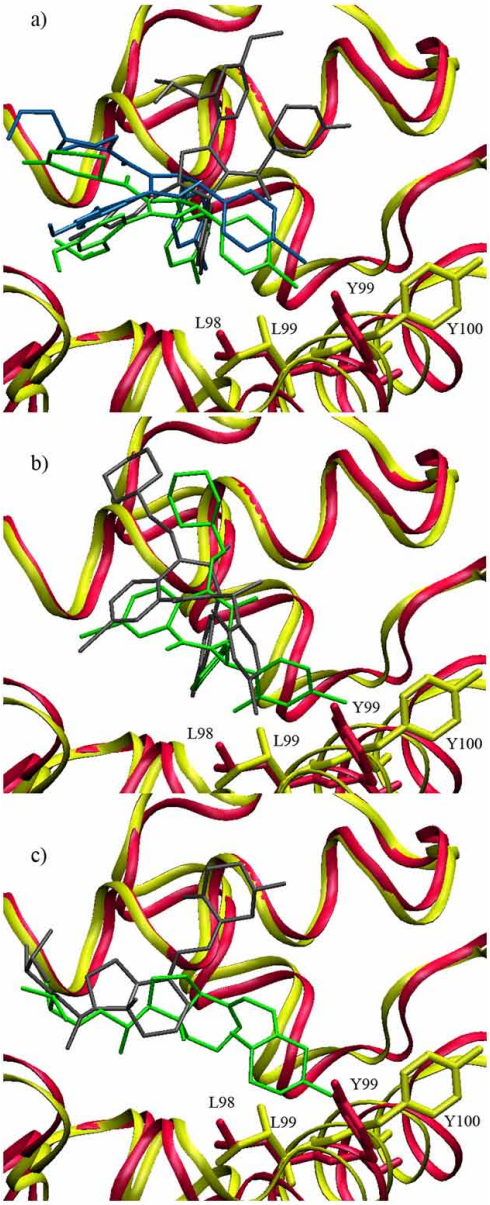
Docking

Molecular docking is a standard constituent of many SBDD studies described in the literature (Abagyan & Totrov, 2001; Halperin, Ma, Wolfson, & Nussinov, 2002; Mihasan, 2012). The idea of docking and scoring as a virtual screening tool has been proposed since the birth of docking methods (DesJarlais et al., 1990; Kuntz, 1992; Kuntz, Blaney, Oatley, Langridge, & Ferrin, 1982). The main problem which all docking algorithms try to solve can be stated as follows: given two interacting molecular structures, what is the most probable binding configuration to form a stable three-dimensional protein-ligand complex? In order to address this problem, docking procedures can be divided into two major steps. First, explore the conformational space of the ligand within the binding site of the target. At this stage, numerous conformations are generated for the ligand. Second, select the optimal target-ligand alignment by scoring their interactions and ranking the docking results (poses) according to their predicted binding affinity. Today, there are at least 30 docking programs commercially (or publicly) available with different conformational sampling algorithms and a variety of scoring functions (Bissantz, Folkers, & Rognan, 2000). The most commonly used programs are AUTODOCK (Goodsell & Olson, 1990), GOLD (Jones, Willett, & Glen, 1995b), GLIDE (Friesner et al., 2004), DOCK (Kuntz et al., 1982), ICM (Totrov & Abagyan, 1997), IFREDA (McGann, Almond, Nicholls, Grant, & Brown, 2003) and FlexX (Rarey, Kramer, Lengauer, & Klebe, 1996). These programs differ mostly in the way they deal with protein-ligand flexibility or their scoring and ranking methods.

Regardless of the poor representation of the receptor flexibility during docking, most docking methods can handle the flexibility of ligands efficiently (Bissantz et al., 2000). In other words, for most of the cases, docking algorithms can reproduce the protein-ligand binding modes that have been observed experimentally using X-ray crystallography. As an example, Figure 1a shows the successful docking of nutlin, a well-known p53-MDM2 inhibitor, to the p53-binding site within MDM2 using AUTODOCK 4.0. In general, the degree of success for docking methods can be measured by comparing the predicted binding mode to the experimental conformation (the native binding mode) (Goodsell & Olson, 1990; Jones et al., 1995b; McGann et al., 2003). This assessment can be evaluated quantitatively by calculat-

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Figure 1. Structural variations between MDM2 (yellow) and MDMX (red) and their effect on the binding modes of nutlin-3 (a) and two selected hits form the predicted MDM2/MDMX inhibitors (b and c). Tyr100 and Leu99 of MDM2 and the same residues in MDMX are shown in Licorice representations with the same color as that of the two proteins. For each compound, the binding mode within MDM2 is shown in green and within MDMX is shown in gray. Tyr99 and Leu98 prevent nutlin-3 from binding to MDMX with the same binding conformation adopted by nutlin-2 within the MDM2-pocket (blue). The conformation of nutlin-2 was extracted from the MDM2-nutlin crystal structure 1RV1. On the other hand, compounds Pub#11952782 (b) and ZINC04629876 (c) from the suggested MDM2/MDMX inhibitor list can tolerate the structural variations in the two binding sites in order to maximize their interactions with the proteins.



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ing the root-mean-square deviation (RMSD) between the two structures. However, in certain systems, where unexpected flexibility of the receptor is crucial for the binding reaction or the interaction of the ligand and protein is mediated by water molecules or metal ions, docking may fail to predict the correct binding conformation of the complex, leading to improper and unrealistic interactions.

Ligand Flexibility

The binding reaction between a ligand and a particular target involves numerous conformational changes in the two molecules as well as water molecules and ions located in their interface. Each entity in this reaction adapts its shape and distribution in order to maximize its interactions with the other entities, forcing the whole system to reach the global minimum. This binding interaction is somehow similar to a folding problem of a protein, comprising a huge number of degrees of freedom. Consequently, the majority of docking programs avoid this combinatorial explosion problem by implementing almost full-flexibility for the ligands while keeping the target completely rigid with no flexibility allowed (Abagyan & Totrov, 2001; Bissantz et al., 2000). According to the nature of the search method, the conformational sampling techniques that have been adopted by all docking programs to introduce ligand flexibility can be classified into three main categories: (1) systematic search routines, (2) stochastic exploration, and (3) simulation techniques.

Systematic Search

In a typical systematic search, all rotatable bonds in the ligand are gradually rotated in order to cover all possible combinations among the dihedral angles. Evidently, the number of generated structures using this method increases dramatically with the number of rotatable bonds involved, leading to the problem of combinatorial explosion. In this way, applying a standard systematic search to explore the entire conformational space of a ligand requires massive calculations and considerable computational time. Although, a number of docking programs such as FLOG (Miller, Kearsley, Underwood, & Sheridan, 1994) get around this obstacle by limiting the created structures to a pre-generated set of conformations recorded in structural databases, other docking algorithms adopt an incremental procedure to reconstruct the ligand within the binding site of the target. The main objective of these methods is to limit the number of degrees of freedom for the ligand, allowing for a less-expensive and rapid conformational search.

Essentially, there are two main approaches for the incremental reconstruction methods. First is the one that has been employed by LUDI (Bohm, 1992), FlexX (Rarey et al., 1996), DOCK (Kuntz et al., 1982), ADAM (Mizutani, Tomioka, & Itai, 1994) and Hammerhead (Welch, Ruppert, & Jain, 1996), where the ligand is split into a rigid core fragment that is docked first and a number of flexible regions that are subsequently and successfully added. This method is commonly referred to as the "incremental approach". The other method, known as "place and join", is to break the ligand into several fragments, dock them within the binding site of the target and finally connect them together in order to rebuild the final ligand conformation.

Stochastic Exploration

Stochastic exploration samples the conformational space of a ligand by generating random variations in the orientation of all rotatable bonds and in some cases random translations for the whole ligand within

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the binding site. This is done mainly to enable crossing the energy barriers and searching for local minima enclosed by the rugged energy surface of the ligand. This procedure can be applied to a single ligand or a population of conformations derived from the same molecular structure of the ligand. Each resultant conformation is then evaluated according to a probability distribution or by estimating its binding affinity with respect to the target. In fact, there are three methods that are derived from this technique, namely: Monte Carlo simulations, Genetic Algorithms and Tabu Search methods (Schneider & Bohm, 2002).

The Monte Carlo (MC) method of simulations is one of the most powerful techniques ever developed to allow crossing potential energy barriers and sampling the conformational space of a complex system. Since this method has been successfully implemented to solve several molecular modeling problems, it was regarded as one of the natural options to explore ligand flexibility in docking. The method starts with a randomly generated conformation for the ligand by randomly changing one or more dihedral angles or even the whole orientation or position of the ligand with respect to the target. This new conformation (pose) is accepted or rejected according to a Metropolis algorithm that follows the Boltzmann probability distribution. Programs like ICM (Totrov & Abagyan, 1997), MCDOCK (Liu & Wang, 1999) and DockVision (Hart & Read, 1992) employ this approach in exploring the flexibility of ligands during docking.

Genetic algorithms (GA) exploit the biological concepts of natural selection introduced by Darwin in order to explore all possible conformations of the ligand and predict its native structure. In contrast to MC-based algorithms, instead of manipulating a single ligand, GA generates a random population of the same molecular structure of the ligand (Goodsell & Olson, 1990; Jones et al., 1995b). Each member of this population is unique in terms of the internal orientation and the global placement and alignment within the binding site of the target. This random population forms the initial generation (seed) of a set of non-interacting ligand species. These poses are further subjected to a number of biological operators that add up more diversity to the generated structures. Among these operators the most important ones are the mutation operator (generates new ligands from earlier ones by altering a rotatable bond or moving the whole ligand to a new position), and the crossover operator (merges two ligands in order to create a new structure comprising their common features). The fitness of each newly generated structure is evaluated by calculating its binding affinity to the target. The pose, which retains the most predominant interactions with the binding site survives and becomes the parent of the new generation. This iterative procedure terminates after reaching a predefined number of generations or energy evaluations, or if no more improvement to the binding affinity has been observed (converged solution). Examples of programs that incorporate genetic algorithms in conformational sampling include AUTODOCK, GOLD and DARWIN (Taylor & Burnett, 2000).

As a memory-based stochastic exploration method, Tabu Search (TS) prevents the searching machinery from revisiting the same conformation more than once. PRO_LEADS is one of the most popular programs that employ this searching technique (Baxter, Murray, Clark, Westhead, & Eldridge, 1998). This is generally achieved by creating a list that records all previously visited solutions, which acts as a memory for the algorithm. A decision to accept or reject a new conformation is made after comparing its RMSD to the other recorded conformations in order to ensure that no conformation is visited twice.

Simulation Techniques

Simulation techniques employ a deterministic approach that either: (1) passes through both time and space giving rise to an evolving trajectory describing the biological behavior of a typical system, or (2)

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reconfigures the system by rearranging its particle composition towards a more stable state. Molecular dynamics (MD) simulations and energy minimization methods are the most widely used techniques in a number of docking programs. Although the two approaches can handle the full-flexibility of both the ligand and target, their foremost disadvantage is that they can be readily trapped within a local energy minimum, which in turn precludes them from efficiently sampling the conformational space of the complex. Therefore, simulation techniques are usually used as complementary methods and a refining step subsequent to GA or MC simulations (Bissantz et al., 2000).

Protein Flexibility

Docking a ligand against a crystal or relaxed receptor structure is a commonly used approach in structure-based drug design (Abagyan & Totrov, 2001). However, in many cases, the degree of success that may be achieved in a typical docking experiment depends on the characteristics of the target and how important the protein flexibility is in the simulation. Most of the successful cases reported in the literature were either related to nearly rigid proteins or proteins having a real binding mode of their respective ligands, or complexes whose structure was determined experimentally (Reddy, Pati, Kumar, Pradeep, & Sastry, 2007). In spite of these studies, there are cases where the binding interaction has been shown to induce significant conformational changes in the target protein, ranging from local reorganization of side-chains to hinge movement of domains. Sampling these conformational changes during docking is impractical, as they involve too large a number of degrees of freedom. To address such problems, a number of docking packages like AUTODOCK, GOLD, FlexE and IFREDA, manage to include a modest amount of flexibility in the target during the docking simulations. These approaches include soft docking (Osterberg, Morris, Sanner, Olson, & Goodsell, 2002), sidechain flexibility (Schneider & Bohm, 2002), combined protein grid and united descriptors of the target (Knegtel, Kuntz, & Oshiro, 1997).

Soft docking algorithms allow the ligand to penetrate through the surface of the protein in order to approximate and predict the dynamical changes that may take place within the active site as a result of ligand binding. This is generally achieved by attenuating Lennard-Jones repulsive parameters in the potential energy function that describes the system (Osterberg et al., 2002). Another commonly used technique to introduce active site dynamics in the context of docking is to allow key side chains that have been shown to mediate the interactions with the ligand to rotate freely and search for their preferred conformation. These side chain rotations are usually restricted to a number of pre-defined experimental conformations stored in rotamer libraries or predicted from a prior MD simulation. While this method reduces the risks associated with the lack of flexibility to some extent, it neglects backbone dynamics, which may affect the ultimate docking results (Reddy et al., 2007).

In order to account for a larger degree of receptor flexibility at a reasonable computational cost, a number of dominant protein conformations can be combined simultaneously to generate a comprehensive model that describes the essential dynamics of the binding site (Knegtel et al., 1997). This approach is generally termed as “combined protein grid” and is usually implemented in two steps. First, for each conformation, all possible protein-ligand atomic interactions are calculated and recorded in what is called a docking grid. Second, a combined grid is created by applying a weighted average for all the resulting grids that represent various conformations. Alternatively, the averaging procedure may be applied to the atomic coordinates to generate an average structure for the protein.

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Scoring Methods

As scoring of the poses is crucial in prioritizing and ranking of the compounds, it is important to use sensitive and accurate scoring functions that can not only reproduce but also predict experimental results. This is normally achieved using an objective scoring function that directs the conformational search algorithm in predicting the native conformation and ultimately estimates the binding affinity of the protein-ligand complex. Nevertheless, it has been broadly demonstrated that docking scoring functions are less successful at predicting the actual binding affinities and at discriminating true binders from inactive (decoy) compounds (Abagyan & Totrov, 2001; Schneider & Bohm, 2002; Shoichet et al., 1993). These puzzling results are direct outcomes of many factors that have been mistreated while analyzing the binding interactions of the resulting poses as a compromise to achieve a speed-up of the docking process. These factors mostly comprise the lack of proper solvation, the neglect of protein flexibility and the bias toward the training set of structures that have been used in optimizing the scoring process (Reddy et al., 2007). In fact, developing new scoring functions and innovative ranking schemes is a widely open area of research in the field of docking. Although a more precise scoring method can be practically implemented within docking, a large computational cost that is associated with such a function will be the actual barrier to using it. In this way, many assumptions have been proposed in the currently used docking scoring functions in order to reduce the complexity and computational time required to evaluate a particular pose. Overall, a typical scoring function includes among its ingredients at least the following ones: a descriptor of the hydrophobic effects, van de Waals dispersion interactions, hydrogen bonding, electrostatic interactions, and solvation effects. Based on their scoring functions, all docking programs that are in use today (Bissantz et al., 2000; Kitchen, Decornez, Furr, & Bajorath, 2004; Reddy et al., 2007) can be divided into four major categories: force field-based, empirical, knowledge-based and consensus methods.

According to the energy landscape theory, the native conformation for a ligand within the binding site of its target is correlated with a pronounced deep minimum on the energy surface. Therefore, potential energy (force-field) functions have been used to describe protein-ligand interactions and assess their binding affinity by exploring the energy surface and locating these minima. As docking algorithms usually deal with a single target conformation, the internal protein interactions are typically neglected. Accordingly, force field methods approximate the ligand-protein binding interactions by adding the interaction energy between the protein and the ligand to the ligand internal energy. These internal interactions are approximated by harmonic springs that describe the vibrations and rotations of the different bonds forming the ligands. The non-bonded interactions between the ligand and its target are estimated by van de Waals, hydrogen bonding, and electrostatic terms. Consequently, the major drawback of standard force field scoring functions is the lack of solvation and entropy contributions to the binding energy. Examples of force-field-based scoring functions include D-Score (B. Kramer, Rarey, & Lengauer, 1999) and GoldScore (Verdonk, Cole, Hartshorn, Murray, & Taylor, 2003).

Another widely used scoring approach is to hypothesize an empirical scoring function that has been optimized to reproduce a collection of experimental data (Bohm, 1992). These data may include binding affinities or native conformations for known active compounds. Notable examples include F-Score (Rarey et al., 1996), ChemScore (Eldridge, Murray, Auton, Paolini, & Mee, 1997), SCORE (Tao & Lai, 2001) and Fresno (Rognan, Lauemoller, Holm, Buus, & Tschinke, 1999). The basic concept behind this type of scoring functions is that the binding energies can be approximated by a summation of unrelated contributions. Each element of this summation describes a certain binding interaction such as hydrophobic,

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hydrogen bonding, electrostatic or solvation effects. Some functions may comprise an approximation for the loss of entropy due to binding, which is proportional to the number of rotatable bonds included in the ligand. Overall, the terms that build up a typical empirical scoring scheme are simple enough to be rapidly evaluated in order to speed up the docking process. A fairly accurate estimate for the coefficients pre-multiplying these terms can be obtained by performing regression analysis and fitting the whole function against the set of experimental data. Although several empirical functions have been successfully used in many cases, they are generally biased toward the experimental data that have been used in their optimization (Bissantz et al., 2000; Reddy et al., 2007).

Similar to empirical scoring functions, knowledge-based scoring methods attempt to reproduce experimentally determined structures using simpler atomic interaction-pair potentials. These potentials are based on the frequency of occurrence of all possible interactions between the ligand and its target. Using statistical analysis, knowledge-based models implicitly describe binding effects that are hard to represent explicitly during docking. Therefore, the accuracy of these methods depends on the extent of the manipulated protein-ligand data set and the diversity of atomic interactions included in these complexes. An example of such functions is DrugScore (Gohlke, Hendlich, & Klebe, 2000).

A more recent scoring technique, consensus scoring collects assessments from several scoring functions in order to evaluate a particular docking result (Charifson, Corkery, Murcko, & Walters, 1999). Hence, the method is expected to reduce the errors that result from the individual scoring functions and to improve the probability of selecting true binders. Nevertheless, it has been recommended to use different and uncorrelated scoring functions in constructing a successful consensus scheme. This is because correlated functions tend to produce similar results leading to error amplification and misleading results. Despite these constraints, several studies have pointed to the success of a number of consensus scoring functions when compared to using a single scoring method (Terp, Johansen, Christensen, & Jorgensen, 2001). These methods include X-CSORE (Wang, Lai, & Wang, 2002) and FlexX (Rarey et al., 1996) scoring functions.

Structure-Based Pharmacophore Modeling (SBPM)

In spite of the great efforts of many research groups in both the pharmaceutical industry and academia, there still exist several barriers that prevent the use of docking methods as a leading approach to drug discovery (Abagyan & Totrov, 2001; Ou-Yang et al., 2012; Zoete, Grosdidier, & Michielin, 2009). These barriers include the following: (1) the inclusion of sufficient target flexibility and the simulation of the induced fit mechanism; (2) the druggability of the target; (3) the modeling of water-mediated interactions at the interface of the target-ligand complex; (4) the effect of metal ions located within the binding site of interest, and; (5) the fine-tuning of the protonation states of both the receptor and the ligand. Consequently, these difficulties motivated researchers to develop novel rational drug design approaches that are far removed from standard docking methods. In this context, structure-based pharmacophore modeling (SBPM) originated as an alternative SBDD technique that can describe the essential interactions behind the binding of a ligand to its target (Z. Chen et al., 2009; Kim, Lee, Kang, Kim, & Lee, 2006; Leach, Gillet, Lewis, & Taylor, 2009; Sun, 2008).

It is important to keep in mind that the concepts of pharmacophore modeling have a long history and date back to their introduction by Ehrlich as early as in 1909 as "a molecular framework that carries (*phoros*) the essential features responsible for a drug's (*pharmacon's*) biological activity". These features are generally classified into two major categories, namely, chemical-based and shape-based features.

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The former include hydrogen bond acceptors or donors, charge centers, metal binding regions, aromatic rings and hydrophobic regions, while the latter mainly include volume-excluded regions and geometrical constraints like distances, angles and dihedral angles. By allocating the different features and including their three-dimensional distributions within the binding site, one can understand the essential properties required for bioactivity of known true binders (Sun, 2008).

Currently, there are two approaches to generate a pharmacophore model depending on the accessibility of the target structure. If the structure of the target is available, one can build a pharmacophore hypothesis that would complement the chemical features within the binding site of the target (Krovat et al., 2005). These pharmacophore models can be further improved if there is any active compound that has been co-crystallized with the target. This approach is called "binding site-based pharmacophore models" or "structure-based pharmacophore models". The second approach, that is more widely used, relies only on the known active compounds and no information about the target is required. This technique is commonly referred to as "ligand-based pharmacophore modeling" and will be explained in detail below. In this section, we focus on the "structure-based pharmacophore models".

The most straight-forward approach in designing a SBPM is to analyze the experimentally determined crystal structures of protein-ligand complexes. The program LigandScout (Wolber & Langer, 2005) introduced by Wolber and co-workers is very effective at manipulating these structures and automatically interpreting the various interactions between a particular macromolecule and its co-crystallized ligands into functional pharmacophore models. The program starts by cleaning up the structures of the ligands by assigning hybridization states and bond characteristics that are missing in the crystal structure. This is accomplished by using an extended heuristic approach combined with template-based numeric analysis. Following this step, pharmacophore models are created by analyzing the atomic interactions between the ligand and all residues located within the binding site of the target. These interactions are classified into complementing groups in terms of hydrogen bonding, electrostatic charges and hydrophobic contacts. Moreover, the flexibility of both the target and ligand can be incorporated by aligning several models in order to generate a common-feature pharmacophore model.

When only the structure of the active site is available, programs such as structure-based focusing (SPF) aid in building up complementing pharmacophore hypotheses (Hoffren, Murray, & Hoffmann, 2001). The process starts by mapping favorable regions or "hot spots" for protein-ligand interactions within the binding site of the target. These regions are then clustered into hydrogen bond donating and hydrogen bond accepting vectors and hydrophobic interaction sites. The clustered groups are then used to build the pharmacophore model. Other algorithms that can construct SBPMs in addition to LBPMs are Unity (Tripos. http://www.tripos.com/) and MOE (Molecular Operating Environment; http://www.chemcomp.com/). Once a pharmacophore hypothesis is created, the model can be converted to a query that is used to screen chemical databases for molecules that satisfy these proposed hypotheses.

CASE STUDIES FOR SBDD

Condensing all the great discoveries and outstanding developments that took place in the last twenty years of SBDD studies is an unattainable task for the purpose of this review. However, here we will try to summarize the most important studies that helped to advance the field and guide the drug discovery community towards the highest impact techniques. These studies were not only centered on uncovering

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new drug entities but also directed towards validating the methods developed, reducing their shortcomings and adding new approaches to the computational arsenal.

The initial evaluations of docking programs were focused on their ability to reproduce the native structures of co-crystallized ligands (Jones, Willett, Glen, Leach, & Taylor, 1997; Verdonk et al., 2003; Warren et al., 2006). It is only during the last 10 years that this focus has shifted towards determining the ability of docking techniques to filter chemical databases as a VS tool (Chen, Lyne, Giordanetto, Lovell, & Li, 2006; Z. Zhou, Felts, Friesner, & Levy, 2007). Nevertheless, even with the many studies published in the literature that ranked and benchmarked docking programs, there are still many practical problems preventing the robust and fair assessment of these programs (Chen et al., 2006; Cole, Murray, Nissink, Taylor, & Taylor, 2005). Warren and Chen carried out two independent studies to assess different docking programs considering a number of crucial factors. These factors included the variety of the screened compounds, the diversity of the targets and the effect of intrinsic docking parameters on the outcome of the VS protocol. The docking algorithms that were tested in these studies comprised Glide, GOLD, ICM, FlexX, DOCK, Dockit, Flo, Fred, LigFit, MOE and MVB. As a principal conclusion resulting from these two studies, there is no universal docking algorithm or scoring function that can work well for every target (Chen et al., 2006; Warren et al., 2006). In particular, most used scoring functions were inaccurate in predicting the actual binding affinities for a number of ligands. Unsurprisingly, recent studies suggested that consensus scoring might form a successful approach in reducing the imprecision of single scoring methods (Feher, 2006). However, this statement still requires more validation (Oda, Tsuchida, Takakura, Yamaotsu, & Hirono, 2006). On the other hand, it is worth mentioning that many improvements have been implemented for a number of well-established scoring functions. Relevant examples include potential of mean force (PMF) (Muegge, 2006), DrugScore (Velec, Gohlke, & Klebe, 2005) and Glide XP (Friesner et al., 2006).

Above and beyond scoring functions, protein flexibility and its effect on hit ranking is the other most important factor in SBVS (C, Subramanian, & Sharma, 2009; Mishra et al., 2009; Nettles et al., 2007). The importance of protein flexibility has been demonstrated in many studies and shown to have a remarkable influence on the final results. Bouzida et al. (1999) investigated the docking of sb203386 and skf107457 inhibitors to HIV-1 protease using MC simulated. These authors compared docking against a single protein conformation to using an ensemble of structures. Their recommendation was to use different protein structures in order to improve the docking results and enhance the final protein-ligand conformation. In another study by Murray et al. (1999) only 49% of the ligands were cross-docked correctly to another target that was co-crystallized with a different ligand. On the other hand, this showed that inducing small movements of the side chains in the binding site resulted in large variations in the predicted binding affinities. These early studies have drawn the attention to the importance of target flexibility and provided a motivation to implement various techniques to include this factor in the context of docking.

One way to accommodate receptor flexibility with more accurate scoring techniques is to implement a hybrid between docking and MD simulations. Originally, the use of MD simulations in VS studies was intended to create a set of receptor conformations (Broughton, 2000; Carlson et al., 2000). However, it was always debatable whether to use structures derived from MD simulations or NMR data. For example, Philippopoulos et al. suggested NMR structures as the most effective source for protein conformations (Philippopoulos & Lim, 1999). A set of 15 NMR conformations for ribonuclease HI was compared to a trajectory obtained in a 1.7 ns MD simulation. The NMR data explored the conformational space of the protein more efficiently than the conventional MD simulation. In spite of their findings, it should

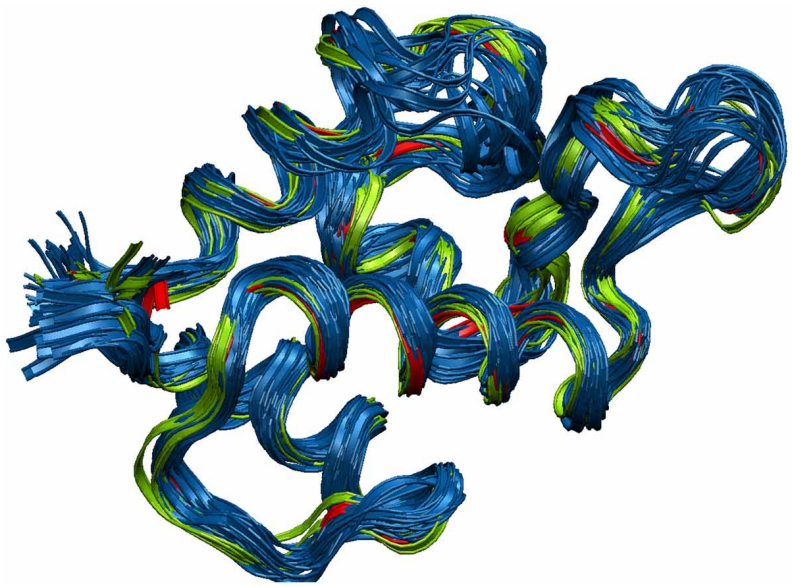
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be noted that Philippopoulos et al. used standard single trajectory MD simulations for a relatively short simulation time. In generating such ensembles, one should employ multiple trajectory MD simulations, commonly termed the replica exchange molecular dynamics (REMD), or run the simulation over longer times. For example, Figure 2 shows 28 dominant conformations representing the major backbone dynamics of the MDM2 target as extracted from ~100 ns of an MD simulation. Another benefit of incorporating MD simulations with docking is to use more accurate scoring functions like molecular mechanics/ Poisson-Boltzmann surface area (MMPBSA) (Thompson, Humblet, & Joseph-McCarthy, 2008) or linear interaction (Aqvist, Medina, & Samuelsson, 1994) methods to re-rank the docking results.

In this context, a successful approach reported by Perryman and McCammon et. al.(Lin, Perryman, Schames, & McCammon, 2002) is the relaxed complex scheme (RCS). MD simulations are applied to explore the conformational space of the protein receptor, while docking is subsequently used for the fast screening of drug libraries against an ensemble of receptor conformations. This methodology has been successfully applied to a number of cases (Lin et al., 2002). An excellent example of such a case is an HIV inhibitor, raltegravir (Markowitz et al., 2006), which became the first FDA approved drug targeting HIV integrase. MD simulations played a significant role in discovering a novel binding site, and compounds that can exchange between the two binding sites have formed a new generation of HIV integrase inhibitors.

In our recent work, we have applied an improved RCS to uncover dual-inhibitors for the MDM2/ MDMX- p53 protein-protein interactions (Barakat, Mane, Friesen, & Tuszynski, 2009). In this study we first filtered a set of ~6000 different ligands against 28 distinct MD-based conformations for the p53-binding site within MDM2 using AUTODOCK 4.0. Top 300 hits were redocked to the same binding site within MDMX. We finally rescored the top hits from both of the two screening experiments using MMPBSA. In this study, although the binding sites were fairly similar (see Figure 1), the MDMX pocket

Figure 2. Twenty-eight dominant conformations for MDM2. This ensemble comprised the crystal structure (red), five structures extracted from the holo (p53-bound)-trajectory (green) and twenty-two structures extracted from the apo (p53-free)-trajectory (blue).



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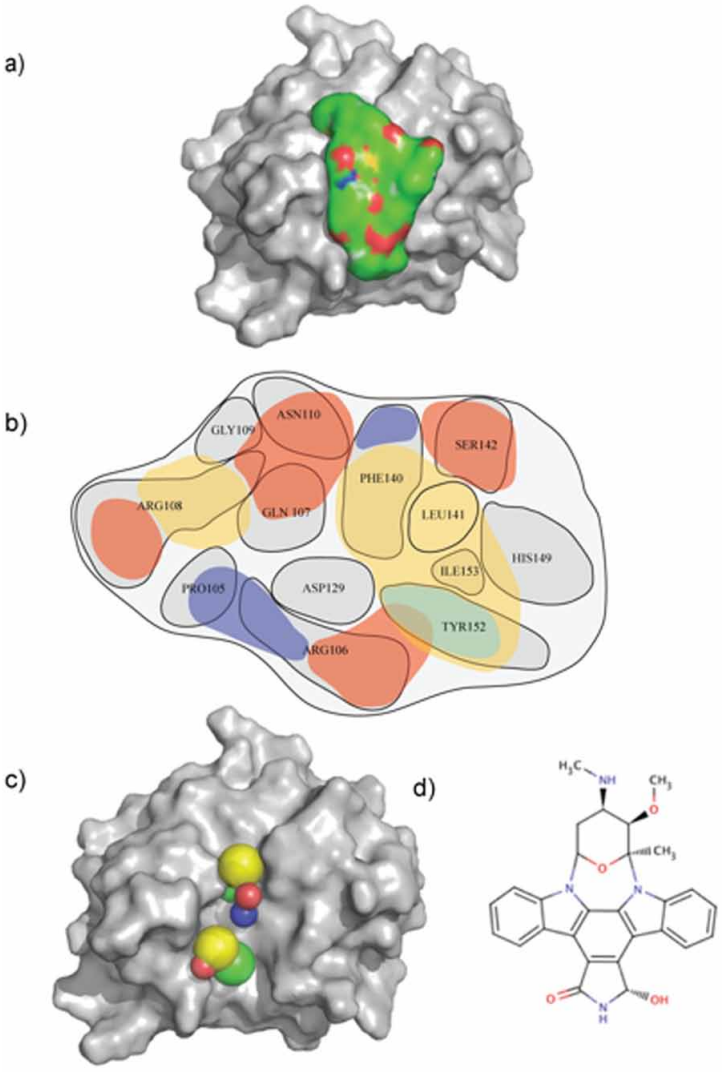
seemed to be more compact than that of MDM2. This is mainly due to the three residues Pro95, Ser96 and Pro97 in MDMX that have been replaced by His96, Arg97 and Lys98 in MDM2. These substitutions shifted a helical domain in MDMX relative to MDM2 and caused Lys98 and Tyr99 to protrude into the p53-binding cleft within MDMX, making it shallower and less accessible to many of the MDM2 top hits we found. This observation is clear when comparing the binding modes of nutlin within the two pockets (see Figure 1a). While Tyr100 and Leu99 of MDM2 extend the binding site allowing nutlin to intimately bind to MDM2, while the same residues in MDMX clash with the drug preventing it from taking the normal conformation that was adopted within MDM2. On the other hand, Figure 1b-c show how two compounds from the list of proposed MDM2/MDMX inhibitors were able to tolerate the structural variations between the two binding sites. This is apparent in Figure 1, where the compounds took on different conformations within the two binding pockets in order to maximize their interactions with the proteins. Recently, we used similar approaches to identify inhibitors for the XPA-ERCC1 (see Figure 3) (Jordheim et al., 2013) and XPF-ERCC1(K. H. Barakat et al., 2012) protein-protein interactions (Barakat, Gajewski, & Tuszynski, 2012).

In another interesting study, Doman *et al.* examined protein tyrosine phosphatase-1B (PTP1B) using both docking-based VS and HTS. In the course of the HTS run (Doman et al., 2002), approximately 400,000 compounds were screened against the target and only 85 compounds resulted in IC_{50} values less than 100 μM. Applying the docking-based protocol against the X-ray crystal structure and using 165,581 compounds, the top 350 hits were tested which resulted in 127 compounds with IC_{50} values less than 100 μM. While docking based VS yielded better results than HTS, it should be mentioned that the screened libraries and assay conditions were different in the two approaches. In a more difficult study, Vangrevelinghe et al. (2003) carried out a VS experiment using DOCK 4.0 in discovering the most potent inhibitor for human casein kinase II (CK2). This work started by building a homology model using a crystal structure template that shared 85% similarity with the human protein (Vangrevelinghe et al., 2003). Then, 400,000 different compounds were screened against the target followed by post-processing of the 12,428 top hits. The processing procedure followed two levels of iterations. Since the interaction with the hinge domain of the binding site was determined to be crucial for any potential activity, the top ranked compounds were first filtered for molecules that have at least two hydrogen bonds with this hinge structure. The second filter employed a more sophisticated scoring function that included an additional solvation term. This two-step filtering scheme revealed 1592 promising compounds. Only 12 compounds were finally tested experimentally and resulted in 4 hits that inhibited more than 50% of the enzyme activity. The most potent compound exhibited an IC_{50} of 80 nM and it turned out to be the best CK2 inhibitor discovered so far.

Using the induced-fit conformation for aspartic protease renin protein, Krovat et al. (2004) employed shape-based VS using the docking program LigandFit/Cerius to uncover novel renin-inhibitors and test a number of scoring schemes. Seven different scoring functions (LigScore1, LigScore2, PLP1, PLP2, JAIN, PMF, LUDI) were used to rank the compounds (Krovat & Langer, 2004). A collection of 990 diverse and drug-like compounds was used along with 10 known active renin inhibitors. Individually, all seven scoring functions that were used recovered at least 50% of the active compounds within the first 20% of the entire database. Using a consensus scoring that incorporated only four scoring functions (LigScore2, PLP1, PLP2, and JAIN), a hit rate of 90% in the top 1.4% was recorded. Finally, a more focused database was created and these compounds were docked to the binding site of the protein. A hit rate of 100% in the top 8.4% resulted from the use of the triple consensus scoring of PLP1, PLP2, and PMF.

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*Figure 3. ERCC1-XPA-inhibitor pharmacophore determination. (a) The equilibrated ERCC1 (grey surface) showing the excluded volume occupied by atoms from ligands obtained in the virtual screening experiments (green surface). Atoms included in this image were obtained by clustering the top ligands, from virtual screening experiments, and omitting those that were outside of a 90% RMSD cutoff. (b) Pharmacophores from each of the top 30 ligands were created with their interactions in the ERCC1 binding site. The type of pharmacophore interactions, with each residue were scored and are represented schematically. Yellow patches indicate hydrophobic interactions with the pocket, red and blue patches represent hydrogen bond acceptor and donors respectively, while green patches indicate aromatic interactions. Orientation of the binding site is the same as in panel a. Tyr145 and His149 (indicated by an asterisk *) do not lie on the bottom of the pocket but are observed within a lip that overhangs the pocket (see panel a). (c) The averaged pharmacophore model obtained from the docked poses from virtual screening. Each sphere represents a specific chemical entity with the size being representative of the overall contribution at each position. Coloring is identical to that described for panel a. (d) The chemical structure of UCN-01, a weak ERCC1-XPA small molecule inhibitor.*



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Although the above-mentioned success cases integrated docking as the key and principal ingredient of the VS protocol, other studies employed pharmacophore search techniques to reduce the size of the chemical database and extract a more focused library of compounds (Brenk et al., 2003; Cavasotto & Phatak, 2009; Rollinger, Haupt, Stuppner, & Langer, 2004; Rollinger, Hornick, Langer, Stuppner, & Prast, 2004; Sirois, Wei, Du, & Chou, 2004; Varady et al., 2003; Westerfors et al., 2003). Varady et al. introduced an exciting example of the method in their screening of 250,251 compounds for dopamine 3 (D_3) inhibitors (Varady et al., 2003). Similar to the Vangrevelinghe approach mentioned above, Varady et al. started by building up a homology model for D_3 followed by applying a hybrid, stepwise computational screening approach. Then, a pharmacophore model was developed and used to identify promising compounds from a chemical database of approximately 250, 000 synthetic compounds and natural products. As a result, 6,727 molecules were identified which satisfied the pharmacophore hypotheses. These molecules were further screened through structure-based searching using multiple receptor models obtained from MD simulations via computational docking and scoring. Top-ranked potential D_3 inhibitors were further subjected to structural novelty screening by comparing them to the known D_3 ligands. Finally, the most promising 20 potential D_3 ligands were tested for their D_3 binding affinities. As a result, 11 of these 20 compounds showed IC_{50} values greater than 3 μM and 8 compounds showed a nanomolar affinity.

In contrast to Varady's case, instead of initially filtering the database for potential hits, a pharmacophore model was used as a post-docking filter to validate the results and reduce the number of hits submitted to experimental verification (Betzi et al., 2007). Betzi used this technique to identify the first set of drug-like ligands that bind to the HIV-1 Nef SH3 surface. To accomplish this, the whole chemical database was filtered for drug-likeness properties which reduced the set of compounds to ~1,400 molecules. These structures were then docked to the binding interface between Nef and SH3 proteins. Top 335 hits were then filtered using a structure-based pharmacophore model derived from the binding site within the SH3 domain. This post-filtering procedure yielded 33 compounds that were visually inspected and reduced to 10 hits for experimental validation. Out of the final 10 tested compounds, one molecule resulted in a dissociation constant, K_D, of 1.8 μM.

Rollinger et al.(2004) used structure-based pharmacophore screening for the discovery of novel inhibitors for acetylcholinesterase (AChE). This study generated a pharmacophore model using the protein and one of the known inhibitors. Then, the in-house 3D database was screened which included about 110, 000 natural products within a molecular mass of 140−700 Da (Rollinger, Hornick, et al., 2004). Finally, two compounds from the top VS hits were subjected to experimental validation and progressed to *in vitro* and *in vivo* testing. On another large scale SBVS study and using a pharmacophore searching strategy (Sirois et al., 2004), Sirois et al. screened over 3.6 million compounds against SARS (severe acute respiratory syndrome). About 0.07% of the initial database of compounds satisfied at least five of the original six pharmacophoric points. Furthermore, a subsequent evaluation for the druggability of the top ranked compounds retrieved 17% that had a perfect score of 1.0.

LIGAND BASED DRUG DESIGN (LBDD)

Despite the advances in macromolecular structure prediction methods, the number of protein structures that have been determined experimentally is still lagging compared to that of their sequenced counterparts. In this case, homology modeling may play a major role in understanding and predicting

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the three-dimensional structure of the target (Bordner, 2012; Levit et al., 2012; Ravna & Sylte, 2012). However, homology modeling has its own limitations and the degree of success of incorporating the method within the context of VS depends mainly on the quality of the predicted structure (Cavasotto & Phatak, 2009). Therefore, it is important to seek alternative routes that depend merely on known active compounds and in which no information about the target is required (Speck-Planche, Luan, & Cordeiro, 2012; Wilson & Lill, 2011). These ligand-based filtering techniques have played a significant role in discovering potent inhibitors for many targets. In fact, ligand-based screening methods use known active and inactive compounds as templates and employ comparative algorithms to identify new compounds that are similar to the templates. Overall, one can classify the different LBDD methods into two main approaches, namely, similarity search (Willett, 2006) and pharmacophore search (Sun, 2008).

Similarity Search

The fundamental theory behind this approach is Maggiora's "similar property principle" (Johnson & Maggiora,1999), which states that similar molecules are more than likely to have similar properties. While not universally correct (Kubinyi, 1998), there are many cases where this simple idea showed great success and helped in the discovery of novel active molecules (Martin et al., 1993; Patterson, Cramer, Ferguson, Clark, & Weinberger, 1996). According to this concept, one can use known active compounds as reference structures and filter a given chemical library for ligands that are structurally similar to the active molecules. The filtered compounds are expected to display some activity that in some cases could be greater than the original reference structures. In fact, there are mainly two ways to assess the similarity between two molecular structures. These methods comprise molecular alignment and molecular descriptor algorithms (Lemmen & Lengauer, 2000).

Molecular alignment algorithms such as FlexS (Lemmen, Lengauer, & Klebe, 1998) or GASP (Jones, Willett, & Glen, 1995a) typically align the filtered compounds with the reference structure and rank them according to their degree of similarity. During this superposing process, the two aligned molecules can be treated either as rigid or flexible. Similar to docking methods, flexibility can be introduced by employing an incremental construction approach (FlexS) or a genetic algorithm procedure (GASP). Other algorithms like Fflash (A. Kramer, Horn, & Rice, 2003; Pitman et al., 2001) apply fragment-based techniques to incorporate ligand flexibility during the filtering process. Other algorithms adopted more complicated approaches to carry out more accurate molecular alignment. These methods include incorporation of Gaussian functions as in the program MIMIC (Mestres, Rohrer, & Maggiora, 1997) or constructing interaction potential grids around molecules (Goodford, 1985).

A major drawback of molecular alignment techniques is that the time required for a single molecule comparison is long enough to discourage a user from employing the method in screening large databases (Willett, 2006). As a result, more efficient and accurate techniques have been developed to describe the information inherited in the molecular structure of a given ligand along with its physiochemical and topological properties. These molecular descriptors are generated on-the-fly and compared to the reference structure very rapidly. Based on the dimension of the information that is used, molecular descriptors can be classified into 1D-, or 2D-descriptors. Evidently, the higher the dimension of the descriptor approach, the lower its computational time will be and the higher the accuracy one can expect from the searching protocol. Generally speaking, bulk properties like molecular weight, molar refractivity or log P values are adequate to construct a 1D-molecular descriptor (Lipinski, Lombardo, Dominy, & Feeney, 2001). However, since there is no information about the structural properties or chemical features of

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the ligand, it is impossible to only rely on such descriptors in filtering a typical chemical library for active molecules. Consequently, one should draw on a higher level of information and include structural properties as an additional descriptor in order to increase the accuracy of the method. This introduced molecular fingerprints as the most successful and widely used similarity search approach in LBVS.

Molecular fingerprint is a bit-string representation that reflects structural features and other properties of a molecule given its chemical structure (Willett, 2006). Key advantages of this approach over direct comparisons of molecules are that it is very simple to implement, remarkably fast to be calculated and the final outcome is expressed as a single number that quantifies the degree of similarity. According to the complexity level and designing scheme, one can recognize two basic approaches in generating a molecular fingerprint for a specified structure. The first approach is what is known as "keyed" representation (McGregor & Pallai, 1997). In this case, an individual bit within the string can be set as "on" or "off" reflecting the presence or absence of a pre-defined functional group (pattern) in the sub-structural space of the ligand. While the order of the bit-string map is the same for each molecule, the individual bits are turned on or off if their representative substructure exists or not. A widely used VS algorithm that employs this procedure is MACCS (McGregor & Pallai, 1997) whose bit-strings may include up to 166 bits representing commonly known fragments. The second approach is known as the "hashed" representation (James & Weininger, 1995). This method resembles human fingerprints by not restricting the definition of bits to describe a pre-specified set of patterns. That is, similarly to human fingerprints, which are very characteristic of individuals, a pattern's fingerprint characterizes the pattern, but the meaning of any particular bit is not well defined. To do so, a typical hashed representation algorithm starts by generating a pattern for each atom. Then it creates a pattern representing each atom and its nearest neighbors in addition to the bonds that join them. This hierarchal construction evolves to include higher order nearest neighbors until the complete structure is recovered.

At the heart of these similarity-based VS techniques lays a similarity measure, usually termed a similarity coefficient that is used to quantify the degree of resemblance between two molecules. In fact, the most commonly used parameter is the Tanimoto (Jaccard) coefficient (defined in equation 1) (Willett, 2006) To understand the concept behind this parameter, let us consider the case of 2D fingerprints representing two molecules A and B that have a and b bits that are set as true, respectively. Now, if there are c common bits that are mutually set as true in the two molecules, where c is the intersection subset of a and b, one defines their Tanimoto coefficient as:

$$T = \frac{c}{a + b - c} \tag{1}$$

The Tanimoto coefficient gives values between zero (no similarity) and one (completely similar structure). Although this coefficient is the most popular choice for both in-house and commercial screening packages, a number of studies have indicated that it is dependent on the size of the fingerprint of the reference structure and on how many bits are set in its bit-string representation (Flower, 1988). However, other studies suggested that 85% of compounds that have a Tanimoto coefficient value of 0.85 or greater relative to an active compound are predicted to be active as well (Patterson et al., 1996).

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Pharmacophore Search

While the basic concepts behind the pharmacophore search approach have been introduced in previous sections, here we focus on pharmacophore modeling techniques that have been broadly followed in the literature when no target structure is available (Barnum, Greene, Smellie, & Sprague, 1996; Z. Chen et al., 2009; Ou-Yang et al., 2012; Sun, 2008; Z. L. Zhou et al., 2013). In this case, the only information that can be exploited is a set of known active compounds that are recognized experimentally and the general procedure can be summarized in two fundamental steps. First, this set of molecules is analyzed in order to identify all chemical features within their structures. Then, for each molecule an ensemble of different conformations is generated and used to produce the best alignment between the different compounds to overlay their corresponding features. Although the main approach seems feasible and simple to implement, searching the conformational space is the most important and most difficult part of the method. This is because it is hard to predict the active conformer of a given ligand without understanding how it interacts with the target, with the solvent molecules and other elements of the binding environment. Nevertheless, there are several programs that have been successfully used in building ligand-based pharmacophore models for many targets. These programs differ mostly in the way they handle ligand flexibility and in the method of searching a typical chemical database for promising hits. The most popular programs are Catalyst (Barnum et al., 1996), DiscoTech (Martin et al., 1993) and GASP (Jones et al., 1995a).

Catalyst introduces ligand flexibility very efficiently and additionally is extremely fast in searching 3D chemical databases (Barnum et al., 1996). In brief, the program extensively explores the conformational space of a ligand by using a random search algorithm along with a poling function, which creates a large number of low-energy conformations. Catalyst follows two alternative algorithms in building up pharmacophore models. The first algorithm, HypoGen, is a quantitative approach in which each chemical feature allocated to the ligand structure is associated with a particular weighting factor that is related to its relative importance in describing the bioactivity of the molecule. Following this procedure, the algorithm builds up a number of pharmacophore hypotheses and ranks them based on their ability to explain available experimental data. In the other approach, Catalyst follows a qualitative procedure that is termed the HipHop algorithm. In this process, for each ligand, the algorithm checks for the surface accessibility for receptor interactions. Then, chemical features are defined based on their absolute coordinates in the different conformations of the molecule rather than by their inter-feature distances. This procedure usually starts with the most active compounds in the training set followed by highlighting their matching features from other less-important molecules. This results in a considerable number of proposed pharmacophore hypotheses that is significantly reduced by rejecting models that cannot explain the bioactivity of these molecules. Regardless of which approach is used, Catalyst can merge different models in order to generate a more comprehensive pharmacophore hypotheses.

Disco not only suggests pharmacophore models that demonstrate the important features in a ligand, but it also predicts their potential complementary regions that should be located within the binding site (Martin et al., 1993). This is accomplished by breaking up a pharmacophore to groups of ligand points and binding pocket interaction sites. Ligand points include atoms with hydrogen bonding properties, charge centers and hydrophobic characteristics. Binding pocket interaction sites are predicted to be complementary regions within the target and are calculated using the coordinates of the heavy atoms of the ligand. Similarly to Catalyst, the conformational flexibility of the ligands is explored using a set of pre-calculated conformations for each ligand in the training set. However, one pitfall of using Disco

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is that all chemical features that make up the final pharmacophore model must be identified in every molecule, which may result in the exclusion of talented models.

In contrast to both Catalyst and Disco, the program GASP (Jones et al., 1995a) handles the ligand conformational flexibility in a very sophisticated manner. Instead of using a pre-calculated set of ligand conformations, the program uses a genetic algorithm to explore the conformational space of the ligand during the pharmacophore generation process. GASP algorithm starts by detecting all possible chemical features in the structure of each ligand. The molecule with the least number of features is selected as a reference structure. Every structure in the training set is then fitted to the reference structure using a genetic algorithm that is similar to its counterparts in docking programs (see above). However, in this case, the fitness of a particular model is measured based on a combination of similarity, the number of overlaid features and the volume integral of the overlay. One more advantage for GASP over DISCO and Catalyst is that, models generated by GASP account for the steric clashes between the ligands in generating the final pharmacophore model. On the other hand, the other two programs propose their models by only matching the chemical features of the ligands without taking their overall shape into account.

Regardless of which approach is used to generate a pharmacophore model, which includes SBPM, there are two main ways to screen chemical libraries for compounds that satisfy the constraints of the pharmacophore hypotheses (Z. Chen et al., 2009; Sun, 2008). First, one can use a database file format that includes a set of pre-defined conformers for each compound in the database. Although this approach remarkably speeds up the search process, it requires massive storage of the different conformations. Alternatively, a single conformation can be used as a precursor for generating an ensemble of conformations followed by fitting these structures to the pharmacophore query during the screening process. While this procedure eliminates the need for substantial storage, it is much slower than the former method.

Case Studies for LBDD

Similar to SBDD, a plethora of LBDD studies has been published in the literature in the past few years including methodology-development papers focused on enhancing the accuracy of current models or devising new methods (Bender et al., 2009; Hert et al., 2004; Selvaraj & Singh, 2013; Speck-Planche et al., 2012). Others represent pure applications for the various techniques attempting to recognize bioactive molecules or identifying new scaffolds by screening chemical databases.

In an important assessment study Bender et al. (2009) compared the performance of 37 different molecular descriptors. The judgment was based on the ability of the different descriptors to rank active compounds from a database using a number of randomly selected query molecular structures. The basic question in this study was which descriptors contain independent (orthogonal) information and which descriptors are redundant because they are correlated with each other. Consequently, Bender et al. identified four independent broad descriptor classes: (1) circular fingerprints; (2) circular fingerprints considering counts; (3) path-based and keyed fingerprints and; (4) pharmacophoric descriptors. In 2004, Hert et al. (2004)investigated the performance of a new similarity searching methodology, known as data fusion. This technique is similar to the consensus scoring approach that is widely used in current docking protocols. However, similarly to consensus scoring, data fusion is mainly dependent on the combination(s) of similarity measures that are used to rank the compounds, which, if selected properly, will lead to a significant enhancement in searching performance. In another interesting study, Tresadern et al. (2009) compared a number of LBVS methods with a careful application to corticotropin releasing factor 1 receptor (CRF1). This list of assessment and development studies continues to grow and includes

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many entries that are committed to improve the LBVS techniques and increase their accuracy (Burton, Ijjaali, Petitet, Michel, & Vercauteren, 2009; Neves, Dinis, Colombo, & Sa e Melo, 2009).

In addition to the aforementioned evaluation studies, there have been many successful applications of the methods. For example, following a two-step ligand-based approach, Franke *et al.* filtered a database of natural product and natural-product-derived compounds for potential inhibitors of human 5-lipoxygenase (5-LO) activity (Franke et al., 2007). In the first step, a similarity searching was performed using a topological pharmacophore model constructed from 43 known inhibitors. Out of the 430 top hits, 18 compounds were selected that satisfied the pharmacophore features of the original reference inhibitors. Experimental investigation of these compounds retrieved two novel structures that showed significant activity. Using these two molecules a second round of virtual screening was performed through different ligand-based virtual screening methods. The top ranked molecules from the final set of screening potently inhibited 5-LO activity in intact cells representing a novel class of 5-LO inhibitors. Using a similar approach, Carosati *et al.* used LBDD to identify novel activators for Kir6.2/SUR1 K_{ATP} channels (Carosati et al., 2007). Firstly, using pharmacokinetic filtering via the VolSurf program, a set of 65,208 commercially available compounds was reduced to 1,913 compounds. Subsequently, six molecules were selected to serve as reference structures in a similarity search procedure using different scoring methods. As a result, 32 hit candidates were identified and tested for their biological activity. This procedure resulted in the discovery of 3 novel compounds that were able to inhibit insulin release with micromolar potency.

In order to identify compounds that can block NAADP (nicotinic acid adenine dinucleotide phosphate) signaling, Naylor *et al.* used NAADP as the query ligand to screen the chemical library ZINC for compounds with its 3D-shape and electrostatic similarity (Naylor et al., 2009). By testing the top-ranking hits in a sea urchin egg bioassay it was found that one hit, Ned-19, blocked NAADP signaling at nanomolar concentrations.

Sciabola *et al.* developed a new 3D pharmacophore fingerprinting approach (TOPP, Triplets Of Pharmacophore Points) to identify inhibitors for a specific P450 target, polymorphic CYP2D6 (Sciabola, Morao, & de Groot, 2007). Their new 3D pharmacophoric fingerprints were as accurate as other 3D descriptors and 2D features. Interestingly, it was found in the 3D models that the use of more realistic substrate conformations, and by an additional docking step using GOLD on a homology model structure, did not significantly improve the statistical results. Using a hybrid between LBPM and docking-based screening, Li *et al.* screened for inhibitors against Raf-1 kinase (Li et al., 2009). First, a 3D pharmacophore model was developed from a training set of structurally diverse ligand structures. Then a virtual database searching was performed with the pharmacophore model as a 3D query. Subsequently, molecular docking was carried out on the selected hits whose estimated IC_{50} was less than 1 μM, which yielded 29 hits that were identified as potential leads against the Raf-1 kinase target. In another study, Neves *et al.* developed a new ligand-based strategy combining important pharmacophoric and structural features based on a known an aromatase-inhibitor (Neves et al., 2009). The NCI database was screened and new potent aromatase inhibitors were found to be active in the low nanomolar range. Interestingly, all the molecules found exhibited a common binding mode.

Chen and co-workers carried out a very exciting study to look for dual inhibitors for both neuraminidase (NA) type 1 (N1) and haemagglutinin type 1 (H1) (C. Y. Chen et al., 2009). An N1- pharmacophore model was constructed and used to screen the NCI database for N1-inhibtors. Top hits resulted from the initial screening were docked into haemagglutinin type 1 (H1) in order to find out dual-inhibitors for both N1 and H1. It was suggested that the compound NCI0353858 could be effective in curing the worldwide disease N1H1 influenza.

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CONCLUSION

Under a growing social and economic pressure directed at discovering new and improved drugs and lowering the cost of current brand names, the pharmaceutical industry is struggling to develop innovative, fast and economical drug discovery techniques. Although HTS still remains the main approach driving the identification of new lead compounds, the overall methodology is still largely phenomenological and technology driven (Lahana, 1999). Moreover, the basic cost of a typical HTS laboratory includes collecting and maintaining screening libraries of thousands, if not millions, of compounds. In contrast, using rational drug design methods for drug discovery, the compounds that are tested need not physically exist or be chemically synthesized as long as they can be synthesizable in principle. In addition, experimental difficulties that are associated with HTS such as limited solubility or aggregate formation are not relevant to these tools and do not need to be considered (Stahura & Bajorath, 2004; Zoete et al., 2009).

Even when used perfectly, no computational method developed thus far can predict all possible solutions. It is important to use a combination of all possible approaches in order to substitute a weakness of one technique with strengths in the others (Muegge, 2006). Although SBDD is more computationally demanding than LBDD (Reddy et al., 2007), it is easier to rationalize and to aid in designing more specific drugs by targeting a precise domain or binding pocket within a particular target. Additionally, recent advances in crystallography allowed the automation of structure prediction for parallel crystallization of different protein targets. These new rapid techniques introduce protein crystallography as a powerful high throughput approach in reducing the gap between sequenced and structured proteins (Sharff & Jhoti, 2003). Moreover, new co-crystallized ligands, binding affinity assays and high performance computing facilities add more promise for improving and validating SBDD protocols. Nevertheless, as the majority of potential therapeutic targets, especially membrane proteins, continue to be hard to crystallize (Drews, 2000), LBVS will remain a dominant alternative means in identifying new lead compounds and designing new drugs. On the other hand, SBDD on its own cannot elucidate the functional activity of a typical ligand. Examples of such problems include agonism, antagonism (Mailman & Murthy, 2009) and side effects, which are hard to capture and are difficult to model. Hence, in our opinion both SBDD and LBDD taken together will remain strongly positioned as two optional and complementary methods in drug discovery and development. However, based on the discussion presented here, rational drug design as a whole is still under development, and far from forming a mature of field of science. This is apparent in the fact that the number of strategies followed in the field is nearly as considerable as the number of reported screening campaigns.

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Chapter 45
Smart, Innovative and Intelligent Technologies Used in Drug Designing

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ABSTRACT

Smart and intelligent computational methods are essential nowadays for designing, manufacturing and optimizing new drugs. New and innovative computational tools and algorithms are consistently developed and applied for the development of novel therapeutic compounds in many research projects. Rapid developments in the architecture of computers have also provided complex calculations to be performed in a smart, intelligent and timely manner for desired quality outputs. Research groups worldwide are developing drug discovery platforms and innovative tools following smart manufacturing ideas using highly advanced biophysical, statistical and mathematical methods for accelerated discovery and analysis of smaller molecules. This chapter discusses novel innovative applications in drug discovery involving use of structure-based drug design which utilizes geometrical knowledge of the three-dimensional protein structures. It discusses statistical and physics based methods such as quantum mechanics and classical molecular dynamics which can also play a major role in improving the performance and in prediction of computational drug discovery. Lastly, the authors provide insights on recent developments in cloud computing with significant increase in smart and intelligent computational power thus allowing larger data sets to be analyzed simultaneously on multi processor cloud systems. Future directions for the research are outlined.

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INTRODUCTION

Rapid and steady growth of low-cost computer power in discovering and designing new drugs has become a central topic in modern biology and medical chemistry. These includes methods and techniques ranging from high-throughput screening of compounds in databases to protein inhibition through molecular dynamic and thermodynamic approaches using distributed and cloud computing. Computational drug design approaches can be divided into structure and ligand-based approaches (Reddy et al., 2007). Former approach focuses on available X-ray crystallographic protein structure, NMR-based structure or homology model obtained from known template from protein databank. Once a rigid structure of the target is available, ligand-based virtual screening identifies novel ligands by three-dimensional (3D) similarity searching or by pharmacophore protein matching (Reddy et al., 2007). Ligand based virtual screening detects compounds that are similar to the active known compounds. Next, the target compounds are identified through virtual screening following which the docking algorithms are applied to position the available compounds in the binding sites derived from several biochemical studies (Reddy et al., 2007). These compounds are then ranked according to their steric and electrostatic interactions between the receptor and ligand (Reddy et al., 2007). On the other hand, ligand-based approach is useful in absence of experimental 3D structure. Under such circumstances, known ligands are explored to understand the basic structural and chemical properties correlating with specific pharmacological properties. Design of new ligands is performed in several steps mostly by manual inspection and qualitative interpretation of ligand-binding site interactions. Ligand-based approaches use Quantitative Structure Activity Relationship (QSAR) method and pharmacophore modeling. The QSAR is a computational methodology for quantifying correlation between structures of chemical compounds and chemical/biological process (Acharya et al., 2011).

The QSAR model is applied to optimize active compounds to increase their relevant biological activities to the maximum possible level. The success of this model is closely related to the selection of appropriate molecular descriptors and its ability to generate mathematical relationships between the molecular descriptor and biological activity (Acharya et al., 2011). Some of the major statistical methods used to select molecular features are multivariate linear regression analysis (MLA), Principle Component Analysis (PCA) and Partial Least Square Analysis (PLS), all of which will be discussed in this chapter review (Acharya et al., 2011). Structure-based approaches also involve virtual screening of compound libraries which can be manifested using molecular docking approach and Molecular Dynamics (MD) simulations (Okimoto et al., 2009; Durrant & McCammon, 2011). Molecular docking allows prediction of different complex protein-ligand conformations and estimation of binding affinity using mathematical algorithms for faster conformational search of ligands. MD simulations allow the estimation of the effect of explicit and implicit water molecules around the binding site of ligand and calculation of binding free energy. The techniques involved are thermodynamic integration (TI), free energy perturbation (FEP) and molecular mechanics/Poisson-Boltzmann surface area (MM/PBSA) (Okimoto et al., 2009; Durrant & McCammon, 2011).

One of the robust methodologies currently being developed and extensively used is cloud computing in computational drug discovery. Cloud computing is a term which involves services provided over the Internet. This idea has been implemented from distributed/grid computing that integrates several individual computers to generate a super computer system of superior computational abilities (Shudong et al., 2004). Cloud computing utilizes computing resources allocated on-demand over the Internet. Use

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of massive computational power can thus be applied for virtual screening of principal compounds and undergoing complex calculations within a very short time span.

In this chapter we will discuss all the computational methods developed and currently used in drug discovery and design. We will focus on the ability of these smart, intelligent and innovative methods and approaches for identifying novel drug candidates and latest advances in intelligent computing power and their interconnection and smart manufacturing applications in computational biochemistry.

Computational Drug Design Strategies

Application of computational tools in drug discovery has grown steadily over the past several years and there have been significant improvements over the methods. A number of computational techniques are used to resolve complex chemical and biological problems including statistical analysis of large datasets, structure-based drug designs, computational genomics, molecular simulations and pharmacophore based molecular library designing. Computational chemistry tools are regularly employed to resolve detailed atomic structures of different ligand-receptor complexes; and for evaluating small-molecule drug characters. Statistical and information sciences are being increasingly used nowadays to generate and manage chemical and biological activity databases. With the aid of computers, new techniques have been developed for automated derivation of drug derivatives. Libraries of compound derivatives can be assembled by targeted structure-based combinatorial chemistry. Improved methods have developed for rapid generation and prediction of potential drug binding to proteins. In addition, refinement of drug candidates can also be performed in most effective manner. Therefore, use of computers has become an important asset for development of new therapeutics for most common and complex diseases.

Structure-Based Drug Designing

SBDD is one of the powerful approaches in drug discovery as it combines both combinatorial chemistry and high-throughput screening for synthesis of potential drug candidates. First step in drug development is to find small molecule which can bind to target proteins and alter its biological function by increasing or inhibiting its activity (Wilson & Muftuoglu, 2012). Development of small molecule drug candidate against a given disease requires proper understanding of metabolic pathways. Therefore, first step in drug designing is identification of potential biological target from the given metabolic pathway. Therefore, identification and development of potential ligands specific to the biological target is the second step in Structure-Based Drug Design (SBDD) (Reddy et al., 2007). There are many automated screening tools and software that allow screening, testing and selection of large number of compounds from online Web databases which is based on their ability for interaction with biological targets. Some of the tools such as PyRx developed by The Scripps Research Institute allow screening libraries of compounds against potential drug targets (http://mgltools.scripps.edu/documentation/links/pyrx-virtual-screening-tool). The VSDocker is an important tool allowing parallel high-throughput virtual screening on windows based computer cluster systems (Prakhov, Chernorudskiy, & Gainullin, 2010).

The biological target has pocket where the ligand binds and alters the activity of the target. The pocket comprises of different types of possible hydrogen bond donors and acceptors and hydrophobic characteristics (Reddy et al., 2007). Compounds (or fragments of compounds) from the available databases are then positioned over a selected region or in other words, the active site of the structure. These are

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next scored and ranked depending on the steric and electrostatic interactions with the target site. Top selected compounds are next screened using biochemical assays (Anderson, 2003).

Molecular Docking Approach from Virtual Screening of Lead Compounds

Structure-based computational methodology involves computational screening of compounds which can be performed by molecular docking. It is often useful when the 3D structure of the biological target is available. Molecular docking involves knowledge of putative binding sites where the ligand can bind and initiate a biological response. The knowledge can either be acquired from biochemical studies or obtained from co-crystallizing the target protein with a ligand. Once the target binding sites are known, correct binding modes (binding pose) of a ligand molecule is conducted by running number of trials by searching different translational as well as rotational degrees of freedom. The ligand-receptor interaction energy is calculated for each binding pose and a '**score**' is assigned for each pose. Score is calculated mainly by three scoring function techniques: force-field, knowledge and empirical based scoring functions (Kroemer, 2007; Cheng et al., 2010).

Force field based functions employ a force-field to compute non-covalent ligand-receptor interactions in an implicit solvent. Generalized Born (GB/SA) or Poisson-Boltzmann (PB/SA) methods are used for computing the interactions. Knowledge-based scoring functions are generated from the inter-atomic distances available in the protein-ligand complex structures databases. Frequency distributions are computed from the distances and the distribution is converted to potential of mean force or knowledge-based potentials. Empirical based method is based on the principle that free energy change upon ligand binding (ΔG_{bind}) can be decomposed into the summation of the internal energy (ΔG_{int}), the solvation energy (ΔG_{solv}), the conformational energy (ΔG_{conf}) and the free energy of motion (ΔG_{motion}).

Virtual Screening

Small-molecule compound library is prepared. Table 1 illustrates a list of databases which can be used for library preparation. Both the biological target and the compound library require preprocessing such

Table 1. Number of compounds (entries) in the commonly screened databases (Note: All are approximate figures)

Databases	Number of compounds
PubChem	30 million
ChEMBL	1 million
NCI (National Chemical Institute)	250,000
ChemSpider	26 million
ACX	140,000
ASINEX	140,000
ChemBridge	900,000
ChemDiv	1.2 million
ChemNavigator	55 million

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as assigning proper tautumeric, stereoisomeric, and protonation states (Cheng et al., 2010). Each compound in the library is docked to the biological target molecule using a docking program or tool which models the ligand-receptor interactions for achieving optimal complimentarity between the active site of target and the compound. Scoring function evaluates the fitness between the target and compound and calculates a score for each binding pose. Binding poses are ranked based on their scores. Lee et al. (2010) performed virtual screening for determining vascular endothelial growth factor receptor (VEGFR)-2 kinase inhibitors using pharmacophore modeling and docking studies.

Recent Advances in Docking

4D Docking

Giovanni et al. utilized a novel procedure of ligand docking by exploiting the receptor flexibility (Bottegoni et al., 2009). In four dimensional (4D) docking procedure, an ensemble of receptor conformers are generated. Generated structures are converted to conformational stack and matching structure with complete sequence in Uniprot database is selected as reference structure. All the other structures are superimposed to the reference structure by an iterative weighted process. This procedure of making a conformational stack with one reference molecule is called 4D grid. Ligands are placed in the 4D plane and allowed to move from one 4D plane to another allowing transfer between the different receptor conformations. Receptor-ligand poses with lowest energy conformation then undergoes all atom scoring procedures. This approach was tested on 99 therapeutically relevant proteins and 300 diverse ligands. The approach reproduced correct ligand-binding geometry matching the success rate of the traditional process.

Drug Repositioning by Structure-Based Virtual Screening and Molecular Docking

Drug repositioning is a strategy which involves generation of new or additional value from drug by targeting diseases. In this approach, candidate compounds are screened against the biological targets for higher potency and selectivity against the targets. Combination of structure-based virtual screening and drug repositioning represents highly efficient approach for development of novel medicines. For drug repositioning, specialized virtual libraries are available through databases which contain information about existing drugs and compounds which have made through later stages of clinical trials and commercialization (Ma et al., 2013). When potential compounds are identified by virtual screening, molecular docking can be performed using "single target" approach which identifies potential interactions between the drug candidates and biological target. Molecular docking involves three main steps: (1) generation of molecular model of the biological target and pre-treatment of ligands, (2) conformational sampling of the ligands on the biological target binding site, (3) Assignment of score for the poses of the ligand-receptor complexes. Docking can be performed by various algorithms. Some of the commonly used algorithms are Monte Carlo (MC) or Genetic Algorithms (GA) (Hart & Read, 1992; Morris et al., 1998). Drug repositioning has been applied in several applications. A well-known example is its use in discovery of sildenafil for the treatment of erectile dysfunction in human males (Ashburn & Thor, 2004). Sildenafil was originally known for its anti-anginal activity for inhibition of phosphodiesterase family of proteins. By performing drug repositioning, male erectile dysfunction was treated in phase I clinical trials.

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Drug Docking via Systems Theorem

Computational docking of drug candidates is performed by various docking approaches in which the flexible drug compound is docked to the rigid receptor and various receptor-drug conformational poses are generated. Score is calculated for each pose of the complex. However the docking algorithms considers receptor structures as rigid which does not provide accurate docking scores. Flexible protein and ligand motions are required for efficient docking at the docking site. The Lyapunov approach provides focused stability during protein folding for drug docking calculations (Sung & Chen, 2012). Applying the Lyapunov equation and the molecular dynamics approaches, lowest energy protein structure can be determined. Ligand-receptor docking procedure consists of 3 major steps, namely, (1) creation of docking box around the receptor binding site, (2) placement of ligands in the docking box, (3) generate various poses and score the poses based on various interacting energy terms. The Lyapunov function provides dynamic stability in both linear and non-linear systems which are based on Hess's law by which free energy of binding of ligand and receptor is calculated in solvent system. Lyapunov stability function illustrates relationship between minimum energy and modeling. Reaction coordinates are generated and stability basins of the energy landscape for system of interest are characterized using biomolecular simulations. This approach is based on stability theory which decomposes the molecular systems into subsystems. Then it constructs a suitable vector Lyapunov function. Authors have tried this approach on Cyclooxygenase-2 protein by docking with Flurbiprofen. However, practical application of the approach remains unexplored.

Molecular Dynamics Approach

Macromolecular structures (target biological receptors) play a vital role in drug discovery. For novel drug discovery prediction, virtual screening of the compounds is performed using drug database. This is followed by selection of potential compounds and docking to the receptor active site. However, the docking algorithms were unable to take into consideration the role of protein flexibility in ligand binding involving complex calculations essential for describing quantum-mechanical motions and chemical interactions of large molecular systems. MD simulations were able to remove this problem by applying approximations based on Newtonian physics for simulating atomic motions. By undertaking such approach, computational complexity can be further reduced. First, a crystal structure or homology model of the receptor is taken and forces acting on each atom are calculated from the equation (Durrant, & McCammon, 2011):

$$E_{Total} = \sum_{bonds} K_r (r - r_{eq})^2 + \sum_{angles} K_\theta (\theta - \theta_{eq})^2 + \sum_{dihedrals} \frac{V_n}{2}[1 + \cos(n\varphi - \gamma)] + \sum_{i<j} \left[\frac{A_{ij}}{R_{ij}^{12}} - \frac{B_{ij}}{R_{ij}^{6}} + \frac{q_i q_j}{\epsilon R_{ij}} \right]$$

Forces are calculated from contributions from bonded and non-bonded atom types. Bonded atom contribution is composed of chemical bonds and atomic angles from virtual springs and dihedral angles generated from rotations around the bonds. However, the non-bonded contribution is estimated from Van der Waals interactions that are modeled applying the Lennard-Jones potential and the electrostatic

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interactions based on the Coulomb's law (Leonnard-Jones, 1924). For reproducing the behavior of the target molecule in motion, the energy terms are derived from experimental and quantum-mechanical data. To perform MD simulations, force fields from CHARMM (Brooks et al., 1983), GROMACS (Christensen et al., 2009) and AMBER (Wang et al., 2009) are generally used. Once the forces on each are calculated, each atom is moved to different position according to Newton's laws of motion. Simulation is advanced by a femtosecond and is repeated till the simulation time specified by the user, usually 40-50 nanoseconds. Results from MD simulations explain various conformational changes of protein and ligands. Experimental studies involving protein and ligand have shown good agreement between the experimental and computational measurements using MD simulations (Markwick et al., 2009; Showalter, & Brüschweiler, 2007).

MD simulations provide structural insights into the identification of drug binding sites of the β-adrenergic receptors. MD simulations were performed for 0.5 microseconds which produced distinct conformational changes. MD simulations revealed binding pockets at both the solvent and lipid-exposed areas which serves for undertaking various structure-based approaches for designing new compounds (Ivetac & McCammon, 2010). Using β-adrenergic receptor protein, MD simulations were performed for testing the association of several ligands such as alprenolol and propranolol (Dror et al., 2011). The long MD simulations performed for ~20 microseconds revealed the entire process of association of drug molecules to the GPCR binding site. The process of drug binding explained the energetic barriers required for drug binding and unbinding kinetics.

Statistical Mechanics and Thermodynamic Approaches

The MD calculations utilize virtual screening of ligands for a receptor protein by screening drug databases for available compounds/libraries. However, docking fails to perform in certain cases which limit its use in drug designing. Methods like MM/PBSA and MM/GBSA are applied for calculating binding free energies using molecular mechanics and implicit solvation models. Several thermodynamic approaches have been identified recently which can be used for calculating binding free energy of ligands. Pathway method employs a technique which transforms the system from one state (complex) to another state (unbound) (Shirts, Mobley, & Chodera, 2007). Using FEP method, the system is transformed from one thermodynamic state to another. The two states (A and B) can be characterized by potential energy functions where state A corresponds to unbound ligand state and state B corresponds to complex state. The free energy difference between the two states is calculated by:

$U_{AB} = U_A - U_B$ (Shirts, Mobley, & Chodera, 2007)

where U_{AB} is the free energy difference between the two states. By solving the above equation, we obtain the final *free energy perturbation (FEP) formula* (Shirts, Mobley, & Chodera, 2007):

$$U_{AB} = -kT \ln \left\langle e^{-\beta(U_B - U_A)} \right\rangle$$

where β is the Boltzmann constant and free energy difference between states A and B needs to be calculated by natural log of average Boltzmann factor of potential energy difference between states A and B over equilibrium configurations from the state A. Such a transformation between two end states is

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termed as *alchemical free energy calculation*. A recent application of the approach was demonstrated the use of FEP coupled with MD and MC used for developing and optimizing lead compounds for HIV-1 protease and non-nucleoside reverse transcriptase inhibitors (Acevedo et al., 2012).

Another widely used approach for binding energy calculation are the MM/PBSA or MM/GBSA methods (Kaukonen et al., 2008). Free energy calculations using MM/PBSA or MM/GBSA methodology are conducted on collective assemblage of structures that are sampled during MD simulations using implicit solvent. By the application of energy minimized structure of receptor; the ligand binding free energies can be estimated. Implicit solvation approximations speed up the calculations, reduce overall completion time and utilize lower computational power in comparison to conventional MD simulation. The free energy is calculated applying PB implicit solvent technique based on SASA using thermodynamic variables based on experimental conditions for regulating specific parameters (for *e.g.* ionic strength, external and internal dielectric constants) (Suenaga et al., 2012; Miller et al., 2012). Free energies calculated using GB also uses implicit solvent method based on SASA. GB method controls other variables such as salt concentration. The biding free energies between the ligand and receptor are calculated as (Hou et al., 2012):

$$\Delta G_{bind} = \Delta H - T\Delta S \approx \Delta E_{MM} + \Delta G_{sol} - T\Delta S$$

where (Hou et al., 2012),

$$\Delta E_{MM} = \Delta E_{internal} + \Delta E_{electrostatic} + \Delta E_{vdw}$$

and (Hou et al., 2012),

$$\Delta G_{sol} = \Delta G_{\frac{PB}{GB}} + \Delta G_{SA}$$

where ΔG_{sol}, ΔE_{MM} and $-T\Delta S$ are solvation energy, change in gas phase molecular mechanical energy and conformational entropy upon binding of ligand. The ΔE_{MM} is calculated as sum of internal energy, electrostatic solvation energy and Van der Waals energy. The ΔG_{sol} is calculated as contribution of polar (PB/GB) and non-polar (surface area) components (Hou et al., 2012).

PBSA and GBSA methods are integrated in MD simulation software suites such as GROMACS (Brooks et al., 1983), CHARMM (Christensen et al., 2009) and AMBER (Wang et al., 2009). Both the methods have been successfully applied for drug binding to the receptor where 59 ligands were tested for binding to 6 different proteins (Hou et al., 2012). The sensitivity of these approaches was evaluated by applying MD simulations for a timescale of 400-4800 picoseconds. The simulation results demonstrated that the length of the simulations did not impact the binding energies seriously. However the solute dielectric constant impacted protein-ligand binding more significantly. The results clearly showed that PBSA and GBSA are important tools in drug designing that can carefully estimate energies and rank inhibitors depending on interactions within the targeted biomolecules.

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Ligand-Based Drug Design

Ligand-based drug designing is an indirect method for generating of pharmacologically active compounds interacting with target biological compounds. The first step in drug development will be proper identification of the target molecule associated with a disease present in the biochemical pathway. In the absence of experimental structure or homology model, ligand molecules are analyzed to comprehend the structural and physico-chemical characteristics of the ligand that correlates with targeted pharmacological activity of the ligand.

Ligand-based drug design is generally performed using QSAR and pharmacophore modeling. QSAR is an approach for correlating structures of chemical compounds and biological activities. The first step of QSAR involves identification of the active ligands. The ligands are screened from the drug databases such as PubChem, ChEMBL, NCI, etc by a process called "virtual screening." Next suitable molecular descriptors are identified for the ligand. The molecular descriptors can be characterized into 1D, 2D, 3D or 4D that comprises of the description of shape, volume, density, dipole moment, solvation energy, water accessible surface area, potential energy, torsion energy, electronic energy, electrostatic energy, Van der Waals energy, etc. describing the general characteristics of the ligand.

In the following step a mathematical expression is written for correlating the molecular descriptors and the biological activity. Finally, the QSAR model is constructed and validated for its statistical and predictive abilities (Acharya et al., 2011). The application of QSAR using HypoGen/Discovery Studio softwares for constructing pharmacophore models with hydrophobic and hydrogen bond acceptor features for cathepsin D inhibitors for prevention of various degenerative diseases such as Alzheimer's disease and neuronal ceroid lipofuscinosis (Sakkiah, Thangapandian, & Lee, 2012). Fischer's randomization method was used for identifying correlation between experimental and predicted activity. They obtained 49 compounds as potent cathepsin D inhibitors based on consensus scoring values (Sakkiah, Thangapandian & Lee, 2012).

QSAR approach can be performed using three major statistical methods, namely: MLR, PCA and PLS. The MLR is performed using stepwise regression for finding the best model. MLR has also been used in developing Web-based software (PreADME) which performs *in silico* ADMET (Absorption, Distribution, Metabolism and Toxicity) predictions which is dependent on relationship between molecular descriptors and activity profiles of the compounds (Khan, Mahmud, & Ingebrigt, 2009). One such study using MLR analysis was performed on thermolysin protein responsible for several bacterial infections such as cholera, gastritis and peptic ulcer (Jin et al., 1996; Winfield, Inniss, & Smith, 2009). Thermolysin inhibitors were constructed applying ADMET parameters using QSAR models derived from MLR analysis. This was performed using a Web-based tool called PreADME (http://www.bmdrc.org/04_product/01_preadme.asp). The MLR has also been used successfully in prediction of lead compounds in the development of anti-proliferative agents (Winfield, Inniss, & Smith, 2009). Celecoxib, an FDA approved drug used for treatment of colon cancer was used for development of pyrazole-based molecules as celecoxib was found to cause cardiovascular and cerebral vascular diseases.

There are various steps required for QSAR analysis using MLR. In the *first step*, database of molecules is built and the initial conformations of molecules are energy minimized using a force field. Then molecules are aligned using an alignment tool. In the *second step*, relationship between anti-proliferative activity and structure of compounds is calculated which can include several 2D and 3D molecular descriptors such as *pKa, protein binding, volume of distribution, etc*. From a subset of descriptors, those compounds are selected which shows significant association with anti-cancer activity performed using

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Contingency coefficient, Cramer's V, uncertainty coefficient and correlation coefficient (Winfield, Inniss, & Smith, 2009). In the *third step*, selected compounds are subjected to conformational analysis and alignment. Alignment generates an overlap score and the compounds with highest overlap score are selected. Compounds which best fits the pharmacophore are separated into training and test sets. In the *fourth step*, multiple linear regression equation is determined using statistical software such as SAS for anti-cancer activity prediction. Then, stepwise regression is performed in which descriptors are added or removed based on p-values of F-statistics. Those descriptors which satisfy the minimum significance criteria are considered as 'predictors'. PCA on the other hand generate data from multiple redundant variables into smaller uncorrelated variables (Wold, 1987). This method is used to find collinearities and multicollinearities in multiple and multivariate QSAR. This has been used previously for finding trimethoprim derivatives (Mager, 1982). PLS is an improvised technique which uses combination of MLR and PCA techniques. An improved method of PLS called the non-linear PLS (NPLS) overcomes the inability of PLS for obtaining nonlinear model between principle component of dependent and independent variables (2; Tang & Li, 2008).

QSAR

2D-QSAR

In the QSAR method, several hydrophobic, electronic and steric features are correlated with the biological activities represented by (Acharya et al., 2011):

$$\log\left(\frac{1}{c}\right) = k_1\pi - k_2\pi^2 + k_3\sigma + k_4E_s + k_s$$

C is the concentration of compound needed for biological activity, π is partition coefficient, σ is Hammett electronic substituent constant and E_s is the steric substituent constant. Different chemical groups of the compound mediate specific biological activity. When the chemical group is substituted, the specific biological activity of the substituted compound is evaluated by the summation of parent molecule activity and individual substituent contribution. The molecular descriptors used (for correlation with the biological activity) represent fragments of the parent molecule. The advantages of using fragment-based descriptors include computational ease, diversity of available substituents and their convenient mathematical implementations (Acharya et al., 2011).

3D-QSAR

3D-QSAR uses 3D properties of ligands for correlating and predicting the biological activity. This can be performed using several statistical approaches such as PLS and MLR described above. Several 3D-QSAR techniques such as CoMFA and CoMSA methods have been developed which utilizes the data from the compounds stored in drug databases and derive correlation between the activity of a ligand and its 3D features.

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CoMFA

CoMFA (Comparative Molecular Field Analysis) is a technique for developing correlation between 3D properties of ligands and biological activities. Several optimized conformations of a ligand are generated. The optimized conformations of a ligand are placed in 3D grid and a probe atom is used for calculation of steric, electrostatic, potential and Van der Waals energies (Kubinyi, 2008) A cutoff value for the energies is specified and the conformations which exceed the cutoff values are rejected. Probe atom not only helps in determining the energies but it also helps in determining the activity of the ligand using PLS method (Acharya et al., 2011). Result of the analysis is represented by contour maps which show favorable and unfavorable steric regions around the molecules. The CoMFA has been successfully applied in the development of novel N1-amino-acid substituted 2,4,5-triphenyl imidazoline derivatives for inhibition of p53-MDM2 binding which is responsible for malignant tumor growth in humans (Hu et al., 2012).

CoMSA

CoMSA (Comparative Molecular Surface Analysis) is a method similar to CoMFA (Acharya et al., 2011) and calculates similarity indices by comparison between individual ligand molecule with a common probe having charge, radius, hydrophobicity and hydrogen bond properties similar to (Reddy et al., 2007).. The similarity indices describe receptor-ligand binding interaction energy. CoMSA has also been successfully used for designing molecules for synthesis of new HIV-1 integrase inhibitors which uses Kohonen Self-Organizing Maps (SOM) which allows projection of 3D molecular data into 2D maps (Niedbala et al., 2006).

Cloud Computing Advances in Drug Discovery

Cloud computing is a technology for delivering computational resources using Web technology. Molecular docking and MD simulations require extensive computational power and time for performing high throughput analysis. Cloud computing technology reduces the huge investment in computer power and time by providing same resources over the Internet. The end user can utilize such resources on-demand which means that user can demand amount of computational power for performing high throughput analysis. Such technology has been recently incorporated by InhibOx (http://www.inhibox.com). It has developed next generation drug discovery platform and delivers cloud-based drug development services. Virtual screening of active site of biological receptor protein has been performed by molecular docking tools such as AutoDock (Morris et al., 2009). It applies distributed/grid computing technology that integrates several individual computers generating a super computer with huge computational power (Shudong et al., 2004). Similar research has been performed by cloud computing using Internet version of AutoDock. Using 100 million compound databases of 3D candidate compounds with pre-calculated shape, charge and stereochemistry can be used to guide the process of virtual screening. Using such massively computer intensive and rigorous approach, cloud computing makes the whole approach flexible.

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CONCLUSION

In this review, we discussed various computational approaches for drug design from virtual screening of ligands from drug databases to computation of ligand binding free energies using statistical and thermodynamic methods. We clearly outlined both the structure and ligand-based approaches requiring known target 3D structure or clear description of 3D features of the ligand for optimal binding to the receptor for QSAR approach, respectively. Ligand binding to the receptor active site can be performed using long MD simulations, however it requires the extensive processing and graphical power which states current limitation of the approach for larger macromolecules. However, by employing certain molecular mechanics approaches such as PBSA or GBSA can speed up the computation otherwise done for classical all-atom MD approach. Development of cloud-based technology can also help drug discovery projects with higher computational power delivered at a reduced cost.

FUTURE DIRECTIONS

Advances in computational techniques and resources have led to the development of smart and innovative methods in drug designing. However, current research and developments are targeted mostly towards a single receptor protein responsible for causing disease. This approach however creates a targeted drug but neglects the potential effects caused due to various interactions with other proteins and biomolecules which creates a plethora of potential side effects. Current approaches outlined can only be used for designing derivatives of known compounds by various statistical and thermodynamic approaches which completely fail to consider several design parameters aimed at targeting causal biomolecule at targeted site. Certain design parameters and smarter algorithms which can consider the toxicological and other side effects of the compounds during pharmacophore modeling can be manufactured which can specifically target the protein of interest. Also, development of nanoparticles can also enhance the specificity of the ligand by allowing the nanoparticle to carry the drug concentration to the specific target site. Such smarter approaches and innovative thinking can help in the development of novel therapeutic compounds for combating target diseases such as cancer.

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KEY TERMS AND DEFINITIONS

Cloud Computing: A term that involves services provided over the Internet. This idea has been implemented from distributed/grid computing that integrates several individual computers to generate a super computer system of superior computational abilities utilizing computing resources allocated on-demand over the Internet.

Computational Drug Design: Drug designs using highly specialized and sophisticated software.

Drug: Chemical substance(s) that can have either medicinal, hallucinogenic or performance improving properties administered as an external/foreign agent to either humans or animals.

Ligands: Small molecules forming complexes with other complex biomolecules such as nucleic acids and proteins to perform highly specialized biochemical reactions and/or transformations.

Molecular Docking: Molecular docking involves knowledge of putative binding sites where the ligand can bind and initiate a biological response.

Molecular Dynamics: Specialized software used for simulating physical movements of atoms and molecules.

Pharmacophore: Molecular characteristics of ligands for identifying specific target biomolecuels.

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APPENDIX: ABBREVIATIONS USED IN THE CHAPTER

3D: Three-Dimensional;
4D: Four-Dimensional;
ADMET: Absorption, Distribution, Metabolism and Toxicity;
CADD: Computer Aided Drug Design;
CoMFA: Comparative Molecular Field Analysis;
CoMSA: Comparative Molecular Surface Analysis;
FEP: Free Energy Perturbation;
GB: Generalized-Born;
GBSA: Generalized-Born Surface Area;
HTS: High-throughput Screening;
MC: Monte Carlo;
GA: Genetic Algorithm;
MD: Molecular Dynamics;
MLA: Multivariate Linear Regression Analysis;
MM/GBSA: Generalized-Born Surface Area;
MM/PBSA: Molecular Mechanics/Poisson-Boltzmann Surface Area;
MM: Molecular Mechanics;
NMR: Nuclear Magnetic Resonance;
NPLS: Non-Linear PLS;
PB: Poisson-Boltzmann;
PBSA: Poisson-Boltzmann Surface Area;
PCA: Principle Component Analysis;
PLS: Partial Least Square Analysis;
QM: Quantum Mechanics;
QSAR: Quantitative Structure Activity Relationship;
SASA: Solvent Accessible Surface Area;
SBDD: Structure-Based Drug Design;
SOM: Self-Organizing Maps;
TI: Thermodynamic Integration.

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Section 5
Nanostructures and Technology

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Chapter 46
Hybrid Plasmonic Nanostructures:
Environmental Impact and Applications

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ABSTRACT

Plasmonic hybrid nanostructure including Semiconductor-metallic nanoparticles, and graphene-plasmonic nanocomposites have great potential to be used as photocatalyst for hydrogen production and for photodegradation of organic waste. Also, they are potential candidate as active materials in photovoltaic devices. Plasmonic-magnetic nanocomposites could be used in photothermal therapy and biomedical imaging. This chapter will focus on the environmental impact of these materials and their in-vitro and in-vivo toxicity. In addition, the applications of these hybrid nanostructures in energy and environment will be discussed in details.

1. INTRODUCTION

Recently, population growth and fast urbanization and industrialization have been increased dramatically. Therefore, several serious environmental issues such as; climate changes, global warming and pollution of water resources were still a main challenge for the whole world. These issues may become even worse if no efficient/sustainable solutions has been developed to reduce their reasons.(Li et al. (2015)) Due to these tremendous advances, the search for the suitable platforms, and materials to be used in wide-variety of environmental applications became the main target for several research groups.

Plasmonic materials and their hybrid nanostructures have unique and intriguing physico-chemical and biological properties compared to their bulk counterparts. This is attributed to their high *surface-to-volume* ratio, shape and size dependent optical properties and their localized surface Plasmon.(Li et al. (2001), Sharma et al. (2008), Iglesias-Silva et al. (2007), Huang and Yang (2008) and El-Nour et al.

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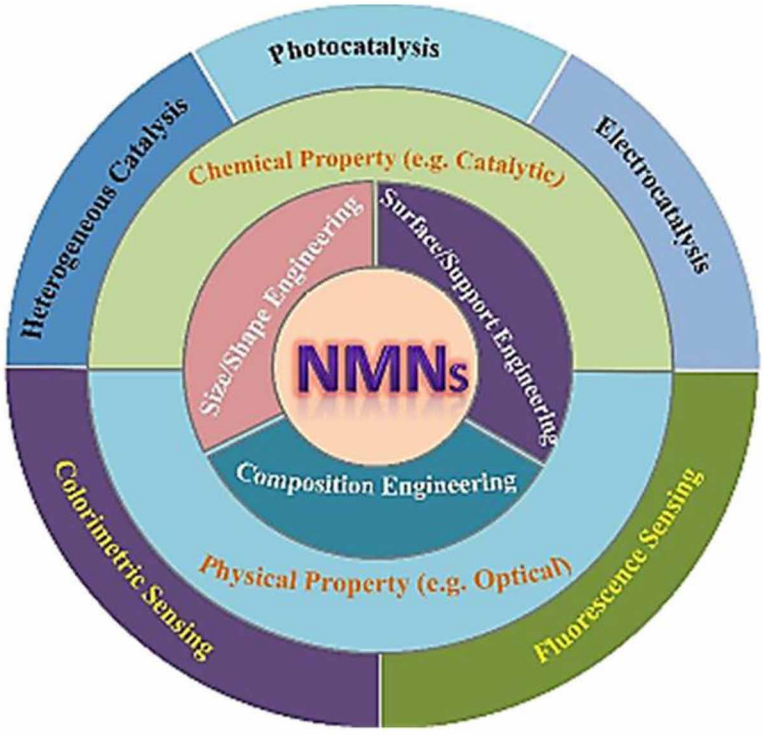
(2010)) Additionally, the ease of surface modification functionalization to be utilized in different applications.(Qu et al. (2013), Cuenya (2010), and Zhang et al. (2010)) Therefore, plasmonic nanomaterials and their hybrid nanocomposites represent new class of materials which could be good candidates to provide promising solutions for the most environmental issues.(Guo and Wang (2011), Cao et al. (2013), and Corma and Serna (2006)) These possible applications can varies either from their catalytic and photocatalytic applications, light harvesting, sensing, and antimicrobial applications (Figure 1). (Li et al. (2015))

2. ENVIROMENTAL APPLICATIONS OF PLASMONIC NANOPARTICLES

a. Silver Nanoparticles

Silver nanoparticles exhibit unique optical, catalytic, sensing and antimicrobial characteristics which made them have huge industrial demands, pegged at 16,000 to 24,000 tons per year. As an antimicrobial agent, silver nanoparticles are expected to have annual demand of 2800 tons/year in the fields of 'food, hygiene and water purification, in addition to 3125 tons/year for medicine. Only 36% of the silver consumed in these industrial processes is being recycled and the rest is let out to the environment. The widespread

Figure 1. Schematic illustration of the possible applications od plasmonic nanoparticles in the environmental field. Reproduced from[Li, J., Zhao, T., Chen, T., Liu, Y., Ong, C.N. and Xie, J., 2015. Engineering noble metal nanomaterials for environmental applications. Nanoscale, 7(17), pp.7502-7519] Published by The Royal Society of Chemistry



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application of Ag NPs in our daily life will unavoidably increase the human and ecosystem exposure. Furthermore, during their production, transport, erosion, washing or disposal of Ag NP containing products, results in their release to the environment. Though the long historical use of silver has not shown obvious adverse effects, there is concern about the potential risks of Ag NPs in the environment.(Lima et al. (2012) and Yu et al. (2013))

i. Antimicrobial Applications

Ag NPs could be used as antimicrobial agents in the health industry, food storage, textile coatings, cosmetics and a large number of environmental applications. Silver nanoparticles were applied in a wide range of applications from disinfecting medical devices and home appliances to water treatment (Bosetti et al. (2002), Cho et al. (2005), Jain and Pradeep (2005), and Li et al. (2008)) Textile industry use Ag NPs in different textile fabrics. In this direction, silver nanocomposite fibers were prepared containing silver nanoparticles incorporated inside the fabric.(Montazer et al. (2012), Xue et al. (2012), Khalil-Abad et al. (2010)), In addition, Ag NPs might be used as surface disinfection agent.(Kumar et al. (2008), Roe et al. (2008), Jain et al. (2009), Gottesman et al. (2011), and Freeman et al. (2012)) This property can be achieved through different ways, such as embedding into bactericidal coating,(Kumar et al. (2008)), surface functionalization of plastic catheters and other medical devices,(Roe et al. (2008)), gel formulation for topical use,(Jain et al. (2009)) coating of packing papers to be used in food preservation,(Gottesman et al. (2011)), or by impregnating fabrics for clinical clothing.(Freeman et al. (2012)).

The mechanism of silver nanoparticles as antimicrobial has been studies against many types of bacteria and biofilms. Table 1 summarizes the mechanism of action and Figure (2) describes how silver nanoparticles can be toxic to bacteria.

Table 1. Details of Ag NPs and their mechanisms of action against bacteria and biofilms

Bacterial Strain	Mechanism of Action
Acinetobacterbaumannii	Alteration of cell wall and cytoplasm
Escherichia coli	Alteration of membrane permeability and respiration
Enterococcus faecalis	Alteration of cell wall and cytoplasm
Klebsiellapneumonia	Alteration of membrane
Listeria monocytogenes	Morphological changes, separation of the cytoplasmic membrane from the cell wall, plasmolysis
Micrococcus luteus	Alteration of membrane
Nitrifying bacteria	Inhibits respiratory activity
Pseudomonas aeruginosa	Irreversible damage on bacterial cells; Alteration of membrane permeability and respiration
Proteus mirabilis	Alteration of cell wall and cytoplasm
Staphylococcus aureus	Irreversible damage on bacterial cells
Staphylococcus epidermidis	Inhibition of bacterial DNA replication, bacterial cytoplasm membranes damage, modification of intracellular ATP levels
Salmonella typhi	Inhibition of bacterial DNA replication, bacterial cytoplasm membranes damage, modification of intracellular ATP levels
Vibrio cholera	Alteration of membrane permeability and respiration

Reprinted from [Franci, G., Falanga, A., Galdiero, S., Palomba, L., Rai, M., Morelli, G. and Galdiero, M., 2015. Silver nanoparticles as potential antibacterial agents.Molecules, 20(5), pp.8856-8874.] Published by MDPI AG, Basel, Switzerland

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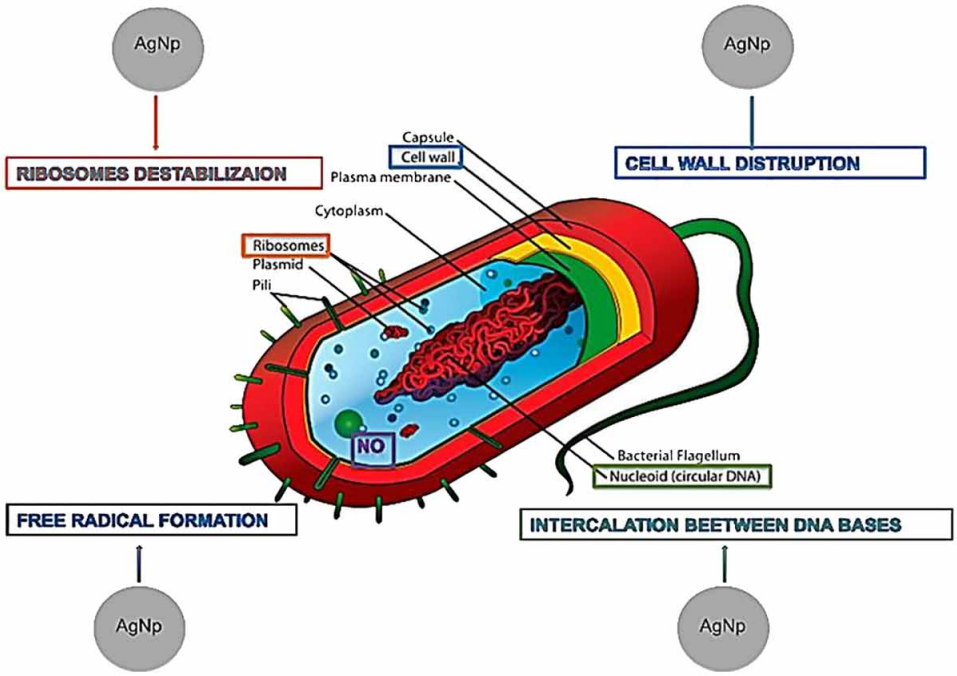
Several products include containing Ag NPs have been approved by a range of accredited authorities such as, FDA, EPA, and SIAA of Japan.(Azonano, El-Nour et al. (2010), Jia et al.(2008) and Bhattacharya and Mukherjee(2008))

For these purpose, the toxicity of silver nanoparticles has been studied in different mammalian cell systems, including rat and human cells. (i.e. rat liver cells, (Hussain et al. (2005)) human keratinocytes and fibroblasts cultures,(Burd et al. (2007)) and human spermatogonial stem cells.(Braydich-Stolle et al. (2005)) *In vitro* studies shows that an elevated dose of nano silver induces oxidative stress (liberation of reactive oxygen species) as a mechanism of cytotoxicity.(Arora et al. (2008)) At the safe concentration range, silver nanoparticles have been described to cause an exert anti-inflammatory effects as: acceleration of wound healing,(Tian et al. (2007)), inhibition of allergic contact dermatitis in mice, suppression of the expression of TNF-α and IL-12, and induction of apoptosis of inflammatory cells.(Bhol and Schechter (2005)), modulation of cytokine production and induction of peripheral blood mononuclear cells proliferation (Figure 2),(Shin et al. (2007)).

ii. Water Purification and Disinfictents

Water is one of the most important substances on Earth and is essential to all living things. Although 70% of the Earth is covered with water, only 0.6% is suitable for human uses. According to the WHO, at least 1 billion people do not have access to safe drinking water. Contamination of drinking water are the leading cause of death in many developing nations.(Pradeep (2009), Tran et al. (2013) and Gusseme et al. (2010))

Figure 2. Mechanisms of Ag NPs' toxic action
Reprinted from [Franci, G., Falanga, A., Galdiero, S., Palomba, L., Rai, M., Morelli, G. and Galdiero, M., 2015. Silver nanoparticles as potential antibacterial agents. Molecules, 20(5), pp.8856-8874].Published by MDPI AG, Basel, Switzerland



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Complexity of contaminants, population growth, presence of resistive pathogens and their impact on the environment create a demand for advanced technologies for clean and safe drinking water. Several chlorine and halo-compounds such as tri-halo methanes, halo-acetic acids, halo-acetonitrile, halo-ketones and other DBPs were found to cause colon, rectal and bladder cancers and adverse reproductive disorders. Ag NPs are very ideal to be used in water disinfection. It could be easily incorporated to polymeric membranes to disinfect the water contaminated from bacteria and viruses.(Gusseme et al. (2010 and 2011), Lv et al. (2009), Yakub and Soboyejo (2012), Jain and Pradeep (2005), Gangadharan et al. (2010), Zhang et al. (2012), and Sheng and Liu (2011)) Moreover, the addition of silver-based NPs could prevent bacterial/viral attachment and biofilm formation in filtration medium.(Sintubin et al. (2011) and Fernández et al. (2015)). The application of silver-based NPs is of utmost importance to prevent outbreaks of waterborne diseases related to poor treatment of drinking water.

b. Gold Nanoparticles

Gold nanoparticles have unique optical and electronic properties due to their surface Plasmon resonance. Thus, many researchers developed the use of gold nanoparticles and their composites in many areas such as biological tagging, chemical and biological sensing, optoelectronics, photo thermal therapy, biomedical imaging, DNA labeling, microscopy and photo-acoustic imaging, drug delivery, surface-enhanced Raman spectroscopy, tracking and drug delivery, catalysis and cancer therapy and imaging.(Kim and Jon (2012), Khlebtsov and Dykman (2010), and Tedsco et al. (2010))

Environmental applications of gold nanoparticles has been limited to fabrication of gold nanoparticles based sensors to detect various metal ions by working on the principle of colour change due to the aggregation of gold nanoparticles. Such types of sensors have been widely used for the detection of copper, mercury lead and arsenic in water (Nalawade et al. (2012)). Another very promising application is using them in catalysis to convert harmful gasses into less toxic or environmental friendly gasses. In particular, using gold nanoparticles, or nanoalloy of gold and another metal loaded into metal oxides are catalytically very active for a wide range of reactions, (Kim et al (2004)) including CO oxidation,(Yoon et al. (2005) and Gluhoi and Nieuwenhuys (2004)) water gas shift reaction,(Liu et al. (2005)) NO_x reduction, CO and CO_2 hydrogenation, NO oxidation, alkene epoxidation and selective oxidation of hydrocarbons. (Hughes et al. (2005))

c. Enviromental Appliations of Plasmonic Hybrid Nanocomposites

Magnetic nanoparticles, especially iron (Fe^0), magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) nanoparticles, have sparked an enormous interest in applications for treatment of polluted water or subsurface environments.(Yantasee et al. (2007), Shen et al. (2009 and 2011), Tang and Lo (2013)). Magneto-plasmonic nanostructures open an opportunity for some many environmental applications, such as sunlight photocatalytic inactivation of Bacteria (Zhai et al. (2011)). Zhai and co-workers illustrated a convenient method to fabricate noble metals based super-paramagnetic nano hybrid hetero-structures (i.e. Ag-Fe_3O_4 and Au-AgCl@Fe_3O_4). They reported in their study that Au-AgCl@Fe_3O_4 nanotubes was an excellent candidate for photocatalytic inactivation of bacteria upon irradiation with sunlight (Figure 3). (Zhai et al. (2011))

Sun and co-worker (Sun et al. (2015)) developed another system based on dumbbell-like structure α-Fe_2O_3/Ag/AgX heterostructures via *in-situ* oxidation reaction. In such system showed a higher photocatalytic activity than both of α-Fe_2O_3 and α-Fe_2O_3/Ag systems as shown Figure (4).

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Figure 3. Schematic depiction of the synthesis of petal-like Ag/Fe_3O_4, Ag nanocube/Fe_3O_4, Ag nanowire/Fe_3O_4, and Fe_3O_4/Au-AgCl nanotube composites. And Survival rate of E. coli under direct sunlight (700 W/m^2) without hybrids and with 0.016 mg/mL Fe_3O_4/Au/AgCl nanotubes

Reprinted with permission from (Zhai, Y., Han, L., Wang, P., Li, G., Ren, W., Liu, L., Wang, E. and Dong, S., 2011. Superparamagnetic plasmonic nanohybrids: shape-controlled synthesis, TEM-induced structure evolution, and efficient sunlight-driven inactivation of bacteria. ACS nano, 5(11), 8562-8570.Copyright (2011) American Chemical Society

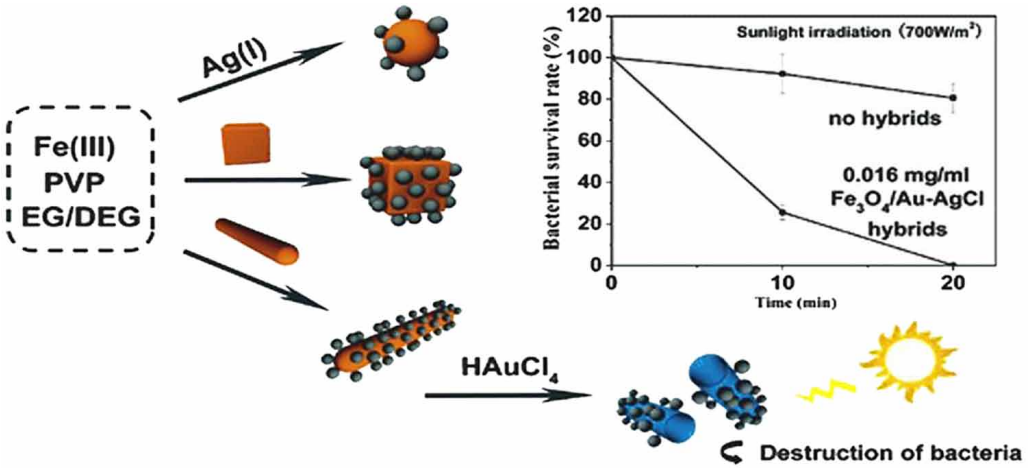
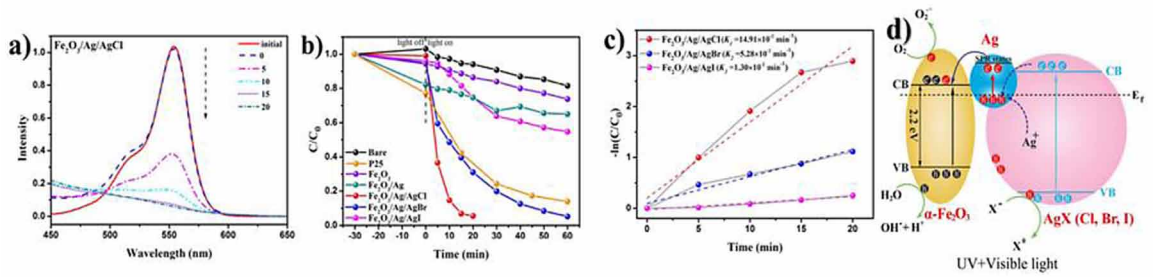


Figure 4. (a) UV–vis spectral evolution of RhB as a function of light irradiation time over α-Fe_2O_3@Ag/AgCl ; (b) Photocatalytic degradation of RhB solution over as-prepared samples as a function of irradiation time under simulated sunlight irradiation; (c) kinetic linear simulation curves of the α-Fe_2O_3/Ag/AgX (X = Cl, Br, I)photo-catalysts for the photo-degradation of RhB under simulated sunlight irradiation. (d) Schematic illustration of the photocatalytic process for dumbbell-like α-Fe_2O_3/Ag/AgX (X = Cl, Br, I)

Adopted with permission from (Sun, L., Wu, W., Tian, Q., Lei, M., Liu, J., Xiao, X., Zheng, X., Ren, F. and Jiang, C., (2015). In situ Oxidation and Self-Assembly Synthesis of Dumbbell-Like α-Fe2O3/Ag/AgX (X= Cl, Br, I) Heterostructures with Enhanced Photocatalytic Properties. ACS Sustainable Chemistry & Engineering..Copyright (2015) American Chemical Society



Metal oxide semiconductors nanoparticles hold great promise for wide range applications, such as energy conversion and storage, environmental remediation, and etc... However, critical factors including the high charge – carrier recombination rate and narrow light absorption have restricted more practical and viable applications. ((Pelaez et al. (2012)) Particularly, TiO_2 is one of the most candidates which got a lot of interests from several research groups, due to its low cost and high stability. (Fang et al. and Pelaez et al. (2012), Chehadi et al. (2016)). However, TiO_2 has large energy band-gap of 3.2 eV and only absorbs solar radiations in the ultraviolet (UV) region, while the UV lights only contribute to less than

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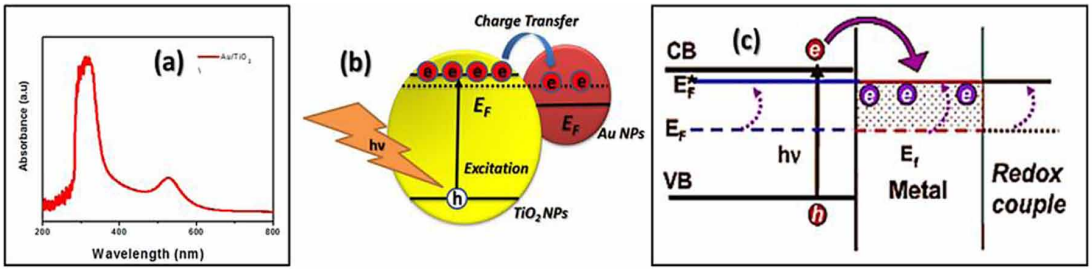
10% of total solar radiations; the visible lights. On the other hand, contribute to 50% of the solar radiations.(Pelaez et al. (2012)) Furthermore, the photo-catalytic activity of TiO_2 decreases substantially with prolonging the service time; and the deactivation of TiO_2 – based photo-catalysts is a crucial drawback for the application.(Wang et al. (2012)) There have been many research efforts devoted to improve the photocatalytic activity by incorporating metals or ions (i.e. Ag, and Au NPs),(Arunachalam et al. (2012) and Suvith and Philip (2014), Bhakya et al. (2015)) to broaden the absorption of solar radiations and to re-activate and regenerate TiO_2-based photocatalysts.(Sangpour et al. (2010), Wu et al. (2010), Linic et al. (2011), Kochuveedu et al. (2013))

Over the past decade, the idea of fabricating hybrid nano-objects that composed of plasmonic metals and semiconductors was emerged, due to their localized surface Plasmon resonance and the formation of effective Schottky junctions.(Subramanian et al. (2003 and 2004), Hirakawa and Kamat (2005)). In such nano-hybrid system; plasmonic nanoparticles are able to absorb visible light intensely due to the surface plasmon resonance (SPR) effect. Through the SPR effect, the electromagnetic field of incident light couples with the oscillations of conduction electrons in the plasmonic nanoparticles, resulting in surface plasmon absorption and an enhancement of the local electromagnetic fields close to the surface of plasmonic nanoparticles. Therefore, plasmonic nanostructures of noble metals in combination with semiconductors offer a promising future for the next generation of energy and environmental needs (Figure 5).(Subramanian et al. (2003 and 2004), Sangpour et al. (2010), Wu et al. (2010), Linic et al. (2011), Kochuveedu et al. (2013))

Contamination of waste water with organic compounds (i.e. dyes, pesticides, drugs, phenolic compounds and polymers) has huge hazardous effect in the environment and spontaneously affects human health. Conventional treatment method of organic pollutant is based upon removing large amounts of organic compounds from waste water streams by adsorption. The disadvantage of this method is that it is non-destructive for the organic pollutants, which simply transfers the pollutants from one phase/substance to another. Photocatalytic degradation of organic pollutants using UV or sun light in presence of nano-catalyst has been a great interest because it has the capability of converting these organic hazards into

Figure 5. (a) Absorption Spectra of Au-TiO_2 Hybrid Nanocomposites. Copyright © D M Fouad and M B Mohamed, "Comparative Study of the Photocatalytic Activity of Semiconductor Nanostructures and Their Hybrid Metal Nanocomposites on the Photodegradation of Malathion," Journal of Nanomaterials, vol. 2012, Article ID 524123, 8 pages, 2012. (b) Fermi level equilibrium in semiconductor-metal nanocomposite system, (c) Equilibrium of semiconductor –metal nanocomposites with redox couple after irradiation

Adopted with permission from (Subramanian, V., Wolf, E.E. and Kamat, P.V., 2004.Catalysis with TiO2/gold nanocomposites. Effect of metal particle size on the Fermi level equilibration. Journal of the American Chemical Society, 126(15), pp.4943-4950 Copyright (2004) American Chemical Society



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non-toxic compounds. In the recent years, numerous metal oxides, plasmonic and plasmonic graphene hybrid nanocomposites have attracted growing attentions for photo-degradation of organic pollutants. (Wu et al. (2010), Linic et al. (2011), Pelaez et al. (2012), Wang et al. (2012), Kochuveedu et al. (2013))

Phenolic based – organic hazardous compounds are considered one of the most and highly spread highly toxic wastes. This is due to the difficulty of their biological degradation, leading to a setting up of restricted level of phenol in the environment.(Hu et al. (2009)) Phenolic based water pollutant compounds, include 2-chlorophenol (2-CP), 2,4-dichlorophenol (2,4- DCP), and trichlorophenol (TCP) have been used in photo-catalytic activity assessment, upon irradiation via sunlight.(Hu et al. (2009)) Several reports has been devoted to use a hybrid nano-objects based on combination between plasmonic nanostructures and metal oxide as a catalyst supports.(Hu et al. (2009) and Chehadi et al. (2016)) Chehadi and co-worker demonstrated the influence of Au NPs as an example of the plasmonic nanostructure on the photocatalytic efficacy of various metal oxide (i.e. TiO_2, ZnO, and Al_2O_3) as a catalyst supports on the photo-degradation of Bisphenol A (BSA) upon laser irradiation. In addition, investigate the effect of different parameter such as, irradiation time, laser power, and catalyst type. They reported that Au/TiO_2 showed a fastest degradation rate than other composite systems. (Chehadi et al. (2016)) Another study has been illustrated by Hu et al. (Hu et al. (2009)). They developed metal-semiconductor nanocomposites base on coupling between plasmonic nanostructures Ag-AgI with mesoporous alumina using deposition-precipitation, and photo-reduction techniques.(Hu et al. (2009)) They reported that their configuration showed higher photo- sensitivity, stability and catalytic activity for the degradation and mineralization of toxic persistent organic pollutants especially Phenolic based organic pollutants upon irradiation using simulated sun light.(Hu et al. (2009)) in addition the capability to be used not only as a photo-catalyst, but also photovoltaic fuel cells (Figure 6).(Hu et al. (2009))

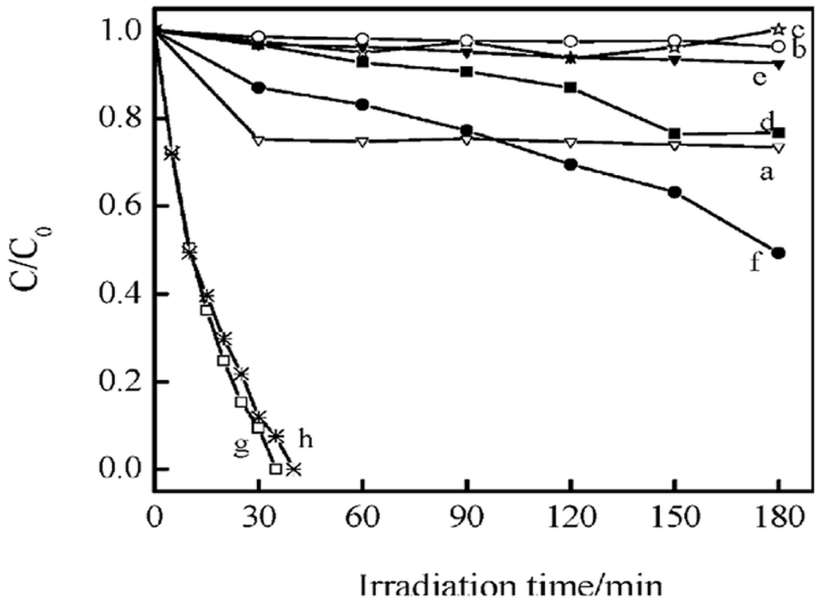
Different shapes of hybrid CdSe/Au penta-pods and nano-dumbblls shows photocatalytic activity for the photo-degradation of organic dyes by irradiation using UV light. Presence of plasmonic at the interface of CdSe Semiconductor nanoparticles enhances the photocatalytic activity three times more (Figures 7 and 8). This is due to the electron transfer from CdSe to Au Fermi level. (Costi et al. (2008), Gao et al. (2011), Tongying et al. (2012) and Haldar et al. (2012))

Recently, Zhou et al. (Zhou et al. (2015)) developed a ternary hybrid nanocomposites of semiconductor-plasmonic hetero-nanostructures loaded into graphene has been developed and display extraordinary photocatalytic efficiency induced by the plasmonic energy that operates in the Ag@CdSe-rGO hybrid ternary composites. The obtained plasmonic photo-catalysts in nanoscale were fabricated by using a one-step hydrothermal method, during which the in situ nucleation of Ag@CdSe core−shell nanoparticles and the reduction of GO to rGO occurred simultaneously. The enhanced photocatalytic and optical properties toward photo-degrading of organic pollutant under visible light irradiation can be ascribed to plasmonic enhancement of Ag by charge and energy transfer and to the carrier rGO further enhancing the separation of photo-generated e^-/h^+ pairs. Three major roles of Ag core, electronic acceptor by the Schottky barrier formed in Ag@CdSe hetero-structure and induced by the local Plasmon resonance of Ag, have facilitated the separation of photo-generated e^-/h^+ in CdSe QDs, and the plasmonic energy transfer from Ag to CdSe has strengthened the excitation and charge separation of CdSe QDs. The introduction of rGO carrier in the Ag@CdSe-rGO system has enhanced the photocatalytic activity for its high charge carrier mobility and ambipolar field effect Figure (9).

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Figure 6. Temporal course of photodegradation of 2-chlorophenol (10 mg L; 60 mL) in aqueous dispersions (containing catalyst: 1.6 g L-1): (a) Ag-AgI/Al2O3 in dark, (b) no catalyst; (d) AgI/Al2O3, (e) Ag/Al2O3 (f) TiO2-xNx, (g) Ag-AgI/Al2O3 with λ > 420 nm; and (c) AgI/Al2O3, (h) Ag-AgI/ Al2O3 with λ > 450 nm

Adopted with permission from (Hu, C., Peng, T., Hu, X., Nie, Y., Zhou, X., Qu, J. and He, H., (2009). Plasmon-induced photodegradation of toxic pollutants with Ag− AgI/Al2O3 under visible-light irradiation. Journal of the American Chemical Society,132(2), 857-862.Copyright (2009) American Chemical Society



3. CONCLUSION

Plasmonic nanoparticles such as Ag NPs and Au NPs have unique optical, catalytic and electronic properties which made their uses in industrial applications grow tremendously in the last few years. The super-antiseptic and antimicrobial effect of silver nanoparticles lead to new generation of disinfectant, infection control and antibiotics based nano-silver products. The catalytic and photothermal properties of gold nanoparticles developed several based applications in medicine and environment such as drug delivery, cancer therapy and imaging, sensors for detection of heavy metals and bacteria in environment Plasmonic-hybrid nanocomposites including magnetic-plasmonic, metal oxide-plasmonic, or quantum dots/plasmonic opens a new class of photo-catalysts used for the photo-degradation of organic pollutant and waste water purifications, light harvesting and hydrogen productions. Extraordinary photocatalytic activity had been achieved via loading these plasmonic hybrid nanostructures on graphene sheets.

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Figure 7. TEM (upper panels) and HRTEM (lower panels) images of (a) green (QD1), (b) yellow (QD2), and (c) red (QD3) emitting QD and QD/Au. (d) Schematic representation of the entire decay process for CdSe and CdSe/Au

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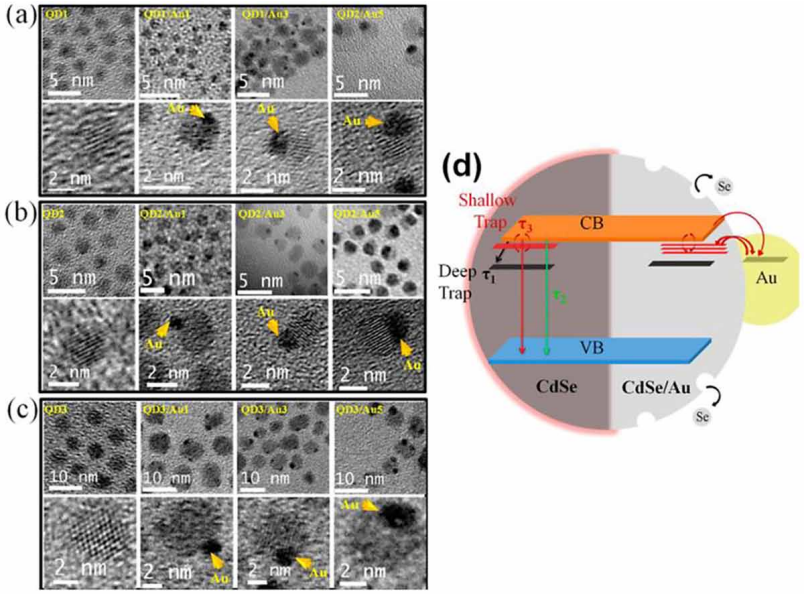
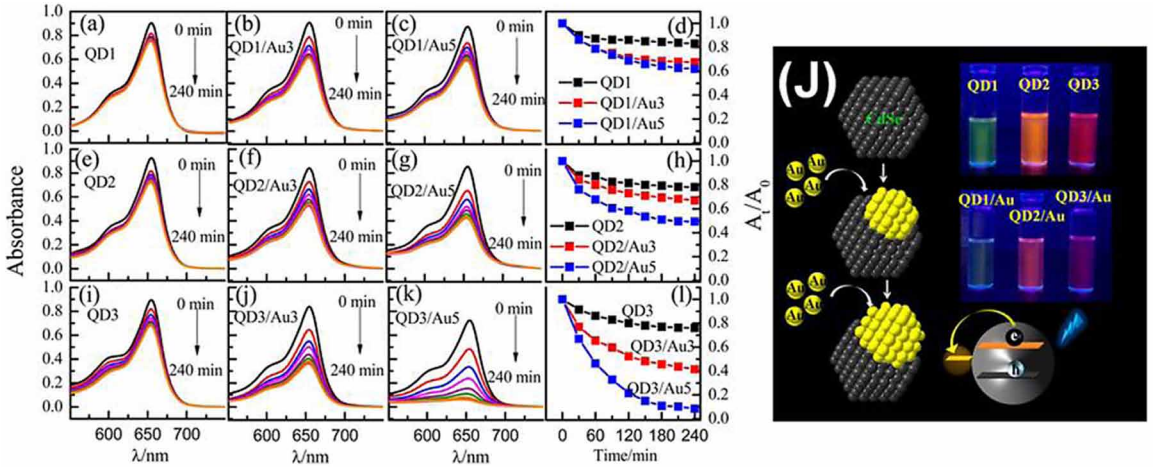


Figure 8. Set of absorption spectra for photocatalytic reduction MB by using (a) QD1, (b) QD1/Au3, (c) QD1/Au5, (e) QD2, (f) QD3/Au3, (g) QD3/Au5, (i) QD3, (j) QD3/Au3 and (k) QD3/Au5. Change in normalized absorption with respect to time for (d) QD1, QD1/Au3, QD1/Au5, (h) QD2, QD2/Au3, QD2/Au5, and (l) QD3, QD3/Au3, QD3/Au5

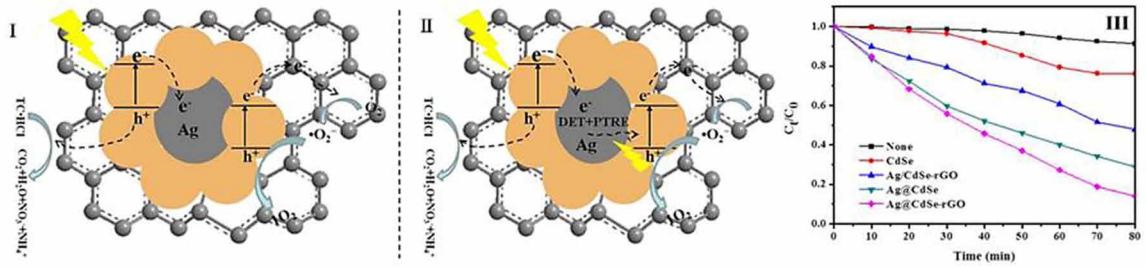
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Figure 9. Illustrations of three different roles of Ag core in enhancing the separation of the photogenerated e^-/h^+ transfer process in the Ag@CdSerGO system: (I) electronic acceptor; (II) DET and PIRET effect induced by the LPR effect of Ag core, (III) The decrease (C/C_0) of 20 mg/L TC·HCl versus irradiation time for the TC·HCl without catalysts, CdSe, Ag@CdSe, and Ag@CdSe-rGO

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Chapter 47
Implementation of Nanoparticles in Cancer Therapy

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ABSTRACT

Cancer is a wide group of diseases and generally characterized by uncontrolled proliferation of cells whose metabolic activities are disrupted. Conventionally, chemotherapy, radiotherapy, and surgery are used in the treatment of cancer. However, in theory, even a single cancer cell may trigger recurrence. Therefore, these treatments cannot provide high survival rate for deadly types. Identification of alternative methods in treatment of cancers is inevitable because of adverse effects of conventional methods. In the last few decades, nanotechnology developed by scientists working in different disciplines—physics, chemistry, and biology—offers great opportunities. It is providing elimination of both circulating tumor cells and solid cancer cells by targeting cancer cells. In this chapter, inadequate parts of conventional treatment methods, nanoparticle types used in new treatment methods of cancer, and targeting methods of nanoparticles are summarized; furthermore, recommendations of future are provided.

1. CANCER BIOLOGY

Cancer, although one of the world's most widely studied medical conditions, remains as the leading cause of death after the heart failure, accounted for 7.6 million deaths (around 13% of all deaths) in 2008. According to comprehensive, 184 country survey of WHO's International Agency for Research on Cancer (IARC), about 70% of all cancer deaths occur in low- and middle-income countries. Worldwide cancer mortalities are envisioned to continue to rise to over 13.1 million in 2030 (http://globocan.iarc.fr/).

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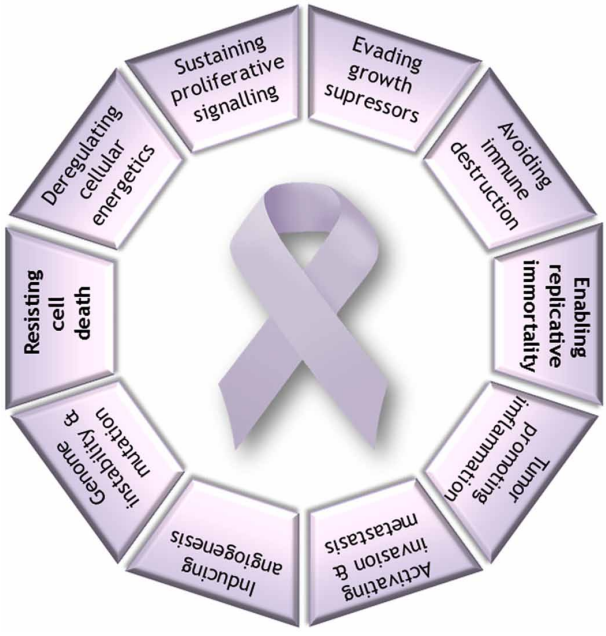
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Normal eukaryotic cells tend to increase their number by mitosis and meiosis, and the cell division process is triggered by activation of the metabolic pathways when the cell size and the environmental conditions are favorable. The metabolic pathways that are necessary for cell division include the specific signaling pathways, the transmitting signal molecules, enzymes and proteins (Cooper & Hausmann, 2013). After completion of division process, the cell enters into a new cycle again and the cycle is repeated approximately 50 times before the cell goes to apoptosis. Apoptosis is the death of a cell programmed by specific genes. The reason for the limited number of cell divisions is telomeres, regions of repetitive nucleotide sequences at each end of a chromatid, which protect the ends of the chromosomes from deterioration or from fusion with neighboring chromosomes. Telomeres shorten with each cell division and eventually the cell cannot be divided further, progresses into senescence or dies (Counter et al., 1992).

Some cells, due to hereditary and environmental factors, continue to grow and proliferate even in the presence of cell growth and division inhibitory factors. Thereby, cells start division without special signals. Cell growth is disrupted, cells begin to divide too quickly and telomere shortening stops due to telomerase activity (Yoshida et al., 1997).When unnatural telomerase activity is observed, cell structure changes, adhesion property increases, abnormal signal transduction occurs, tumor suppressor genes are silenced and growth factors and their receptors are generated irregularly. These are typical characteristics of cancer cells. Therefore, the researchers specify that cancer originates in fast-growing and immortal cells. Abnormal cells affect the cells around them and they also lead to deterioration of their functions. The other most important feature of these cells is that they may maintain their lives regardless of the specific microenvironment. Thereby, they can easily spread to the surrounding tissues, blood and entire body (metastasis). These abnormal cells generate new blood vessels around them by secreting vascularization factors, and they can make the metastasis process more quickly and efficiently (Sooriakumaran & Kaba, 2005) (Figure 1).

Figure 1. Emerging hallmarks of cancer for therapeutic targeting (Adapted from Hanahan & Weinberg, 2011)



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Formation of such a cell abnormality is caused by mutations due to environmental factors and/or heredity. X-rays, UV light, tobacco products, diet and obesity, environmental pollutants and viruses are considered the most common environmental carcinogens.

Although many methods have been developed in the treatment of cancer, the WHO figures suggest that a highly effective treatment technique is yet to be found. Surgery, radiotherapy, chemotherapy, immunotherapy and hormone therapy, which are the most commonly used conventional methods have specific advantages and disadvantages. The chosen treatment type depends on the location, the progress status and the type of cancer. The limitations of status quo treatments are commonly due to non-specific bio-distribution and insufficient targeting of the therapeutic agents, poor oral bio-availability, low therapeutic indices, dose-limiting toxicity to healthy tissues, and almost invariably emerging drug resistance and subsequent challenge of providing remissive therapy. Multimodal therapy methods are under investigation, and researchers are looking into developing alternative therapies. Oncology is one of the disciplines that will potentially benefit the most from nanotechnology. The nanoparticle based targeted delivery vehicles can effectively overcome the major challenges in successful clinical translation, including specificity, proper dosage, overcoming various biological barriers and demonstrating better therapeutic efficacy than that of the current clinical standards.

Conventional Approaches for Cancer Therapy

Surgery

Surgery is the oldest treatment method used to treat cancer (Wu et al., 2006). Initially patients who have an operation had died because of excessive blood loss, difficulties of anesthesia and respiratory insufficiency. Thanks to the advances in the drugs and medical sector, the number of deaths caused by operation is significantly reduced and the therapeutic outcomes are increased.

Surgery is the preferred treatment method for initial stages of solid cancer tumors and benign (non-metastatic)tumors. The efficacy of treatment is inversely proportional to the ability of tumor metastasis. Despite the surgery eradicates solid tumors, it generates low success rate on non-solid and spreading tumors (Rani et al., 2012).

Primarily the tissue specimen is removed by surgical cutting and examined by microscopic analysis. The tumor tissue can be removed by surgery if the structure of the tumor is suitable to be removed. Regional lymphatic nodes are excised during the operation to prevent metastasis. To eradicate the metastatic cells, the surgery is supplemented by another treatment modality. One of the most important disadvantages of surgery is that the surrounding tissues can also be damaged besides the tumor tissue during the operation. The entirety of cancer cells must be cleaned during the operation, because even a very small number of remained cancer cells may lead to recurrence of the tumor. To achieve this, some parts of the healthy tissues around the tumor are also removed during the surgery. Substantial tissue loss can lead to physiological and psychological issues for patients. In addition, locations in the body where operation is very dangerous is an extra limitation for the surgery (Haskell & Berek, 2001).

Radiotherapy

Radiotherapy is a treatment method used to destroy the cancer cells that cannot be removed by surgery from tumor tissue (Love et al., 1999; Haskell & Berek, 2001). In some cases, it can also be used to reduce

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tumor size. In radiation therapy, X-rays, gamma rays and electrons are used. Radiation therapy is used at certain time intervals to provide a period for recovery of the normal cells that are damaged beside the cancer cells during the treatment. Generally, normal cells are less sensitive than tumor cells to radiation. Nevertheless, the dose and location of radiation given to the patient must be arranged very accurately in order to minimize the damage to normal cells (Love et al., 1999). The sensitivity of tumor cells to radiation is also different. Therefore, the effectiveness of radiation therapy is directly proportional to the sensitivity of the tumor to radiation.

Radiation therapy can be used for therapeutic purposes; it can also be used in palliative therapy. Palliative care is also called "comfort care" or "supportive care" given to a patient who has a serious or life-threatening disease, such as cancer, and it can be given at the time of diagnosis and throughout the course of illness. There are two kinds of radiation therapy according to the mode of administration. Beams are generated outside the patient and are targeted at the tumor site in "External Beam Radiation" method (Murray & Robinson, 2011). In "The Internal Radiation" method, the radioactive materials are placed directly into the tumor tissue. The most important parameters for treatment planning and dose calculations are energy loss of radiation, range and scatter of radiation, dose and isodose of radiation (Chopra, 2011).

Radiation therapy kills the cancer cells by damaging their DNA. This treatment damages cells' DNA either directly or by forming free radicals. Damaged cells which are not repaired stop dividing or die. Radiotherapy has serious side effects related to radiation dose, volume of the tissue and administration rate. Radiobiological effects critically depend on the pattern of ionization at the level of the biomolecule. The most common side effects are fatigue, nausea, vomiting, inflammation and edema of the irradiated organ in the early days, and possibly chronic blood supply disorders, neutropenia (sudden drop in white blood cell count), and anorexia (Soussain et al, 2009; Shafiq et al., 2009).

Chemotherapy

Chemotherapy is the use of chemical agents to kill or suppress the growing cancer cells. In contrast to surgery and radiotherapy, chemotherapy is not local and affects the entire body. The main target is rapidly dividing cells in chemotherapy since most cancer cells are characterized by the ability of rapid division. Intestinal epithelial cells, bone marrow cells, mucosal and hair follicle cells and stem cells are rapidly dividing cells in the body and these cells are damaged as well by chemotherapy (Rani et al., 2012). On the other hand, all of the cancer cells are not capable of dividing rapidly. Types of tumors with doubling rate more than 70 days to less than 30 days exist. Due to this non-specificity, selective and/or targeted agents are necessary to expand the effectiveness of chemotherapy. The main target of traditional chemotherapy is prevention of replication and transcription by targeting DNA and thus inhibition of cell growth and division by suppression of protein synthesis. In addition, nowadays, specific protein synthesis pathways, mitotic vehicles, specific antigens and protein kinase inhibitors are studied to target with drugs that may be effective in different cell division phases, and impair the formation of mitotic spindles (Miglarese & Carlson, 2006).

The biggest problem in chemotherapy is toxicity. The toxic effects are often observed because the chemotherapeutic agents have high toxicity and elevated doses are used for the treatment of many cancer types. It is possible to classify these effects under two headings as long and short term toxicity. The short-term toxicity usually emerges quickly and occurs in tissues that have rapid division capability. Vomiting, nausea and dizziness are the main side effects of short-term toxicity (Poli-Bigelli et al., 2003). Long-term

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toxicity usually leads to carcinogenesis, teratogenesis (defect births) and infertility. In addition to these problems, cancer cells can develop resistance to chemotherapy drugs and this constrains effectiveness of chemotherapy. Drug resistance may occur because of over expression of multidrug resistance genes, membrane uptake reduction and the increasing of tolerance to cytoplasmic detoxification, DNA repair and DNA damage. This situation makes it necessary to have new active substances in cancer treatment continuously (Tiwari et al, 2011; Yap etal. 2009).

All these treatments can be used single or in combination. However the point to be considered is that the damage to the healthy cells may be increased with combined and/or repeated therapies since they are not specifically targeted. In recent years, new methods of treatments are applied in order to reduce exposure rates to normal cells. The most prominent of these methods are immunotherapy and hormone therapy (De Schryver et al., 1992; Fong et al., 2001; Burch et al., 2004). The patient's own immune system fights cancer and reduces treatment-related side effects in immunotherapy. The hormones that help the development of tumor may be stopped, or the activities of the hormones that can fight cancer cells, make cancer cells grow more slowly are increased.

2. NANOPARTICLES IN CANCER THERAPY

Nanotechnology is a research area with the potential to be used in a wide variety of fields from biomedical technologies to textiles and from food packaging to informatics. Nanoparticles (NPs) have been available in all areas of human life for a long history, although the word "nanoparticle" has begun to be used widely with developing nanotechnology in late 20th century. People are exposed to nanoparticles in the air, food and beverages constantly (Buzea et al., 2007). Nanoparticles offer unique physical and biological properties particularly in the medical field to overcome cellular barriers and improve the delivery of various bioactive molecules and drugs as well as to enhance molecular imaging, to facilitate early tumor detection, to implement monitoring of therapeutic response and to minimize side effects. There are different mechanisms for the internalization of nanoparticles by cells influenced by nanoparticles' physicochemical properties (Hillaireau&Couvreur, 2009; Susa et al., 2009). However the biggest obstacle against the development of nanoparticle drug delivery systems is the ambiguity in nano-toxicology issues. Although there are comprehensive studies on nano-toxicology, a definitive concensus has not been revealed yet. Many studies have shown possible negative effects of nano-materials on mammalian cells. However, the number of works to prevail these adverse effects has been limited. NPs need to be analyzed more critically and their effects on organisms must be investigated by well-designed *in vitro* and *in vivo* studies to eliminate this problem.

In this section, general information about NPs that are used in drug delivery and targeting in cancer therapy will be presented with their advantages and disadvantages. Selected nanoparticle-based therapeutics approved by federal agencies or still in clinical trials in cancer treatment are tabulated at the end (Table 1).

Polymeric Nanoparticles

Polymeric NPs are obtained by using natural or synthetic polymers in the core/corona part (Alexis et al., 2008). Polymeric NPs are often used for controlled drug delivery systems. Drugs which are used for therapeutic purposes can be encapsulated in the polymer, or loaded on either the surface or the core

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(MuFei Tang et al., 2010). NPs can be produced from biodegradable or non-biodegradable polymers depending on the purpose. Biodegradable polymer NPs may be removed from the body through the normal metabolic pathways by eroding into its constituent oligomers. Their degradation and drug release rates or kinetics can be adjusted by changing the physicochemical properties of the polymer and thus they can be used for different delivery systems (Alexis et al., 2010).

Polymeric nanoparticles may cause tissue damage depending on the toxicity and dosage of the polymer by accumulating in certain parts of the body. However, polymeric NPs are the most preferred NPs in drug delivery/targeting systems and cancer treatment due to their higher biocompatibility, lower immunogenicity and toxicity, biodegradability, easy preparation methods and surface functionality (Nahar et al., 2006). Drug delivery systems can be adjusted as controlled, delayed or triggered release from these particles. Some polymer based nanosystems such as Oncaspar® (pegaspargase) that is L-asparaginase modified by covalently conjugating units of mono methoxy polyethylene glycol (PEG) to treat acute lymphoblastic leukemia, Opaxio™ (paclitaxel poliglumex) that links paclitaxel active ingredient to a biodegradable polyglutamate polymer for lung cancer and ovarian cancer treatment and also Livatag, NKTR-102, Taxoprexinare approved by FDA and available in the market or on phase 3-4 trials as shown in Table 1.

The surface of the polymer particles can be functionalized with various methods. Hydrophilic polymers such as polyethylene glycol (PEG), dextran and chitosan are most widely used polymeric NPsdue to their stable surface and their high availability of functional groups to be conjugated, grafted or absorbed to other surfaces and bioactive molecules. Thereby, circulatory half-life of the bioactive molecules is increased and hepatic uptake is reduced (Alexis et al., 2010). Not only drugs which have low molecular weight but also therapeutic agents such as nucleic acids, genes, proteins and peptides can be carried with polymeric NPs. Functional groups on the particle and the surface charge have a great importance for avoiding the phagocytic system cells (Yang et al., 2009). Positively charged particles are taken into the phagocytic cells more than the neutral and negatively charged particles (Alexis et al., 2008). Properties which directly affect the treatment such as circulation half-life and release rate can be arranged as requested by adjusting functional groups on the particle and surface charge (Karmali & Simberg, 2011; Fadeel, 2012; Zaki et al., 2011).

Table 1. Selected nanoparticle-based therapeutics approved by FDA or in clinical trials in cancer treatment

Nanoparticle Type	Product Name	Company	Active Pharmaceutical Ingredient	Situation	References
Biological NPs	CALAA-01	Calando Pharmaceuticals	siRNA	Phase 1b	(Yu et al., 2012)
	SGT53-01	SynerGene Therapeutics	p53 gene	Phase 1b	(Yu et al., 2012)
	ALN-VSP	Alnylam Pharmaceuticals	siRNA	Phase 1	(Vaishnaw et al., 2010)
	ABI-009	(Abraxis Biosciences)/Celgene	Rapamycin (mTOR inhibitor)	Phase 1	(Schütz et al., 2013)
	ABI-010	(Abraxis Biosciences)/Celgene	17-AAG	Phase 1	(Schütz et al., 2013)
	ABI-011	(Abraxis Biosciences)/Celgene	Thiocolchicine dimer	Phase 1	(Schütz et al., 2013)
	ASG-22ME	Seattle Genetics	Anti-Nectin-4	Phase 1	-
	ASG-5ME	Seattle Genetics	Human monoclonal antibody directed to SLC44A4	Phase 1	-

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Table 1. Continued

Nanoparticle Type	Product Name	Company	Active Pharmaceutical Ingredient	Situation	References
Liposome NPs	SPI-77	Sequus Pharmaceutical	Cisplatin	Phase 2	(Swami et al., 2012)
	Doxil/Caelyx	Schering-Plough	Doxorubicin	Approved	(Haley & Frenkel, 2008)
	DaunoXome	Galen US Inc.	Daunorubicin	Approved	(Bawa, 2008)
	Myocet	Enzon Pharmaceuticals	Doxorubicin	Approved	(Haley & Frenkel, 2008)
	INGN-401	Introgen Therapeutics	FUS1	Phase 1	(Bawa, 2008)
	MBP-426	Mebiopharm	Oxaliplatin	Phase 2/3	(Yu et al., 2012)
	MCC-465	Mitsubishi Pharma Corporation	Doxorubicin	Phase 2	(Yu et al., 2012)
	Lipoplatin	Regulon	Cisplatin	Phase 3	(Stathopoulos & Boulikas, 2011)
	Thermodox	Celsion	Doxorubicin	Phase 3	(Wood et al., 2012)
	2B3-101	to-BBB technology	Doxorubicin	Phase 3	(Schütz et al., 2013)
	Aroplatin	Aronex Pharmaceuticals	NDDP, Oxaliplatin analog	Phase 2	(Zamboni, 2008)
	CPX-351	Celator Pharmaceuticals	Cytarabine and daunorubicin	Phase 2	(Venkatraman et al., 2010)
	LE-DT	Insys Therapeutics Inc. (NeoPharm)	Docetaxel	Phase 2	-
	LEP-ETU	Insys Therapeutics Inc. (NeoPharm)	Taxol (paclitaxel)	Phase 2	(Zamboni, 2008)
	LE-SN-38	Insys Therapeutics Inc. (NeoPharm)	7-ethyl-10-hydroxycamptothecin	Phase 2	(Heidel & Davis, 2011)
	Sarcodoxome	GP Pharma	Doxorubicin	Phase 2	-
	S-CKD602	Alza Corporation/J & J	CKD602	Phase 2	(Zamboni, 2008)
	SLIT cisplatin	Insmed	Cisplatin	Phase 2	(Wittgen et al., 2007)
	Tocosol paclitaxel	Eagle Pharmaceuticals	Taxol (paclitaxel)	Phase 2	(Constantinides et al., 2008)
	EmPAC	Cornerstone Pharmaceuticals	Taxol (paclitaxel)	Preclinical	(Bremerich et al., 2007)
	Liploxa	LiPlasome Pharma	Oxaliplatin	Preclinical	(Jensen et al., 2004)
	LE-rafAON	Insys Therapeutics Inc. (NeoPharm)	c-Raf antisense oligodeoxynucleotides	Phase 1	(Dritschilo et al., 2006)
	LiPlaCis	LiPlasome Pharma	Cisplatin	Phase 1	(de Jonge et al., 2010)
	NL CPT-11	-	Irinotecan = CPT-11	Phase 1	(Schütz et al., 2013)
	TKM-PLK1	Tekmira Pharmaceuticals	siRNA directed against PLK1	Phase 1	-
	Depocyt	Pacira Pharms/Sigma-Tau Pharmaceuticals	Cytarabine	Approved	(Glantz et al., 1999)
	Mepact	IDM Pharma	Mifamurtide	Approved	(P. Anderson et al., 2010)
	Marqibo	Inex/ Enzon/ Thalon therapeutics	Vincristine sulfate	Approved	(Rodriguez et al., 2009)
	Alocrest	Thalon therapeutics	Vinorelbine	Phase 1	(Zamboni, 2008)
	ANX-514	Adventrx	Docetaxel	Phase 1	(Curran & McCormack, 2008)
	ANX-530	Adventrx	Vinorelbine	Phase 1	(Zamboni, 2008)
	ATI-1123	Azaya Therapeutics, Inc.	Docetaxel	Phase 1	(Zamboni, 2008)
	Atu027	Silence therapeutics	siRNA targeting PKN3	Phase 1	(Aleku et al., 2008)
	Brakiva	Thalon therapeutics	Topotecan HCl	Phase 1	(Zamboni, 2008)
	CNF1010	Conforma Therapeutics Corporation	17-AAG	Phase 1	(Schütz et al., 2013)
	IHL-305	Yakult Honsha Co., Ltd	Irinotecan	Phase 1	(Schütz et al., 2013)

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Table 1. Continued

Nanoparticle Type	Product Name	Company	Active Pharmaceutical Ingredient	Situation	References
Polymeric NPs	BIND-014	BIND Biosciences	Docetaxel	Phase 1	(Yu et al., 2012)
	Prolindac (AP5346)	Access Pharmaceuticals	Oxaliplatin	Phase 2	(Sood et al., 2006)
	Opaxio (Xyotax)	Cell Therapeutics	Taxol (paclitaxel)	Phase 3/4	(Alexis et al., 2010)
	NC-6004	NanoCarrier Corporation	Cisplatin	Phase 1	(Uchino et al., 2005)
	NK-105	Nippon Kayaku Corporation	Taxol (paclitaxel)	Phase 2	(Hamaguchi et al., 2007)
	NK911	Eagle Lifestyle Nutrition	Doxorubicin	Phase 1	(Matsumura et al., 2004)
	Docetaxel-PNP	Samyang Co.	Docetaxel	Phase 1	-
	DOX-OXD	-	Doxorubicin	Phase 1	(Danhauser-Riedl et al., 1993)
	Livatag	BioAlliance Pharma S.A.	Doxorubicin	Phase 3	(Heidel & Davis, 2011)
	NKTR-102	Nektar	Irinotecan	Phase 3	(Vicent et al., 2009)
	Taxoprexin	Luitpold Pharmaceuticals	Taxol (paclitaxel)	Phase 3	(Zamboni, 2008)
	AP5280	Access Pharmaceutical	Carboplatine platine	Phase 2	(Rademaker-Lakhai et al., 2004)
	CRLX101	Cerulean Pharma Inc./Calando	Camptothecin	Phase 2	(Svenson et al., 2011)
	CT-2106	Cell Therapeutics	Camptothecin	Phase 2	(Heidel & Davis, 2011)
	DE-310	Daiichi Sankyo Co. Ltd	DX-8951f, exatecan mesylate	Phase 2	(Shiose et al., 2007)
	FCE28068	Pfizer	Doxorubicin	Phase 2	(Seymour et al., 2009)
	FCE28069	Pfizer	Doxorubicin-galactosamine	Phase 2	(Seymour et al., 2009)
	Pegamotecan	Enzon Pharmaceuticals	Camptothecin	Phase 2	(Scott et al., 2009)
	MAG-CPT	Pharmacia & Upjohn Inc.	Camptothecin	Phase 1	(Heidel & Davis, 2011)
	NKTR-105	Nektar	Docetaxel	Phase 1	-
	PNU166945	Pharmacia & Upjohn	Taxol (paclitaxel)	Phase 1	(Meerum Terwogt et al., 2001)
	XMT-1001	Mersana therapeutics	Camptothecin analog	Phase 1	(Walsh et al., 2012)
	XMT-1107	Mersana therapeutics	Fumagillin analog	Phase 1	-
Inorganic NPs	Aurimmune (CYT-6091)	CytImmune Sciences	Gold —— TNF-α	Phase 2	(Bawa, 2008)
	AuroLase	Nanospectra Biosciences	Gold nanoshell	Phase 1	(Bawa, 2008)
	AuriTol	CytImmune Sciences	Gold – Paclitaxel + recombinant TNF-a	Preclinical	-
	AntiEpCAM	Triton biosystems	SPION + antibody	Preclinical	(Morrow Jr et al., 2007)
Protein NPs	Abraxane	Abraxis Bioscience	Taxol (paclitaxel)	Approved	(Haley & Frenkel, 2008)
	Oncaspar	Enzon Pharmaceuticals	Pegaspargase	Approved	(Davis, 2008)
	ABI-008	Abraxis Biosciences	Docetaxel	Phase 2	(Schütz et al., 2013)

continued on following page

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Table 1. Continued

Nanoparticle Type	Product Name	Company	Active Pharmaceutical Ingredient	Situation	References
Micelles	Taxol	Bristol Myers Squibb	Paclitaxel	Approved	(Heidel & Davis, 2011)
	Taxotere	Sanofi-Aventis	Docetaxel	Approved	(Heidel & Davis, 2011)
	Genexol-PM	Samyang Co.	Paclitaxel	Approved	(A. Z. Wang et al., 2012)
	Docetaxel-PM	Samyang Co	Docetaxel	Phase 1	(Simon, 2006)
	Paxceed	Angiotech	Paclitaxel	Phase 2	(J. A. Zhang et al., 2004)
	SP1049C	Supratek Pharma	Doxorubicin	Phase 2	(Valle et al., 2011)
	SP1060C-O	Supratek Pharma	Topotecan	Preclinical	(Kabanov & Alakhov, 2002)
	Nanoxel	Fresenius Kabi Oncology Ltd/ Dabur Pharma	Taxol (paclitaxel)	Phase 1	-
	NC 4016	NanoCarrier	Oxaliplatin	Phase 1	-
Polymer–protein conjugates	ADI-PEG 20	Polaris Group - Phoenix Pharmacologics	Arginine deaminase	Approved	(Shen & Shen, 2006)
	CDP 791	UCB S.A.	Anti-VEGFR2 Fab fragment	Phase 2	(Pasut & Veronese, 2009)
Antibody-drug conjugates	Trastuzumab emtansine	Genentech	Mertansine (DM1)	Phase 3	(Mathew & Perez, 2011)
	Glembatumumab vedotin	Celldex Therapeutics	Human mAb to GPnMB-auristatin conjugate	Phase 2	(Naumovski & Junutula, 2010)
	SGN-CD19A	Seattle Genetics	Antibody anti-CD19	Preclinical	-

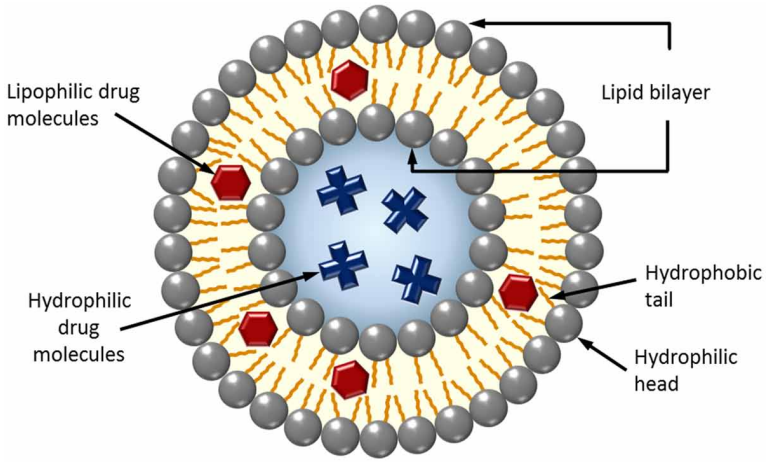
Liposomal Nanoparticles

Liposomes are artificial spherical vesicles that allow encapsulation of drugs in liquid phase owing to their lipid bilayer structure with sizes varying between 50 nm – 5 μm (Malam et al., 2009). Hydrophilic drugs are transported in the center of liposome whereas hydrophobic drugs are transported within the lipid bilayer (Sharma & Sharma, 1997) (Figure 2). It was put forward that liposomes could be used in drug delivery systems in the early 1970s (Gregoriadis, 1995). Despite more than 40 years have passed after the discovery of liposomes, liposome based systems still has not been exploited adequately today.

Liposomes are classified as conventional liposomes (CL), pH-sensitive liposomes, cationic liposomes, long-circulating liposomes (LCL) and immune-liposomes according to their structures (Sharma & Sharma, 1997). Liposomes help to increase pharmacokinetics, efficiency and bioavailability, and decrease the toxicity of the drug or bioactive agent in the body because these particles can be adopted to target-driven treatments by functionalizing (Drulis-Kawa & Dorotkiewicz-Jach, 2010). However, their use is limited because liposome NPs are not very stable, and do not always remain in desired site for long periods of time to show the therapeutic effect, although local deposition of the drugs is higher when encapsulated with liposomes since liposomes can be internalised quickly by macrophages and other cells in the body (Hatefi & Amsden, 2002). The drug delivery systems based on liposomes are among the most studied nanoparticles such as Myocet, DaunoXome, DepoCyt, liposomal doxorubicin/Caelyx, Mifamurtide (Mepact), Marqibo (vinCRIStine sulfate liposome) have been approved by FDA among other liposomal medicines in pre-clinical or clinical trials as summarized in Table 1.

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Figure 2. Liposomes can be used in dual drug delivery. Hydrophilic drugs are transported in the center of liposome whereas hydrophobic drugs are transported within the lipid bilayer



The basis for properties of liposomes is their lipid bilayer structure which forms hydrophilic space inside. Therefore, the instability disadvantage can be eliminated with appropriate modification of this membrane's internal and external structure (Felnerova et al., 2004). Liposomes should be produced with prudently selected methods to be able to load proper amounts of the drugs and adjust the drug release rate (Koudelka & Turanek, 2012).

Dendrimer Nanoparticles

Dendrimers with hollow internal structure are tree-like nanostructures which have been discovered in the 1980s (Tomalia et. al, 1986 (Dang et al., 2012). Due to their three-dimensional structure which is smooth and highly branched, and easy functionalization of their surface, dendrimers are commonly used in medicine, in host-guest chemistry and as catalysts (Luo & Sun, 2007). Dendritic structure can be examined in four sub-classes, (i) randomly hyper-branched polymers around the inner core structure (ii) dendrigraft polymers, (iii) dendrons and (iv) dendrimers. More than a hundred different dendrimers and more than a thousand different surface chemistries are known (Oliveira et al., 2010).

Owing to properties such as high degree of molecular uniformity, monodispersity and multifunctional surface, dendrimers are distinguished from other linear and branched polymers (Devarakonda et al., 2005). One of the most widely used dendrimers is poly(amidoamine) (PAMAM) dendrimer. PAMAM dendrimers possess important properties such as biocompatibility, non-immunogenicity, water solubility and manipulability of their functional groups for binding to variety of guest molecules (Strydom et al., 2013, Ouyang et al., 2010), and can be used for controlling the release rate and increasing dissolution of the drugs with low water solubility (D'Emanuele & Attwood, 2005). In addition to PAMAM; poly(propylene imine) (PPI), poly-lysine (PLy), arborols (ARB), poly(benzyl ether) (PBzE), polyphenylene (PHEN), thiophosphorylphenoxymethyl (methylhydrazono) (PMMH) are also widely used (Caminade et al., 2005).

It has been shown that dendrimers can be used for theranostic applications as well as drug delivery systems due to their compatibility with many nanostructures such as genetic and/or bioactive materials, fluorescent molecules etc. Despite all the potential, the accumulation, redistribution and the adverse

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effects to other tissues have not been fully revealed (Oliveira et al., 2010), and there is eminent need for large scale *in vivo* studies.

Protein Nanoparticles

Proteins can be used as drugs themselves as well as carriers for drug delivery systems since they are biodegradable, non-antigenic and can reduce unwanted side-effects. Another distinctive advantage of protein NPs is their functional diversity (Vázquez & Villaverde, 2010).Desired proteins can be produced easily by using classical biotechnological methods or recombinant technologies by macro/microorganisms. Although studies on protein nanoparticles have accelerated relatively recently, there are some nano-systems such as Abraxane, Oncaspar and ABI-008 that are FDA approved or are ongoing phase trials as shown in Table 1. Their biocompatibility is high because these protein NPs are synthesized using biological building blocks like amino acids and peptides, and toxic chemicals are not used in provision.

Protein-based drug-loaded NPs produced by albumin have received FDA approval and have been used in clinical applications for some time (Pazdur, 2012; Elzoghby et al., 2012). Enhanced permeability and retention (EPR) effect has an important role for accumulation of albumin in tumor site. The EPR effect, a universal phenomenon in solid tumors, is the property by which certain sizes of molecules (typically liposomes, nanoparticles, and macromolecular drugs above 40 kDa, even larger than 800 kDa) tend to accumulate in tumor tissue much more than they do in normal tissues (Maeda et. al., 2009). On the other hand, some studies show that albumin has a tumor targeting ability by using special pathways to overcome the endothelial barrier in biological environment (Alexis et al., 2010).

Protein-based NPs produced by protein engineering can minimize the toxicity of solvent-based systems. Hydrophilic drugs may be directly toxic when they are given into the body because of their solubility in the water. Protein-based NPs allow loading hydrophilic drugs into hydrophobic formulations (Hawkins et al., 2008).

Polymersome Nanoparticles

Polymersomes are self-assembled vesicles and synthetic amphiphilic block copolymers, like natural phospholipids and liposomes (Discher, 1999; Sachsenhofer et al., 2007). Although they have similar structure to natural membranes, properties of polymersomes are quite different than the liposomes. To exemplify, their weight, mechanical and thermal stability is higher than those of biological lipids (Sachsenhofer et al., 2007). In addition, polymersomes possess lower permeability compared to liposomes; and the chemical composition, structure and sizes of polymersomes can be changed by adjustment of the block copolymers (Amstad et al., 2012).The size and shape of the polymerosomes directly affect their pharmacokinetics and bio-distribution properties. Under 10 nm, they can be quickly cleared in capillary beds and lymph nodes, while those above 200 nm are rapidly removed from circulation *via* splenic filtration (Ho & Shoichet, 2013). In addition to removal from the body, polymerosomes' shape and size also affecttheir degradation and release behavior, transportation in the body, targeting ability, uptake by immune cells or the targeted cells, thus biocompatibility (Champion et al., 2007;M. Anderson et al., 2008).

Owing to their amphiphilic structure, they can be loaded with hydrophilic and hydrophobic molecules at the same time and targeted toward desired cell types (Surovtseva et al., 2012). The hydrophilic molecules are captured in the core and hydrophobic molecules in their thick bilayer membrane (Amstad et al., 2012). The specific structure of polymersomes allows theranostic drug carrier systems that include

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both water soluble and insoluble substances such as therapeutic and imaging agents together (Levine et al., 2008). Additionally the surface of polymersomes can be modified by specific biological molecules for targeting the desired tissue sites (Johnson et al., 2011).

Inorganic Nanoparticles

Although it is possible to produce organic nanoparticles such as liposomes, polymersomes and polymeric nanoparticles to design nanoscale drug delivery systems, and many of them are in clinical and preclinical stages, the research on inorganic nanoparticles increase day by day because of their unique optical, electrical, magnetic, and/or electrochemical properties (H. C. Huang et al., 2011). The biological delivery systems (viral carriers) are the most effective carriers for drug delivery, but they tend to cause immune response or mutagenesis so that the success of the cellular delivery of these systems is low. Liposome and polymersome based devices may prevent these problems, but they are often instable and may show toxicity to the cells. Owing to their low toxicity and highly controllable release rates, inorganic nanoparticles are alternatives to organic nanoparticles (Z. P. Xu et al., 2006; Mironava et al., 2013).

Metallic nanoparticles such as gold, silver, iron, titanium, platinum etc. have been used for medical applications like imaging, targeting and treatment of cancer. Easy control of their shape and size, as well as their easy surface functionalization are the main advantages of inorganic nanoparticles as compared to organic nanoparticles (Son et al., 2007).Generally super-paramagnetic iron oxide nanoparticles (SPIONs), due to their magnetic properties and gold nano-vehicles due to their high X-ray absorption coefficient have been widely used as contrast agents. Also SPIONs and gold nano-vehicles can be used in hyperthermia and photothermia treatment where these inorganic particles are heated selectively by application of an external high frequency magnetic field, microwave irradiation, radiofrequency pulses or ultrasound waves leading to thermal destruction of the tumor when targeted effectively. Metallic nanoparticles can also be used in drug delivery systems, but recently other applications are more popular than drug delivery because loading of the drugs on inorganic NPs is more difficult and laborious. However certain gold nano-systems such as Aurimmune (CYT-6091), AuroLase, Auritol and some SPIONs like AntiEpCAM have been used for treatment.

Silver NPs have unique thermal, electrical, optical and antibacterial properties, and they are investigated for diagnostic and conductive applications such as biosensors, imaging and antibacterial dressings. Although known and used for quite a long history, there is still active debate on toxicity and biocompatibility of silver. Titanium dioxide NPs have interesting dielectric and optical properties, and they are also tested as drug eluting vehicles and in photodynamic therapy where these normally non-toxic, light-sensitive particles are exposed selectively to light, whereupon they become toxic to targeted malignant and other diseased cells (Moreno-Vega et al., 2012).

In addition to metallic NPs, quantum dots (Qdots) and carbon nanotubes are also among the most studied nano-systems. Solutions of Qdots of varying size have different color, and the variation in optical absorption each solution illustrates the particle size dependence for each sample (L. Wang et al., 2010; Pathakoti et al., 2013). Qdots can be used both in *in vitro* diagnostics and in imaging systems due to their specific absorption properties.

The selection of nanoparticles intended for medical applications is very imported. In most cases, toxicity is the more important criteria for the selection if the particles will be used especially in vital systems such as central nervous systems (Suh et al., 2009).To exemplify, although Qdots possess very specific optical features, they cannot be used widely in treatment of diseases because of their Cd/selenide

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core which are very toxic for mammalian cells. In addition to particle chemistry, researchers must also account for other properties of the particles such as geometry, size, dose, surface area and aspect ratio to select the most appropriate delivery system for distribution of the therapeutic agent with minimum adverse effects. For example, many studies specify that size of CNTs' is one of the most important reasons of their toxicity. With increased fiber size, clearance of CNTs by macrophages becomes harder. The surface properties also affect the uptake of CNTs to the cells, their interactions with cell surface molecules and the signaling pathways (X. Zhao & Liu, 2012).

Other Biological Nanoparticles

Some unicellular microorganisms including bacteria with different shapes and sizes are also classified as biological nanoparticles. The different sizes of microorganisms are useful for encapsulating both hydrophobic and hydrophilic molecules. Although biological nanoparticles have been investigated more recently than the other types of NPs, there are some biological nano-systems such as CALAA-01, SGT53-01, ALN-VSP, ABI-009, ASG-22ME on phase 1 trials.Despite some studies carried on animals verify that these nanoparticles show no significant toxicity, there has been a potential for immunological response because of the containment of the microorganism specific lipo-polysaccharides. In addition to unicellular microorganisms, bio-based nanoparticles comprise many agents such as DNAzymes (catalytically active DNA molecules), short interfering RNAs, antisense oligonucleotides, aptamers (oligonucleic acid or peptide molecules that bind to a specific target molecule) and ribozymes (an RNA molecule that is capable of performing specific biochemical reactions). They possess ability of silencing non-desired genes with appropriate design (L. Zhang et al., 2012). In order to obtain an effective medicine for tumors, short synthetic nucleic acid nanoparticles may be designed for the targeted therapy of the genes associated with the disease (Fuessel et al., 2007).

Radiotherapy aims to kill the cancer cells by ionizing radiation and also by regulating the genes that provide resistance to radiotherapy (Ghisolfi et al., 2012). However in many cases, radiotherapy, as well as chemotherapy cannot achieve the success for the treatment due to the presence of cancer stem cells (CSCs) which play a key role in cancer recurrence and resistance (Clarke & Fuller, 2006). It is possible to prevent the development and growth of the cancer cells and even CSCs by using these biological gene silencers. For the most effective treatment, gene silencing and radiotherapy can be used simultaneously. In this case the therapeutic agents can silence the genes that are responsible for radiation resistance and stop the proliferation of the cancer stem cells. Concurrently, radiotherapy may kill the cancer cells that are present in the tumor sites.

3. NANOPARTICLE TOXICITY AND SAFETY

Developments in nanotechnology also caused an increase of concerns about nanotoxicity. Especially the adverse effects caused by nanoparticles on healthy cells must be the priority interest. For this reason, there are many toxicology studies for each type of nanoparticles. *In vitro* toxicity assays used in these studies are different from each other, and their results may sometimes be controversial. European Commission supports Joint Research Center (JRC) Institute for Health and Consumer Protection for safety assessment of nanomaterials, particularly for developing methods for detection of nanomaterials in food and consumer products, validation of analytical methods for nanomaterials and developing standardised

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protocols for toxicity testing (www.jrc.ec.europa.eu). A forty seven institute consortium FP-7 Project, MARINA, Managing Risks of Nanomaterials, aims for integrated and intelligent testing, integrated assessment, and modular interconnection of knowledge and information for validated science-based risk management methods (http://www.marina-fp7.eu/). NanoLINEN is another consortium of seven European laboratories and Indian Institute of Toxicology Research (CSIR Laboratory) from India to strengthen the research ties in the area of Nanomaterial Toxicology. The goal of this project is to develop robust risk assessment methodologies that will be useful for the community manufacturing and using nano-products (http://www.nanolinen.org/).

Some of toxicity tests which can be used for a variety of different nanoparticles include;

- **Morphological Changes:** Light microscopy.
- **Cell Viability and/or Cell Growth:** Neutral red assay, Trypan blue, Calceinacetoxymethyl / ethidiumhomodimer, Lactate dehydrogenase, Tetrazolium salts (MTT, MTS, XTT, WST), Resazurin or Alamar blue, Propidium iodide, (Choi & Wang, 2011).
- **Celldeath:** Lactate Dehydrogenase (LDH) assay.
- **Apoptosis/Necrosis:** Acridine orange/ethidium bromide, Propidium iodide, Caspase-3 activity, DNA laddering.
- **Oxidative Stress:** Levels of reduced (GSH) andoxidized (GSSG) glutathione, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CT), ROS production, Vitamin E, levels of lipid peroxidation, DCF assay.
- **Visualization of Intracellular Localization:** Transmission electron microscopy.
- **Cellular Uptake:** Radiolabelled nanoparticles.
- **Genotoxicity:** *In vitro* micronucleus test, *Allium cepa* chromosome damage test.
- **DNA Damage:** Comet assay.
- **Cytotoxicity:** Colony forming efficiency test.
- **Hemolysis:** Hemoglobin estimation.

Nano-vehicles should be fully characterized and tested in order to pass judgment on toxicity. Different test methods, surface modifications, nanoparticle size, shape, dose, surface charge, targeted cells, organisms, and route of administration may obtain different results on toxicity. For further details on nanotoxicity issues, Won HyukSuh et al. 2009 can be referred for a wide analysis about titanium dioxide (TiO_2), iron oxide (Fe_2O_3), silicon oxide (SiO_2) and cerium oxide (CeO_2) nanoparticles and Xingchen Zhao and Liu 2012 is recommended for the effects of different carbon nanotubes on different mammalian organisms and cell lines (Suh et al., 2009; L. Zhao et al., 2011). Although the toxicity caused by characteristic features of different nanoparticles may be diverse, it is possible to generalize the factors which influence the toxicity as follows:

- Increasing the surface area of nanoparticle increases the toxicity significantly more than increasing nanoparticle mass (Stoeger et al., 2006).
- Nanoparticles smaller than 100nm are more successful in drug delivery but these particles lead to more side effects related to respiration compared to larger particles (Donaldson & Stone, 2003).
- Whether nanoparticles are biodegredable or not does not seem to have a significant effect on toxicity. It is possible to do the classification from less toxic toward the most toxic as: (i) biodegradable and larger than 100 nm, (ii) non-biodegradable and larger than 100 nm, (iii) biodegradable and

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less than 100 nm and (iv) non-biodegradable and less than 100 nm nanoparticles (Keck & Muller, 2013).

- The geometry of nanoparticles may play an important role in nanoparticle toxicity. For example, although single walled or multi walled carbon nanotubes and nano-60 fullerenes are both based on carbon, their toxicity is not the same *in vitro*(Sanvicens& Marco, 2008).
- Generally high concentration of NPs show less toxic effects than low concentration of NPs, because higher concentration of NPs often cause formation of aggregates (Gurr et al., 2005).
- Surface modifications of inorganic nanoparticles can also affect their toxicity (Sanvicens & Marco, 2008).
- Even allotropes of the same material can cause different toxic effects on same organism. For this reason, the chemical structure and crystallinity are highly effective on the toxicity (Buzea et al., 2007).
- Generally it has been strived to form less toxic particles by coating the material surface with various biocompatible polymers. However, in the end it may end up with higher toxicity when the interactions of coating materials with other factors are not known.
- High aspect ratio indicates higher toxicity, because macrophages are unable to phagocytose long fibers (Hoet et al., 2004). Some long fibers break transversely when they degrade in the body and they can be cleared by phagocytosis, but some long fibers such as asbestos break longitudinally and they cannot be cleared by phagocytosis (Oberdörster, 2002).

4. MAJOR CANCER TARGETS FOR NANOPARTICLE SYSTEMS

The recovery rate has not increased significantly on cancer patients in the last twenty years in spite of all the advances in tumor biology, cancer biomarkers, surgical procedures and chemo-radiotherapy. Early diagnosis, pathological characterization of the tumors and personalized treatments are substantially candidates to improve recovery rate figures. Negative side effects on healthy cells, especially rapidly dividing cells besides the tumor cells, are the major problem of conventional therapy methods. Researchers have been investigating new therapy methods that specifically target only tumor cells to overcome this problem.

Targeting systems can be examined in four groups;

1. Cell marker targeting,
2. Signaling pathway targeting,
3. Micro-environment (niche) targeting, and
4. Angiogenesis associated targeting.

The best target for the treatment of cancer cells is the molecules which are found in cancer cells but not found in normal cells. However, in practice, this approach is not possible sincecancer cells actually evolve out of normal cells, and therefore, there are no clear distinctive markers. Some of the more commonly synthesized molecules in cancer cells compared to normal cells are considered good targets for therapy. A greater number of cancer cells can be damaged with adjusting the dosage of chemotherapeutics that target these molecules in comparison with conventional therapies.

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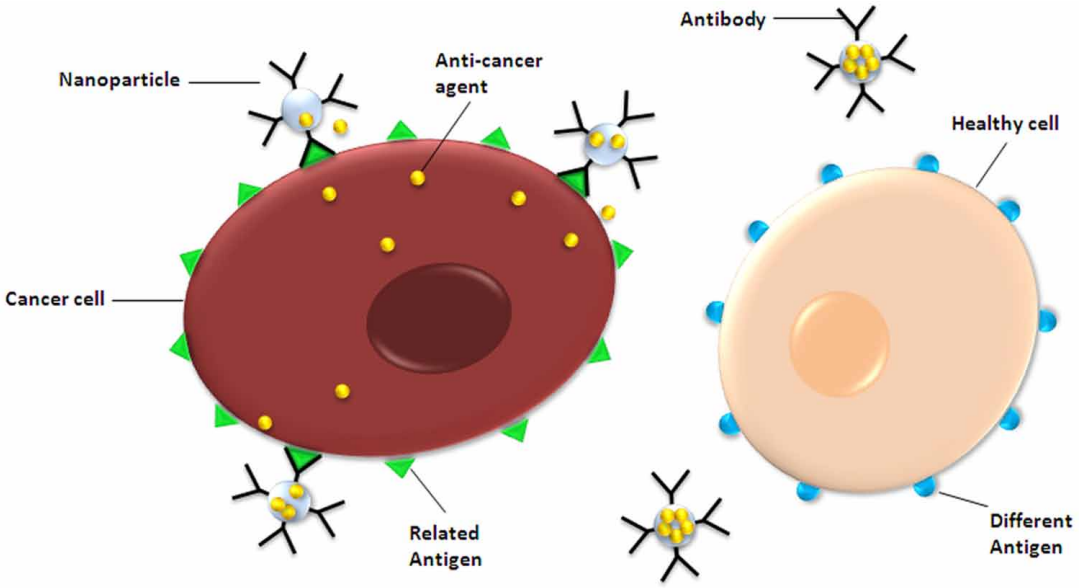
Cell Marker Targeting via Antibodies

An antibody (Ab), also known as an immunoglobulin (Ig), is a large Y-shaped protein produced by B-cells and used by the immune system to identify and neutralize foreign objects such as bacteria and viruses. The antibody recognizes a unique part of the foreign target, called an antigen. The cell membrane contains many surface and trans-membrane molecules such as peptides, proteins, saccharides, antibodies and glycoproteins. Some of these molecules are specific for particular cell types, and can be targeted effectively with monoclonal antibodies for therapeutic purposes. Some surface antigens synthesized by cancer cells are different from normal cells and this circumstance is an important opportunity for targeting *via* antibodies. The main scheme of treatment of cancer by targeting these specific molecules is to attach the nanoparticles only on the cancer cells by integrating these surface markers or affinity molecules to tumor cells, or the molecules that increase the uptake of the nanoparticles into the targeted cells (Figure 3).

Specific antibodies are produced by injecting an antigen into a mammal, such as a mouse, rat, rabbit, goat, sheep, or horse for large quantities of antibody. Monoclonal antibodies (mAb) are monospecific antibodies made by identical immune cells that are all clones of a unique parent cell, in contrast to polyclonal antibodies which are made from several different immune cells (Binyamin et. al., 2006).

Cancer cell signaling pathways are affected by the use of monoclonal antibodies in various ways. Antibodies can be used to prevent communication between the signaling molecules and their receptors. Human tumors express high levels of growth factors and their receptors, and many types of malignant cells are stimulated by these signal molecules. In anti-receptor directed therapies, antibodies targeted

Figure 3. The main scheme of treatment of cancer by targeting via antibodies is to attach the nanoparticles only on the cancer cells by integrating the surface markers (or affinity molecules to tumor cells or the molecules that increase the uptake of the nanoparticles to the targeted cells) that are specific to the cancer cells on the nanoparticles.



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by nanoparticles are bound to cell surface receptors and block their interaction with signal molecules (Baselga& Mendelsohn, 1994; Hicklin et. al., 2001; Capdevila et. al, 2009). MAbs directed against epidermal growth factor (EGF), transforming growth factor (TGF), G-protein, fibroblast growth factor (FGF), IL-2, andIL-6 receptors are examples of this approach. Antibodies can also be used as a vehicle for delivering the chemotherapeutic agents to the tumor sites (Adler &Dimitrov, 2012; Perche et. al., 2012; Cirstoiu-Hapca, 2010). They can damage the targeted tumor cells by releasing their chemotherapeutic agents locally. Another promising approach is to target tumor cells by antibodies that trigger immune response to destroy the cells by body's own defense mechanisms (Miller et al., 2002).

In recent years, targeting studies with surface markers are engineered to target cancer stem cells (Hu & Fu, 2012; Wicha, 2009). For a long time researchers believed that every cancerous cell has the potential to excessively proliferate and regenerate a tumor in consequence of internal and external factors that cause mutations. This model has been called as stochastic model (Wicha et al., 2006; C. Tang et al., 2007; Shackleton, 2010; Chen et al., 2012). Numerous contemporary studies indicate that the formation of tumor depends on the differentiation of a relatively rare cell groupstermed as cancer stem cells (CSCs)due intrinsic and extrinsic inducement (Hanahan& Weinberg, 2011; Clarke & Fuller, 2006; Dean et. al, 2005). The functional capabilities of CSCs are associated with normal stem cells such as the high ability for self-renewal, the capacity to differentiate, active telomerase expression, activation of anti-apoptotic pathways, increased membrane transport activity and immense potential to migrate and evolve (Wicha et al., 2006). Eliminating these cells is very difficult by conventional methods due to their immense resistance to chemotherapy and radiotherapy. Consequently, CSC targeted therapies are escalating fast in literature, and they hold great promise in cancer treatment.

Monoclonal antibodies have some limitations even though they have effective potential in targeted therapies. The most important limitation is that, the antibodies can be used primarily to target surface molecules because they are large proteins and they cannot pass the cell membrane easily. Other main limitation is that different cancer cells possess different markers, and same marker can be found also on some healthy cells causing significant toxic side effects. At present, there is insufficient number of recognized markers and deficient knowledge on them. Antibodies derived from animals can sometimes cause immune reactions. Antibody fragments containing only the variable region of the antibody can also be used for active targeting for less immunogenicity. They retain the specificity for the target, while they result in less foreign body reaction or undesirable interaction with other cells (Byrne et al., 2008).

Targeting Signaling Pathways

Signaling (or signal transduction) pathways are the cascade processes existing in the cells to perform specific cellular tasks. For example, a cell needs signaling molecules and pathways that are activated by these molecules to grow and divide. The signal molecules, including growth factors and inhibitors, must be attached to receiver cell's surface receptors for activation of the signaling pathway (Cho et al., 2007; Lee et al., 2013). When the signaling pathway is activated, the information that comes from receptor reaches into the cell, may even be delivered to DNA. Activation of the signaling pathways leads to accumulation of proteins that are inhibiting or activating the cell response. The amounts of proteins which stop and start cell division are under control in normal cells. On the other hand, the amount of the activator molecules and proteins that promote the cell proliferation are increased in cancerous tissues. Therefore, the inhibition of the aforementioned pathways has a high potential for preventing the uncontrolled proliferation of cancer cells (Kelleher et al., 2012). The main purpose of targeting signaling

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pathways are blocking a molecule in the pathway to stop interaction with other molecules and thus blocking the signal transduction (Leonarduzzi et al., 2010). For instance, targeting of the Mitogen Activated Protein Kinase (MAPK) pathway has a potential for therapy since the dysregulation of this pathway is observed in many cancer types (Wancket et al., 2012). The MAPK pathway is controlled by a complex cascade mechanism through extracellular signals in normal cells. Disruption of this pathway leads to increased cell proliferation, angiogenesis, metastasis and increased cell survival, thus cancer development (R. Xu et al., 2011). The biggest problem in the MAPK pathway inhibition is that, pathway has several signal molecules that activate this pathway, and other signal molecules can activate the pathway even if a specific molecule is inhibited (Lugli et al., 2006). Several studies have shown that more than one signal protein and/or pathway must be targeted simultaneously. The chemical agents are given to the patient orally or intravenously in conventional methods, where the agents can be inactivated before showing any therapeutic effect since they are passing through different compartments in the body such as the gastrointestinal tract. On the other hand, drug doses must be very high to show the desired effect since the drug is distributed throughout the entire body, and inhibition without targeting increases the side effects (Diaz-Moralli et al., 2013). Nanotechnology is used to overcome these problems as it allows the production of multifunctional transport systems that can implement multiple genetic and pharmaceutical agents (Nair et al., 2010). The surface of the nanoparticles can be functionalized with molecules that inhibit signal pathways as well as with chemotherapeutic agents targeting other schemes. The most effective treatment method will emerge when different targeting systems are used in a single nanoparticle. There are several options for pathway selection, including Notch signaling pathway and mitochondrial signaling pathways to overcome drug resistance, phosphoinositide 3-kinase (PI3K) pathway and nuclear factor-κBto induce apoptosis in tumor cells, transforming growth factor-β signalling pathway to attack promotion of tumor growth, invasion and colonization of secondary organs, phosphatidylinositol 3-kinase (PI3K)/Akt pathway to suppress angiogenesis, etc. Recently, targeting apoptosis pathways in cancer stem cells is also under investigation (Signore et. al., 2013; Fulda &Pervaiz, 2010)

Niche Targeting

The local microenvironment, or niche, of a cancer cell plays important roles in cancer development (Hu & Polyak, 2008). Thereby, targeting tumor microenvironment is another commonly used method for targeting studies for cancer therapy, and particularly metastasis prevention (Joyce, 2005). The microenvironment of the cancer has a direct effect on life and behavior of the cancer cells. Cancer cells are fed by the niche and the exchange of necessary molecules can be mediated through this microenvironment. Extracellular matrix (ECM), an important part of the cell niche, contains components such as collagen I-III-IV, decorin, hyaluronan,versican, fibronectin, laminin and osteonect in that can contribute to developing of tumor resistance to chemical agents (Ioachim et al., 2002; Sung et al., 2005). For example, laminin and fibronectin was shown to provide resistance to apoptosis while collagen I-III-IV, versican and osteonectin increase the proliferation of tumor cells (Whatcott et al., 2011).

Desmoplasiais defined as the formation and proliferation of fibroblasts and fibrous connective tissue, especially in solid tumors. Desmoplasia causes a significant increase in the synthesis of ECM proteins (Walker, 2001), preventing perfusion of chemotherapeutic agents. In order to overcome the chemoresistance, targeting of these ECM proteins has a very high potential. For example, hyaluronan, which is a component of ECM and provides protection of connectivity and rigidity of connective tissue, is over-produced in tumor cells due to desmoplasia. Excessive hyaluronan concentration in ECM causes

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increased interstitial fluid pressure and limits entrance of any substance from outside (Whatcott et al., 2011). Chemo-resistance may be reduced by targeting hyaluronan viahyaluronansynthetase inhibitors or through destruction of these molecules by enzymes such as hyaluronase.

The slightest alteration -even a single amino acid replacement- in the components of ECM generates variations in the physicochemical properties of the tissue and also leads to changes in the cellular phenotype and cell-matrix relations. It has been shown in various studies that ECM is needed to protect the structure and polarity of tissues. ECM components of cancer cells differ from normal tissues. Abnormal ECM also influences cancer development indirectly *via* influencing stromal cells' behavior including fibroblast cells, immune cells and endothelial cells. These stromal cells continue synthesis of the abnormal ECM creating the appropriate microenvironment for migration of cancer cells and formation of new vessels(Y. Tang et al., 2005). ECM also has an important role in reaching of the cancer cells to surrounding capillaries and lymph nodes. Local tumors constitute appropriate microenvironment by imposing necessary changes in the ECM in order to maintain their survival. Most of the tumor cells die when they leave their microenvironment, a type of apoptosis called *anoikis*. Therefore, it has been a subject of curiosity how the metastatic cancer cells can adapt and continue to live in other parts of the body. Some studies specify that cancer cells which have receptors for ECM components exhibited a higher survival rate than those without these receptors (Lu et al., 2012). Survival rates of cancer cells can be significantly reduced by targeting these ECM receptors. To prevent the development and spreading of cancer, multiple ECM components can be targeted with appropriate nano-tools.

Angiogenesis-Associated Targeting

All living cells in the body need oxygen and nutrients in order to survive. For this reason, all the living tissues are nourished with capillary blood vessels. Since the cell division in tumors is faster than normal physiological conditions, the capillaries become insufficient for the expanded tumor size. Cancer cells often secrete particular growth factors tore-model the ECM and form a suitable microenvironment to trigger the sprouting and growth of new capillary blood vessels (Chatelain et al., 2011). This phenomenon is defined as angiogenesis, and it is vital for tumor tissue to grow and reach its maximum extent (Nishida et al., 2006). The blood vessel cells (endothelium) only divide about once every 3 years, and do not form new capillaries in normal tissues when they are in a quiescent state. However, they are activated within the tumor site and they form new capillaries due to these secreted angiogenic factors within (Liekens et al., 2001).Once activated, these endothelial cells begin to divide at an accelerated pace and also secrete matrix metalloproteinases(MMPs) that degrade the extracellular matrix (ECM) further allowing other endothelial cells to migrate towards the tumor (Petros&DeSimone, 2010).

A lot of molecules that promote endothelial growth and the formation of new blood vessels are convenient for targeting angiogenesis for cancer therapy (Makrilia et al., 2009). In tumor tissues, the balance between inhibitors and activators that are present in normal tissues is disturbed; the numbers of activators are increased and the formation of new blood vessels is begun by rapid proliferation of endothelial cells. Rapid vascularization also helps metastasis, as the cancer cells find more chance to enter the blood stream. Even when the tumor is removed or destroyed by means of any therapy, the cancer has high risk of recurrence (and/or metastasis) due to these cancer cells that had already entered the circulatory system. The prevention of tumor vasculature before the tumor reaches the critical size is crucial (Bjerkvig et al., 2009). Developments in nanotechnology and discovery of molecules that are capable of inhibiting angiogenesis have led to a boost in studies targeting angiogenesis. Some of these

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studies include targeting molecules and pathways that cause the alteration on the extracellular matrix structuring which provides appropriate environment for the formation of new vessels. Another approach is targeting angiogenesis activators or sending angiogenesis inhibitors directly to the tumor site. The most important criterion to achieve success is detection of the primary tumor before reaching critical size for metastasis. Nanotechnology offers important opportunities for early diagnosis and treatment of the tumor. There are many proteins including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (BFGF) and platelet-derived growth (PDGF) that promote angiogenesis (Ishihara et al., 2002; Li et al., 2006). It has been shown that the installation of the nanoparticle delivery systems with the inhibitor antibodies to these growth factors improve both accumulation of the nanoparticles in the tumor tissue and toxicity of the anti-cancer drug on the tumor cells. The treatment of the tumor by targeting multiple angiogenesis activators along with other anti-cancer drug will ensure the most effective cure.

5. TARGETING SCHEMES

The effectiveness of vehicles used in cancer therapy is measured by their ability to eliminate tumor cells without harming healthy tissues (Byrne et al., 2008). Multi-drug resistance, exposure of the entire body by the treatment and related side effects, insufficient efficacy levels of the drugs given orally or viasystemic circulation due to different parameters such as pH of different body compartments, and the increasing number of cancer patients force researchers to discover alternative therapies. Nanotechnology, by enabling specific targeting to the affected area, offers great opportunities for the elimination of these problems. For instance, the targeted chemotherapeutic agents with pH-sensitive nanoparticles may remain intact until getting into the area of the appropriate pH. Heat-sensitive nanoparticles enable elimination of unwanted cells by generating local heat by external forces such as magnetic field when they reach the desired location (Yu et al., 2012). Although there is a large amount and variety of nanodevices under investigation, the number that received FDA approval and can be used in the medical field is very small (Alexis et al., 2010). Nanomaterials produced for cancer treatment should have several additional properties such as high selectivity, high penetration ability, multi-functionality, appropriate pharmacokinetic and pharmacodynamics characteristics, therapeutic and diagnostic properties, high biocompatibility, causing low immune response, high physical and chemical stability (Chopra, 2011; Rani et al., 2012). Nanotechnological therapeutic devices that are implementing several of these parameters will provide glorious change in the development of new cancer therapeutics (Swami et al., 2012).

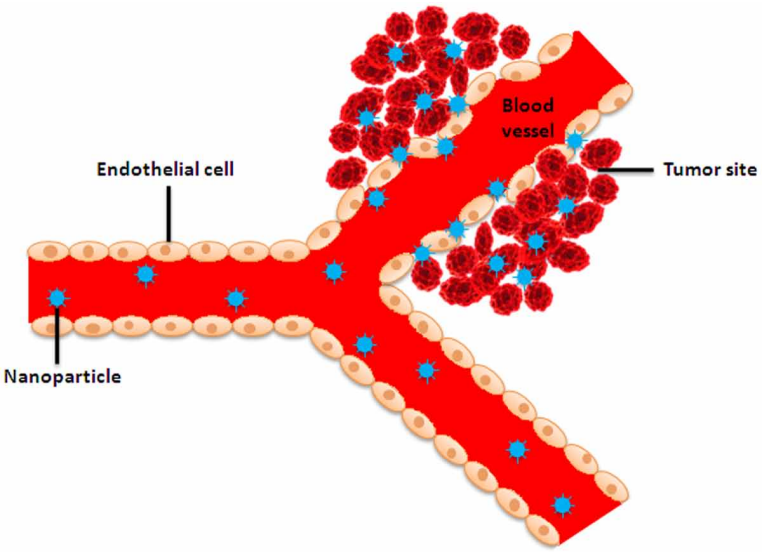
There are three basic methods used to target the nanoparticles to desired tumor sites; *passive targeting*, *active targeting* and *triggered targeting*. In order to develop the most efficient system, these methods used for diagnosis and treatment of cancer must be analyzed thoroughly, and combinations of the three methods may also be used for improved success.

Passive Targeting

Owing to their size, NPs can accumulate in many tumor tissues several times higher than other parts passively *via* the *enhanced permeability and retention* (EPR) effect (Maeda et al, 2009;Wu et al., 2006). This accumulation is a non-selective process that occurs due to "leaky," underdeveloped tumor vasculature that allows macromolecules of a certain size range to accumulate in the tumor(Figure 4).The gap junctions between adjacent endothelial cells in tumor tissues are much larger than those in normal blood

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Figure 4. NPs can accumulate in many tumor tissues at several times higher rates than other parts of the body passively via the enhanced permeability and retention (EPR) effect due to "leaky," underdeveloped tumor vasculature



vessels (Kazuo, 2011). The macromolecules can exit the circulation owing to the leaky vasculature, but remain at the tumor site as a result of impaired lymphatic drainage properties (Petros & DeSimone, 2010; Wang & Thanou, 2010; Mousa & Bharali, 2011; Sailor & Park, 2012).

There is no need for specific biomarkers in passive targeting (Rani et al., 2012; Danhier et al., 2010). The structure of the blood vessels supplying normal tissues is very tight and permeable for particles smaller than 10 nm. In contrast, the blood vessels that feed the tumor tissues lose their tight structure and become permeable as their pore dimensions become 100 nm – 1 μm (Emerich & Thanos, 2006; Cole et. al., 2011). Using these features, drug loaded nanoparticles can be sent to tumor site by the circulatory system and accumulate without needing any cell surface markers; thereby, sufficient amount of medication can be sent to the tumor site without giving high doses of medications to the patients (Kim et al., 2011; Sailor & Park, 2012; Swami et al., 2012). The most important points to consider in this method are the size, surface properties and duration of exposure in the circulation of the used nanoparticles. The nanoparticle surface must be hydrophilic to be protected from macrophages. If otherwise, this can be achieved by modifying the surface with a hydrophilic polymer. The polymer most widely used for this purpose is poly(ethyleneglycol) (PEG),called 'stealth' polymer (Egusquiaguirre et al., 2012). However, hydrophobic surfaces are more suitable for penetrating into the tumor cells and thus increasing efficiency and efficacy. The dilemma may be solved by functionalizing the nanoparticles with a copolymer which has both hydrophilic and hydrophobic domains (Cho et al., 2008).

It has been shown that positively charged nanoparticles are easily taken up by cells, but they cause immune reactions. Therefore, for clinical applications, neutral or negatively charged nanoparticles are preferred (Gratton et al., 2008).

Particles less than 5 nm are rapidly cleared from the circulation through extravasation or renal clearance. Nanoparticles that are smaller than 100 nm can be phagocytosed by the reticuloendothelial system cells (mainly by macrophages). For larger particles sized in the range of up to few micrometers, accu-

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mulation occurs primarily in the liver, the spleen and the bone marrow. Spherical particles in100–200 nm size range have the highest potential for prolonged circulation because they are large enough to avoid uptake in the liver, but small enough to avoid filtration in the spleen (Kim et al., 2011; Petros & DeSimone, 2010; Huile et al., 2011).

Nanoparticle degradation rate and duration of exposure in the circulation should be appropriately engineered to increase the effectiveness of treatment and in order for the concentration of nanoparticles in the tumor site reach the desired level.

In addition to leaky vasculature, the acidic conditions of the tumor microenvironment can also be exploited to deliver antitumor therapeutics selectively to the tumor tissues by designing nanodevices to obtain a pH-controlled drug release (Egusquiaguirre et al., 2012; Danhier et al., 2010; Manchun et al., 2012; Shen et al., 2012).

Passive targeting has some limitations; it may not show the same success in each cancer because of the fact that the EPR effect varies depending on the tumor site, tumor size and the patient, and there can even be huge differences in different areas of a single tumor (Jee et al., 2013). The duration that the nanoparticles will stay in circulation intact (half-life)should be long enough and their structures should not degrade or release the therapeutic agent before reaching the target tumor site (Oh et al., 2013).

Active Targeting

When low nonspecific binding and internalization of the drug by the cells is highly desirable, the carrier system must be functionalized by conjugation of receptors to specific ligands, like monoclonal antibodies (mAb), short peptides or RNA aptamers for *active targeting* (Figure 3).The ligands bind to receptors that are overexpressed on the surface of rapidly dividing cancer cells. For example, many types of cancer cells overexpress transferrin and folate receptors due to the high metabolic demands generated by rapid proliferation, and this makes conjugation of transferrin, folic acid or antibodies to these receptors a successful targeting approach for engineered nanoparticles (Petros&DeSimone, 2010). If the targeted receptors activate endocytosis pathways and are present in excess on the surface of tumor cells, the success of the penetration of nanoparticles and thus the success of chemotherapeutic agents are increased (Gary-Bobo et al., 2012).

Most obvious target is the surface markers expressed by the specific cancer cells. There is tremendous amount of literature trying to identify specific markers for these cells (Hu & Fu, 2012; Adler & Dimitrov, 2012). But the main challenge of active targeting tumors is that the cancer cells are often very similar in characteristics to its surrounding healthy tissue, and finding specific markers is troublesome.

Other popular approaches are to target tumor vasculature via vascular endothelial growth factor receptor (VEGFR), $\alpha_v\beta_3$ integrin, vascular cell adhesion molecule-1 (VCAM-1), and to target cell proliferation viahuman endothelial receptors (HER), transferrin (Tf) receptors, and folate receptors, instead of the tumor cells. As these molecules have less phenotypic variations and they are not specific to cancer type, they are easier targets (Kumar & Li, 2001; Byrne et al., 2008; Goel et al., 2011).

There are several biological barriers at the cellular level that an engineered nanoparticle must overcome. For example, the cell membrane blocks diffusion of complexes larger than ~1 kDa. The blood-brain barrier (BBB) is one of the strongest barriers in terms of delivery of cancer therapeutics. The blood-brain barrier is a physiologic obstruction to the delivery of systemic chemotherapy to the brain parenchyma and central nervous system (CNS), since endothelium in the CNS is distinctly different from the vasculature found in the periphery. Passive delivery does not work in CNS malignancies, because

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the leaky vasculature is not present in CNS, and only highly lipophilic molecules can passively diffuse through BBB to reach CNS. Therefore, nanodevices cannot be accumulated specifically to tumor cells in the brain without the active targeting (Zhan & Lu, 2012; Zhou et al., 2012; Chen & Mitchell, 2012).

Concentration of surface ligands is another parameter in active targeting scheme that affects the ligands' targeting efficiency. Higher ligand densities are used as a method of increasing the probability of nanoparticle interactions with cell receptors (Wang &Thanou, 2010).

The immobilization of the ligands to the nanoparticles is another important parameter of concern since several ligands have three dimensional, tertiary structures. If the immobilization is random, it can alter the ligand's 3-D configuration and reduce or completely block its binding capacity to the target cell. Therefore ligands need to be selected or engineered to bind onto the nanoparticles in a site specific manner in order for their activity to be retained.

Triggered Targeting

Certain nanoparticles can be activated by the external forces for diagnosis and treatment after given to the patient's body owing to their unique characteristics. This targeting type is named *triggered targeting*. Magnetic nanoparticles are used commonly in this targeting scheme and magnetic field is used as the trigger. Magnetic NPs given into the body can be positioned to the desired tumor site by applying external magnetic field (Pradhan et al., 2010).

This method also includes triggering nanoparticles targeted with active or passive methods for drug release with stimulus. Potential stimuli can be electromagnetic or electric fields, pH, solvents, heat, ultrasound, mechanical stress, irradiation and chemical or biological agents. Nanodevices which are sensitive to these stimuli can be reacted by changing their chemical, electrical, optical, superficial or permeation properties (Schmaljohann, 2006). Drugs can be released from heat-sensitive nanodevices within specific temperature ranges. The systems that are designed for higher temperatures than the body temperature are used in this method. After the accumulation of the nanoparticles in the tumor site, this area is heated locally until it reaches the necessary temperature and drug release is provided (May & Li, 2013; Liu et al., 2010). The biggest disadvantage of this system is that, the healthy tissue around the tumor can also be damaged because this region reaches hyperthermia temperature. Therefore, studies are being directed to non-thermal treatment methods. One of the most promising methods is photodynamic therapy, where light-sensitive nanosystems are exposed to irradiation after sent to tumor site. Drug delivery system is activated by irradiation and the drug can be released in a controlled manner (Derycke& de Witte, 2004; Keall, 2011).Using ultrasound sensitive nanodevices is another promising approach (Mo et al., 2012; Paliwal&Mitragotri, 2008).The biggest advantages of triggered targeting are that they do not need any invasive operation and they potentially can overcome the problem of drug resistance since they release the therapeutic agent only in the desired region of the body (Rapoport et al., 2004).

Triggered targeting systems mainly consist of four steps;

1. Giving nanoparticles into the systemic circulation or locally to particular area,
2. Accumulation in the tumor site (actively or passively),
3. Activation of the vehicles by external forces, and
4. Processing to apoptosis of undesired cells (Chatterjee et al., 2008).

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Thermal therapies have been used to treat cancer for a long time and are based on killing cancer cells by heating them up to 45°C (Martirosyan, 2012). There are basically two types of thermal treatments; magnetic nanoparticle-based hyperthermia and photothermia. Inorganic nanoparticles used in these systems can be utilized for both tumor imaging and treatment. Gold NPs are good contrast agents due to strongly absorbing and scattering capability in the near-infrared (NIR) region of the radiation spectrum. Absorption band can be adjusted by tuning gold nanoshell thickness, changing shape and size of the NPs. Recently, carbon nanotubes have been also used in photothermal therapy due to their naturally NIR absorption property. Long-wavelength laser irradiation is applied in the body in photothermal therapy and NPs (particularly gold NPs) absorb irradiation and generate heat to kill cancer cells (X. Huang et al., 2006). The efficiency of the treatment depends on nanoparticle concentration and laser power. In order to ensure death of cancer cells without killing normal cells these two criteria should be standardized (Lal et al., 2008).

First attempt to hyperthermia had been done by Gilchrist and his coworkers in 1957 and iron oxide particles were distributed in lymph nodes of dogs and after exposure to alternating magnetic field,5°C increase in temperature was observed. Magnetic nanoparticle-based hyperthermia is based on the heating of nanoparticles under exposure to alternating magnetic field by energy absorbing. However main disadvantage of hyperthermia is that healthy tissues can also be affected because of the nonselective heating (Hilger et al., 2005; Cassim et al., 2009; Johannsen et al., 2010; L. Zhao et al., 2011). Therefore, only cancer cells must be heated by using selective systems such as active targeting. The therapy also can be achieved by not only killing cancer cells but also activating anti-cancer immunogenicity with hyperthermia (L. Zhao et al., 2011). Low pH and hypoxic conditions are more effective for hyperthermia.

Hyperthermia has fewer side effects than chemotherapy and radiotherapy. Hyperthermia increases perfusion and oxygenation, therefore the chemotherapeutic agents are taken into the tumor cells easier and formation of free radicals may be enhanced (Cassim et al., 2009). For this reason, hyperthermia gives better results in therapy if it is used in conjunction with chemotherapy and/or radiotherapy (Martirosyan, 2012). Magnetic properties of the particles, magnetic field strength, frequency of oscillation, and cooling capacity of the blood flow in the tumor site have effect on the extent of temperature rise (Monica Tang et al., 2009). However, heating rate and time should be controlled to avoid damaging of healthy cells.

6. NANOPARTICLE-MEDIATED GENE THERAPY

The main objective in cancer gene therapy is the development of efficient, non-toxic gene carriers that can encapsulate and deliver foreign genetic materials into cancerous cells. This genetic material can inhibit the expression of a specific defective gene or express a normal gene. The classical media for gene delivery is viral vectors, but these have great disadvantages in terms of their safety, immunogenicity, and limited capacity of carrying transgenic materials. Nanotechnology has enabled the development of new, non-viral vectors for gene therapy like cationic polymers such as poly(ethylenimine) (PEI) and poly (L-lysine) (PLL), cationic peptides and cationic liposomes. Yet, these non-viral vectors are less efficient than viral vectors, but they have great promise for improvement (El-Aneed, 2004).

There are four major problems to be solved for success of cancer gene therapy (Scanlon, 2004):

1. Identification of key target genes critical for disease pathology and progression;
2. Identification of the appropriate therapeutic gene to inhibit disease progression;

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3. Optimal trans-gene expression for suppressing the target gene;
4. Delivery of therapeutic product to the target tissue at an efficient dose.

Small interfering RNAs (siRNAs) are new tools for gene therapy. They silence the expression of specific target genes by mediating RNA interference (RNAi) in mammalian cells. They have demonstrated great potential in cancer therapeutic applications caused by abnormal gene over-expression or mutation (Wang et al., 2010).Chemical modifications as well as nanoparticle conjugation of siRNAs, aptamers, ribozymes, antisense (AS) oligonucleotides (ONs), and microRNAs (miRNAs) are shown to improve the pharmacokinetic and pharmacodynamic properties, reduce immunogenicity, increase target affinity and specificity, decrease susceptibility to nuclease degradation (Singh et al., 2010; Zheng et al., 2012; Lam et al., 2013; El-Aneed, 2004).

As in previous schemes, tumor vasculature is another viable target for gene therapy simply on the basis of anatomical accessibility. Particularly, combination of transductional and transcriptional targeting should allow a higher selectivity against tumor vasculature. Antiangiogenic molecules such as angiostatin and endostatin regulating proliferation of normal endothelium are suitable as therapeutic mediators. Several cytotoxic gene products like ricin and diphtheria toxin can also be targeted and may be used to destroy tumor vascular endothelium directly (Bazan-Peregrino et al., 2007).

7. FUTURE PERSPECTIVES

Nanotechnology is beginning to allow scientists, engineers, and physicians to work at the cellular and molecular levels to produce major advances in life sciences and healthcare. Nanotechnology holds the promise of novel and more effective treatments for serious human health issues. Utilisation of nanoparticles in diagnosis and treatment is becoming widespread, and these particles are en route to become indispensable in the medical arena. The diagnosis and the treatment of diseases can be combined by nanoparticles (*theranostics*). This combinatorial approach provides "point of care" cure without needing repeated treatment steps such as biopsy which damage the patient physiologically and psychologically. Although many nanodevices and production strategies have been produced to date, the challenge still remains in creatively integrating the different functionalities into single full-featured tools.

Another challenge is that, there are no reliable control mechanisms or standards of nanoparticles' biocompatibility. The results may differ from method to method or even from laboratory to laboratory. To achieve the implementation of nanoparticles in clinical or preclinical level studies, international agencies must accelerate focused work on the development of *in vitro* and *in vivo* validation assays as well as standards for production of nanodevices that possess desired properties.

At present, the main cell types that are targeted in the tumor vasculature are endothelial cells and stem cells. However, tumor vasculature also includes pericytes and smooth muscle cells that could be important targets for nanoparticle based drug therapies.

Like normal stem cells residing in a "stem cell niche" that maintains them in a stem-like state, CSCs also require a special microenvironment to control their self-renewal and undifferentiated state. The "CSC niche" is likely to be the one of the most compelling targets in the nanoparticle based treatment of cancer.

Advanced delivery strategies are critical to fully harness the power of RNA and the flexibility of RNA-based therapeutics. The future prospects of RNA-based drugs and gene delivery will require biochemical refinements to minimize off target toxicity and immunogenicity.

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Each type of nanoparticles has its own distinguishable advantages and disadvantages. Utilisation of individual characteristics of different types of nanoparticles will help improve multifunctional nanotheranostics and development of personalized medicine. Every person has a unique metabolism, genetic background and health history, and is affected differently by the internal and external factors. Therefore, even if each disease has same or similar symptoms, it progress diversely in each individual, and diagnosis and treatment must be performed on the basis of patient's own information. This difference is more prominent in diseases such as cancer caused by body's own cells' mutations and differentiation. Thanks to nanoparticle based personalized approaches it will be possible to develop patient-specific therapies in near future. It is anticipated that the patient's own antibodies can be used and concentrations of drugs and targeting systems may be adjusted according to the individual needs.

The production of controlled, uniform and functionalized nanoparticles and systems are developing very quickly. Comprehensive investigations have been made to overcome existing problems such as toxicity, non-biodegredibility, insolubility, non-effective targeting and more promising results are obtained each day. Future nano-systems will include the necessary tools for both diagnosis and treatment, and they will be activated from outside in order to ensure local and specific treatment. Thus, using unnecessary amounts of toxic chemicals will be prevented. Advanced nano-systems can be engineered to determine pathogen microorganisms and harmful cells that are in motion and they can be incorporated protective and preventive properties supplementary to the body's own immune system.

With an ideal hybrid nano-device, it may be possible to treat different diseases simultaneously and effectively without causing any adverse effect on the patient by efficiently controlling the release rate and concentration of the therapeutic agents. Improved sensitivity and selectivity will allow diagnosis of the disease at a very early stage, or even reveal the disease before it commences. To attain these goals, research groups must be multidisciplinary so that engineers, doctors, fundamental scientists and materials scientists can collaborate.

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KEY TERMS AND DEFINITIONS

Cancer: A serious disease generally characterized by uncontrolled proliferation of cells whose metabolic activities are disrupted.

Conventional Therapies: Used to describe traditional cancer treatment methods used in clinic today.

Gene Therapy: A technique that uses genetic modification to treat or prevent disease.

Nanoparticle: A small particle whose two or three dimensions are between 1 – 100 nm.

Nanoparticle Drugs: Drugs or drug delivery systems, which are approved and/or under phase trials.

Nanoparticle Toxicity: The possible threats of nanoparticles to the patients.

Targeted Therapy: A relatively new method for the treatment of diseases by targeting specific organs, tissues, cells, and even biomolecules.

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Chapter 48
Nanomedicine:
Therapeutic Applications and Limitations

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ABSTRACT

Nanomedicine, an offshoot of nanotechnology, is considered as one of the most promising technologies of the 21st century. Due to their minute size, nanomedicines can easily target difficult-to-reach sites with improved solubility and bioavailability and reduced adverse effects. They also act as versatile delivery systems, carrying both chemotherapeutics and imaging agents to targeted sites. Hence, nanomedicine can be used to achieve the same therapeutic effect at smaller doses than their conventional counterparts and can offer impressive resolutions for various life-threatening diseases. Although certain issues have been raised about the potential toxicities of nanomaterials, it is anticipated that the advances in nanomedicine will furnish clarifications to many of modern medicine's unsolved problems. This chapter aims to provide a comprehensive and contemporary survey of various nanomedicine products along with the major risks and side effects associated with the nanoparticles.

INTRODUCTION

Nanotechnology has been proved significantly beneficial to society by producing major advances in areas of energy, food and agriculture. However, the role of nanotechnology in the advancement of healthcare is most promising. Nanomedicine is an application of nanotechnology to healthcare which utilizes improved physicochemical and biological properties of nanoscale structures for better diagnosis and treatment of diseases. Nanostructures usually possess unique properties. They allow the miniaturization of biomedical devices, leading to quicker and integrated operations. Again, nanometer size of these structures permits them to efficiently interact with the biology of living organism. These aspects keep

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the promise to render achievements in nanomedicine, resulting in big impact on preventive medicine, diagnosis, therapy and follow-up care.

Nanotechnology could provide improved *in vitro* diagnostic tests with more sensitive detection technologies or advance nano-labels detectable with high sensitivity after binding to disease-specific biomarker present in the sample. Moreover, nanotechnology allows size reduction of biomedical lab tests leading to significant reduction of the sample volume, potentially expensive reagents like monoclonal antibodies and most importantly time required. These advantages of nanomedicine in the area of diagnostics render quick and cost effective detection of a disease.

Since last decade, the role of nanotechnology in therapeutics has been studied extensively. Nanotechnology based delivery systems has shown promising results in targeting only diseased tissue and hence increasing the efficacy and limiting the side effects of therapeutics. Moreover, nanotechnology based delivery systems also have the ability to combat with drug solubility, permeability and early clearance issues associated with small molecules and biologics. Multifunctional nano-delivery systems evolved recently have the capability of simultaneous targeting, diagnostic and therapeutic action. Therefore, targeted delivery systems and nanotechnology-assisted regenerative medicine will play the central role in future therapy. This chapter discusses about various nanostructures and their application in diagnosis and therapy of diseases.

NANOSTRUCTURES IN MEDICINE

Nanoparticles are materials with overall dimensions in the nanoscale i.e., under 100 nm and offers exiting prospects for the development of novel, clinically pertinent diagnostic and therapeutic multifunctional systems. In recent years, these materials have emerged as imperative players in contemporary medicine, with clinical applications extending from carriers for drug and gene delivery into tumor to contrast agents in imaging (see Figure 1). Indeed, there are few instances where nanoparticles facilitate analyses and remedies that merely cannot be implemented otherwise. The aim of this chapter is to highlight the major categories of nanoparticles employed in modern medicine and their uses.

Polymer Based Nanocarriers

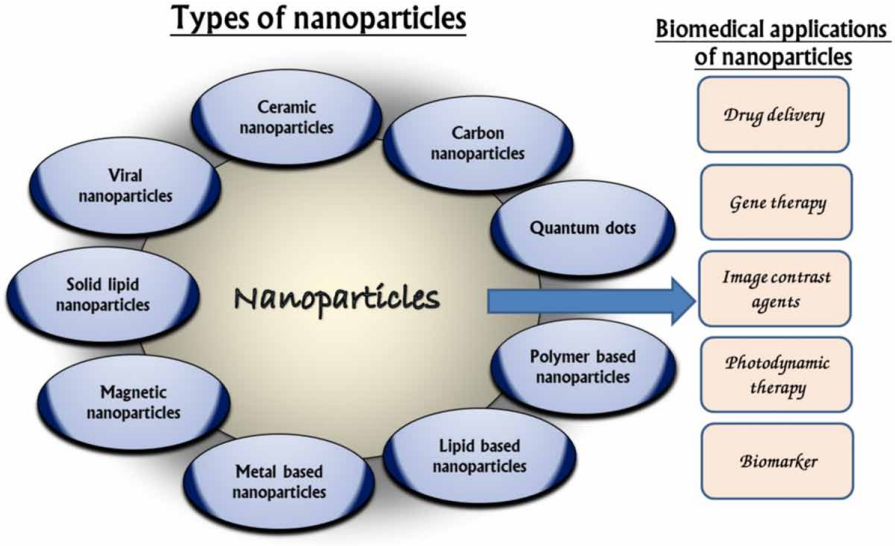
Polymeric nanoparticles are biodegradable systems in the range of 10-200 nm. Ease of surface modification and ligand functionalization renders them excellent pharmacokinetic control and can be used to deliver a plethora of chemotherapeutic agents.

Polymeric Drug Conjugates

Polymeric nanoparticles comprises of nanosized solid particles or capsules consisting of natural or synthetic polymers such as albumin, chitosan, poly ethylene glycol (PEG) etc. to which the drug is attached. Drug molecule can either be adsorbed or encapsulated on the polymeric nanoparticle structure (Luo et al., 2010; Mora-Huertas et al., 2010) and the release occurs via desorption, diffusion or nanoparticle erosion in target tissue (Torchilin, 2008). Recently Abraxane nanoparticles (albumin bound paclitaxel) have been employed in clinics for metastatic breast cancer treatment (Gradishar et al., 2005). HPMA and PEG are the most extensively used non-biodegradable synthetic polymers (Duncan, 2003). PEG coating

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Figure 1. Types of nanoparticles and their biomedical applications



on PLGA (polylactic-co-glycolic acid) nanoparticles have shown to improve their stability (Torchilin, 2005) and stealth properties. These drug-biodegradable polymeric nanocarrier conjugates are non-toxic and non-thrombogenic. They are also non-immunogenic as well as non-proinflammatory, and they neither activate neutrophils nor affect reticuloendothelial system (des Rieux et al., 2006). PLGA conjugates have been widely used as representative chemotherapeutic agents in clinics both *in vitro* and *in vivo* and have displayed encouraging capabilities to evade the shortcomings of their free drug counterparts (Li, 2002).

Polymeric Micelles

Self-assemblage of amphiphilic block copolymers such as poly (ethylene oxide)-poly (β-benzyl-L-aspartate) and poly (N-isopropylacrylamide) polystyrene, in aqueous media results in the formation of a nano-sized core/shell structure documented as polymeric micelles. The outer shell confers stability to the hydrophobic core region which serves as a drug reservoir, thereby rendering the polymers water solubility. Micelles can be readily altered with small functional groups, enhancing their targeting capability and their specific accumulation in pathological tissue is attributed to its small size (Qiao et al., 2010). External stimulus such as pH, temperature, ultrasound etc. can be used to control the drug release (Rapoport, 2007) but low drug incorporation stability and low loading efficiency limits the targeting ability of the polymeric micelles (Yamamoto et al., 2007; Seow et al., 2007).

Dendrimers

Dendrimers are highly branched synthetic polymeric molecules emerging radially from a central core. Dendrimers can be easily customized for optimal activity, mode of action, pharmacokinetics, steadiness and biocompatibility. The precision with which their size and surface functionality can be tuned makes them highly promising nanoscale molecules for pharmaceutical applications. Dendrimers offer colossal scope for solubilization of hydrophobic compounds, and can be readily altered with guest molecules (Yang

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et al., 2009). The easily malleable surface properties of dendrimers enables their simultaneous conjugation with various molecules such as therapeutic drugs, targeting ligands and/or imaging contrast agents etc., yielding a dendrimers-based multifunctional drug delivery system (Svenson and Tomalia, 2005).

Lipid Based Drug Carriers

Liposome

Liposomes are 80–300 nm size range nano-scale spheres comprising of a lipid layer surrounding the drug (Sunderland et al., 2006). They are spherical vesicles which form simultaneously on dispersion of lipids in aqueous medium. Liposomes have been noted to increase the solubility of drugs and augment their pharmacokinetic properties, such as the therapeutic index of chemotherapeutic agents, fast metabolism, and reduction of deleterious side effects and increase of *in vitro* and *in vivo* anticancer activities. A drug is incorporated in liposomes by the encapsulation process and its release from liposomes depends on the liposome composition, pH, osmotic gradient, and the surrounding environment (dos Santos Giuberti et al, 2011).

Surface modification of liposomes using a biocompatible polymers such as PEG decrease their recognition by macrophages thereby increasing the stability of the liposomal formulations as well as their circulation half time (Torchilin, 2005). Moreover, to promote accretion in the desired destination, liposomes are fabricated with ligands enabling them to identify and bind to precise cell clusters. The herceptin/trastuzumab antibody is employed to target the Her-2 antigen expressing breast cancer cells (Colbern et al., 1999). RGD is utilized to target integrins that are upregulated by proliferating endothelial cells of the tumor vasculature (Lee and Low, 1994). Customized nanoscale liposomes have been indicated to possess excellent pharmacokinetic profiles for the transport of DNA, antisense oligonucleotide, siRNA, proteins and chemotherapeutic agents. First liposomal formulations of the anti-cancer drug doxorubicin were launched under the trade names such as Doxil®/Caelyx® or Myocet® in beginning of the 1990s.

Solid Lipid Nanoparticles (SLNs)

SLN are particles made of solid lipids i.e. Lipids that are solid at room temperature and also at body temperature, (e.g., highly purified triglycerides, complex glyceride mixtures or waxes) and stabilized by various surfactants (Kovacevic et al., 2011). SLN`s possess substantial physical stability, good tolerability, protect the accumulated drug from degrading and act as a meticulous drug delivery vehicle.

PEGylation of SLN`s checks their clearance from circulation by liver and spleen (stealth property) and drug-tumor targeting is assisted by PEG-coated polymeric NPs (Parveen et al., 2012). Stealth SLN escalates the tumor accumulation, augmenting the activity of antiparasitic and antifungal drugs incorporated with the nanoparticles, and also permit brain delivery of anticancer drugs incapable of traversing the Blood Brain Barrier (BBB) (Zara et al., 2002). Thus, these modified SLNs may have great potential for targeted gene delivery (Yu et al., 2010).

Metal Based Nanoparticles

Gold, nickel, silver, iron oxide, zinc oxide, gadolinium, cerium and titanium dioxide particles constitute most common metallic nanoparticles (Doria et al., 2012). Metallic nanoparticles are extremely

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small (~50 nm) and thus a large surface area facilitates integration of relatively higher doses of drugs (Tourinho et al., 2012; Xu et al., 2006; Wong and Liu, 2012). These nanostructures can be synthesized and refashioned with discrete functional groups allowing their easy association with drugs of interest, ligands and antibodies thus paving the way for a broad range of potential applications in targeted drug delivery including carriers for gene and drug delivery and more importantly diagnostic imaging.

One of the most significant biomedical applications of metal nanoparticles are being utilized as contrast agents which lead to the development of various imaging modalities such as MRI, CT, PET, ultrasound, SERS and optical imaging as an aid to image various disease states (Qian et al., 2008) as well as photo thermal and thermo lytic laser therapies. Gold nanoparticles (AuNP`s) are the most commonly used metal based nanoparticles as they are nontoxic and biocompatible and because of their negative charge they are easily functionalized by various biomolecules (Bhattacharya et al., 2007; Hainfeld et al., 2006). AuNP`s also have a diverse drug delivery applications. They have been proved to be potent drug delivery vehicles for the delivery of anticancer drugs such as oxaliplatin (Brown et al., 2010) and trans-mucosal insulin delivery (Joshi et al., 2006). Despite the metallic nanoparticles being biocompatible and inert vehicles their use for drug delivery is a concern as a considerable portion of metal particles can be accumulated in the body following drug administration, possibly triggering toxicity (Wang et al., 2012).

Magnetic Nanoparticles

Magnetic nanoparticles are a class of nanoparticles which can be manipulated using an external magnetic field. Magnetic nanoparticle based drug delivery systems have gained importance because of their ability to target a specific site and enhance uptake at the destination thereby reducing the systemic distribution of cytotoxic compounds *in vivo* resulting in efficient treatment at therapeutically optimal doses. Iron oxide nanoparticles are most commonly used magnetic nanoparticles for therapeutic purposes. The super paramagnetic properties of iron (II) oxide particles facilitate directed delivery of microcapsules by external magnetic fields. Another advantage of using magnetic NPs is their hyperthermic properties, which allows heating the particles after internalization, triggering drug release (Brazel et al., 2006). The paramagnetic particles offer a powerful strategy for directed and localized heating of cancerous cells thereby reducing the risk of destruction of the surrounding healthy tissues caused by conventional heating techniques.

Surface coating of the super paramagnetic nanostructures with materials such as silica and PEG renders stability as well as biocompatibility and surface customization with appropriate ligands like oligonucleotides, proteins and antibodies enables targeted delivery and efficient cellular uptake (Bryant, 2007). Super-paramagnetic nanoparticles are widely utilized as contrast agents in magnetic resonance imaging (MRI) as they possess superior magnetic susceptibility than traditionally employed MRI contrast agents, such as gadolinium (Sonvico et al., 2005), can be used for multiplex immunoassays (Ibraimi et al., 2006), and also for separation of pathogenic cells from normal cells (Bryant, 2007). Thus it can be concluded that ease of accessibility to hidden tumors, minimal side effects and minimal invasiveness gives the magnetic nanoparticles a better effect over classical cancer therapies.

Viral Nanoparticles (VNPs)

VNPs are regarded as one of the most versatile nanomaterials produced by nature. Viruses deliver their cargo of genetic materials by infecting specific host cells with great accuracy, thereby providing an ideal

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foundation for the development of targeted drug delivery vehicles and tissue specific imaging reagents. Virus like particles (VLPs) are the non-infectious counterparts of VNPs expressed in heterologous systems lacking any genomic nucleic acid. VNPs are extraordinarily robust self-assembling organizations that form exceedingly symmetrical, polyvalent, and monodisperse structures. They present programmable scaffolds and can be generated in substantial quantities in a short time span.

VNP`s of bacteriophage and plant virus origin are particularly advantageous as they are less likely to be pathogenic in humans thereby less likely to induce detrimental side effects. Biocompatibility and biodegradability of the virus nanoparticles offers further advantage over synthetic nanomaterials. The basic assembly of the nanoparticles can be engineered in a number of ways to confer diverse functions to the VNPs. Drug molecules/imaging reagents/quantum dots and/or other nanoparticles can be crammed into the internal void, whereas alteration of the external surface with specific ligands allows targeted delivery and their specific tumor targeting ability *in vivo* is mediated by conjugating them with several ligands including folic acid, transferrin and single chain antibodies (Manchester and Singh, 2006; Singh et al., 2006).

VNPs have been engineered for the targeted delivery of drugs and also for targeted photodynamic therapy (PDT). Fluorescent VNPs could be coalesced with targeting moieties to formulate a potent novel ploy for non-invasive disease specific imaging (Lewis et al., 2006; Wu et al., 2005) and more recently many VNP`s have been developed as a contrast agent platforms for non-invasive imaging techniques such as MRI and PET (Liepold et al., 2007). Although mammalian viruses (e.g. adenovirus) have also been scrutinized in the context of nanotechnology, but presently they are only employed in gene delivery, not drug delivery or imaging (Parker et al., 2008; Singh and Kostarelos, 2009; Steinmetz, 2010).

Ceramic Nanoparticles

Ceramic NPs are particles fabricated from inorganic compounds such as silica and other materials like alumina, titania, etc., which are vastly consistent with biological systems and are inert in nature. Their ultra low size, porous framework, high surface area for adsorption of therapeutic or diagnostic agents and ease of functionalization attributes to their becoming important drug delivery vehicles (Amato, 2010).

Because of their small size (~50 nm) they easily evade uptake by RES and also do not demonstrate any pH related conformational change (leading to change in porosity or swelling). Therefore, these particles can efficiently shield diverse bio-macromolecules, such as enzymes, against denaturation induced by changes in the external pH and temperature. Further these nanoparticles have easily modifiable surface characteristics which aid their conjugation to target specific ligands such as monoclonal antibodies (Lal et al., 2000; Bradley et al., 1990).

Diverse types of drugs, such as anticancer drugs, antibiotics etc. have been incorporated into (Di Pisqua et al., 2009; He et al., 2011) a ceramic nanoparticle by chemical or physical absorption and the drug release is usually controlled by diffusion (Li et al., 2010). The amorphous structure of ceramic nanoparticles enables incorporation of both small and large molecules (Di Pisqua et al., 2009; Kim et al., 2011), DNA absorption and gene transfer (Slowing et al., 2008) permitting their use in combination therapy (He et al., 2011). Ceramic nanoparticles are also used as non-viral vectors for efficient *in vivo* gene delivery (Losic et al., 2010; Mainardes et al., 2010) and as a drug carrier for photodynamic therapy (Losic et al., 2010).

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Carbon Nanoparticles

Carbon nanotubes (CNT`s) and nanohorns (CNH`s) are the most commonly used carbon based nano-assemblies. They belong to fullerenes, a family of structures made exclusively of carbon (Sanvicens and Marco, 2008). Carbon nanotubes are cylinders made up of coaxial graphite sheets and can be classified into single-walled carbon nanotubes (SWNT) (one graphite sheet) or multi-walled carbon nanotubes (MWNT) (several concentric graphite sheets) (Qiao et al., 2010). Nano horns are a type of the only single-wall nanotubes– demonstrate properties analogous to nanotubes, are highly pure and can be easily prepared with very low cost (Shiba et al., 2006).

Certain health concerns may be raised by the insolubility of carbon based nanoparticles in most solvents but they can be made water soluble by chemical alteration of their surface qualities and multiple covalent functionalization link them to a wide array of molecules such as nucleic acids, peptides and other therapeutic agents (Vardharajula et al., 2012).

Immobilization of drug in carbon nano-carrier can be obtained by encapsulation of drug in the carbon nanotubes (Arsawang et al., 2011), chemical adsorption on the surface or in the spaces between the nanotubes (Chen et al., 2011), and attachment of active agents to functionalized carbon nanotubes. Encapsulation being advantageous over the other two methods as the drug is protected from degradation during its transport and released only in specific conditions. Drug release from carbon nanoparticles can be electrically or chemically controlled.

Carbon nanoparticles are biocompatible and have unique electronic, structural, and thermal physiognomies making them appropriate vehicles for drug delivery systems. Biological application of CNP`s include their use as drug carriers (anticancer drugs, antifungal agent), fluorescent agents (Wu et al., 2005; Pastorin et al., 2005) and biosensors for DNA and protein detection (Vardharajula et al., 2012). However with the increasing evidences exhibiting the toxicity of carbon NPs, the interest to develop carbon NPs for drug delivery has lessened considerably in recent years (Schipper et al., 2008; Drabu et al., 2010).

Quantum Dots

Quantum dots are semiconducting nanocrystals with diameters ranging from 2-10 nm. They consist of a metalloid crystalline core wrapped inside a semiconductor shell having a large spectral band gap. An electron–hole pair is generated when a QD absorbs energy greater than the spectral band gap of the core semiconductor. The exited nanomaterials on reverting back to its ground state emits a photon and produces a strong fluorescence signals which can be detected by diverse techniques like confocal microscopy, total internal reflection microscopy, fluorescence microscopy, wide-field epifluorescence microscopy as well as fluorometry (Michalet et al., 2005).The QD`s have a broad absorption spectrum, narrow emission spectrum and a high photo stability (Azzazy et al., 2007; Azzazy and Mansour, 2009; Michalet et al., 2005).

Quantum dots can facilitate the detection of numerous cancer biomarkers in blood assays or on cancer tissue biopsies. Presently, one of the fastest growing fields of nanotechnology is the use of highly stable, water soluble, functionalized QDs for biological and biomedical applications. QDs offer new prospects for live cells, *in vivo* imaging and diagnostics aided by their stable fluorescent properties. QDs can also be adapted with diverse biomolecules, such as antibodies, streptavidin, and oligonucleotides for specific target recognition. They have also shown displayed substantial usefulness in genomic investigation and

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nucleic acid detection (Azzazy and Mansour, 2009; Michalet et al. 2005) and are also utilized for surgical guidance applications (Kim et al., 2004). Regardless of its extensive applications their high toxicity and ambiguous defrayal mechanisms from the body possess a major obstacle for their use in *in vivo* imaging.

APPLICATIONS OF NANOMEDICINE

Diagnostics

Disease diagnosis is an essential element of disease care process. Early detection of disease can lead to less severe therapeutic requirements and better clinical results. To achieve advance disease diagnosis, better understanding of biology and medicine at the molecular level with accurate quantification need to be perceived. Nanotechnology combined with advanced instrumentation has the ability to fulfill this goal. A variety of nanomaterials can be used as labels in diagnostics and detection by virtue of a unique combination of chemical and physical properties that allow detection of low concentrations of disease biomarkers. Nanoparticles like gold nanoparticles (AuNPs) and quantum dots (QDs) are most widely used in diagnosis but other nanotechnological devices like nanocantiliver, nanowire as well as nanobiosensers have promising clinical functions. A brief overview of various types of nanoparticles which are being developed for improved diagnostics is given in Table 1.

Table 1. Nanomedicine in diagnosis of diseases (Roy et al.)

Type of Nanomaterials	Diagnostic Strategy	Advantages
Aptamer-AuNP conjugates	Conjugation of aptamer with AuNPs provides selectivity and specific affinity of aptamers and spectroscopic advantages of AuNPs to detect diseased cells	Sensitive detection and differentiation of different types of target and control cells based on the aptamer
Magnetofluorescent particle systems	Bimodal contrast agents with magnetic and fluorescent properties	Noninvasive breast cancer diagnosis
Antibody conjugated AuNPs	Identification by specific targeting of cell surface proteins with specific antibodies conjugated with AuNPs	Quick and efficient identification and quantification of tumor cells
Semiconductor fluorescent QDs	The fluorescence emitted by these biomarkers enables efficient cancer diagnostics	Facilitates speedy and accurate cancer diagnostics
Semiconductor QDs	Detection of cancer biomarkers aided by intense stable fluorescence	Detection of cancer at molecular levels
Aptamer conjugated NPs	Magnetic NP-aptamer conjugates can be used for selective targeted cell extraction and their fluorescent counterparts can play a vital role in cancer detection with high sensitivity	Enables the collection and detection of multiple cancer cells
Fluorescent europium(III)-chelate-doped NP	Europium nanoparticles along with antihexon (high affinity monoclonal antibodies) provides a sensitive immunoassay for adenovirus detection	Has potential in sensitive screening of viral analytes

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Imaging

Nanoparticles can also be successfully exploited to enhance the service of fluorescent markers for medical imaging of diseased tissue and therapeutics. Current fluorescent markers used in research and clinical diagnostic applications have various drawbacks, such as need of color matched lasers, fluorescence bleaching and lack of discriminatory capacity of multiple dyes, etc. Fluorescent nanoparticles have great ability to overcome these problems and a major advancement of these nanoparticles toward clinical applicability is *in vivo* imaging of tumors and other diseases. Currently, functionalized QDs are highly used for the purpose of imaging and diagnostics. These QDs are highly stable against oxidation for biological and biomedical applications and manifest stable fluorescent properties offering new promises for live cells, *in vivo* imaging and diagnostics. Magnetic iron oxide nanoparticles are also attracting novel contrast agents for biomedical imaging considering their ability of deep tissue imaging, noninvasiveness and low toxicity. AuNPs have also been extensively used for cellular imaging. A brief account of different studies involving use of functionalized nanoparticles for imaging of diseased cells is given in Table 2.

Table 2. Nanoparticles as contrast agents (Roy et al.)

Type of Nanomaterials	Imaging Strategy	Advantages
Poly(alkyl cyanoacrylate) NPs	Poly(alkyl cyanoacrylate) amphiphilic copolymer nanoparticles tagged with the fluorescent rhodamine B for imaging	These NPs can be used for human brain endothelial cell imaging
AuNPs	Surface functionalization of NPs with cancer specific monoclonal antibodies or surface antigen specific RNA aptamer enables specific imaging of healthy and cancerous tissue	These multifunctional NPs can be used for quick and efficient identification and quantification of tumor cells
Streptavidin NPs	Biotinylated anti-Her2 Herceptin antibody and biotinylated Cy5.5 fluorophore to a streptavidin NP provides specific imaging of the tumor	Effective for multimodal imaging of tumor in mice
Multifunctional superparamagnetic iron oxide NPs	The superparamagnetic iron oxide NPs serve as excellent MRI contrast agent.	MRI based cancer monitoring
CNPs	Tumor targeted CNPs can be used as single or dual-modality cancer imaging agents (near-infrared fluorescent dye, Cy5.5, and gadolinium (Gd(III)) ions)	Effective as a dual imaging agent (optical/MR) for cancer treatment
QD-loaded micelles	Lipid conjugated QDs together with herceptin augments tumor cell uptake and hence can be used for concurrent tumor therapy and imaging	Can be used for targeting, imaging and cancer management in the early stages
Carboxyl-functionalized silica-coated QDs	The stable fluorescent property of QDs enables specific imaging	Carboxylation renders stability to the QDs in aqueous solution. These nanoparticles provides specific tumor targeting and can be easily fabricated and thus serve as efficient targeting probes for cell imaging
Fluorescent silica NPs	Fluorescent dye loaded silica NPs can be used for cancer cell imaging. In addition these NPs can also be conjugated to specific biomolecules overexpressed on cancer cells	Cancer targeting and imaging
Polymer-Ag@SiO2 Hybrid Fluorescent NPs	Cationic surface and suitable size allows the nano-formulations to be readily internalized into cells, thus aiding cellular imaging	These nanoparticles are useful for efficient cellular imaging as they produce bright fluorescence and are cyto-compatible

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Disease Therapy

Cardiovascular Disease

Cardiovascular disease accounts for largest cause of mortality in the general population. The increasing trends in industrialized and developed country have contributed for the cardiovascular disease. Additionally, aging that alter the cardiovascular function which leads to increase in cardiovascular disease even in the healthy asymptomatic individuals (Dantas et al., 2012). The diverse complexity involved in cardiovascular disease has triggered researchers to develop a novel therapeutic concept for the future. The cost and time duration taken to cure this disease has been a challenge for researchers to develop a therapeutics drug. From 1975-2005 there has been a significant decline in the cardiovascular related deaths (Jemal et al., 2009). This is due to improvement in surgical interventions and diagnostics, as well as awareness and change in life style. More effective treatment has to be implemented to improve the patient outcome, as the reason behind cardiovascular disease is unknown.

Nanotechnology has been extensively developed in last decade. The rapid development in nanoscience has shown a new direction for biotechnology research by studying the properties of biological materials in nanometer which is different from properties of both macro and micro particles. In recent year the wide range work in nanotechnology and nanomedicine has been an advantage to synthesize a new material and device for therapeutic purpose, which are applicable in nanodiagnostics, molecular diagnostics and improvements in the drug delivery. The cardiovascular disease can be promisingly addressed by nanopharmaceuticals products (Lanza et al., 2006). These products have wide application in the treatment of patients suffering from cardiological and vascular diseases.

Worldwide, many researchers have been synthesizing the wide range of therapeutic nanoparticles for cardiovascular disease. Liposome is extensively studied for delivery of therapeutic and bioactive agents in vascular system. Liposome encapsulated with phosphotidylcholine will enrich the high density lipoprotein (HDL) at the lesion. These HDL will remove the cholesterol from the foam cells. This was confirmed by injecting the liposomal formulation in cholesterol fed rabbit for 1 week and it showed a significant decrease in atherosclerotic plaque volume. This suggests that liposome can be a therapeutic formulation for cardiovascular treatment (Barter, 2005). Micelle based strategies can be directed towards the atherosclerotic and thrombotic sites in vascular disease (Ding et al., 2006). Gadolinium (GD) loaded in micelle tagged with anti-CD36 antibody was able to target the macrophages in atherosclerotic human and also helped for MR imaging of artherosclerosis. The efficiency of this nanoparticle can be enhanced by conjugating the specific protein or antibody which helps in targeted delivery. Vascular cell adhesion molecule-1 (VCAM-1) is over expressed on endothelial cells in artherosclerosis which can be account for targeted delivery. The poly (sebacic acid)-co-PEG (PSAPEG) microparticles a polymer based nanoparticle tagged with VCAM-1 antibodies exhibited increased adhesion, binding and accumulation at atherosclerotic lesion sites in ApoE-/-mice (Deosarkar et al., 2008). Polymer based nanoparticles have been used because of its biocompatibility and biodegradable property and provide shapes, surface, size modifications and exhibited good drug loading and drug release parameters for targeted delivery to cardiovascular disease. Metal has been playing an important role in our life. Metal nanoparticles have developed a great interest in biomedical application. The unique properties exhibited by gold make it a noble metal to synthesize nanoparticles which can be applicable to treat the cardiovascular disease. Hence gold based nanoparticles by themselves or in combination with other modalities can be implemented for therapeutic and diagnostic strategies on cardiovascular disease (Gupta, 2011).

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The potential of nanoparticles for biomedical application in cardiology is promising to develop fundamentally new methods of diagnosis and treatment. The nanoparticle mediated therapy can offer surgeons and cardiologists a new ways to treat the cardiovascular diseases with higher efficiency. In future, nanoparticle–drug formulation is anticipated to enter clinical trials and will improve the diagnosis and treatment of atherosclerosis and cardiovascular diseases.

Cancer

Cancer is a broad group of disease involving unregulated growth of cells and forming malignant tumors by invading nearby parts and distinct parts throughout the blood stream. It is leading cause of death out of 58 million deaths that occurred in the world in 2005, 7.6 million is due to cancer. Currently cancer has been treated through chemotherapy, radiotherapy and surgical resection.

Initially, many cancer react to currently available chemotherapeutic and chemo preventives agents, but later it develop several side effect, such as anemia and importantly the generation of cellular resistance. Multiple drug resistance is the important factor for drug resistance in cancer cells. Efflux of most of the hydrophobic anticancer drug through ATP Binding Protein (ABC) causes MDR and thus limits the efficiency of treatment (Gottesman, 2002).

Nanotechnology has provided an innovative and promising strategy to overcome the side effect of chemotherapeutic drugs. The ideal diameter of nanoparticle is 1-100 nm which can carry multiple drugs and target a specific cancer. The nanoparticle mediated drug delivery provides an exclusive advantage for cancer therapy over free drug by enhancing the solubility, stability, pharmocodynamic and pharmokinetic properties of drug. Nano-encapsulated drug increases the drug concentration in tumor tissue through passive and active targeting which reduces the toxic side effect in normal tissue (Alberts and Garcia, 1997; Gabizonn et al., 1994; Uziely et al., 1995).

The efficiency of nanoparticle towards tumor tissue has been increased due to enhanced permeability and retention effect (EPR). The unique physiological property of the tumor microenvironment has revolutionized the anticancer therapy through EPR effect (Maeda et al., 2006). This EPR effect driven passive drug delivery occur only in tumor and limited drug penetration in normal tissue results in reduced side effects (Maeda, 2001). The efficiency of nanoparticle can be significantly increased through active targeting. Several strategies can be applied for active targeting. The receptor mediated targeting in which nanoparticle are surface functionalized with ligands to recognize a specific receptor which is over expressed in tumor tissue. The surface sensitive drug targeting designed to respond to environmental changes in microenvironment of tumor which trigger the drug release from nanocarrier (Torchilin, 2006; Couvreur and Vauthier, 2006). The delivery of therapeutic DNA into tumor cells is another application of Nano therapy to cancer therapy. The nanoparticle should be able to protect active DNA from hydrolytic enzyme and deliver the DNA in to the nucleus in transcriptionally active form. Many Nano compounds such as polymer base and porphyrins have been used to deliver the ssDNA in to the nucleus. The stability of ssDNA increased in physiological relevant condition when assembled with meso-tetra-4-pyridyl-porphyrin-DNA nano-complexes (PDN) induce light dependent cytotoxicity *in vitro* and *in vivo* in bladder cancer concluding the therapeutic property of PDN (Gosh et al., 2014). The *in vivo* tumor imaging can be done by cyanin 5.5 conjugated Nano bubble using ultrasound fluorescence imaging (Mai et al., 2013). The side effect of anticancer drug can be reduced by shielding the drug with nanoparticle. Many researchers have been working on this area. Recently it has been studied that bladder cancer can be treated by inhibiting the histone deacetylase in cancer cells. Belinostat,

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a histone deacetylase inhibitor was loaded in poly (lactide-co-glycolide) (PLGA) nanoparticles and these nanoparticles were coated with novel cell penetrating polymer poly(guanidinium oxanorbornene) (PGON). The result showed that NP-Bel-PGON illustrated a significantly lower IC50 in bladder cells compare to unencapsulated belinostat. Xenograft tumor showed 70% reduction in tumor compare to unloaded NP-PGON (Martin et al., 2013). The two different nanoparticles were used to treat the breast cancer. Silver nanoparticles possess cytotoxic effect with apoptotic feature and suggest the reactive oxygen species generated by silver nanoparticle have significant role in apoptosis (Gurunathan et al., 2013). The polymer based approach was applied to overcome the MDR effect of cancer cell. Doxorubin encapsulated chitosan-graft-D-α-tocopheryl polyethylene glycol 1000 (TPGS) (CT) nanoparticles were found to have IC 50 value of 1.5, which is 199-fold higher than Adriamycin. Both pharmacokinetic and *in vivo* anticancer properties were increased in doxorubicin loaded nanoparticle compare to Adriamycin (Guo et al., 2014). In attempt to increase the drug concentration in colon cells pH mediated drug delivery system were used. The synthetic methacrylic acid-ethyl hexyl acrylate (MAEHA) based copolymer indicated pH-sensitive drug-release behavior in the colon. 5 Fluorouracil drug loaded MAEHA Nano gels accumulated in the tumor microenvironment and induce cell death by apoptosis (Ashwani Kumar et al., 2012). The application of star shaped micelle showed a good stability against dilution with water. Star shaped micelle (PEG/PCL) were less toxic, monodisperse and biodegradable and shown to enhance anticancer activity *in vitro* and *in vivo* in colon cancer (Gao et al., 2013). Human epidermal growth factor receptor-2 (HER-2) mediates number of cellular activity in cancer cells so this receptor is highly expressed in cancer. The antibody against this receptor can be used for targeted delivery. Using HER-2 as a marker for targeted delivery a wide variety of imaging techniques can be developed for early detection of cancer and localization of tumor, as well as monitoring of drug delivery and tissues response to drug treatment. Application of HER-2 is rapidly moving towards clinical research (Wang et al., 2013).

Considerable efforts have been taken by researchers to develop multifunctional nanomedicine with high efficacy and minimized adverse effects. Researchers have developed a nanoparticle which will target highly complex disease pathology of cancer and also reduced the toxic effect of anticancer drug to normal cells. They have also designed and developed strategies to improve the cellular uptake of the therapeutic molecules and their delivery to an organelle of interest. The experimental studies suggested that nanoparticles were capable to deliver a high concentration of drug to tumor site in animal model. The high concentration of drug persists in the tumor site for a long period and had a more potent effect less toxicity when compare to free drugs. This research showed that nanoparticle and other nanostructure appeared to hold great promise for future of cancer treatment.

Neurodegenerative Disease

The neurodegenerative disorders (NDs) is a term for the persistent and progressive loss of structure and function of neuronal subtype. The most widely and commonly occurring diseases due to neural degeneration is Alzheimer's disease (AD) and Parkinson's disease (PD). It is a significant challenge to 21st century scientists to discover a medicine which specifically affects the clinical outcome for mind, motor and behavioral abnormalities. The researchers have discovered many similarities, which relate this disease to one another at sub-cellular level. This discovery offers hope to design a new therapeutic model for many neurodegenerative disorders simultaneously (Rubinsztein, 2006; Bredesen et al., 2006). The nanotechnology-directed method that can specifically target to damaged CNS and rule out the unwanted

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side effect of the drug. Polymeric nanoparticles are currently used to deliver several drugs, including doxorubicin in to the central nervous system (CNS) (Calvo et al., 2001; Alyaudtin et al., 2001; Kreuter et al., 2003). Nanogels are commonly used to increase the drug and oligonucleotide loading efficiency. *In vivo* experiments suggest higher uptake of nanogel in brain when compare to liver and spleen (Friedrich et al., 2005; Kabanov and Gendelman, 2007). In attempt to increase the encapsulation of drugs in nanoparticle, polymeric nano-liposome was synthesized. The surface modification of nano-liposome increases the systemic circulation time, reduces opsonization in plasma and decreases its recognition and removal by the liver and spleen (Shi et al., 2001; Voinea and Simionescu, 2002). Various application of nano-liposome has been studied to target the central nervous system (Mora et al., 2002; Schmidt et al., 2003). Scientist believe the further advance research will provide a scientific resource need to develop innovative nanomedicine. The improved drug delivery system and early detecting method to neurodegenerative disease will certainly change the clinical outcomes.

Diabetes

Diabetes mellitus is rapidly growing problem often referred as diabetes. In 2030 it is expected that 360 million people will be affected by diabetes (Wild et al., 2004). It is a lifelong progressive disease with serious complication such as blindness and cardiovascular disease (US Department of Health and Human Service, 2008). Permanent cure for diabetes is not available but patient can reduce the disease associated complications through tight control of blood glucose level and insulin therapy. The drawbacks like insulin resistance and chronic treatment causes anaeroxia nervosa, brain atrophy and fatty liver limits the insulin therapy for potent treatment of diabetes (Pickup et al., 2008). To overcome the drawbacks of insulin therapy, nanotechnology can be an efficient tool to treat the diabetes. Nanotechnology has a tremendous potential to make important contribution in diabetes prevention and monitoring blood glucose level. Nanotechnology offers some significant advantage owing to their small size to develop a nanosensor for monitoring the blood glucose level. These properties of nanoparticle offer a research to improve the accuracy, size, life time and usability of nanosensor (Wang, 2008). The oral delivery of protein drug such as insulin encounter major difficulty with low pH of gastric medium and digestive enzyme is a major barrier for protein drug to reach the targeted cell for specific action (Clore and Thurby-Hay, 2004). The nanomedicine can be employed for oral insulin delivery includes prodrugs, (insulin–polymer conjugation) micelles, liposome and nanoparticle of biodegradable polymers. Nanotechnology has a greater potential for the management of diabetes in future and likely to be solving many diabetic related problem.

Musculoskeletal Disorder

The musculoskeletal disorder covers mostly injuries (damage or disorder to muscle joints, tendons, ligaments and nerves). This comprises of hundreds of diseases and syndromes including osteoporosis, osteoarthritis, spinal disorders, severe trauma, and crippling disease and deformities in children. Recent advances in technology made it easier to understand the musculoskeletal disorder at molecular level. The muscle loss can be successfully replaced by suitable skeleton muscle grown through advance research in tissue engineering and regenerative medicine. Nanomedicine has the potential to improve significantly the prevention, detection, diagnosis and treatment of musculoskeletal disease. Recent studies showed multinucleated myotubes are more readily formed on carbon nanotube containing scaffolds and skeleton

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muscle regeneration was determined using electrospun polyurethane carbon nanotubes scaffolds (Sirivisoot and Harrison, 2004). Thus, different strategies have been applied for treatment of musculoskeletal disorder and have a great potential for monitoring and supporting tissue regeneration.

TOXICOLOGICAL AND ETHICAL ISSUES IN NANOMEDICINE

Today, almost fifty years after Richard Feynman's famous talk significant progress has been achieved in the area of nanomedicine. But, it is still facing many challenges regarding toxicological, ethical and social aspects of nanomaterials employed in diagnostics and treatment of disease. There are various issues which need to be resolved to achieve safe and sustainable development of the nanotechnologies for medicine. Since, this book chapter is focused on nanotechnology implemented in medicine; we will discuss the toxicity and ethics of only engineered nanostructures used in biomedical applications.

Toxicity Issues

With the development of nanotechnology in the area of medicine, concerns about the possible adverse health effects of nanomaterials have been increased (Gulumian and Vallyathan, 2010; Kim et al., 2010). These nanomaterials when exposed to biological system may produce oxidative stress, inflammation, immunotoxicity, genotoxicity and carcinogenicity. Generally, researchers do not evaluate the potential toxicological outcomes of the nanomaterials during the development of a nanomedicine product. The chemistry, solubility, degree of agglomeration, size, shape, and charge of particles may influence the toxic properties of the particles. Again, the toxicity profile of nanoparticles is speculated to be different from that of larger particles of the same chemical composition, by virtue of their extremely small size and high specific surface area (Donaldson et al., 2001; Oberdorster et al., 2005). Furthermore, toxicity may also vary with the route of administration of particles. Although, the systemic assessment of acute and chronic toxicity of nanoparticles, especially those used in nanomedicine, such as drug delivery carriers, has not yet been performed, but the adverse health effects of some of the nanomaterials used in nanomedicine has been reported in literature. It has been demonstrated that crystalline SiO2 may cause carcinogenesis, as well as produce inflammation, irritation, fibrosis, and other adverse health effects (Castranova et al., 2002; Nishimori et al. 2009) investigated that the toxicity of SiO2 nanoparticles is size dependent.

In vitro studies on toxicity of gold nanoparticles (AuNPs) have shown that AuNPs could induce apoptosis and necrosis in different cell types (Goodman et al. 2004; Nagender et al. 2009). Goodman et al. (2004) also proved that anionic functionalized AuNPs exert higher cytotoxicity compared to cationic functionalized AuNPs. Moreover, *in vivo* studies demonstrated that AuNPs impart size dependent toxic effects in mice. Cho et al. (2009) revealed that 13 nm sized PEG-coated AuNPs induce acute inflammation and apoptosis in the liver. Chen et al. (2009) found that AuNP ranging from 8 to 37 nm induced severe distress, fatigue, and change of fur color, loss of appetite, and weight loss. Similarly, toxic consequences of silver nanoparticles (Roberts et al. 2011) and fullerene have been investigated by researchers (Kolosnjaj et al. 2007; Chen et al. 1998). The toxic effects of fullerene were found to be dependent upon the route of administration as orally administered fullerene was considered nontoxic in comparison of intraperitoneally injected fullerene where all the rats died within 30 h post injection (Chen et al. 1998).

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Research evidence demonstrates that both single walled carbon nanotubes (SWCNT) and multi walled carbon nanotubes (MWCNT) induce cytotoxicity, oxidative stress, apoptosis, and necrosis *in vitro* (Casey et al. 2008; De Nicola et al. 2009; Hu et al. 2010; Kim et al. 2010). Various studies in rat and mice indicated that pulmonary exposure to CNT by inhalation, intratracheal instillation, or pharyngeal aspiration may lead to granulomatous inflammation and persistent interstitial fibrosis (Han et al. 2010; Mercer et al. 2011). It was recommended that CNT induced fibroblast proliferation and collagen production might be the possible reason for fibrosis (Wang et al. 2010).

Various studies in recent years also revealed the genotoxic and carcinogenic outcomes of engineered nanoparticles (Donaldson et al. 2010; Xie et al. 2011). The mechanism of nanoparticle induced genotoxicity is still not clear and specificity of nanoparticle action on DNA is not well interpreted. Nanoparticles can directly damage the genetic material by interacting with DNA, or indirectly by generation of reactive oxygen species (ROS), or by releasing toxic ions from soluble nanopartilces (Kisin et al. 2007; Barnes et al. 2008). It has been observed that small sized nanoparticles (diameter between 1 nm to 10 nm) can interact with DNA directly through diffusion or by transportation through nuclear pore complex (Barillet et al. 2010). Further, large sized nanoparticles may reach out to DNA only in dividing cells, during mitosis where nuclear membrane dissolves (Shukla et al. 2011; Hackenberg et al. 2011; Asharani et al. 2009; Hackenberg et al. 2010). After reaching to the nucleus, nanoparticles could induce genotoxicity depending upon phase of cell cycle. Nanoparticles could interrupt the DNA replication and transcription by directly interacting with DNA during interphase (An et al. 2010). During mitosis the interaction of nanoparticles with chromosomes could generate clastogenic or aneugenic effects. Nanoparticles might introduce breaks into chromosomes or disturb the process of mitosis, mechanically or by chemical binding. In addition to this, nanoparticles could induce genotoxicity by interacting with various nuclear proteins involved in replication, transcription, and repair of genetic material. In silico analysis exhibited that nanoparticle could interact with DNA mismatch repair proteins like PMS2, RFC3 and PCNA, which might disrupt their function and lead to mutation (Gupta et al. 2011). Interaction of nanoparticles with the mitotic spindle apparatus, centrioles or their associated proteins could result in abnormal multipolar spindle formation, chromosomal alignment and segregation during anaphase and telophase (Huang et al. 2009). Moreover, nanoparticles might disturb the cell cycle checkpoint functions by interacting with and affecting the activity of mitotic check point proteins and create aneuploid or multinucleated cells (Huang et al. 2009). Another mechanism of genotoxicity by nanoparticles is generation of reactive oxygen species (ROS). Free radicles may generate from nanoparticle surface in aqueous suspensions or they can be created by toxic transition metal ion released from soluble nanoparticles (Barillet et al. 2010; Kruszewski et al. 2011). In response to stress build by nanoparticles, cell componants like mitochondria can also produce ROS (Asharani et al. 2009). ROS production can also be triggered by activation of inflammatory cells in response of nanoparticle induced inflammatory reaction (Trouiller et al. 2009). ROS attack may lead to oxidative damage to DNA by oxidation of purine and pyrimidine bases giving rise to DNA base lesions and DNA strand breaks. DNA base lesions can cause mispairing during replication resulting in mutations, thus carcinogenicity (Cooke et al. 2003). It is also reported that nanoparticles may inhibit the antioxidants *in vitro* which consequently enhances the accumulation of ROS and lead to DNA damage (Barillet et al. 2010).

In recent years, carcinogenicity and reproductive and developmental toxicity of engineered nanoparticles has also been studied but the data is still limited. It has not yet been established whether human exposure to engineered nanoparticles produces cancer and adverse reproductive or developmental effects.

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As the nanomaterial applications are being introduced increasingly in industry, medicine, and pharmacology, elucidation of potential adverse health effects of nanomaterials is necessary. Moreover, with escalating applications of nanomaterials in medicine, urgent systematic and well-designed toxicological studies are further needed.

Ethical Issues

The booming advancements in nanotechnology have made commentators to urge government agencies and private companies to proactively address the ethical, social and regulatory aspects of nanotechnology and develop judicious policies to curtail its detrimental impacts on the environment and public health. Issues arising from the application of nanomedicine have been discussed below in brief.

Before commercialization of nanomedicine products they must first undergo extensive pre-clinical and clinical testing. Scientist suggests that, if society has to take advantage and benefits of nanotechnology first safety and risk issue of nanotechnology must be understood.

The nanoparticles should pass through stringent and multiple clinical trials to minimize risks pertaining to their use. Despite of the numerous preclinical and clinical trials the ambiguity pertaining to the first exposure of a human subject to a particular category of nanomedicine products cannot be eradicated. Substances that are innocuous at particular dose to a particular species may not be safe to human being and vice versa. It is important for a clinical researcher who is conducting the clinical trial of these substances to keep the above point in mind when they begin testing on human subject. All clinical investigations must incorporate a data and safety monitoring board (DSMB) to identify any precarious trends and minimize potential damage to human subjects and also conduct long term follow ups and assessments to monitor the safety of the nanomedicine products. Further, the researchers should educate people about how nanotechnology can be used in medicines and the benefits and risks associated to it.

Once the product is released into the market another set of ethical issues comes to light and needs to be addressed appropriately. Firstly, a nanomedicine product should be priced so that it is within the reach of general public. For this regulatory bodies should be set up by the government to ensure that the manufacturer of a nano product does not enjoy market monopoly and secondly it is to be decided whether it is fair to use nanomedicine for physical enhancement rather than therapy as this may intensify the already existing socioeconomic inequalities providing an unfair advantage or competitive edge to the rich who can afford enhancements.

CONCLUSION

Nanomedicine has shown great potential to address clinical needs in significant diseases. Nanomedicine can make the care process more effective in terms of clinical outcome for the patient and more affordable for society, by enabling prior diagnosis, improved therapy. There is a wide variety of engineered nanostructures which have been studied and proved efficacious in imaging, diagnosis and treatment of various diseases. However, toxicity and ethical issues of nanomedicine are of particular concern but are often ignored. Fortunately, with the outburst of public and scientific awareness of nanotechnology there is a growing intensity of discussions on these ethical and toxicological issues. The potential applica-

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tions provided by nanotechnology for diagnosis, prevention, and treatment of diseases are currently very broad. Therefore, to pursue the practical application of nanomedicine there is a requirement of simple approaches, and systematic development along with creativity and visionary power.

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KEY TERMS AND DEFINITIONS

Cardiovascular Disease: Class of diseases involving heart and blood vessels.

Disease Diagnostics: Identification of nature and cause of a disease.

Drug Delivery: Process of transporting a pharmaceutical compound safely in the body with desired therapeutic effect by using various approaches, formulations and systems.

Genotoxicity: Property of chemical agent to damage the genetic information (genetic material).

Musculoskeletal Disorder: Disorder related to body's muscles, joints, tendons, ligaments and nerves.

Nanomedicine: Applications of nanotechnology for treatment, diagnosis, monitoring, and control of biological systems.

Nanoparticle: Particles or structures having dimensions of 1-1000 nm.

Nanoscale: Scale of one billionth of a meter or 10^{-9} m.

Neurodegenerative Disease: A disease occurred due to loss of structure and function of neuron.

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Chapter 49
Nanosuspensions in Nanobiomedicine

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ABSTRACT

The tremendous success in developing new nanomaterials and fostering technological innovation arises from the focus on interdisciplinary research and collaboration between physical and medical scientists. The concept of nano-medicine is one of the most important and exciting ideas ever generated by the applications of nanoscience. One of the most challenging tasks in the pharmaceutical industry is the formulation of poorly soluble drugs. The implication of conventional techniques for improving the solubility has gained limited success. Nanoparticles facilitate formulation with improved solubility and efficacy mainly through nanosuspension approach. Techniques such as media milling, high-pressure homogenization, and use of microemulsion have been used for production of nanosuspensions for a novel delivery system. Moreover, they are manoeuvred to patient-acceptable dosage forms like tablets, capsules, and lyophilized powder products. Nanosuspension technology has also been studied for active and passive targeted drug delivery systems, which the chapter highlights on various formulational perspectives and applications as a biomedicine delivery system.

INTRODUCTION

In recent years various technological advances have led to unprecedented improvements in lifestyle, productivity and life span. Moreover there has been a significant burden on global economies and public health as shown by the increases in the outbreak of emerging and re-emerging infectious diseases. Transmission of infectious pathogens to the community has caused outbreaks such as influenza (*A*/*H1N*1 – Swine flu), Dengue fever, Diarrhoea *(Escherichia coli),* Cholera *(Vibrio cholera)* etc throughout the world. Societal changes resulting from mass urbanization (with the mechanisation of agricultural practices and industrialization) and population explosion along with poor water supply and environmental

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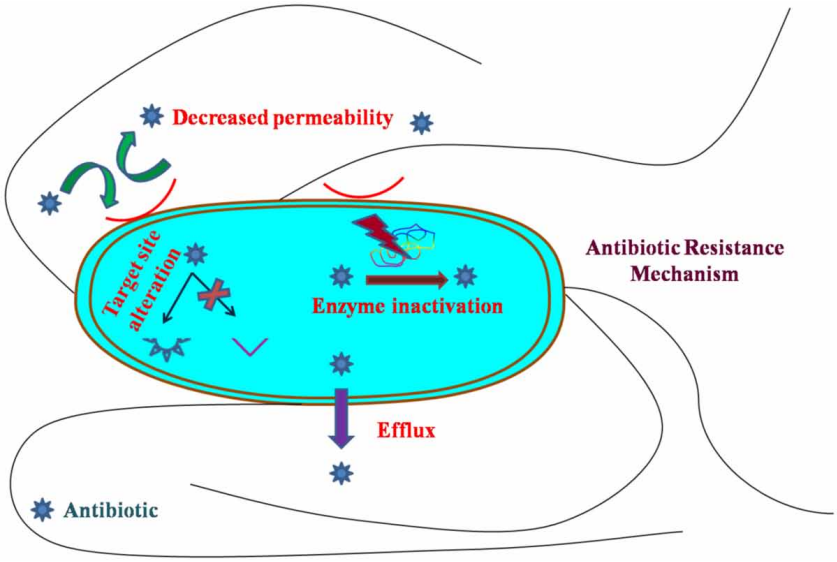
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hygiene are the main reasons for the increase in outbreak of infectious pathogens. These events have led to the widespread use of illicit intravenous drugs, which in turn has contributed to the spread of serious blood-borne pathogens like human immunodeficiency virus (HIV) and hepatitis viruses (HBV), and to increased incidence of sub-acute bacterial endocarditis (Hawkey 2008).

The impact of technology on biomedical and clinical practice is among the most valuable changes that have occurred during the twentieth century, notably in the area of prevention and treatment of infectious diseases. The introduction of antibiotics, penicillin (Flemming, 1929) as an antimicrobial agent has pioneered the era of chemotherapy by bringing down the mortality rate with a breakthrough in medicinal field. The different classes of antibiotics that followed served as broad range *wonder drugs* inhibiting the growth of infectious agents by disrupting cell wall synthesis, interfering with DNA replication and protein synthesis (Finberg et al 2004). Furthermore, inappropriate antibiotic usage and inability to produce new antibiotics have introduced extreme selection pressure on bacterial survival. However, the difficulty in identifying the newly proposed crucial bacterial targets by the antibiotics has culminated in the emergence of resistance in the context of antibiotic-mediated stress, an important criterion in bacterial evolution and pathogenesis. The resistance phenomena have several potential mechanisms such as (a) spontaneous / induced mutation, (b) horizontal DNA transfer and (c) alteration of bacterial surface (Figure 1). In addition, bacteria themselves produce a number of antimicrobial products, the most common being the peptide antibiotics. The best studied are the colicins produced by a gram negative bacterium *E. coli* (Riley and Wertz 2002).

The emergence of antibacterial resistance has prompted restrictions on antibacterial use in developed countries like the United Kingdom in 1970 (Swann report 1969), and banning the use of antibacterials as growth-promotional agents by the European Union since 2003. According to US centers for Disease control and Prevention in Atlanta, Georgia drug misuse plays a key factor in the emergence of antibiotic resistance. A survey published earlier this year by the Center for Disease Dynamics, Economics and Policy, found that in developing countries sales of carbapenems ("last resort") increased by nearly six times

Figure 1. The antibacterial resistance pattern exhibited by a bacterium



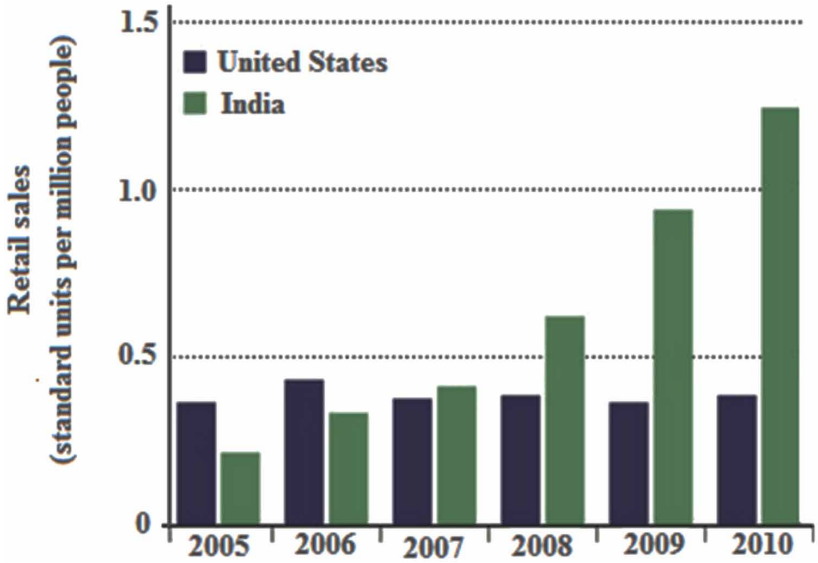
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between 2005 and 2010 (Figure 2). It is noteworthy to mention that due to limited / poor basic medical facilities, nearly 70% of 1.2 billion rural Indian citizens rely entirely on local pharmacists rather than a Physician and have access to drugs of their sort. The fact that antibiotics have revolutionised medical care in the 20th century has been toppled up by the emergence of superbugs winning the battle against the medical profession. According to reports from Infectious Disease Society of America (IDSA), the containment of emerging superbugs especially the Gram negative bacilli pose a major threat. As a matter of fact, since 2009 only 2 new antibiotics were approved in US and the numbers approved for marketing continues to decline where only few antibiotics were identified in phase 2 or phase 3 trials to contain *E. coli, Salmonella, Shigella* and Enterobacteriaceae members (Boucher et al, 2013).

The increase in bacterial strains that are resistant to conventional antibacterial therapies has prompted the development of bacterial disease treatment strategies which are alternatives to conventional antibacterials. One strategy to address bacterial drug resistance is the discovery and application of compounds that modify resistance to common antibacterials. For example, some resistance-modifying agents may inhibit multidrug resistance mechanisms, such as drug efflux from the cell, thus increasing the susceptibility of bacteria to an antibacterial. Further, phage therapy (Mattery, M 2008), bacteriocins, chelation (Jones 1977), vaccines, biotherapy (Nacar et al 2008), Probiotics (Ljungh et al 2009), Silver (Morones-Ramirez et al, 2013), host defense peptides, antimicrobial coatings – copper alloy surfaces (Samuel 2012) and targeting the trans-translational pathway (Keiler et al 2013) remain some of the strategies in combating the drug resistant microflora.

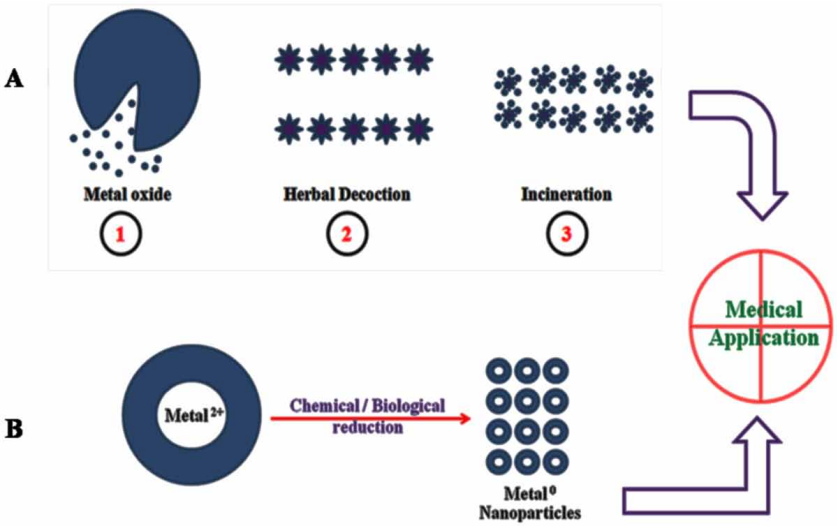
'Bhasmas' one of the ancient and unique ayurvedic metallic / mineral preparations prevailing since Charak Samhita worked similar to the nanoparticles of modern nanotechnology with the concept of reduction in particle size. The common properties like immune-modulation and anti-aging quality (rasayana) and targeted drug delivery (yogavahi) were observed in all bhasmas that are comparable to the novel medical strategies of modern era (Sanjeetha Paul et al. 2011) (Figure 3). In pharmaceutics, nanoparticles are typically defined as a discrete internal phase consisting of an active pharmaceutical

Figure 2. Sales scenario of carbapenem antibiotics in the United States and India



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Figure 3. (A) showing different steps involved in 'Bhasmikarana' converting the zero valent metal to oxide with high medicinal value (1) and mixing it with herbal decoction (2) thereby turning into ashes by incineration (3); (B) shows the metal oxide being reduced by any chemical or biological reducing agent converting it into a zero valent metal nanoparticles



ingredient having physical dimensions, less than 1 micron in an external phase. The use of nanoparticles as a drug-delivery approach for various difficult-to-formulate reagents is an age old practice (Poste et al., 1976; Poste and Kirsh, 1983; Davis et al., 1987; Douglas et al., 1987; Papahadjopoulos, 1988) wherein they can be designed to form *de novo* when exposed to appropriate biological fluid (Shott, 1995).

Nanosuspensions consist of the pure poorly water-soluble drug without any matrix material suspended in dispersion. For a successful formulation, entire range of parameters like solubility, stability, compatibility and photostability need to be considered. Alongside, the switching over to nanosuspensions confers following advantages: enhanced solubility and bioavailability, suitability for hydrophilic drugs, higher drug loading, dose reduction, enhancement of physical and chemical stability and possible passive drug targeting (Vishal R Patel et al. 2011) those of which breach the barriers and resolve issues related to solubility and bioavailiablity.

The current chapter describes the insights about nanosuspensions and also the methods of preparation, merits & demerits, characterization and evaluation parameters. A nanosuspension not only solves the problems of poor solubility and bioavailability but also alters the pharmacokinetics of drug and thus improves drug safety and efficacy.

NANOBIOMATERIALS

Nanostructures

Nanoparticles are gaining scientific interest as they effectively bridge the gap between bulk materials and atomic or molecular structures. A bulk material exhibits a constant physical properties regardless of

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its size, whereas at the nano-scale level size-dependent properties could be observed: quantum confinement in semiconductor particles, surface plasmon resonance in some metal particles (Ag, Au, Zn) and superparamagnetism (Nanocrystals, quantum dots) in magnetic materials.

- **Nanotubes:** Buckminsterfullerene or bucky balls, single layer of carbon atoms arranged in a cylinder with 1 – 2 nm in diameter and 1mm in length.
- **Nanowires:** Small conducting or semiconducting nanoparticles with a single crystal structure with a large aspect ratio.
- **Quantum Dots:** The number of atoms in a quantum dot range from 1000 to 100,000, makes it neither an extended solid structure nor a single molecular entity.
- **Other Nanoparticles:** It includes a wide range of primarily spherical or aggregated dendritic forms of nanoparticles.

Nanoparticles can be synthesised using variety of methods in order to optimise specific properties of the materials. The properties include, but are not limited, to size (diameter, length, volume) & distribution, symmetry, surface properties, surface coating, purity, ease of manipulation, yield and suitability for scaling up. Nanoparticles used in commercial or deliberate purpose employ methods that are divided into four main groups. These are gas phase processes including flame pyrolysis, high temperature evaporation and plasma synthesis, vapour deposition synthesis, colloidal or liquid phase methods in which chemical reactions in solvents lead to the formation of colloids and mechanical processes including grinding, milling and alloying.

Nanotechnology and Biological Bridge

Most preparations of the building blocks of nanotechnology involve hazardous chemicals, low material conversions, high energy requirements, and difficult, wasteful purifications; thus, there are multiple opportunities to develop greener processes for the manufacture of these materials. Many of the green chemistry principles apply readily to the synthesis or production of nanoscale materials. Green chemistry provides a number of advantages in process development and manufacturing as well as product design. As a result, researchers in the field of nanoparticle synthesis and assembly are focussing their attention on biological systems.

With the bioremediation of toxic metals using bacteria (Stephen and Maenaughton 1999) or yeasts (Mehra and Winge 1991), detoxification often occurs via reduction of the metal ions or formation of metal sulfides. Such microorganisms may then be used as possible nanofactories. Gold particles of nanoscale dimensions can, indeed, be readily precipitated within bacterial cells by incubation of the cells with Au^{2+} ions (Fortin and Beveridge 2000). *Pseudomonas stutzeri*, isolated from a silver mine, when placed in concentrated aqueous solution of $AgNO_3$ formed silver nanoparticles of well-defined size and distinct morphology within the periplasmic space of the bacteria (Klaus-Joerger et al. 2001). See Table 1.

Nanosuspensions: Scope

An important challenge that is being faced by the pharmaceutical industry is the poor solubility of drugs which is now considered to be an area of prime importance in the field of biomedical research. There are about 40% new molecular entities (NMEs) synthesized with advanced drug discovery tools incorporated

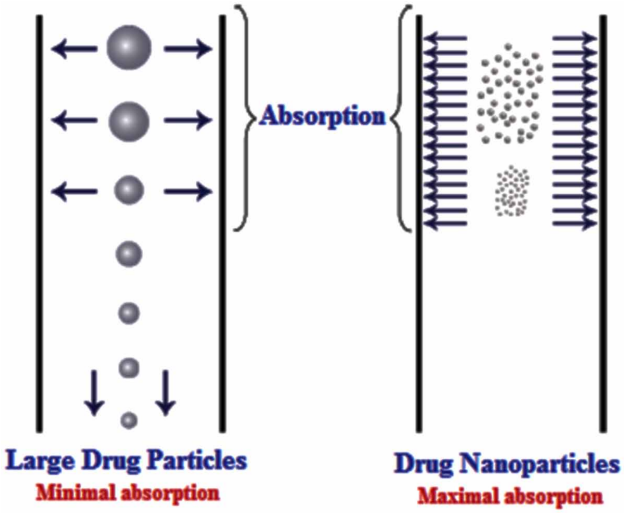
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Table 1. Bio-nanofactories in the synthesis of various metal nanoparticles

Biotic Counterpart	Nanoparticles	Size	Extracellular / Intracellular	Reference
Magnetotactic bacteria	Magnetic (Fe3O4) Greigite (Fe3S4)			Roh et al (2001)
Pseudomonas stutzeri	Ag	Upto 200 nm		Jeorger et al. (2000)
Ps. aeruginosa	Au	15 – 30 nm	Extracellular	Husseiny et al (2007)
Desulphovibrio desulfuricans	Palladium			Yong et al. (2002)
Thermomonospora sp.	Au	8 nm	Extracellular	Ahmad et al. (2003b)
Plectonema boryanum UTEX 485	Octahedral Au platlets	6 µm to 10 nm	At the cell wall	Lengke et al. (2006)
Schizosaccharomyces pombe	CdS	20 Å	Intracellular	Dameron et al. (1989)
Fusarium oxysporum	Zirconia Barium titanate	3 – 11 nm 4 – 5 nm	Extracellular	Bansal et al. (2004; 2006)
Tamarind leaf extract	Au nanotriangles	20 – 40 nm	Extracellular	Ankamwar et al. (2005b)

with combinatorial chemistry even suffer from solubility and bioavailability related issues (Lipinski 2002). Despite bacterial resistance, tight junctional epithelial cells, transporters and enzymatic barriers also reduce the absorption of drugs. In order to make both ends meet, advanced disinfectant nanomaterials have been proposed as a prime source of prophylaxis. The techniques for solubility enhancement have some limitations for which nanotechnology can be used to resolve the problems associated with these conventional approaches for solubility and bioavailability enhancement (Figure 4).

Figure 4. Absorption rate of drug molecules at nanoscale level (Chaubal 2004)



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METHODS OF NANOSUSPENSION PREPARATION

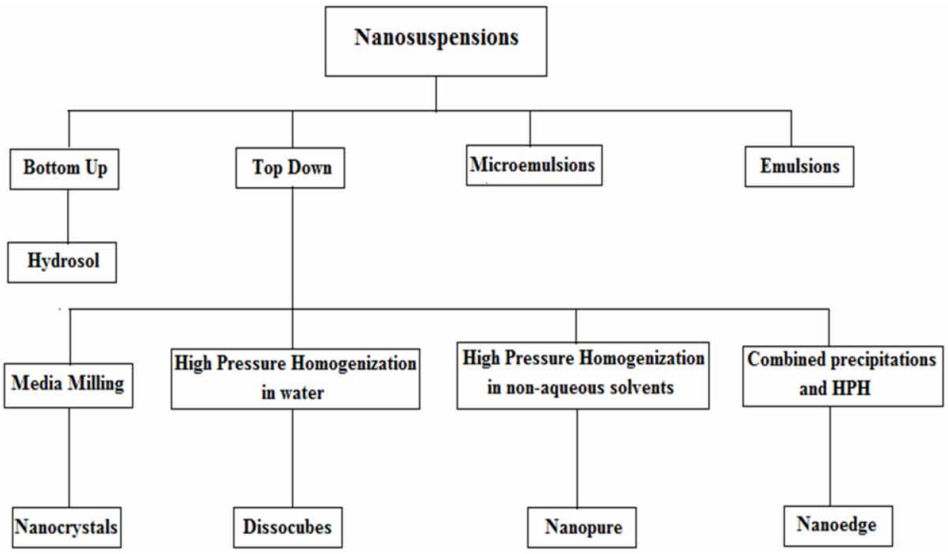
There are two methods for preparation of nanosuspensions. The conventional methods of precipitation (Hydrosols) are called 'Bottom Up technology'. In Bottom Up Technology the drug is dissolved in a solvent, which is then added to non-solvent to precipitate the crystals. The basic advantage of precipitation technique is the use of simple and low cost equipments. The basic challenge of this technique is that during the precipitation procedure the growing of the drug crystals needs to be controlled by addition of surfactant to avoid formation of microparticles. The limitation of this precipitation technique is that the drug needs to be soluble in atleast one solvent and this solvent needs to be miscible with nonsolvent. Moreover precipitation technique is not applicable to drugs, which are simultaneously poorly soluble in aqueous and nonaqueous media (Muller et al 2000).

The 'Top Down Technologies' are the disintegration methods and are preferred over the precipitation methods. The 'Top Down Technologies' include Media Milling (Nanocrystals), High Pressure Homogenization in water (Dissocubes), High Pressure Homogenization in nonaqueous media (Nanopure) and combination of Precipitation and High-Pressure Homogenization (Nanoedege) (Chowdary et al 2005; Cornelia et al 2006). Few other techniques used for preparing nanosuspensions are emulsion as templates, microemulsion as templates etc (Patravale et al 2004) (Figure 5).

Media Milling (Nanocrystals or Nanosystems)

The method is first developed and reported by Liversidge et.al. (1992). The nanosuspensions are prepared by using high-shear media mills. The milling chamber charged with milling media, water, drug and stabilizer is rotated at a very high shear rate under controlled temperatures for several days (at least 2-7 days). The milling medium is composed of glass, Zirconium oxide or highly cross-linked polystyrene resin. The high energy shear forces are generated as a result of the impaction of the milling media with the drug resulting into breaking of microparticulate drug to nanosized particles.

Figure 5. Flow chart representing different methods of preparation of nanosuspensions



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Homogenization in Water (Dissocubes)

R. H. Muller developed Dissocubes technology in 1999. The instrument can be operated at pressure varying from 100 – 1500 bars (2800 –21300psi) and up to 2000 bars with volume capacity of 40ml (for laboratory scale). For preparation of nano suspension, we have to start with the micronized drug particle size less than 25μm to prevent blocking of homogenization gap hence it is essential to prepare a presuspension of the micronized drug in a surfactant solution using high speed stirrer (Cornelia et al 2006).

Homogenisation in Nonaqueous Media (Nanopure)

The drugs that are chemically labile can be processed in such nonaqueous media or water-miscible liquids like polyethyleneglycol-400 (PEG), PEG1000 etc. The homogenization can be done at room temperature, 0^{o} C and below freezing point (-20^{o} C).

Combined Precipitation and Homogenization (Nanoedge)

The precipitated drug nanoparticles have tendency to continue crystal growth to the size of microcrystals. They need to be processed with high-energy forces (Homogenisation). They are completely amorphous, partially amorphous or completely crystalline which create problems in long term stability as well as in bioavailability, so the precipitated particle suspension is subsequently homogenized which preserve the particle size obtained after the precipitation step.

NANOSUSPENSIONS AND PROPERTIES

Physical Long-Term Stability

Dispersed systems show physical instability due to Ostwald ripening which is responsible for crystal growth to form microparticles. Ostwald ripening is defined as the tendency for a particle dispersion to grow in diameter over time; by a process in which the smaller particles dissolve because of their higher solubility, with subsequent crystallization onto larger particles to form microparticles. Ostwald ripening is caused due to the difference in dissolution velocity/ saturation solubility of small and large particles.

In nanosuspensions all particles are of uniform size hence there is little difference between saturation solubility of drug particles. The difference in the concentration of the saturated solutions around a small and large particle leads to the diffusion of dissolved drug from the outer area of the large particles. As a result the solution around large particles is supersaturated leading to the drug crystallization and growth of the large crystals or microparticles. Ostwald ripening is totally absent in nanosuspensions due to uniform particle size, which is also responsible for long-term physical stability of nanosuspensions.

Increase in Saturation Solubility and Dissolution Velocity of Drug

Dissolution of drug is increased due to increase in the surface area of the drug particles from micrometers to the nanometer size. According to Noyes-Whitney equation (equation no.1) dissolution velocity increase due to increase in the surface area from micron size to particles of nanometer size.

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$$Dx/dt = [(D \times A/ h] [Cs\text{-}X/V]$$

where D is diffusion coefficient, A is surface area of particle, dx/dt is the dissolution velocity, V is volume of dissolution medium and X is the concentration in surrounding liquid.

According to the Prandtl equation, for small particles the diffusional distance h decreases with decreasing particle size. The decrease in h increases Cs (saturation solubility) and leads to an increase in gradient (Cs-Cx)/h and thus to an increase in the dissolution velocity. According to Ostwald-Freunddlich equation decrease in particle size below 1μm increases the intrinsic solubility or saturation solubility (Patravale et al 2004; Muller et al 2000).

Internal Structure of Nanosuspensions

The high-energy input during disintegration process causes structural changes inside the drug particles. When the drug particles are exposed to high-pressure homogenisation particles are transformed from crystalline state to amorphous state. The change in state depends upon the hardness of drug, number of homogenisation cycles chemical nature of drug and power density applied by homogeniser.

CHARACTERIZATION OF NANOSUSPENSIONS

The essential characterization parameters for nanosuspensions are as follows. Mean particle size and particle size distribution. The mean particle size and the width of particle size distribution are important characterization parameters as they govern the saturation solubility, dissolution velocity, physical stability and even biological performance of nanosuspensions. It has been indicated by Muller & Peters (1998) that saturation solubility and dissolution velocity show considerable variation with the changing particle size of the drug.

1. **Photon Correlation Spectroscopy (PCS) (Muller & Muller 1984):** Can be used for rapid and accurate determination of the mean particle diameter of nanosuspensions. Moreover, PCS can even be used for determining the width of the particle size distribution (polydispersity index, PI). The PI is an important parameter that governs the physical stability of nanosuspensions and should be as low as possible for the long-term stability of nanosuspensions. A PI value of 0.1–0.25 indicates a fairly narrow size distribution whereas a PI value greater than 0.5 indicates a very broad distribution. Although PCS is a versatile technique, because of its low measuring range (3nm to 3 μm) it becomes difficult to determine the possibility of contamination of the nanosuspension by microparticulate drugs (having particle size greater than 3 μm). Hence, in addition to PCS analysis, laser diffractometry (LD) analysis of nanosuspensions should be carried out in order to detect as well as quantify the drug microparticles that might have been generated during the production process.
2. **Laser Diffractometry:** Yields a volume size distribution and can be used to measure particles ranging from 0.05 – 80 μm and in certain instruments particle sizes up to 2000 μm can be measured. The typical LD characterization includes determination of diameter 50% LD (50) and diameter 99% LD (99) values, which indicate that either 50 or 99% of the particles are below the indicated size. The LD analysis becomes critical for nanosuspensions that are meant for parenteral and pulmonary

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delivery. Even if the nanosuspension contains a small number of particles greater than 5–6 μm, there could be a possibility of capillary blockade or emboli formation, as the size of the smallest blood capillary is 5–6 μm.

3. **Crystalline State and Particle Morphology:** Assessment of the crystalline state and particle morphology together helps in understanding the polymorphic or morphological changes that a drug might undergo when subjected to nanosizing. Additionally, when nanosuspensions are prepared, drug particles in an amorphous state are likely to be generated. Hence, it is essential to investigate the extent of amorphous drug nanoparticles generated during the production of nanosuspensions. The changes in the physical state of the drug particles as well as the extent of the amorphous fraction can be determined by X-ray diffraction analysis (Muller & Bohm 1998; Muller & Grau 1998) and can be supplemented by differential scanning calorimetry (Shanthakumar et al 2004). In order to get an actual idea of particle morphology, scanning electron microscopy is preferred (Muller & Bohm 1998).
4. **Particle Charge (Zeta Potential):** Determination of the zeta potential of a nanosuspension is essential as it gives an idea about the physical stability of the nanosuspension. The zeta potential of a nanosuspension is governed by both the stabilizer and the drug itself. In order to obtain a nanosuspension exhibiting good stability, for an electrostatically stabilized nanosuspension a minimum zeta potential of ±30mV is required whereas in the case of a combined electrostatic and steric stabilization, a minimum zeta potential of ±20mV is desirable (Muller & Jacobs 2002b).
5. **Saturation Solubility and Dissolution Velocity:** The determination of the saturation solubility and dissolution velocity is very important as these two parameters together help to anticipate any change in the *in vivo* performance (blood profiles, plasma peaks and bioavailability) of the drug. As nanosuspensions are known to improve the saturation solubility of the drug, the determination of the saturation solubility rather than an increase in saturation solubility remains an important investigational parameter. The investigation of the dissolution velocity of nanosuspensions reflects the advantages that can be achieved over conventional formulations, especially when designing the sustained-release dosage forms based on nanoparticulate drugs. The dissolution velocity of drug nanosuspensions in various physiological buffers should be determined according to methods reported in the pharmacopoeia.
6. ***In-Vivo* Biological Performance:** The establishment of an *in-vitro/in-vivo* correlation and the monitoring of the *in-vivo* performance of the drug is an essential part of the study, irrespective of the route and the delivery system employed. It is of the utmost importance in the case of intravenously injected nanosuspensions since the Nanosuspensions: a promising drug delivery strategy *in vivo* behaviour of the drug depends on the organ distribution, which in turn depends on its surface properties, such as surface hydrophobicity and interactions with plasma proteins (Blunk et al 1993, 1996; Luck et al 1997a,b). In fact, the qualitative and quantitative composition of the protein absorption pattern observed after the intravenous injection of nanoparticles is recognized as the essential factor for organ distribution (Muller 1989; Blunk et al 1993, 1996; Luck et al 1997a,b). Hence, suitable techniques have to be used in order to evaluate the surface properties and protein interactions to get an idea of in-vivo behaviour. Techniques such as hydrophobic interaction chromatography can be used to determine surface hydrophobicity (Wallis & Muller 1993), whereas 2-D PAGE (Blunk et al 1993) can be employed for the quantitative and qualitative measurement of protein adsorption after intravenous injection of drug nanosuspensions in animals.

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NANOTECHNOLOGY IN MEDICINE: NANOMEDICINE

Applying nanotechnology for treatment, diagnosis, monitoring, and control of diseases has been referred to as "nanomedicine". Although the application of nanotechnology to medicine appears to be a relatively recent trend, the basic nanotechnology approaches for medical application date back several decades. The first example of lipid vesicles which later became known as liposomes were described in 1965 (Bangham et al); the first controlled release polymer system of macromolecules was described in 1976 (Langer et al); the first long circulating stealth polymeric nanoparticle was described in 1994 (Gref et al); the first quantum dot bioconjugate was described in 1998 (Bruchez et al); and the first nanowire nanosensor dates back to 2001 (Cui et al). Recent Studies on new targeted nanoparticle contrast agents for early characterization of atherosclerosis and cardiovascular pathology at the cellular and molecular levels that might represent the next frontier for combining imaging and rational drug delivery to facilitate personalized medicine (Wickline et al 2002) (Table 2).

Nanotechnology-based highly efficient markers and precise, quantitative detection devices for early diagnosis and for therapy monitoring will have a wide influence in patient management, in improving patient's quality of life and in lowering mortality rates, in diseases like cancer and Alzheimer's disease.

Multi-Functional Nanoparticles

Biodegradable chitosan nanoparticles encapsulating quantum dots were prepared by D. K. Chatterjee and Y. Zhang, with suitable surface modification to immobilize both tumor targeting agent and chemokine on their surfaces. The interactions between immune cells and tumor cells were visualized using optical microscope. Use of Quantum dots in the treatment of cancer is a great advancement in this area. Quantum dots glow when exposed to UV light. When injected they seep into cancer tumour. The surgeon can see the glowing tumour. Nanotechnology could be very helpful in regenerating the injured nerves. During the last decade, however, developments in the areas of surface microscopy, silicon fabrication, biochemistry, physical chemistry, and computational engineering have converged to provide remarkable capabilities for understanding, fabricating and manipulating structures at the atomic level. The rapid evolution of this new science and the opportunities for its application promise that nanotechnology will become one of the dominant technologies of the 21st century.

Table 2. Different categories of Nanoparticles and their applications

Nanostructures	Material	Applications
Nanotubes	Carbon (fullerenes)	Synthetic muscles, Body armor, Nanotube membrane filters, Scaffolds (Haddon et al; Sholl et al 2006;
Nanowires	Metals, semiconductors, oxides, sulphides, nitrides	Real time sensing of biomarker proteins for cancer, Diagnosis of myocardial infarction (Elnathan et al)
Nanocrystals, quantum dots	Insulators, semiconductors, metals, magnetic materials	Fluorescent probes for cellular imaging – Real time tracking
Other nanoparticles	Ceramic oxides, metals	Gene delivery and Tissue engineering

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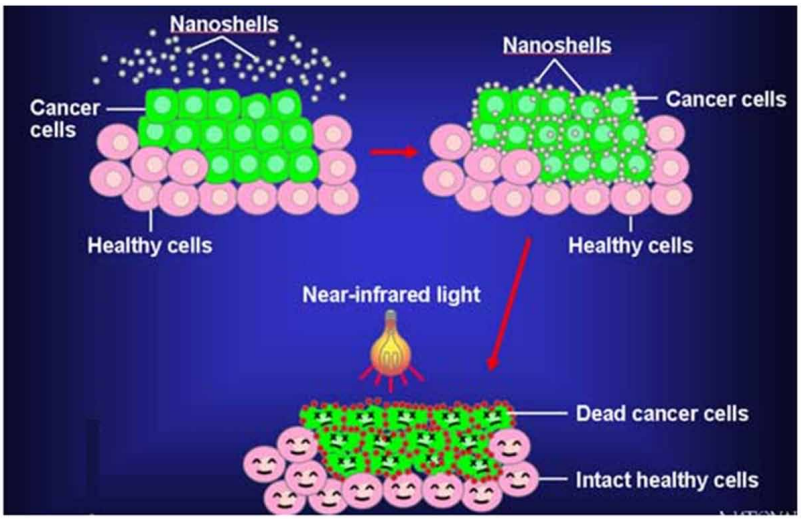
Nanoshells

Nanoshells are miniscule beads coated with gold. By manipulating the thickness of the layers making up the nanoshells, scientists can design these beads to absorb specific wavelengths of light. The most useful nanoshells are those that absorb near-infrared light, which can easily penetrate several centimeters of human tissue. The absorption of light by the nanoshells creates an intense heat that is lethal to cells. Researchers can already link nanoshells to antibodies that recognize cancer cells. Scientists envision letting these nanoshells seek out their cancerous targets, then applying near-infrared light. In laboratory cultures, the heat generated by the light-absorbing nanoshells has successfully killed tumor cells while leaving neighboring cells intact (Figure 6).

Dendrimers

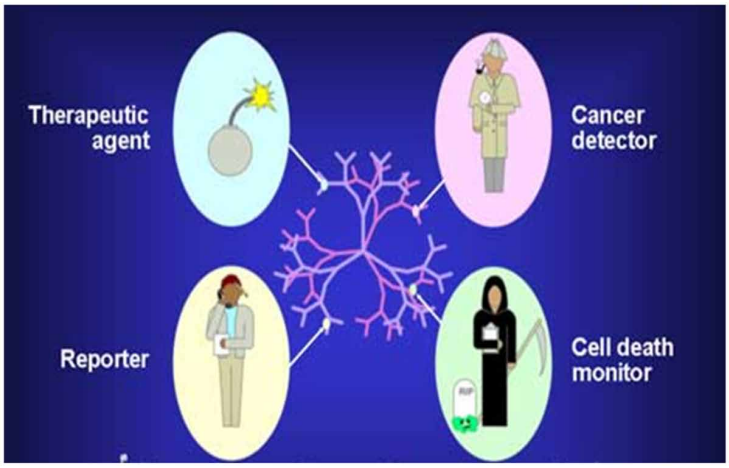
Dendrimers are man-made molecules about the size of an average protein, and have a branching shape. They are used to facilitate drug delivery as they behave as a potent diagnostic tool. The shape gives them vast amounts of surface area to which therapeutic agents or other biologically active molecules can be attached. A single dendrimer can carry a molecule that recognizes cancer cells, a therapeutic agent to kill those cells, and a molecule that recognizes the signals of cell death. It has been manipulated in such a way that they release their contents only in the presence of certain trigger molecules associated with cancer. Following drug release, the dendrimers may also report back whether they are successfully killing their targets (Figure 7).

Figure 6. Nanoshells as cancer therapy. (Courtesy: National Cancer Institute at the National Institutes of Health http://www.cancer.gov/cancertopics/understandingcancer/nanodevices).



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Figure 7. Dendrimers in cancer therapy. (Courtesy: National Cancer Institute at the National Institutes of Health http://www.cancer.gov/cancertopics/understandingcancer/nanodevices).



Early Detection of Alzheimer's Disease

Brain represents one of the most complex systems in biomedicine. With an improved understanding of brain functioning, better diagnosis and treatment for neurodegenerative diseases like Alzheimer's is offered by nanotechnology. Presently, the prevailing problem is early detection for effective treatment of the disease. An ideal diagnostic tool for Alzheimer's disease (AD) must have specificity & sensitivity more than 80% for its early diagnosis & excluding other causes. Since the neurodegeneration process begins well before AD becomes symptomatic the potential for early detection is another important characteristic of an ideal diagnostic tool.

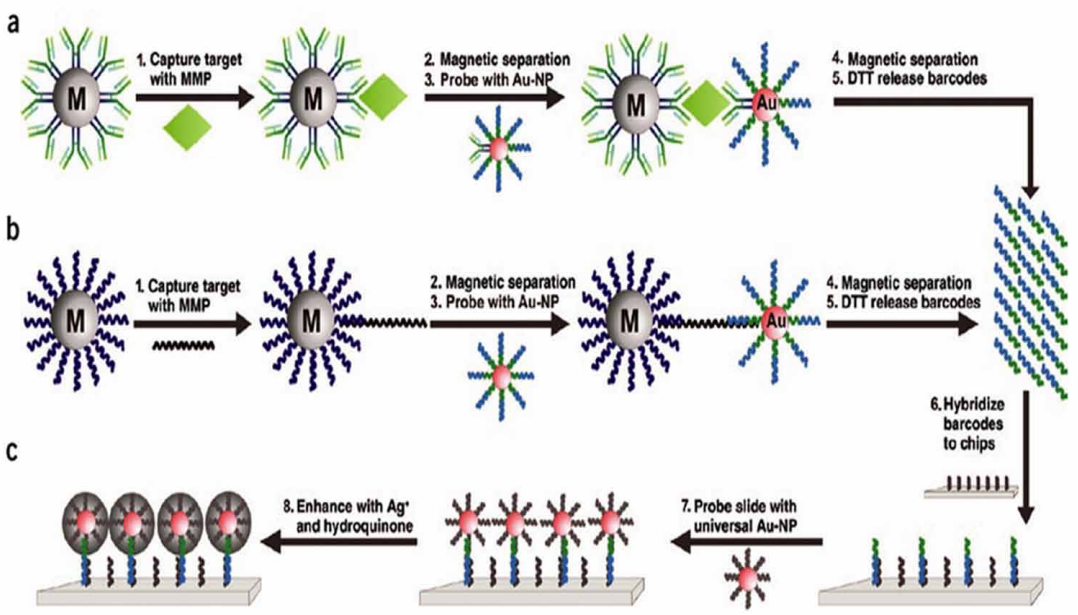
Nanotechnology can be the basis of new tools for very early detection of AD. Nanotechnology in the diagnosis of AD came into light after two articles were published in February 2005. The two detection approaches proposed are the Bio-barcode assay (BCA) & Localized surface plasmon resonance (LSPR) technology.

Bio-Barcode Assay

One of the recently developed bio-barcode assay used for the detection of nucleic acid and protein targets without PCR showing high sensitivity for both nucleic acid and protein targets. Two types of particles are used in the assay: (i) a magnetic microparticle with recognition elements for the target of interest and (ii) a gold nanoparticle (Au-NP) with a second recognition agent (which can form a sandwich around the target in conjunction with the magnetic particle) and hundreds of thiolated single-strand oligonucleotide barcodes. After reaction with the analyte, a magnetic field is used to localize and collect the sandwich structures, and a DTT solution at elevated temperature is used to release the barcode strands. The barcode strands can be identified on a microarray via scanometric detection or *in situ* if the barcodes carry with them a detectable marker (Figure 8).

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Figure 8. Schematic representation of protein and nucleic acid detection using bio-barcode assay (Hill et al. 2006)



Localized Surface Plasmon Resonance (LSPR) Technology (Heas et al. 2004)

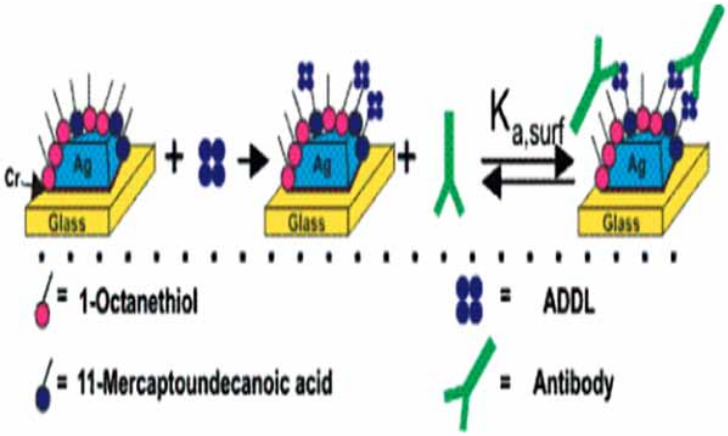
Surface-confined Ag nanoparticles are synthesized using Nanosphere lithography (NSL). Nanoparticle adhesion to the glass substrate is promoted using a 0.4 nm Cr layer. The nanoparticles are incubated in a 3:1 1-octane-thiol / 11-mercaptoundecanoic acid solution to form a self assembled monolayer. Then the samples are incubated in 100 mM 1-ethyl-3-[3-dimethyl-aminopropyl] carbodiimide hydrochloride / 100 nM amyloid-derived diffusible ligands (ADDL) solution. Finally, incubating the ADDL-coated nanoparticles to varying concentrations of antibody completes an anti-ADDL immunoassay. By varying the concentration of anti-ADDL antibody, a surface confined binding constant of 3.0×10^{7} M^{-1} for the interaction of ADDLs and anti-ADDLs measured (Figure 9).

Nanosuspensions in Drug Delivery

Nanosuspensions are also advantageous in achieving quick onset of action for drugs that are completely but slowly absorbed. A study involving a comparison of the pharmacokinetic profiles of a drug in the form of nanosuspension, suspension and tablet forms revealed that the time required to achieve C_{max} was reduced by approximately 50% for the nanosuspension compared to the suspension and tablet. Additionally, the nanosuspension resulted in 2.5 to 4.5 fold increase with improved dose proportionality, reduced variability and increased bioavailability.

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Figure 9. Design of LSPR sensor based on optical properties of Ag nanoparticle (Heas et al., 2004)



Oral Drug Delivery

The oral route is the most preferred route for drug delivery. The efficacy or performance of the orally administered drug generally depends on its solubility and absorption through the gastrointestinal tract. When a drug candidate exhibits poor aqueous solubility or rate limited absorption, it is believed to possess low and/or highly variable oral bioavailability. In such cases, the drug candidate would have to be administered in a larger excess than actually required in order to achieve a therapeutically active concentration, thus making the therapy costly. This could very well be appreciated in case of antibiotics such as atovaquone and bupravaquone.

Nanosizing and Nanosuspensions of such drugs can lead to a dramatic increase in their oral absorption and subsequently bioavailability. The adhesiveness of the drug nanosuspension, increased surface area (due to reduction in particle size by 10–50-fold), increased saturation solubility, leading to an increased concentration gradient between the gastrointestinal tract lumen and blood, and increased dissolution velocity. This enhancement in bioavailability will lead to a subsequent reduction in drug dose, rendering the therapy cost-effective and undue drug dumping in the body.

Parenteral Drug Delivery

The parenteral route is an invasive route and the administration of drugs is critical and often associated with safety issues and biological problems such as allergies and thrombophlebitis (thrombus - blood clot; phlebitis - vein inflammation). Despite all these limitations, the parenteral route still retains its value with special advantages, such as quick onset of action in case of emergency, reduction in dose of the drug and the ability to target the drug quickly to the desired site of action, especially in the case of severe infections. This route is also employed as an alternative to oral administration in case of poor absorption. For administration by the parenteral route, the drug either has to be solubilized or have particle/globule size below 5 μm to avoid the capillary blockade.

The current approaches for parenteral delivery include salt formation, solubilization using co-solvents, micellar solutions (Kim et al 2001), complexation with cyclodextrins and recently liposomes (Dupont 2002). However, there are limitations on the use of these approaches because of limitations on their

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solubilization capacity and parenteral acceptability. In this regard, liposomes are much more tolerable and versatile in terms of parenteral delivery. However, they often suffer from problems such as physical instability, high manufacturing cost and difficulties in scale-up.

Nanosuspensions appear to be a unique approach to solving the problems mentioned above. From the formulation perspective, nanosuspensions meet almost all the requirements of an ideal drug delivery system for the parenteral route. The nanosized drug particles and absence of any harsh solvents or any toxic ingredient makes the nanosuspensions bypass the limitations leading to improved therapeutic performance. A therapeutic efficacy of paclitaxel nanosuspension against Taxol on mammary 16-C murine tumour model revealed the superiority of paclitaxel nanosuspension over Taxol in reducing the median tumour burden (Merisko-Liversidge et al 2003).

Ocular Drug Delivery

Nanosuspensions support the drugs that exhibit poor solubility in lachrymal fluids. Even though delivery approaches such as suspensions and ointments have been recommended, the therapeutic performance of the water-soluble drugs depends on the intrinsic solubility of the drug in lachrymal fluids. Thus, the intrinsic dissolution rate of the drug in lachrymal fluid (constant inflow and outflow) governs its release and ocular bioavailability. However, nanosuspensions, by their inherent ability to improve the saturation solubility of the drug, represent an ideal approach for ocular delivery of hydrophobic drugs. Moreover, the nanoparticulate nature of the drug allows its prolonged residence in the cul-de-sac, giving sustained release of the drug. To achieve sustained release of the drug for a stipulated time period, nanosuspensions can be incorporated in a suitable hydrogel base or mucoadhesive base or even in ocular inserts. An approach that has recently been investigated to achieve the desired duration of action of the drug is the formulation of polymeric nanosuspensions loaded with the drug. The bioerodible as well as water soluble/ permeable polymers possessing ocular tolerability (Pignatello et al 2002a) could be used to sustain the release of the medication. The polymeric nanosuspensions of flurbiprofen and ibuprofen have been successfully formulated using acrylate polymers such as Eudragit RS 100 and Eudragit RL 100 (Bucolo et al 2002; Pignatello et al 2002b,c).

Pulmonary Drug Delivery

Nanosuspensions compete with the poor soluble drugs delivered as dry powders or as suspension aerosols via pulmonary secretions. The suspension drugs are initially jet milled to achieve particle sizes of microns. As a result, there is a limited diffusion and dissolution of the drug, rapid clearance from the lungs because of ciliary movements, less residence time and deposition of the drug thereby affecting the bioavailability.

The nanoparticulate nature of the drug allows the rapid diffusion and dissolution of the drug at the site of action. At the same time, the increased adhesiveness of the drug to mucosal surfaces (Ponchel et al 1997) offers a prolonged residence time for the drug at the absorption site. This ability of nanosuspensions to offer quick onset of action initially and then controlled release of the active moiety is highly beneficial and is required by most pulmonary diseases. Nanosuspensions could be used in all available types of nebulizer. However, the extent of influence exerted by the nebulizer type as well as the nebulization process on the particle size of nanosuspensions should be ascertained. Budesonide, a poorly water-soluble corticosteroid, has been successfully formulated as a nanosuspension for pulmonary delivery (Muller & Jacobs 2002b).

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Targeted Drug Delivery

The need to target specific sites remains one of the major challenges in the drug delivery system. Nanoparticulate systems have shown tremendous potential in targeted drug delivery, especially to the brain (Schroeder et al 1998; Kreuter 2001). Successful targeting of the peptide dalargin to the brain by employing surface modified polyisobutyl cyanoacrylate nanoparticles has been a major achievement in targeted delivery (Kreuter et al 1997).

The shift and drift strategy exhibited by the nanosuspensions makes them a potent candidate for targeted delivery as their surface properties and *in-vivo* behaviour can easily be altered. Natural targeting of macrophages by nanosuspensions has been achieved but could pose an obstacle when macrophages are not the desired targets. Hence, in order to bypass the phagocytic uptake of the drug, its surface properties need to be altered, as in the case of stealth liposomes (Gregoriades 1995; Allen 1997; Lasic et al 1997; Papisov 1998; Woodle 1998; Vyas et al 2000).

The engineering of stealth nanosuspensions by using various surface coatings for active or passive targeting of the desired site is the future of targeted drug delivery systems.

Example 1: *Cryptosporidium parvum*, the organism responsible for cryptosporidiosis was targeted by using surface-modified mucoadhesive nanosuspensions of bupravaquone (Kayser 2001; Muller & Jacobs 2002a). Mucoadhesive bupravaquone nanosuspensions, because of their prolonged residence at the infection site, revealed a 10-fold reduction in the infectivity score of *Cryptosporidium parvum* as compared to the bupravaquone nanosuspensions without mucoadhesive polymers.

Example 2: Similarly, conditions such as pulmonary aspergillosis can easily be targeted by using suitable drug candidates, such as amphotericin B, in the form of pulmonary nanosuspensions instead of using stealth liposomes (Kohno et al 1997). With the advent of polymeric nanosuspensions loaded with drug, it should be possible to target sites, such as the colon, or bacteria, such as *Helicobacter pyroli*, by suitable modifications in the formulation strategy. Overall, nanosuspensions have indicated a good potential in targeted drug delivery.

Pharmacological and Toxicological Aspects

1. **Inhalation:** Exposure to fine particulate dusts leads to pulmonary diseases. The idea that any poorly soluble particles administered in sufficient quantities to the lung can cause an inflammatory reaction has arisen following the demonstration of rat lung overload (Mauderly et al 1994). An important challenge in particle toxicology is to define the quantity that produces inflammation i.e. define the effective dose. Since the late 1980's, toxicological evidence has been emerging indicating that the health effects associated with inhaling ultrafine particles may not be closely associated with particle mass. Early studies with PTFE (polytetrafluoroethylene) particles around 20 nm in diameter showed that airborne concentrations of a supposedly inert insoluble material lower than 50μg m^{-3} could be fatal to rats (Johnston et al., 2000). Since then, many studies have indicated that the toxicity of insoluble materials increases with decreasing particle size, on a mass for mass basis (Faux et al., 2003).

Traditionally the dose of particle administered to the lung, e.g. in an instillation or inhalation situation, is measured in mass. A number of recent studies indicate that the inflammatory response depends on the

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surface area of particles deposited in the proximal alveolar region of the lungs (Tran et al., 2000; Faux et al., 2003). Thus, particle surface area is a better metric than mass for relating the particle dose to the inflammatory reaction. The precise mechanisms by which these materials exhibit higher levels of toxicity, at smaller particle sizes, have yet to be elucidated, although it is now understood that oxidative stress plays an important role in initiating the chain of events, at the molecular and cellular level, leading to the observed health effects. This was demonstrated in a recent study in which in vitro experiments with a 12 range of poorly soluble particles were conducted (Faux et al., 2003). The data generated by this study were able to demonstrate a relationship between the surface area dose and inflammatory response *in vitro* and the earlier *in vivo* results. This discovery has important practical and theoretical implications.

At the theoretical level, it has highlighted the role of the epithelial cells in the proximal alveolar region, their interaction with the deposited particles and the ensuing molecular events leading to inflammation. At the practical level, it has offered a new approach to interpreting *in vitro* data, a cost effective way of screening new materials for toxicity and an alternative way to animal testing. But particles also exist in a wide range of surface reactivity. Upon deposition in the pulmonary region, such reactivity exerts an oxidative stress on the cells via contact between particle/cell surface area. Consequently, both highly reactive particles with a low surface area and low toxicity particles with a high surface area can exert the same oxidative stress level on the cells they come into contact with. When this inhaled dose reaches a critical level, an inflammatory response occurs. For low toxicity dusts, inflammation will diminish with the cessation of exposure, while for high toxicity dusts, inflammation will persist (Tran et al., 2000). Persistent inflammation is likely to lead to diseases such as fibrosis and cancer. Thus it is important to control inflammation. This can be done by (i) determining the critical dose of particles that initiates inflammation and (ii) setting up exposure limits, according to the relevant metric, so that such a dose cannot be reached within a lifetime exposure scenario. Regulations for the control of airborne particle pollution in the workplaces are currently mass based and vary between particulate types (HSE, 2002b). A proper description of the metric of the particle dose would provide a coherent and consistent body of knowledge on particle toxicity and would, in turn, facilitate a coherent and rational policy for regulating dust exposure in the workplace. Thus, it is apparent that measuring exposures against mass alone is not sufficient. Of the three primary exposure metrics (mass, surface area, and number), there is compelling evidence to suggest that occupational nanoparticle aerosols should be monitored in terms of surface area.

The concentration of nanoparticles might be very small in terms of mass, quite large in terms of surface area and huge in terms of particle number. However, the estimate of surface area does depend on the measurement technique used. Depending on how much detail of the surface is inspected, the measurement can increase significantly. For example, if a prune is seen as approximately a sphere then it has approximately the surface of a smooth sphere of the same diameter; but if the surface of the wrinkles is included then its surface area becomes notably larger. Similarly, with techniques that use adsorption of gas molecules on to the surface of the particle to measure the surface, the smaller the gas molecule chosen, the more it can enter the wrinkles on the particle surface. Nitrogen as the gas molecule for adsorption and measurement of surface area may be a good choice, but other gases (or other surface measurement techniques) are worth considering.

Biologically relevant surface area is likely to be that accessible to biologically relevant molecules in the body. So far, we have assessed the risk of pulmonary disease from inhaled particles. However, it has also been suggested that due to their small diameter, nanoparticles are capable of penetrating epithelial cells, entering the bloodstream from the lungs (Gilmour et al., 2004), and even translocating to the brain via the olfactory nerves (Oberdorster et al., 2004). The ability of inhaled nanoparticles to disperse be-

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yond the lungs suggests that the health effects of nanoparticles may not be confined to the lungs. Also, nanoparticles cleared by macrophages via the mucociliary escalator, can be swallowed and therefore available for transfer to other body organs via the gastro-intestinal compartment. There is also some evidence that smaller particles can be transferred more readily than their larger counterparts across the intestinal wall (Behrens et al., 2002). Little is currently known about the health effects of nanoparticles on the liver and kidneys as well as the correct metric for describing the nanoparticle dose in these organs. Another area which merits further research is the transfer of nanoparticles across the placental barrier.

Exposure to nanoparticles during the critical window of foetal development may lead to developmental damage in the offspring. While a strong case may be made for using aerosol surface area as a dose metric, this may not be universally so. For example, for fibrous aerosols such as asbestos and glass fibre, the currently accepted best approach is to count fibres (i.e. the metric is particle number). Some types of nanoparticle could be considered fibres. Carbon nanotubes for example have high aspect ratios and are durable. They can be manufactured with lengths well within the range conventionally considered to be a fibre (although their diameters are much smaller than we could detect with current standard counting methods which are based on optical microscopy). In any case, it is also necessary to consider characterising exposures against aerosol mass and number concentration until further information and improved methods are available. For each of these exposure metrics, but particularly in the case of mass concentration, size-selective sampling need to be employed to ensure only particles within the relevant size range.

2. **Dermal Exposure:** The importance of dermal exposure to hazardous substances continues to increase. The UK HSE estimate that there are around 85,000 cases of work related skin disease at any one time, with people employed as bricklayers, laboratory technicians and hairdressers being at particular risk (Hodgson et al; 1993). Substances which are considered to be potentially harmful include pesticides such as methyl parathion and solvents such as carbon tetrachloride. Harmful effects arising from skin exposure may either occur locally within the skin or alternatively the substance may be absorbed through the skin and disseminated via the bloodstream, possibly causing systemic effects. Because of this there have been considerable efforts made to develop quantitative methods for monitoring skin exposure.

All of these techniques essentially measure the mass of material deposited onto the skin, either per body part or per unit area of skin exposed. Difficulties in this area are compounded by a multitude of measuring methods which are not easily comparable. The development of a conceptual model of dermal exposure has been beneficial in clarifying some of these issues (Schneider et al; 2000). Systemic effects in particular are unlikely to depend only on the mass on the skin but are more likely to be related to uptake through the skin. Uptake is the flux through the skin and will depend on the concentration through the skin, the area exposed and the duration of exposure. Because of this some authors have suggested that uptake is a more appropriate metric than mass (Robertson and Cherrie, 1995).

A recent review of dermal exposure issues concluded that there was no evidence to indicate that specific health problems are currently arising from dermal penetration of ultrafine particles (HSE, 2000). However, the review conceded that dermal absorption of ultrafine particles (nanoparticles) has not been well investigated and suggested that ultrafine particles may penetrate into hair follicles where constituents of the particles could dissolve in the aqueous conditions and enter the skin. Direct penetration of the skin has been reported by Tinkle et al (2003) for particles with a diameter of 1000 nm, much

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larger than nanoparticles. It is reasonable to postulate that nanoparticles are more likely to penetrate, but this has not yet been demonstrated. Several pharmaceutical companies are believed to be working on dermal penetration of nanoparticles as a drug delivery route. Any metric proposed to assess dermal exposure to nanoparticles should be biologically relevant and should relate to health effects. Further work including workplace studies and *in vitro* assessment of penetration is required. Based on the current level of knowledge, measurement approaches should include assessment of mass, particle number, concentration, area exposed and duration of exposure.

3. **Ingestion Exposure:** In occupational settings very little work has been done up to now on ingestion exposure. Lead is one of the few materials where the ingestion route has received some attention. Lead paint removal activity can produce high ingestion exposures via hand-mouth contact and food contamination in certain workplaces. A recent study has shown that workers involved in the supply and removal of scaffolding can have particularly high blood lead levels as a result of hand contamination and subsequent ingestion (Sen et al; 2002). No work was identified in relation to ingestion exposure of nanoparticles. Metrics to assess ingestion exposure to nanoparticles should be biologically relevant and (probably) in the first instance should be measures of mass uptake.

In conclusion, scientific evidence, so far, has demonstrated that particle surface area and surface reactivity is likely to be the metric of choice to describe the inflammatory reaction to deposited particles in the proximal alveolar region of the lung. For nanoparticles, their potential dispersal to other organs as well as the possibility of exposure by other routes such as dermal or ingestion mean that possible health risks beyond the lung cannot be ruled out. Further research to generate vital data on the possible mode action of nanoparticles in the extra-pulmonary system is needed in order to assess realistically the health risks to nanoparticle exposure.

CONCLUSION AND SUMMARY

Nanosuspensions appear to be a unique and yet commercially viable approach to combating problems such as poor bioavailability that are associated with the delivery of hydrophobic drugs, including those that are poorly soluble in aqueous as well as organic media. Production techniques such as media milling and high-pressure homogenization have been successfully employed for large-scale production of nanosuspensions. The advances in production methodologies using emulsions or microemulsions as templates have provided still simpler approaches for production but with limitations. Further investigation in this regard is still essential. Attractive features, such as increased dissolution velocity, increased saturation solubility, improved bioadhesivity, versatility in surface modification and ease of post-production processing, have widened the applications of nanosuspensions for various routes. The applications of nanosuspensions in parenteral and oral routes have been very well investigated and applications in pulmonary and ocular delivery have been realized. However, their applications in buccal, nasal and topical delivery are still awaiting exploration. The development of stealth nanosuspensions laced with functionalized surface coatings capable of eliciting passive or active targeting as per the requirement can be regarded as the future step in the nanosuspension research.

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KEY TERMS AND DEFINITIONS

Alzheimer's Disease: Alzheimer's disease is a neurological disorder in which the death of brain cells causes memory loss and cognitive decline. A neurodegenerative type of dementia, the disease starts mild and gets progressively worse.

Atovaquone: It is a chemical compound belonging to the class of naphthoquinones with anti-pneumocystic activity.

Bhasmikarana: It is a process of reducing to ashes or calcinations. It is a method employed to convert a metal to its oxide form for a more medicinal application.

Bioadhesive: A property of natural polymeric materials to act as an adhesive or in simple terms any synthetic material designed to adhere to biological tissues.

Bioavailability: It is the measurement of rate and extent to which the administered drug reaches the systemic circulation. It is also one of the essential tools in pharmacokinetics.

Horizontal Gene Transfer: It refers to the transfer of genes between organisms in contrast to the transfer from parent to offspring (vertical). It is considered to be an important factor in the evolution and antibiotic resistance.

Micro-Emulsion: Dispersion made of water, oil and surfactant(s) i.e. an isotropic an thermodyamically stable system with dispersed domain diameter varying approximately from 1 to 100nm, usually 10 to 50nm.

Nanosuspensions: They are defined as a biphasic system consisting of any pure drug particles dispersed in an aqueous vehicle in which the diameter of the suspended particles is less than 1μm.

Phage Therapy: It is defined as the therapeutic use of bacteriophages (virus infecting bacteria) to treat pathogenic bacterial infections. They are more specific in their mode of action compared to antibiotics.

PTFE (Polytetrafluoroethylene): It is a synthetic hydrophobic fluoropolymer used as a lubricant, coating material and a graft material in surgical interventions.

Sustained Release: It is the process of releasing any drug for a predetermined rate and maintaining a constant drug concentration for a specific period of time at minimal side effects.

Targeted Drug Delivery: A method of delivering the drug in such a way that increases the dose in some parts of the body relative to others. It is also an effective and a protective drug interaction strategy against diseased tissue.

Tumor: A condition resulting from the abnormal growth or division of cells forming a mass of tissues (Neoplasm).

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Chapter 50
Quantitative Nanostructure–Activity Relationship Models for the Risk Assessment of NanoMaterials

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ABSTRACT

In the last few decades, nanotechnology has been deeply established into human's everyday life with a great number of applications in cosmetics, textiles, electronics, optics, medicine, and many more. Although nanotechnology applications are rapidly increasing, the toxicity of some nanomaterials to living organisms and the environment still remains unknown and needs to be explored. The traditional toxicological evaluation of nanoparticles with the wide range of types, shapes, and sizes often involves expensive and time-consuming procedures. An efficient and cheap alternative is the development and application of predictive computational models using Quantitative Nanostructure-Activity Relationship (QNAR) methods. Towards this goal, researchers are mainly focused on the adverse effects of metal oxides and carbon nanotubes, but to date, QNAR studies are rare mainly because of the limited number of available organized datasets. In this chapter, recent studies for predictive QNAR models for the risk assessment of nanomaterials are reported and the perspectives of computational nanotoxicology that deeply relies on the intense collaboration between experimental and computational scientists are discussed.

INTRODUCTION

Due to their small size, nanoparticles, exhibit unique physicochemical properties compared to their bulk counterparts and have already found numerous applications in several biomedical and consumer products. Since the production of nanomaterials increases day by day, the probability that they could

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cause adverse effects to human health and the environment rapidly rises. In order to reduce the cost and time demanded for the experimental evaluation of toxicity, computational methods, such as Quantitative Structure-Activity Relationships (QSARs) are being used to predict the potential toxic effects of nanoparticles. In this chapter, the use of Quantitative Nanostructure-Activity Relationship (QNAR) modeling is discussed as a useful tool to predict the biological and toxicological effects caused by diverse types of manufactured nanoparticles (MNPs). Recent studies are reported summarizing the available predictive QNAR models for the risk assessment of nanomaterials (NMs). Finally, the perspectives and the future of computational nanotoxicology that strongly depend on the intense collaborations between experimental and computational scientists are discussed.

BACKGROUND

According to the U.S. National Nanotechnology Initiative (NNI), nanotechnology is defined as the understanding and control of matter at dimensions of roughly 1 to 100 nanometers, where unique phenomena enable novel applications. At this level, the physical, chemical, and biological properties of materials differ in fundamental and variable ways from the properties of individual atoms and molecules or bulk matter (NNI, 2000).

Nanotechnology has become one of the most promising areas of science and it has fairly emerged as one of the central new technologies in the 21st century, drawing worldwide attention (Haase, Tentschert, & Luch, 2012; Fourches, Pu, & Tropsha, 2011). Hitherto, nanotechnology is an important industrial sector in industrialized countries and it has been referred to as the next industrial revolution. The first national nanotechnology program (NNI) was launched in USA in 2000, to serve as the central point of communication, cooperation, and collaboration for all Federal agencies engaged in nanotechnology research, bringing together the expertise needed to advance this broad and complex field (Bondarenko, 2013). Nanotechnology has found numerous applications in materials science, medicine, cosmetics, or clothing, bioimaging, sensing and electronics (Fourches et al., 2011; Makarucha, Todorova, & Yarovsky, 2011). Nanomedicine, for instance, an emerging application of nanotechnology, provides the possibility of delivering small drug molecules to specific cells, by developing novel diagnostics and therapeutics (Wang, Byrne, Napier, & DeSimone, 2011).

Up to date, several classifications of nanomaterials have been proposed. According to the European Committee for Standardization (CEN) Technical Specification, nanomaterials can be classified into two broad categories, namely nanoobjects and nanostructured materials (Figure 1) (Haase et al., 2012).

According to Hansen, Larsen, Olsen and Baun (2007) and depending on the location of the nanoscale structure in the system, nanomaterials are organized into three broad categories, as it depicted in Figure 2.

For simplicity, nanomaterials can generally be organized into four types (Messina, Olsina, & Stege, 2013):

1. Carbon-based materials,
2. Metal-based materials, including metal oxides and quantum dots,
3. Dendrimers, and
4. Composites.

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Figure 1. Classification of nanomaterials according to CEN/ISO TS 27687; Nanoplates have one external dimension, nanorods two orthogonal dimensions, and nanoparticles all three orthogonal dimensions in the nanoscale.
(ISO 2008).

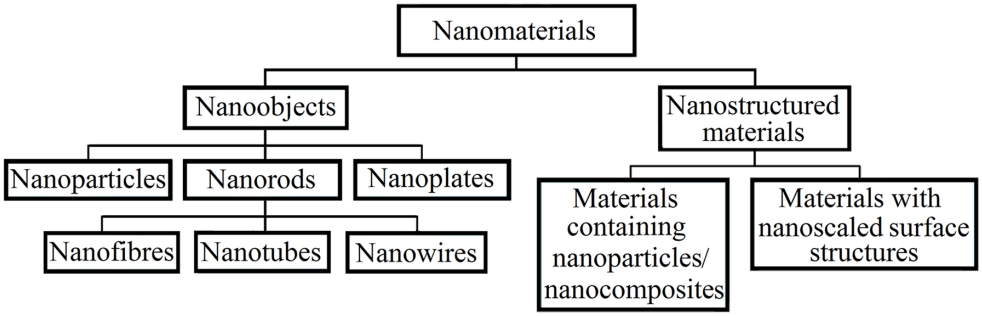


Figure 2. Classification of nanomaterials according to Hansen et al.

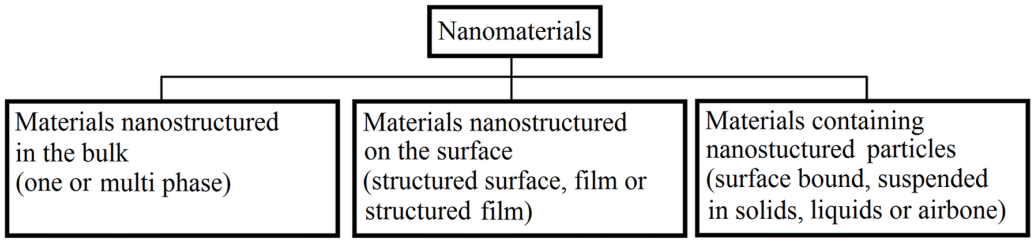
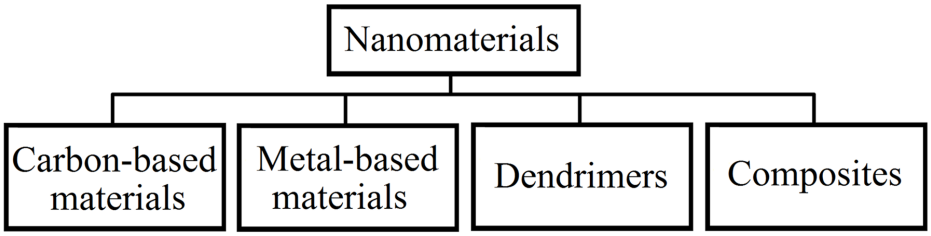


Figure 3. Classification of nanomaterials according to Messina et al.



Nanomaterials can be naturally present as dust from forest fires and volcano's activities, biogenic magnetite, viral particles, and even protein molecules, such as ferritin. Nano-sized materials can also be produced unintentionally by anthropogenic sources, such as from combustion by-products. Engineered nanomaterials (ENMs) designed for a specific purpose or function are useful due to their small size and large surface-area to volume ratio, which makes them important as catalysts in chemical reactions, and they have desirable properties as drug delivery devices, as imaging agents in medicine, and in consumer products such as sunscreens, textiles and cosmetics (Clark, White, & Silbergeld, 2011).

A limited number of nanoparticles are known about their toxicity, including possible mutagenic and/or carcinogenic effects (Dreher, 2004). Recent studies have shown evident toxicity of some nanoparticles to living organisms, including human body, as well as their potentially negative impact on environmental

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ecosystems and highlight the potential risk related to the development of nanoengineering (Gajewicz et al., 2012). It is expected that nanomaterials can become present in the environment, including air, water and soil, during their life cycle of production, distribution, or usage. In the last decade, a new subdiscipline of ecotoxicology has been developed, that is called nanoecotoxicology with the first scientific papers emerged in the literature in 2006. The ecotoxicological problems derived from the use of NMs and their present in the natural environment and the ecosystems are challenging tasks for environmental toxicologists and ecological risk assessment specialists (Kahru, & Ivask, 2013). It is commonly accepted that there is insufficient knowledge regarding the harmful interactions of nanoparticles with biological systems as well as with the environment.

Since the manufacture and use of nanomaterials are increasing, humans are more likely to be exposed occupationally or via consumer products. Workers in nanotechnology-related industries and small workshops have an increasing risk to be exposed to ENMs. Likewise and as any new technology, the main concerns are about environmental pollution, the toxicity in aquatic organisms and their environmental impact in ecosystems. Lack of available data and low adequacy of experimental protocols prevent comprehensive risk assessment. Toxic effects of nanomaterials on different organisms have already been described (Klaine, 2008; Baun, Hartmann, Grieger, & Kusk, 2008), but measurements of environmental concentrations of ENMs are almost completely absent. The high cost and the long time of the *in vivo* toxicological studies are barriers on testing all of the marketed consumer products incorporating nanoparticles. Our knowledge of the human health effects and environmental concentrations of engineered NMs or NPs is incomplete and also the mechanisms of the interatomic interactions between nanoparticles and biological molecules are not sufficiently understood.

Natural and manufactured/engineered nanoparticles due to their unique properties and biological activity gain the access into the human body through the main ports of body's entry, such as the skin, the gastrointestinal tract and the lungs. Starting from these entries and through the fine capillaries, NPs may also permeate deeper in the organism and tissues, and travel throughout the body interacting with other tissues and organs. Several pharmacokinetic studies have showed that nanoparticles can be found in various cells, in mitochondria, fibroblasts, lipid vesicles, nuclei and macrophages. They have direct contact with cell surface and may lead to the formation of reactive oxygen species (ROS) that results in oxidantive stress and inflammation. Furthermore, they can cause peroxidation of lipids in cellular membranes, resulting in cell damage, and the up-/down-regulation of genes encoding a specific protein involved in inflammatory processes (Gajewicz et al., 2012). Nanoparticles are associated with genotoxicity and neurotoxicity. The effects of nanoparticles on the immune system are still unclear.

Concerning to the ecosystem, there are numerous review articles on the behavior of nanoparticles in aquatic environments (Delay, & Frimmel, 2012), whereas, up to date there are no published reports on the concentration of manufactured nanoparticles in the air (Handy, & Shaw, 2007). Nanoparticles in aquatic systems can be formed by natural abiotic or biotic processes, of natural origin but induced by human activity (e.g. mining), engineered NP that enter aquatic systems incidentally, or engineered NP that are intentionally added to these systems, including nanoscale zero-valent iron ($Fe^{(0)}_{(NP)}$) for removal of As(III) from groundwater (Delay, & Frimmel, 2012). Toxicology studies on animals showed that some types of nanoparticles in the air could cause lung disease.

The potential negative health effects of particles depend on some key parameters, such as the dose, dimension, and durability. Nonetheless, it has been showed that the astonishing physical and chemical properties of nanoparticles that are attributable to their small size differ from these properties of particles at the micro or macro scale (Gajewicz et al., 2012; Nel, Xia, Madler, & Li, 2006). Especially,

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ultrafine particles are known to have greater adverse health effects than larger particles. In addition, by decreasing for instance the size of gold samples induces color changes from bright yellow through reddish–purple up to blue. The novel and unique properties of nanoparticles (i.e. physicochemical and/or biological characteristics) are often determined by the relationships between their size, surface, and structure. Recent studies have shown that the reduction of particle size increases the surface area and so, smaller nanoparticles have greater surface area (Gajewicz et al., 2012). The toxicity of nanoparticles according to Oberdörster *et al.* (2005) may depend not only on the particle size but on other features with vital importance, such as size distribution, agglomeration state, shape, porosity, surface area, chemical composition, structure-dependent electronic configuration, surface chemistry, surface charge, and crystal structure. To date, the interrelationship between nanoparticles properties and their biological effects remains unclear.

The fact that nanomaterials consist of many substances (primarily composed of carbon, silver, zinc, silica, titanium and gold) and can be found in many sizes and shapes and with a variety of surface coatings, complicates the risk assessment. Studies have showed that nanomaterials after their release of into the environment may tend to agglomeration, aggregation, adhesion, diffusion, dissociation, degradation, adsorption of different species, and bioaccumulation in organisms as well as biomagnifications in trophic pyramids, processes that depend not only on their size/shape, but also on the local environmental and cellular conditions (Gajewicz et al., 2012). Therefore, an assessment of the physical, chemical and biological properties of nanoparticles in each state and case must be made in order to consider their exposure, fate, transport, and transformation in the environment.

Most researchers have focused on the adverse effects to human health and the environment of one single type of particle or several particle types of the same substances, such as,

1. Metal oxide nanoparticles and
2. Carbon nanoparticles, respectively.

Metal oxides (MO_x) is an important group of engineered NPs due to their limited size and a high density of corner or edge surface sites, which allow them wide applications. They can adopt a vast number of structural geometries with an electronic structure that can exhibit metallic, semiconductor or insulator character. They are produced in large quantities and used frequently in cosmetics, sensors and sunscreens, self-cleaning coatings, and textiles. In addition, MO_x's can be used as catalysts for redox reactions in engineered systems and natural environments, water-treatment agents and materials for solar batteries (Puzyn et al., 2011). The most studied oxide systems are Al_2O_3, MgO, ZrO_2, CeO_2, TiO_2 and SiO_2. Less studied nanostructured oxide systems include Zn, Fe, Cu and Sn.

In the last quarter of century, various carbon nanostructures such as fullerenes, carbon nanotubes (CNTs) and graphene have been given great importance in the field of nanoscience and nanotechnology.

Fullerenes are ball shaped hollow structures composed of carbon atoms. The most important fullerene is known C_{60} that has a football-like structure comprising 12 pentagons located around the vertices of an icosahedron and 20 hexagon rings placed at the centers of icosahedral faces (Figure 4). Fullerenes have numerous applications in personal care products, as well as they have several potential uses in medicine.

Carbon nanotubes' potential applications in medicine are reported in several studies (Zhou et al., 2008). CNTs can enter various organs and cells, so they can function as intracellular probes, drug carriers, imaging agents, DNA modulators, and other medical devices on the condition that they are biocompatible. There are two structural forms of carbon nanotubes (Figure 4):

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1. Single-walled carbon nanotubes (SWCNTs) and
2. Multi-walled carbon nanotubes (MWCNTs).

Graphene, the thinnest but strongest material, is a 2D-sheet composed of carbon atoms characterized by sp^2 hybridization (Figure 4). Some of the applications of carbon nanotubes and graphene are in photodetectors, drug systems, and bioimaging.

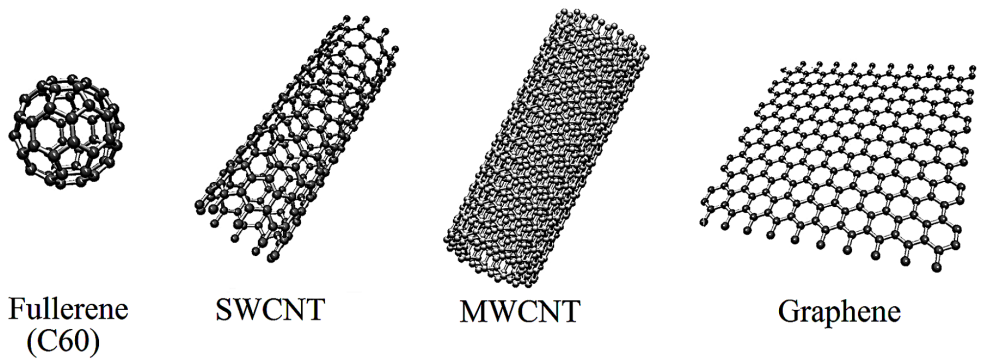
It is interesting that the unique properties of metal oxide nanoparticles can be the cause of potential toxic effects. The general results of a study showed that there was a high variation among different metal oxide nanoparticles concerning their ability to cause toxic effects (Karlsson, Cronholm, Gustafsson, & Möller, 2008). It has been showed that nano-TiO_2 causes ROS stress and DNA damage in lymphocytes (Kang, Kim, Lee, & Chung, 2008). Furthermore, similar to TiO_2 nanoparticles, nano-sized silicon oxide (SiO_2), and zinc oxide (ZnO) can induce pulmonary inflammation in rodents and humans (Gordon et al., 1992; Rehn, Seiler, Rehn, Bruch, & Maier, 2003; Chen, Chen, Dong, & Jin, 2004).

Similarly, the carbon-based nanomaterials have adverse effects to human's health and ecosystem. Potential short- and long-term toxicity of fullerenes, CNTs and graphene should be carefully investigated before full implementation of these in various biological and commercial applications. Although fullerenes, CNTs and graphene are carbon-based nanomaterials, each of them has different distinctive properties. Different studies have showed that fullerene particles C_{60} suspended in water are characterized by antibacterial activity against *Escherichia coli* and *Bacillus subtilis* (Lyon, Fortner, Sayes, Colvin, & Hughe, 2005) whereas they exhibit cytotoxicity to human cell lines (Sayes et al., 2004). In particular, the cytotoxicity of C_{60} is lower than that of SWCNTs, MWCNTs and graphite (Gajewicz et al., 2012). Therefore, risk assessments of carbon nanomaterials are to be taken on a case-by-case basis to assure high safety.

Quantitative Nanostructure-Activity Relationship (QNAR)

As aforementioned, the traditional toxicological evaluation of the different developmental stages involves expensive, time-consuming and labor-intensive technical procedures. To reduce all above factors, computer-based approaches have been widely developed. The computational methods are a significant tool since they can be utilized to predict the toxicological (biological generally) effects of nanoparticles

Figure 4. The structures of carbon nanoparticles: fullerene ($\mathbf{C}_{60}$), single-walled carbon nanotube (SWCNT), multi-walled carbon nanotube (MWCNT), and graphene



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solely from their physical and chemical properties. Subsequently, by screening *in silico* libraries of virtual nanomaterials, the NMs with the most promising predicted properties can be prioritized. Thus, the costly toxicological studies focus only on a small number of selected and/or rationally designed NPs.

Since 2007, REACH (Registration, Evaluation and Authorization of CHemicals) established by European Parliament and Council, is formally a new chemical management system. One of REACH's key goals is to reduce the number of compounds that require toxicity testing in animals (Schaafsma, Kroese, Tielemans, Van de Sandt, & Van Leeuwen, 2009). REACH legislation has encouraged and foresees the use of alternative *in silico* methods, such as the Quantitative structure-activity relationship (QSAR), to avoid using the animals for the risk assessment of chemicals.

The modeling of manufactured nanoparticles (MNPs) is a major challenge for scientists especially due to the high structural complexity and diversity of MNPs. Therefore, it is very difficult to develop quantitative parameters capable of characterizing the structural and chemical properties of MNPs. The difficulty becomes greater with the absence of experimental data that is required for the validation of computational methods. Puzyn Leszczynska and Leszczynski (2009) first suggested that QSAR modeling can be applied in computational nanotoxicology studies but indicated that no universal QNAR model can accurately assess the toxicity of all possible MNPs.

Similar to general QSAR modeling strategies, the overall objective of QNAR modeling is to develop a mathematical and statistically significant model. To enable the modeling of MNPs, every particle should be described by numerical parameters, called descriptors that are calculated or experimentally measured. The model describes the relationship between the measured biological or toxicological activity profiles of MNPs. The properties such as size, shape, morphology, surface area are usually measured experimentally. Unfortunately, these data for the nanostructures are often absent, so there is an obvious need to develop and employ computational descriptors of MNP structure. To date, thousands of zero-dimensional (0D, referred to atom and bond types counts), 1D, 2D, and 3D (even 4D and 5D!) descriptors have been invented and there are several commercially available software packages (i.e. MOE, DRAGON, CODESSA, SYBYL) to compute several descriptors for any single compound. However and at the moment there are no software packages applicable to MNPs due to their complexity of structure (mixture of organic and inorganic patterns in the same molecule, repetition of structural motifs). Therefore, the descriptors for MNPs have to be measured experimentally until novel and more appropriate types of the descriptors will be developed. To enable QNAR studies, there should be sufficiently large amount of data and a well-defined protocol for model selection and validation should be used.

Models can be built using complex machine learning algorithms, linear or non-linear methods, such as Multiple Linear Regression (MLR), Artificial Neural Networks (ANN), Support Vector Machine (SVM), Random Forest (RF), and k Nearest Neighbors (kNN) or Naïve Bayesian Classifier (NBC), Linear Discriminate Analysis (LDA), and Logistic Regression (LGR).

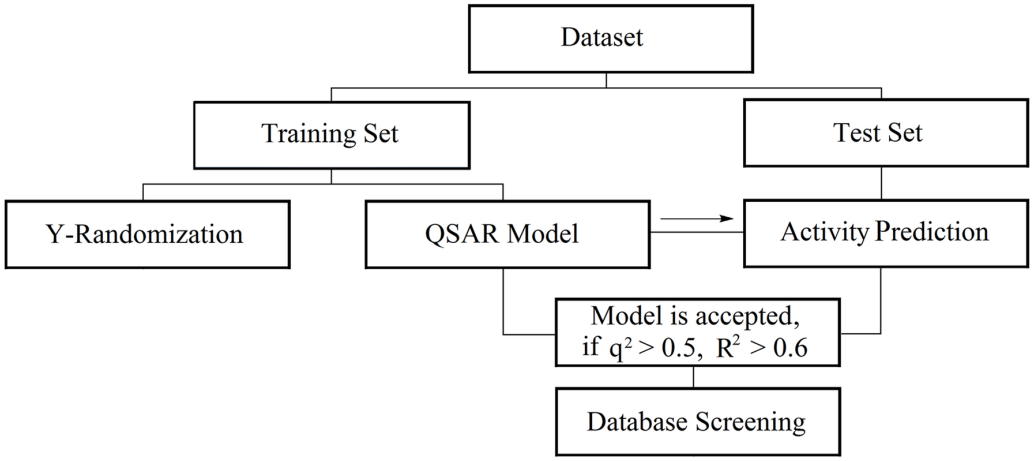
The QSAR modeling workflow consists of three steps (Figure 5):

1. Data preparation (compounds and corresponding descriptors),
2. Model building, and
3. Model validation.

Initially, the set of compounds is divided into training and test set. The training set contains the majority of the data, whereas the test set is smaller (~25-30%). The model is derived using only the compounds of training set, and then it is applied to test set to assess their properties. The splitting of the dataset

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Figure 5. The workflow of QSAR modeling



can be done randomly or using a variety of selection techniques. Leave-one-out (or Leave-many-out), cross-validation and Y-randomization are applied to evaluate both prediction and robustness of model. A good model should predict both training and test set (Golbraikh, & Tropsha, 2002) and satisfy some conditions. Finally, the QSAR model can be used increasingly in chemical data mining and combinatorial library design.

A QSAR model to be appropriate validated in a regulatory environment should comply with the OECD guidelines:

1. A clear definition of the endpoint,
2. The use of an unambiguous algorithm,
3. Necessity of defining applicability domain,
4. Calculating appropriate measures of goodness-of-fit, robustness and predictivity, and
5. Providing a mechanistic interpretation, if possible (retrieved from http://www.oecd.org).

Predicting biological effects of MNPs prior to their synthesis is of outmost importance, and therefore the development of efficient computational approaches is very significant. With the QSAR methodology, if toxicological data are available only for some compounds in a group, the toxicological properties of the other compounds in the same group can also be determined using only calculated molecular descriptors and a suitable mathematical model.

Analyzing data presented in the literature, it is concluded that even if a class of nanoparticles with a similar structure is tested with the same assay and protocol, the number of tested compounds is often insufficient to perform comprehensive internal and external validation of a model and to calculate the appropriate predictivity in QSAR. For instance, Limbach *et al.* (2005) studied the *in vitro* cytotoxicity of seven metal oxide nanoparticles in human and rodent cells and proposed two different rankings: (i) Fe_2O_3 ≈ asbestos > ZnO > CeO_2 ≈ ZrO_2 ≈ TiO_2 ≈ $Ca_3(PO4)_2$ and (ii) ZnO > asbestos ≈ ZrO_2 > $Ca_3(PO4)_2$ ≈ Fe_2O_3 ≈ CeO_2 ≈ TiO_2, respectively. In another study (Brunner et al., 2006), the same research group found that the formation of reactive oxygen responsible for the toxicity of four metal nanoparticles (TiO_2, Fe_2O_3, Mn_3O_4, and Co_3O_4) toward the human lung epithelial cells was their chemical composition. It

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must be highlighted that a dataset containing a significant number of compounds is essential to build an appropriately validated QNAR model.

To date, a limited number of QNAR studies and only few reviews concerning the risk assessment of nanoparticles have been reported in the literature (Puzyn et al., 2009; Cattaneo et al., 2010; Burello, & Worth, 2011; Luan et al., 2014). The majority of published QNAR models focus primarily on the predictions of physicochemical properties of nanoparticles (QSPR), e.g. solubility and Young's modulus of carbon-based nanostructures (Toropov, & Leszczynski, 2007; Toropov, Leszczynska, & Leszczynski, 2007; Martin, Maran, Sild, & Karelson, 2007). In this chapter, only the QSAR models for nanotoxicity are reported. In Table 1 eleven datasets available in the literature for QNAR modeling are listed. From these only three satisfy the criterion of relatively large sample size (n>50) and are highlighted in the Table.

In 2011, Cohen and his co-workers (Liu et al., 2011) developed a classification-based cytotoxicity nanostructure–activity relationship (nano-SAR) using the high-throughput toxicity screening data for transformed bronchial epithelial (BEAS-2B) cells exposed to nine metal oxide nanoparticles. The model was based on a logistic regression and is validated following accepted QSAR validation guidelines. The High Throughput Screening (HTS) data were first processed to identify and label toxic versus non-toxic events relative to an unexposed control cell population. The best-performing model with 100% classification accuracy was based on three descriptors: the atomization energy of the metal oxide (E_{MeO}), the period of the nanoparticle metal (P_{Me}), and the nanoparticle primary size (d), in addition to the nanoparticle volume fraction (θ_{v}) and was expressed by the following equation:

$$ln\frac{\mathrm{P}\left(NP \in T\right)}{\mathrm{P}\left(NP \in N\right)} = 3600.6 \; + \; 103.5 \; \times \; d \; + \; 9.5 \; \times q_v + \; 97.6 \; \times P_{Me} - 58.5 \; \times E_{MeO}$$

where P($NP \in T$) and P($NP \in N$) are the probabilities of a nanoparticle being toxic and nontoxic, respectively.

Table 1. List of available nanoparticles datasets

No.	Dataset	Sample Size	Description	Endpoint
1	Liu et al., 2011	9	Metal oxide NPs	Cytotoxicity
2	Zhang et al., 2012	24	Metal oxide NPs	Cytotoxicity
3	Weissleder et al., 2005	**146**	1 metal core	Cellular uptake in PaCa2
4	Shaw et al., 2008	**51**	4 metal cores	Apoptosis
5	Puzyn et al., 2011	17	Metal oxide NPs	EC_{50} of *E. coli*
6	Sayes et al., 2010	24	MNPs	Cellular membrane damage
7	Gaudin et al., 2012	10	MIL-88B(Fe) MOFs	Caffeine uptake
8	Shinohara et al., 2009	20	fullerene C_{60} NPs	Mutagenicity
9	Zhou et al., 2008	**84**	f-MWCNTs	Protein binding, cytotoxicity, immune responses
10	Tassa et al., 2010	20	MNPs	Ka, Kd of aurora A kinase
11	Luan et al., 2014	41	Metal oxide NPs	Cytotoxicity

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The authors suggested this successful modeling approach on the basis of the HTS data in order to establish metal oxide nanoparticle hazard ranking. However, they recognized that a significantly larger data set is required in order to improve the reliability of and increase confidence in the nano-SAR approach.

Two years later, nano-SARs for metal oxide NPs toxicity were investigated by the same group using metrics based on dose-response analysis and consensus self-organizing map clustering (Liu et al., 2013). A dataset of 24 metal oxide NPs provided a multiparametric toxicity profile for two different cell lines consisting of seven different toxicity assays (Zhang et al., 2012). Initially, 30 descriptors were used and the best performing nano-SAR model built with support vector machine (SVM) was based on two descriptors. These descriptors, namely conduction band energy (EC) and ionic index (Z^2/r), had a high classification accuracy of ~94%. According to the authors, the class probabilities provided by the nano-SAR enabled the construction of decision boundaries with respect to toxicity classification under different acceptance levels of false negative relative to false positive predictions.

Fourches *et al.* (2010, 2011) developed a QSAR model to predict the cellular uptake of 109 NPs in pancreatic cancer cells (PaCa2). Each NP had the same metal core (iron oxide/NH_2 cores) but diverse surface modifiers; an organic small molecule conjugated to the NP surface (Weissleder, Kelly, Sun, Shtatland, & Josephson, 2005). Accordingly, each NP was represented by a unique set of descriptors determined by the organic molecule. In total, 150 MOE descriptors were calculated. For the developed QNAR model k-nearest neighbors (kNN) method was employed to correlate biological activity of NPs with the calculated descriptors. Their average external prediction power was shown to be high (R^2= 0.72). The authors concluded that QSAR is a useful tool to predict the cellular behavior of a common core-based NP library and their obtained model can be utilized to design a specific coating material to control the cellular uptake.

In the same study (Fourches et al., 2011; Fourches et al., 2010), the researchers studied also a dataset of 51 iron oxide- and quantum dot-based nanoparticles with four different metal cores and surface modifications. The various NPs were tested *in vitro* against four cell lines in different assays by Shaw *et al.* (2008) to study their induced biological effects. The model was built using the support vector machine (SVM)-based classification. An arbitrary threshold was utilized to divide the data set into two groups with the same number of NPs, and four experimental descriptors were used to classify the NPs into two clusters: size, relaxivities, and zeta potential. The model was developed for averaged toxicity responses relative to a control (i.e., unexposed) cell population. The data were classified into two equivalent groups, being below or above an arbitrary threshold. A model classification accuracy of 73% was reported, but without a definitive identification of true toxic end points. The authors suggested that QNAR models can be employed for predicting biological activity profiles of novel nanomaterials, and prioritizing the design and manufacture of a whole NM towards better and safer products.

Similarly, Winklert and his co-workers (Epa et al., 2012) performed a first computational modeling study using the two large sets of experimental nanoparticle data by Weissleder *et al.* (2005) and Shaw *et al.* (2008). They studied all possible combinations of biological assays and cell types, and generated robust models of cellular uptake and induction of apoptosis by metal oxide NPs using Bayesian methods. According to the authors, QNAR is a promising modeling technique, and it can be useful for the analysis of the biological effects of NPs and can capture the effects of different environments of body such as serum, plasma, or lung fluids on nanoparticle composition, as well as NP cellular uptake and interaction with cellular biochemical systems.

Chau and Yap (2012) studied the ability of QNAR models to predict the cellular uptake of Weissleder *et al.*'s NPs dataset with a single metal core in pancreatic cancer (PaCa2) cells. They used four different

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modeling methods, naïve Bayesian, logistic regression, k-nearest neighbor and support vector machine, to develop candidate models. A final consensus model was developed using the top 5 candidate models, which was able to accurately predict 86.7 to 98.2% of the nanoparticles with good cellular uptake and 67.3 to 76.6% of the NPs with poor cellular uptake. Thus, the authors suggested that it is practical to use the physical, chemical and/or geometrical activity to develop predictive QNAR models.

In 2011, Puzyn *et al.* (2011) investigated the cytotoxicity of 17 metal oxide NPs towards Escherichia coli bacteria. Based on toxicity data they computed structural descriptors. Although the number of NPs used was small, they built a model to predict the cytotoxicity of other nanomaterials. More specifically, the authors calculated 12 descriptors quantitatively describing the variability of the NPs' structure. The descriptors were calculated in MOPAC software package using the quantum-chemical PM6 method. The dataset was divided into training (10 metal oxides) and validation (seven metal oxides) sets. The EC_{50} values of the studied NPs were consequently predicted using the multiple linear regression (MLR). It is notable that Tthe best MLR model was built using only one descriptor selected by genetic algorithm. The model was expressed by the following equation:

$$\log(1/EC_{50}) = 2.59 - 0.50 \times \Delta H_{Me+}$$

where ΔH_{Me+} represents the enthalpy of formation of a gaseous cation in the same oxidation state as that in the metal oxide structure.

The results indicated predictive ability of the model (R^2=0.85, Q^2= 0.77, externally Q^2=0.83). Thus, the authors suggested that their developed QSAR model can be used reliably to predict toxicity of untested nanomaterials, particularly metal oxides in computational nanotoxicology studies.

Leszczynski and his co-workers (Toropov et al., 2012) continued studying the cytotoxicity of the metal oxide NPs to bacteria *Escherichia coli*, and a year later, using CORAL software and the same data set of metal oxide NPs, they boosted the QNAR modeling. In this approach, the pEC_{50} values of the metal oxide nanoparticles' cytotoxicity to bacteria E. coli were used and six random splits into the training and test sets were examined. All the obtained models were satisfactory. According to the authors, CORAL, a convenient application and an available on the Internet software (http://www.insilico.eu/coral), was proved as a reliable tool for the QSAR analysis of the toxicity of metal oxide NPs.

The CORAL software was also used by Toropov *et al.* (2013) in order to build a QSAR model for prediction of cellular uptake of the 109 NPs in pancreatic cancer cells (PaCa2). The software gave satisfactory and stable predictions of the cellular uptake of NPs in PaCa2 cancer cells for five random splits. The optimal molecular descriptors, provided by CORAL, were calculated directly from simplified molecular input-line entry system (SMILES) and are used to correlate with various endpoints. The SMILES gradually has become the most popular representation of the molecular structure in the databases available on the Internet, and SMILES-based optimal descriptors can become attractive alternative to ''classic'' descriptors. Thus, the authors suggested this concept of QSAR as a random event and the alternative to build up QSAR.

Another attempt was made by Ghorbanzadeh, Fatemi, and Karimpour (2012) to build a model for the prediction of the cellular uptake of 109 magnetofluorescent NPs in pancreatic cancer cells. An artificial neural network was employed and the descriptors were calculated using Hyperchem program and Dragon. Six descriptors chosen by combining self-organizing map and stepwise multiple linear regression (MLR) and multilayered perceptron neural network (MLP-NN) modeling techniques were used to correlate the nanostructure of the studied particles with their bioactivity. For the MLR and MLP-NN

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models, the correlation coefficient and the Root Mean Square (RMS) error were 0.769 and 0.934 and 0.364 and 0.150, respectively. The results obtained after a leave-many-out cross-validation test revealed the credibility of MLP-NN for the prediction of cellular uptake of NPs. Additionally, sensitivity analysis of MLP-NN model indicated that the number of hydrogen-bond donor sites in the organic coating of a NP is the predominant factor responsible for cellular uptake.

Similar to their previous study (Toporov et al., 2013), Toporova and Toporov (2013) worked on TiO_2 nanoparticles. Experimental data on the physicochemical features of TiO_2 nanoparticles and their influence on the membrane damage were taken in the literature (Sayes, & Ivanov, 2010). The optimal descriptors calculated with the Monte Carlo method led to a built up of a predictive model for the membrane damage. The authors suggested that optimal descriptors based on representation of TiO_2 nanoparticles by their physicochemical features can be a tool for modeling of membrane damage.

In a recent study (Sayes, & Ivanov, 2010), nano-SAR classifiers based on nanoparticle properties such as primary size, aggregate size in different media, and zeta potential were developed to predict cell membrane damage induced by TiO_2 and ZnO. The authors compared two approaches to develop mathematical models that have the potential to predict the damage to cellular membranes, as measured by lactate dehydrogenase (LDH) release, of the two metal oxide NPs resulting from several physicochemical features. Given the relatively small number of samples, relatively simple multiple linear regression models were used to probe for possible QSARs related to the endpoint of interest—cellular membrane state. In the cases where the application of the regression models did not produce a good fit, linear discriminant analyses (LDA) classification was applied, and all possible combinations of one, two, and three feature sets were searched and ranked according to how well they are discriminated between different degrees of cellular responses of the biological test system.

A QSAR approach has been undertaken by C. Gaudin *et al.* (2012) for predicting and further interpreting the caffeine uptake, experimentally measured by HPLC and TGA techniques, in a series of functionalized MIL-88B(Fe) materials. Especially, the series of 10 materials consist of MIL-88B(Fe) iron(III) dicarboxylate metal-organic frameworks (MOFs) wherein the organic linker is functionalized by a large variety of polar and non-polar functional groups, such as –H, –Br, –F, $–CF_3$, $–CH_3$, $–NH_2$, $–NO_2$, and –OH. These MOFs were investigated as potential carriers for encapsulating drugs, using the cosmetic amphiphilic caffeine as a model molecule. The two individual but consensus QSAR models were built using the MLR method and a wide range of molecular descriptors calculated for the series of the investigated grafted organic linkers. The obtained models, validated with the leave-one-out cross-validation and the Y-randomization procedures, led to a good correlation between the observed and calculated values of the caffeine loading for the series of modified MIL-88B(Fe) iron dicarboxylate MOFs.

Recently, a study in computer-aided toxicology of nanomaterials was carried out by Luan *et al.* (2014). The aim of the researchers' work was the prediction of the cytotoxicity of diverse nanoparticles using a QSTR (Quantitative Structure Toxicity Relationship) – perturbation model. The measurements were performed against several mammalian lines and under different experimental conditions, such as the chemical composition of nanoparticles, size, shape and time of exposure of the cell lines. The dataset contained 41 nanoparticles and was retrieved from the literature. Four descriptors were measured;

1. Molar volume (*V*),
2. Electronegativity (*E*),
3. Polarizability (*P*), and
4. Size (*L*) of nanoparticle.

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1681 cases (nanoparticle-nanoparticle pairs) were used and randomly divided into training and prediction sets. The training set included 1261 cases (802 cytotoxic and 459 non-cytotoxic), and the prediction set included the remaining 420 cases (156 cytotoxic and 264 non-cytotoxic). The model was obtained using the LDA method and its statistical quality was examined by Wilks' lambda (λ), chi-square (χ^2), and p-level. The best QSTR-perturbation model was given by the following equation:

$$\mathrm{CTox}_i(c_j)_{\mathrm{nw}} = 1.616\mathrm{CTox}_i(c_j)_{\mathrm{rf}} - 0.229\Delta\Delta V(c_a) - 0.322\Delta\Delta P(c_a) + 0.044\Delta\Delta L(p_s) + 0.502\Delta\Delta V(d_m) - 0.004\Delta\Delta L(t_e) - 0.450$$

According to the authors, the LDA statistics ($\lambda = 0.305$, $\chi^2 = 1490.86$ and $p < 10^{-16}$) showed a very good significance and an accuracy higher than 93% was exhibited for both training and prediction sets. It is of paramount importance that authors in order to prove the significance of the model, predicted correctly the experimentally measured cytotoxicity of new nanoparticles not included in dataset.

Most recently, Kar, Gajewicz, Puzyn, Roy and Leszczynski (2014) using the dataset of Puzyn *et al.*'s (2011) with 17 metal oxides developed and validated a QSTR model. In their effort to encode the cytotoxicity profile of metal oxides to bacteria *E. coli*, they used simple molecular descriptors. More specifically, they calculated seven descriptors based only on the molecular formula and periodic table information:

1. Number of metal atoms (N_{metal}),
2. Number of oxygen atoms (N_{oxygen}),
3. The charge of the metal cation corresponding to a given oxide (χ_{ox}),
4. Metal electronegativity (χ),
5. Sum of metal electronegativity for individual metal oxide ($\sum\chi$),
6. Sum of metal electronegativity for individual metal oxide divided by the number of oxygen atoms present in a particular metal oxide ($\sum\chi/nO$), and
7. Molecular weight (MW).

The dataset was randomly divided into training and test sets, including eleven and six metal oxide NPs, respectively. Ten combinations of splitting were performed and the models were developed using stepwise MLR. The best MLR model was built using only one descriptor and expressed by the following equation:

$$\mathrm{pEC}_{50} = 4.781 - (1.380 \times \chi_{ox})$$

with $N = 17$, $R^2 = 0.84$, $Q^2 = 0.81$, and $\mathrm{R}^2_{\mathrm{p}} = 0.82$.

Despite the obtained statistically significant model, the authors considered it necessary to also perform the partial least squares (PLS) method. More specifically, analyzing the results of MLR they found that for two different metal oxides containing same descriptor value, the predicted value of pEC_{50} was the same. According to authors, the prediction of cytotoxicity of metal oxides using only one parameter had limited ability, and an additional descriptor was needed. The obtained final PLS equation is the following:

$$\mathrm{pEC}_{50} = 4.401 - (1.324 \times \chi_{ox}) + (0.176 \times \chi)$$

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with $N = 17$, $R^2 = 0.82$, $Q^2 = 0.75$, and $R^2_p = 0.79$.

The authors were satisfied as they succeeded to build up a significant QSTR model using simple descriptors, in no time, and without any software for calculation of descriptors. Furthermore based on this model, Kar *et al.* attempted to provide a most probable mechanism of action of cytotoxicity of metal oxide NPs to bacteria *E. coli*.

Zhou *et al.* (2008) have discovered functionalized multiwalled carbon nanotubes with reduced protein-binding, cytotoxicity, and immune response. They utilized a combinatorial synthesis technique to decorate CNTs' large surface area with a series of organic molecules and investigated the overall toxicity of obtained series of NPs. All CNTs had the same core structure and so their biological/toxicological behavior could depend only on the surface modification. The set of 84 f-MWCNTs was tested in several *in vitro* toxicological assays at two different doses, including protein binding assays, acute toxicity assays, and immune toxicity assays. The authors observed a significant, high stability of experimental measurements for all f-MWCNTs, and suggested further QNAR modeling investigations.

Three years later, Tropsha and his colleagues (Fourches et al., 2011) developed a statistically significant and externally predictive model based on the previous results. Initially, they calculated 2D Dragon descriptors, proceeding to chemoinformatics analysis of the data set. Their findings validated the hypothesis that any f-MWCNT can be modeled using a single, organic molecule from its surface only if all NPs have the same core. They subsequently proceeded to a binary toxicity class to each NP (1 as toxic and 0 as non-toxic) based on the acute toxicity assay and the toxicity measured for CNT with no surface modifiers. 38 strong toxicants (class 1) *versus* 35 weak toxicants (class 0) were subjected to their QNAR random forest (RF) approach and were carried out in a 5-fold external cross-validation. The results were satisfactory and the authors proposed the virtual screening of a library with 240,000 potential CNT surface modifiers in order to identify those materials with desired properties. For inorganic nanoparticles, such as metal oxides, there are some toxicity data available, mainly: EC_{50}, IC_{50}, LC_{50}, LD_{50}, NOEC, zeta-potential, electrophoretic mobility and surface area or solubility. Carbon-based nanoparticles, such as fullerenes, SWCNTs and MWCNTs can also be characterized by available toxicological data, zeta potential and electrophoretic mobility. For carbon nanotubes, there are significantly more data for endpoints, such as surface area, porosity and solubility (Gajewicz et al., 2012). The number of QNAR studies for metal oxide nanoparticles' toxicity is bigger than the one for carbon-based NPs. This may be justified due to the lack of available in the literature datasets of carbon-based nanoparticles with toxicity data. The multiple linear regression (MLR) is the most popular and more applied method of building a QNAR model followed by the support vector machine.

Recently, Toropov and Toropova (2014) worked as in their previous studies (Toporov et al., 2013; Toporova, & Toporov, 2013) also using used optimal descriptors in order to build a QNAR model to predict the mutagenicity of fullerene C_{60} nanoparticles under various conditions using data taken from the literature (Shinohara, Matsumoto, Endoh, Maru, & Nakanishi, 2009). The descriptors were calculated with the Monte Carlo method and the model of 20 fullerene C_{60} NPs was the mathematical function describing the contributions of

1. Dose (g/plate),
2. Metabolic activation, and
3. Illumination (i.e. dark or irradiation).

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PERSPECTIVES AND FUTURE OF COMPUTATIONAL NANOTOXICOLOGY

To date, many theoretical studies have been reported on the prediction of mechanical, chemical, and electronic properties of NPs, whereas there have been relatively few published studies on the computational modeling of toxicity. Although quantitative structure–activity relationship (QSAR) methods can play an important role in designing novel and potential drugs and predicting their risk to human health and the environment, the unique properties of nanomaterials and their still unknown toxicity make the implementation of these methods more complicated in comparison with the small organic chemicals. QSAR models usually cover the biological profile of small organic molecules and have been proven accurate in predicting the biological effect for a wide range of molecular scaffolds. QSAR models own a great proportion of their success in the presence of organized databases but no such databases are available for the vast majority of NPs. Experimental data on NPs are scarce and produced by different groups of scientists following different protocols and it is often difficult to select and combine the available information from different sources. On top of that, the structural characteristics of NPs cannot be encoded by the "conventional" widely used 2D and 3D molecular descriptors. NPs include organic as well as inorganic elements with sometimes unknown composition and highly complex structures that demand new approaches for developing molecular descriptors.

In this chapter, we attempted to explain the importance of QNAR modeling and we outlined some of the latest and more significant QNAR studies, especially related to toxicity. As it is demonstrated by several studies QNAR methodologies have emerged as important tools in producing reliable models for the evaluation of MNPs' biological properties and toxicity and can provide valuable information for the design of safer products in nanotechnology.

In an effort to computationally explore data on the effects of nanostructures to human health and the environment, several limitations much be addressed. As already emphasized above nanotechnology, and especially nanotoxicology, is an emerging field of science but unfortunately in this new field of science there is a significant lack of systematic experimental data that characterize structural, geometrical and physicochemical properties of large series of NPs and their effects. To assess risk, regulatory authorities need organized experimental data that are produced to assess properties and effects for a wide range of NPs. When such data are available QNAR studies can then emerge as a useful tool in the identification of the most relevant properties for modeling toxicity of NPs and produce robust and predictive models.

The availability of quantitative toxicity studies and models is strictly bonded to the availability of high-quality and well organized nanotoxicity data but unfortunately due to the paucity of such data this task is significantly restricted. However, since these limitations have been well recognized, several efforts are now ongoing to select and organize relevant experimental data that are produced under the same protocol and are organized in a common format. For example ISA TAB Nano is a standard tab-delimited format for describing data related to: Investigations, Nanomaterials, Studies (Specimens) and Assays with the goal to develop a specification to facilitate the import/export of data on nanomaterials and their characterizations to/from nanotechnology resources.

It is important to highlight that the conditions and protocols, under which researchers should work, have not been yet clearly defined. There is a variety of *in vitro* measurement techniques and biological endpoints, and each laboratory has its own criteria to test the MNPs. To overcome this barrier, it is required that researchers working in the fields of chemistry, toxicology, biology and chemoinformatics of MNPs collaboratively work together and establish a set of good practices to characterize MNPs and

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test them in appropriate biological assays under fixed criteria so that data are ready available for in silico exploitation.

As already mentioned, nanomaterials consist of various structures, such as nanotubes, nanorods, fullerenes, crystals, and chemical species of metals, organic compounds, inorganic compounds, etc. This variety could potentially be a problem with the modeling of nanoparticles since we do not know how accurately we can describe the structure of nanomaterials. It is simply impossible to combine all nanoobjects in one description. Since NPs are not structurally homogenous, the properties and the mechanism of toxicity should be studied separately, and thus every group should be analyzed separately. For this reason, sub-classes of structural and physicochemical similarities have been defined and an eight-step procedure is recommended by the Organization for Economic Co-operation and Development (OECD) guidance document on the grouping of chemicals (OECD, 2007).

It is important while developing a QNAR model in order to estimate the potential hazard of ENMs to overcome several common QSAR errors, such as the use of inappropriate tools and methods or use of limited or inappropriate validation procedure. It is important to follow all proposed criteria that can be used to critically assess the suitability of input data and modeling methods and tools for QNAR analysis. Lessons learned from the long standing 'classic' QSAR modeling have to be taken into account from the very beginning when building a QNAR model. It is very important that all five principles recommended by OECD including robust validation and interpretation of results are addressed when the modeling of NPs' biological profile is requested.

Computational methods could address several significant questions emerging from the experimental testing of NPs. An important outcome that needs to be explored is that nanoparticles interact with DNA, proteins, and membranes and establish a series of nanoparticle-biological interfaces. These interactions lead to the formation of protein coronas, particle wrapping, intracellular uptake and biocatalytic processes that could have biocompatible or bioadverse effects. Therefore, it could be a promising approach studying the forces and interactions which occur at the interface between NPs and biological materials to be developed the predictive relationships between structure and activity of nanomaterials.

Up to date, there are several efforts to enable these intensive collaborations between experimentalists, toxicologists, modelers, etc., from both academia and industry. SRC/SEMATECH Engineering Research Program (Retrieved from http://www.erc.arizona.edu/) constitutes a very good example of such collaborative efforts. SRC Engineering Research Center for Environmentally Benign Semiconductor Manufacturing is a dynamic framework with numerous research teams from different U.S. universities and industrial companies. The researchers have synergistic activities by sharing and testing samples of MNPs in different laboratories, creating datasets of them and corresponding QNAR models.

The NanoPUZZLES project (Retrieved from http://www.nanopuzzles.eu/) is another such of activity which focuses on two groups of compounds:

1. Inorganic engineered nanoparticles (metal oxides NPs), and
2. Carbon-based nanoparticles (carbon nanotubes (single-walled and multi-walled), fullerenes and fullerene derivatives).

The project aims to develop a package of computational algorithms for the comprehensive modeling of the relationships between the structure, properties, molecular interactions and toxicity of selected classes of engineered nanoparticles. The *in silico* approaches that are used to predict the harmful effects of NMs are based on mainly QSARs. In addition, NanoPUZZLES is contributing to the development

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of an electronic database of well-characterized NMs consisting of physicochemical and toxicological information.

A research project for safe nanotechnology, called NanoMILE and funded by the European Union's Seventh Framework Programme, has been launched from March 2013 (Retrieved from http://www.nanomile.eu-vri.eu/). The aim of this work is the understanding of the mechanisms of nanomaterial interactions with living systems and the environment in order to lead to the development of a single framework for the classification of NM safety and the creation of a universally applicable framework for nanosafety. The MNMs, both metal and carbon-based NMs, will be characterized physicochemically across their entire life cycle and in a wide range of target species. Using a high throughput screening process, a streamlined testing and selection platform will be developed and applied to refine the MNMs selection. The selected MNMs will undergo focused testing relative to their mechanism(s) of effects on living systems and the environment. An iterative experimental/modeling process will integrate the data obtained into quantitative structure or properties/effects relationships.

NANoREG (Retrieved from http://www.nanoreg.eu/) is an enabling project with the cooperation of 60 partners around the Europe. Its aim is to provide the guidance and tools by which a new nano-based material can be tested, evaluated, and approved for commercial use. The partners emphasized that their QSAR modeling should be clearly defined in order to be used as an *in silico* way to predict the behavior of nanaparticles in the human health and environment.

An important aspect that, in the vast majority of examples presented in literature, remains forgotten is the dissemination of the results to the wider community. It is very crucial that the developed model does not remain within the developers' group but is widely disseminated to the community so that it could immediately serve as an important source of information as it was initially designed to be. We should not omit to highlight recent attempts using open source tools for developing *in silico* models for the risk assessment of NMs and moreover the importance of the dissemination of these tools through web applications that could be easily expanded and adjusted to the special needs of each project.

In 2011, Sushko et al. (2011) created a web-based platform that aims to automate and simplify the typical steps required for QSAR modeling. The Online Chemical Modeling Environment (OCHEM) is free for web users and it is available online at http://www.ochem.eu. The OCHEM database was extended to incorporate nanoscale objects and it will be filled continuously with data on the nanotoxicity of metals and metal oxides. Since then, several changes were made to update the OCHEM to be used as a user-friendly database for the nanotoxicity data collection. For the description of the toxic properties of the nanoparticles an abbreviation was used. More specifically, for each property "Nano" prefix was used to separate the nano-properties from all others. The basic characteristics of nanoparticles are chemical composition, average particle size (APS) and shape of the nanoparticles.

Another attempt in this direction is Enalos InSilicoNano platform (Enalos). In this effort KNIME workflows were made available online through Enalos InSilicoNano platform, a web service based solely on open source and freely available software that was developed with the purpose to make the models available to interested user wishing to generate evidence on potential biological effects in the decision making framework. This web service facilitates the computer aided nanoparticles design as it can serve as a source of activity prediction for novel nano-structures. With this tool in hand external databases can be screened within a virtual screening framework to identify novel potent nanoparticles from a prioritized list of compounds. This online tool could be a useful aid for the decision making of both research groups and regulatory bodies interested in NPs' design and screening.

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CONCLUSION

It is obvious that nanotechnology has the best prospects to evolve but it should be done in the right way. The unique properties of nanomaterials have several applications in science, and significantly in medicine, but the same properties might result in new pathways and mechanisms of toxic action. For safer nanoscale products QNAR modeling can emerge as a useful tool for the understanding of the relationships between the structure and biological activities of MNPs. To enable safety-by-design using predictive QNAR models, some barriers must be addressed. Datasets on the effects of MNPs need to be enriched and extended with well-defined and consistent experimental measurements. Moreover there is a strong need to supplement the existing set of molecular descriptors with novel "nanodescriptors" that can represent size-dependent properties of nanomaterials. The descriptors have to be both theoretical/computational and experiment-based structural descriptors in order to correlate with biological endpoints. An intense collaboration between the experimentalists (chemists, biologists, toxicologists, etc) and QSAR modelers must be established, and, there is no doubt that the use of QNAR modeling will provide critical support to experimental studies aimed to identify safer nanoparticles with desired properties.

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KEY TERMS AND DEFINITIONS

Engineered Nanomaterials: Any intentionally manufactured material/particles, designed with specific properties and for specific purpose.

Nanomaterials: The new materials defined as those with one or more dimensions at the nanoscale, which can exhibit novel characteristics compared to the same materials without nanoscale features.

Nanoobjects: Materials with at least one dimension in the nanoscale (1–100 nm).

Nanoparticles: A subset of nanomaterials, with one or more dimensions at the nanoscale level. One of their dimensions has at least length below 100 nm.

Nanoscale: A scale of measurement that uses nanometers (nm) or microns (μm) as units of measure.

Nanostructured Material: Material with an internal or surface structure in the nanoscale.

Risk Assessment: The process of determining the likelihood of a threat or hazard.

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Chapter 51
QSAR-Based Studies of Nanomaterials in the Environment

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ABSTRACT

Nanotechnology is a newly emerging field, posing substantial impacts on society, economy, and the environment. In recent years, the development of nanotechnology has led to the design and large-scale production of many new materials and devices with a vast range of applications. However, along with the benefits, the use of nanomaterials raises many questions and generates concerns due to the possible health-risks and environmental impacts. This chapter provides an overview of the Quantitative Structure-Activity Relationships (QSAR) studies performed so far towards predicting nanoparticles' environmental toxicity. Recent progresses on the application of these modeling studies are additionally pointed out. Special emphasis is given to the setup of a QSAR perturbation-based model for the assessment of ecotoxic effects of nanoparticles in diverse conditions. Finally, ongoing challenges that may lead to new and exciting directions for QSAR modeling are discussed.

INTRODUCTION

The 21th century has witnessed a fast-dynamic growth of the nanotechnology field, leading in turn to the development of an innumerable number of nanomaterials with unique properties and a variety of applications in many sectors. However, the large-scale production and increasingly likelihood exposure to novel nanomaterials has spurred great concern regarding their potential adverse effects to humans and the environment. This chapter will provide an overview of *in silico* approaches developed so far for the prediction of nanomaterials environmental toxicity; from the use of classical approaches forward-

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establishing Quantitative Structure−Activity Relationships (QSAR) to the applications of more evolved computational models. Particular attention will be paid to the setup of a new *in silico* perturbation-based model aimed at reliable predicting the ecotoxicity of metal-based nanoparticles under a wide range of experimental conditions.

NANOMATERIALS AND ENVIRONMENTAL RISKS

The idea that launched the new field nanotechnology started with the famous lecture "There's plenty of room at the bottom" given by physicist Richard Feynman at the Annual Meeting of the American Physical Society in 1959 (Feynman, 1960). In that meeting, Feynman wondered: "What would the properties of materials be if we could really arrange the atoms the way we want them?", foreseeing the production of materials at the nanometer scale with promising technical, industrial and biological applications. But the term "nanotechnology" was only coined decades later, firstly by Norio Taniguchi in 1974 within his inspections of ultra-precision machining (Taniguchi, 1974), and then by K. Eric Drexler in his 1986 book *Engines of Creation: The Coming Era of Nanotechnology* (Drexler, 1992). Though the real burst of nanotechnology didn't come until the early 1990s, with the development of sophisticated techniques for the characterization and manipulation of individual atoms such as scanning probe microscopy.

Nanomaterials are usually considered to entail materials that have single units with at least one dimension between 1 nm and 100 nm (Oberdörster et al., 2007). Nanomaterials can additionally be classified as one-dimensional (1-D), two-dimensional (2-D) and three-dimensional (3-D), according to the number of dimensions that are confined to the nanoscale range (< 100 nm). 1-D materials include nanofilms, nanolayers, and nanocoatings, while 2-D include nanotubes, nanorods, and nanowires. Finally, the most common 3-D materials are nanoparticles (NPs), the building blocks for nanotechnology applications. Nanomaterials can be amorphous or crystalline, be composed of single or multi-chemical elements, and exhibit various shapes and forms.

The advances in nanotechnology have made possible the development of many new materials and devices with a vast range of applications in medicine, cosmetics, electronics, biomaterials and energy production. Indeed due to the rapid pace at which nanotechnology is evolving and expanding into society via its applications, it is expected that many new innovative products containing manufactured nanomaterials will continue to vastly grow. Commonly, manufactured nanomaterials fall generally into two categories: carbon-based and metal-based materials (Bréchignac et al., 2007). Regarding carbon-based nanomaterials, these usually involve diamond, carbon-nanotubes, graphene and fullerenes, the latter being a class of allotropes of carbon that conceptually are graphene sheets rolled into tubes or spheres. Most of these nanomaterials are of interest for their mechanical strength and electrical properties (De Volder et al., 2013). Moreover, fullerenes in general are under study for potential medical applications, ranging from specific binding of antibiotics on resistant bacteria to even target certain types of cancer cells such as melanoma (Bakry et al., 2007; Simon et al., 2007). Carbon nanotubes for instance, have already been used as drug, gene, or protein carriers (Bianco et al., 2005). Nanoscale materials are being further developed in therapeutic modalities for advanced selective drug delivery systems and targeted nanotherapy (Ravi Kumar, 2000). Many of these modalities include polymeric NPs (Stupp et al., 2005), micelles (Kim et al., 2005), liposomes and dendrimers (Popat et al., 2007; Portney & Ozkan, 2006), hydrogels (Vinogradov et al., 2002), nanoshells (Zahr et al., 2005), and smart surfaces. The key performance characteristics of these materials are the high loading capacity, release kinetics, circulation time,

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bio-distribution, size distribution, and stability (Allen et al., 1999). Furthermore, a recently published review shows that different carbon-based nanomaterials, including nanodiamonds, fullerenes, carbon nanotubes, graphene, carbon nanofibers, carbon nanocones/disks and nanohorns, as well as their functionalized forms, can be used in sample preparation processes in analytical chemistry (Zhang et al., 2013). With regard to metal-based nanomaterials, these include metal and metal oxide NPs based on titanium, zinc, silver, copper, aluminium or cerium among others. For instance, oxides TiO_2 and ZnO have been used in cosmetics, sunscreens, solar-driven self-cleaning coatings, and textiles for enhanced product transparency, as well as in paintings, water treatment and food (Cai et al., 2006; Serpone et al., 2007; Yuranova et al., 2007). As to nanosized silver, it is used today in an increasing number of consumer and medical products. Typical examples include band aids, cosmetics, food complements, anti-odour textiles, dental materials, bone implants, water disinfectants, as well as antibacterials, antivirals, etc. agents (Rai et al., 2014; Wijnhoven et al., 2009). On the other hand, CuO nanocrystals have been found that could replace noble metals in newer catalytic converters for the car industry (Zhou et al., 2006). Further, metal salts including CdSe quantum dots and iron (Fe) NPs have been exploited in many practical applications related to electronics and biomedical imaging techniques (Alivisatos, 2004; Chen & Gerion, 2004). Other specialized industrial applications of nanomaterials involve for instance aluminium (Al) NPs as fuel additives and MnO as a catalyst or in battery technology (Schrand et al., 2012).

Manufactured nanoparticles (MNPs) are receiving a great deal of attention lately both from the basic sciences and industry, as well as from the public at large, since existing and potential applications are almost endless. But along with the benefits, the use of MNPs raises many questions and concerns due to the lack of knowledge regarding their health and environmental impacts (Borm et al., 2006; Olson & Gurian, 2012; Schrand et al., 2012; Walker & Bucher, 2009; Warheit et al., 2008). In fact, uncertainties regarding those are high since the properties exhibited by NPs are often completely different from those of bulk forms, affecting their physicochemical and biological behavior, which may well result in greater toxicity. At the same time, the use, production and disposal of nanoscale materials raises concerns about nano-pollution, taking into account that NPs can be released in the air or water during production or as waste by-product of production, and ultimately accumulate in the soil, water or plant life, endangering the health of living organisms and ecosystems. There is thus an increasingly need to address the overall environmental impact of released NPs to support the development of a sustainable and safe nanotechnology.

IN SILICO STUDIES OF NANOMATERIALS IN THE ENVIRONMENT

As can be judged, while prompt development of nanomaterials will certainly continue, the application of these materials might be limited by their potential health-risks and environmental impacts (Bar-Ilan et al., 2009; García et al., 2011; Gong et al., 2011; Griffitt et al., 2008; Heinlaan et al., 2008; Hund-Rinke & Simon, 2006; Kasemets et al., 2009; Lin & Xing, 2007; Ma et al., 2011; Ma et al., 2010; Marsalek et al., 2012; Sadiq et al., 2011; Zhu et al., 2010; Zhu et al., 2012; Zhu et al., 2009; Zhu et al., 2008). And indeed, there have been already several reports on the deleterious effects of nanomaterials to human health and ecosystems (Arora et al., 2012; Bussy, C. et al., 2013; Liu et al., 2013; Schrand et al., 2012; Tsoy et al., 2013). For example, carbon nanotubes have been shown to produce oxidative stress and being just as toxic as asbestos (Liu et al., 2013; Schrand et al., 2012).

Similarly to any other manufactured chemicals, toxicity evaluation studies of nanomaterials have been carried out on a few cell lines or species that have been accepted by regulatory agencies as models for

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defining toxicological effects. As a rule, an initial screen of toxicity is accomplished by resorting to *in vitro* methodologies, even though the bridge between *in vitro* and *in vivo* remains. Nevertheless, *in vivo* mimicry of higher order organisms is difficult due to the complex nature of biological interactions, and often insurmountable differences between laboratory animals and humans do exist (Eisenbrand et al., 2002; Oberdorster, 2010; Schrand et al., 2012; Shvedova & Kagan, 2010). Alternatively, since *in vivo* experiments are expensive, slow and ethically questionable, low-cost *in vitro* assays without reducing the efficiency and reliability of the risk assessment are usually carried out. One should notice here that some test models and standard testing protocols applied for non-nanoform substances may not necessarily be appropriate or optimal for the testing of MNPs, but nevertheless ongoing efforts in the research community are currently addressing such issues (Holden et al., 2013; Liu et al., 2013; Nel et al., 2013; Schrand et al., 2012).

To date, many important aspects regarding the ecotoxicity profiles of NPs are not well understood, and sometimes experimental assays carried out by different groups on the same NPs have showed a remarkable lack of reproducibility and consistency (Lanone et al., 2009). Powerful experimental techniques focused on high-throughput/content screening (HTS/HCS) could be essential for the fast and efficient evaluation of many toxic effects of NPs (Holden et al., 2013). Nevertheless, even with the use of HTS/HCS, there is a very low probability of covering and filtering the huge "nanotoxicity space". This comes from the fact that there are many combinations of factors such as chemical compositions, sizes and conditions under which the sizes were determined, shapes, coating agents, measures of the toxic effects, living systems (cell lines, crustaceans, algae, bacteria, fungi, plants, fish, etc.), and times at which living systems were exposed to NPs. Computational methods may offer a magnification of the 'sweet spot' regarding the virtual screening of NPs for prediction of toxicity profiles, helping to rationalize the experimental assays by diminishing time and financial resources. Among these methods, Quantitative Structure–Activity Relationships (QSAR) models have been used as promising tools for modeling different biological effects of chemicals (Estrada et al., 2004; Estrada et al., 2006; Estrada et al., 2000; Gonzalez-Diaz et al., 2014; Luan et al., 2013; Prado-Prado et al., 2011; Speck-Planche & Cordeiro, 2014; Speck-Planche et al., 2013a; Speck-Planche et al., 2013b; Tenorio-Borroto et al., 2014), including diverse toxicity profiles (Cruz-Monteagudo et al., 2013; Cruz-Monteagudo et al., 2008; Cruz-Monteagudo et al., 2006; Estrada et al., 2001; Estrada & Uriarte, 2001; Estrada et al., 2003; Helguera et al., 2007; Morales Helguera et al., 2008; Speck-Planche et al., 2012; Tenorio-Borroto et al., 2013). In the field of nanotechnology, QSAR models have provided deeper insights regarding the most relevant aspects of NPs (Poater et al., 2009). Nevertheless, only a few studies have been carried out thus far aiming at the *in silico* prediction of nanotoxicity by resorting to QSAR models (Fourches et al., 2010; Kar et al., 2014a; Kar et al., 2014b; Liu et al., 2011; Liu et al., 2013; Puzyn et al., 2011; Toropov et al., 2012). From now on, this chapter will be devoted to summarize the main progresses in the computational risk assessment of nanomaterials. Towards that goal, prediction of both cytotoxicity (against mammalian cell lines) and ecotoxicity (involving bio-indicator species such as bacteria, algae, fungi, crustaceans, fish, and plants) will be discussed because of their potential direct or indirect implications in environmental risk assessment.

Classical QSAR Models for Prediction of Toxic Effects of Nanoparticles

In general terms, QSAR approaches aim to establish mathematical models disclosing possible parallelisms/relationships between a set of predictor variables X, known as molecular descriptors, and the

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response variable *Y*, which describes a certain type of biological effect (Hansch & Leo, 1995). From one side, molecular descriptors can be viewed as the final result of a defined mathematical procedure that transforms chemical information encoded within a symbolic representation of a molecule into a useful number or the result of some standardized experiment (Todeschini & Consonni, 2000). On the other hand, the biological effect can be associated with the inhibitory activity of a chemical against different types of targets (biomacromolecules, microorganisms, cell lines, etc), the toxicological profile, or any property related with the absorption, distribution, metabolism, and elimination (ADME). QSAR models are obtained by means of statistical methods (van de Waterbeemd, 1995), which include regression techniques such as (but not limited to) multiple linear regression (MLR) and partial least squares (PLS), or classification techniques such as (but not limited to) linear discriminant analysis (LDA). In addition, powerful machine learning methods like artificial neural networks (ANN), support vector machines (SVM), random forest (RF), consensus clustering (CC) and many others can be employed (Hill & Lewicki, 2006).

Classical QSAR models are based on assumptions, which describe the structural dependence of biological activities (or any other biological effect) either by local physicochemical descriptors such as hydrophobicity, molar refractivity, and electronic factors (Hansch analysis), or by indicator variables (discrete descriptors) encoding different structural features (Free-Wilson analysis) (Kubinyi, 1993). Current classical QSAR models maintain the same principles in the way that small datasets of structurally related chemicals are used. However, other groups of local and global molecular descriptors such as those derived from quantum-mechanics calculations or exhibiting graph-theoretical nature have been widely employed (Todeschini & Consonni, 2009).

Several works focusing on predicting nanotoxicity have been reported in the scientific literature, in which the fundamental principles of QSAR studies have been adapted to the computational assessment of different biological effects of NPs. The first of these works was inspired by the idea that the evaluation of biological effects of MNPs is extremely important for nanotechnology sustainability, and there is thus an urgent need for efficient *in silico* approaches able of predicting biological effects of MNPs. Therefore, the authors developed statistical significant QSAR models by establishing relationships between the toxicities of nanomaterials and their corresponding physical, chemical, and geometrical properties, either measured experimentally or computed from the structure of MNPs (Fourches et al., 2010). Towards that end, two representative sets of MNPs involving *in vitro* cell-based assays have been employed. The first set was formed by 51 various MNPs with diverse metal cores (Shaw et al., 2008), and 44 of them were used to derive the nano-QSAR models. Each NP was tested against 4 cell lines, employing 4 doses, and through 4 different assays (64 responses of biological activity). The authors condensed the entire biological effects (cytotoxicities) of each NP into one average response. At the same time, experimental properties such as size, relaxivity, and zeta potential were used as descriptors for the MNPs. Afterwards the SVM technique was used to develop the QSAR models. The whole dataset was split 5 different times, creating 5 different training (80%) and prediction (20%) sets. The best model yielded an external accuracy of 73%. In the case of the second dataset, 109 MNPs with the same core (cross-linked iron oxide with amine groups) but decorated with different organic molecules (Weissleder et al., 2005) was studied. The uptake of the NPs in PaCa2 cells was used as biological response, while 150 different computational molecular descriptors were used to characterize the chemical structures of the different molecules in the surface of the MNPs (coating agents). Then, QSAR models based on the *k*-nearest neighbors (*k*NN) regression technique were setup, and the best model displayed a reasonable external prediction power ($R^2 = 0.72$). The results of this work clearly demonstrate that nano-QSAR models can be used for two

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main purposes: prediction of the biological activity profiles of novel nanomaterials, and prioritization in the design and manufacturing of nanomaterials towards better and safer products.

As a continuation of the work involving the last dataset mentioned above, *i.e.*, that formed by 109 magnetofluorescent NPs, Roy and coworkers built up a new nano-QSAR model using the MLR technique (Kar et al., 2014a). For deriving the model, a pool of 307 descriptors was calculated using different computer programs. The best model contained 6 molecular descriptors, and was rigorously validated internally and externally according to the guidelines established by the Organization for Economic Cooperation and Development (OECD). Accordingly, the values of R^2 and Q^2_{LOO} for the training set (89 cases) were 0.806 and 0.758 respectively, while for the external set (20 cases) R^2_{pred} had a value of 0.879 with a $R^2_{m(test)Scaled} = 0.793$. In this work, the applicability domain was also determined in an attempt to confirm the reliability of the predictions (Dimitrov et al., 2005; Dragos et al., 2009; Fechner et al., 2010; Kulkarni & Zhu, 2008; Netzeva et al., 2005; Weaver & Gleeson, 2008). Additionally, molecular fragments present in the different coating agents were analyzed, and their contributions were calculated. The main purpose underpinning the calculation of fragments contributions was to study the most important molecular features associated with the highest (or the lowest) values of cellular uptake of NPs. One should emphasize here that even when the calculation of quantitative contributions of fragments to different biological effects have been widely reported by different authors (Estrada et al., 1998; Estrada & Peña, 2000; Estrada et al., 2000; Helguera et al., 2005; Helguera et al., 2007; Prado-Prado et al., 2010; Speck-Planche & Cordeiro, 2013), this work introduces for the first time essential and rich information *via* the attained fragment contributions, expanding the horizons for risk assessment in the context of virtual screening of relevant NP libraries.

Another nano-QSAR model has also been developed by employing periodic table-based descriptors to encode cytotoxicity profile of metal oxide NPs (Kar et al., 2014b). In such work, metal electronegativity (χ), the charge of the metal cation corresponding to a given oxide (χ_{ox}), the atomic number, and the valence electron number of the metal were used as simple descriptors to setup QSAR models aiming at predicting the cytotoxicity of metal oxide NPs against the bacterium *Escherichia coli*. As the measure of cytotoxicity, the effective concentration at 50% (EC_{50}) was employed. Then, MLR and PLS were used as statistical regression methods to search for the best QSAR models, which could account for at least 82% of the variance of the cytotoxicity in the training set, with $Q^2_{LOO} \geq 0.75$. In the prediction set, an average R^2_{pred} for the ten derived models (due to random splits) was 0.78 (or higher). Many other statistical indices were calculated, corroborating the quality and predictive power of the nano-QSAR models. A prominent aspect of the latter research work is that the descriptors used to characterize the metal oxide NPs can be obtained easily and quickly from the molecular formula (composition) and the information retrieved from the periodic table. On the other hand, the authors demonstrated that a simple but useful descriptor such as χ_{ox} can efficiently encode the cytotoxicity of metal oxide NPs, leading to models with clear interpretability. Lastly, the authors also hypothesized the possible mechanisms of action of the NPs. Finally, this work served to uncover the fact that the bacterium *Escherichia coli* can be used as a useful target for the study, analysis and prediction of cytotoxicity of NPs. Indeed, two more works have been published previously, and they were focused on modeling the same phenomenon: cytotoxicity of metal oxide NPs against *Escherichia coli* (Puzyn et al., 2011; Toropov et al., 2012). The first of these works was based on the calculation of descriptors derived from quantum-mechanics calculations (Puzyn et al., 2011), while the second one used SMILES-based optimal descriptors, which were calculated from the CORAL software (Toropov et al., 2012). In both cases, the MLR technique was used to derive QSAR models with excellent performance when compared with the research work referred to above.

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Another interesting report focused on the computational assessment of cytotoxicity of NPs by means of nano-QSAR models based on statistical classification methods (Liu et al., 2011). Towards that goal, the authors built up a model to predict the cytotoxicity of 9 metal oxide NPs against bronchial epithelial cells (BEAS-2B), which were exposed to different concentrations of the NPs (0.375-200 mg/L) during a period of time of 24 hrs. The study was realized by using cytotoxicity data from a high-throughput screening assay that was processed to identify and label toxic (through the propidium iodide uptake of BEAS-2B cells) versus endpoints relative to an unexposed control cell population. A total of 14 different physicochemical descriptors were used as input variables. The best nano-QSAR classification model based on logistic regression contained the following descriptors: atomization energy of the metal oxide, period of the metal in the NP, and NP primary size, in addition to NP volume fraction (in solution). This model had 100% classification accuracy in both internal and external validations. Thus, one can undoubtedly infer that nano-QSAR models based on classification tasks can be very useful for risk assessment of NPs. Furthermore, when classification models are used, there is no need to predict the exact value of biological effect of NPs. Consequently, the uncertainty of data can be better controlled.

Finally, a research work dealing with another nano-QSAR classification model has been reported in the last year (Liu et al., 2013). The generation of such a model was inspired by the idea regarding the integration of multi-parametric toxicity/bioactivity assays to arrive at statistically meaningful class definitions, that is, bioactivity/inactivity endpoints. Another purpose of that work was to determine and analyze the decision boundaries and the applicability domain. Towards that end, by applying diverse statistical and machine learning methods, different nano-QSAR models were developed from a set of 44 iron oxide core NPs used in molecular imaging and nano-sensing. These NPs contained bioactivity profiles for four cell types and four different assays. Class assignments were pursued by means of bioactivity identification analysis and self-organizing map based consensus clustering, which permitted the setup of nano-QSAR classification models with acceptable performance (classification accuracy >78%). This is, a naive Bayesian classifier (NBC) containing spin-lattice relaxivity and zeta potential as descriptors was employed. The authors successfully established an applicability domain by using the concept of probability density, and decision boundaries were determined for the above NBC by considering different acceptance levels of false negative to false positive predictions.

Despite the fact that the nano-QSAR models discussed above convey great advances for the regulatory risk assessment of diverse biological effects of NPs, several major drawbacks can be noticed. From one side, some of these models use only experimental descriptors, and taking into account that measurements overall embody experimental errors, this may introduce a great uncertainty when the QSAR models are built up (Fourches et al., 2010). On the other hand, whole QSAR models up to now have been based on limited datasets of NPs, in which toxicity tests have been accomplished by considering only one living system (bio-indicator species, cell line, etc.) and only one reported measure of toxic effects. Additionally, such QSAR models did not provide information regarding important factors such as the shape and medium under which the sizes of the NPs have been measured, as well as other experimental conditions such as the time during which the living systems have been exposed to the NPs. Finally, we would like to emphasize that the previously developed QSAR models in nanotechnology have generally focused on the prediction of cytotoxicity, and thus, no model has been reported so far with the aim of performing virtual screenings of ecotoxic effects of NPs. Therefore, the setup of a QSAR model able to predict the ecotoxicity of NPs by considering different experimental conditions would be of great importance in nanotoxicology, an in general terms, for nanotechnology. Bearing in mind all these ideas, and the fact that many NPs are released into the environment in their bare forms due to processes such as manufacture,

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manipulation and use, we dedicate the next section of this chapter to the analysis of QSAR-perturbation models which are focused on the simultaneous prediction of dissimilar ecotoxic effects of uncoated NPs under multiple experimental conditions.

Perturbation Models: A New Paradigm in QSAR Studies

Recently, it has been reported the possibility to apply perturbation theory to QSAR models (Gonzalez-Diaz et al., 2013). In this context, several QSAR-perturbation models have been developed and thoroughly validated, forward-allowing the prediction of different biological effects of chemicals species in diverse fields such as organic synthesis, computational pharmacokinetic modeling, and the study of process of self-aggregation of drugs into micellar NPs. Taking into consideration the importance of QSAR-perturbation models, we will summarize their main aspects.

These innovative models allow the integration of different kinds of chemical and biological data. One of the principal aspects of QSAR-perturbation models is the use of ontologies, which can be viewed as experimental conditions, *i.e.*, the results of the combinations of different elements or factors. For instance, the ontology c_j can be expressed in the form c_j => (b_t, p_s, d_m, t_e), where b_t characterizes the different biological targets (assay organism, cell line, etc.), p_s describes the diverse shapes (morphologies) of the NPs, d_m represents the conditions under which the sizes of the NPs have been measured (in aqueous solution, culture medium, or for the dry powder), and t_e refers to the different periods of time during which the biological targets have been exposed to the NPs.

The main purpose of the QSAR-perturbation models is to describe two principal aspects. From one side, the model should be sensitive to the variation in both the chemical composition of the NPs and the different sets of experimental conditions. But if the descriptors originally calculated (or experimentally determined) for NPs are used, they will not be able to differentiate the toxic effect of the NPs whenever the experimental condition c_j is modified. In an attempt to accomplish this task, the moving average approach (MAA) can be applied (Hill & Lewicki, 2006), and new descriptors can be generated as follows:

$$DX_i(c_j) = X_i - X_i(c_j)_{avg} \tag{1}$$

In the previous equation, X_i is a defined descriptor or physicochemical property, and $X_i(c_j)_{avg}$ is calculated as the average of all the X_i values, which were tested under the same experimental condition c_j. Equation 1 should be applied to each element of the ontology c_j. In any case, only descriptors of type $DX_i(c_j)$ are employed because they take into account both the chemical composition (or any other property) and the experimental condition c_j under which cases/NPs have been tested.

The second aspect focuses on overcoming the limitations of current classical QSAR models. The main hypothesis assumed by such models is that modifications in the structure and/or composition of the chemicals will produce changes in the activities/toxicities with respect to a hypothetical reference chemical (intercept). This assumption is simple but very weak, due to the fact that, in principle, even more than one chemical could be used as reference at the same time. To overcome such assumption, let us first consider a set of NPs along with their respective toxicities [$TE_i(c_j)$] and descriptors $DX_i(c_j)$. If we generate pairs of NPs, then we can consider the following equation:

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$$TE_i(c_j)_{nw} = a_0 \cdot TE_i(c_j)_{rf} + \sum b_j \cdot \left[DX_i(c_j)_{nw} - DX_i(c_j)_{rf} \right] + d_0 \quad (2)$$

where for each pairs of NPs, $TE_i(c_j)_{nw}$ is the toxic effect a defined NP in the new state (experimental condition to be predicted), while $TE_i(c_j)_{rf}$ is the toxic effect of the NP of the same pair selected as reference. The term in Equation 2 involving the descriptors of type $DX_i(c_j)$ can be viewed as the perturbation term [$DDX_i(c_j)$], which expresses the differences between the two NPs in terms of chemical compositions (physicochemical properties), sizes, shapes, biological targets, conditions under which the sizes have been measured, and times during which the biological targets have been exposed to the NPs. Also, the a_0, b_j, and d_0 coefficients can be determined by resorting to regression or classification statistical techniques. Notice that regression will be used if $TE_i(c_j)_{nw}$ and $TE_i(c_j)_{rf}$ are the exact values of toxic effects of the NPs. If these two variables just mentioned are instead categorical variables (defining classes according to certain cutoff values), then, classification methods are to be used.

The application of perturbation theory to nano-QSAR problems is very recent. For this reason, only two works have been reported up to date. The first of these works was devoted to the prediction of the cytotoxicity of NPs under different experimental conditions (Luan et al., 2014). Therein, the authors developed a QSAR-perturbation model aiming at simultaneously predicting the cytotoxicity of 41 different NPs by considering assays against at least 1 out of 15 mammalian cell lines and within 1 out of 5 exposure times. From the physicochemical point of view, the NPs exhibited at least 1 out of 5 possible shape labels, and their sizes were measured in at least 1 out of 4 conditions. The measure of biological effect was TC_{50}, which is the concentration of NPs leading to 50% reduction in cell viability. The NPs were assigned to 1 out of 2 possible classes named "non-cytotoxic" and "cytotoxic". Thus, the NPs were only considered as non-cytotoxic for $TC_{50} \geq 503.572$ μM, otherwise, they were considered as cytotoxic. The descriptors used were the following: molar volume, electronegativity, polarizability, and NP size. These were then transformed to descriptors of the type $DX_i(c_j)$ (Equation 1). Afterwards, by applying the perturbation approach (Equation 2), the authors generated a database of 1681 cases (pairs of NPs), which was randomly split into training (1261 cases) and prediction (420) sets. Then after, LDA was used as the statistical technique to setup the QSAR-perturbation model. The accuracy of the model was higher than 93% for both training and prediction sets. The practical applicability of the model was demonstrated by predicting the cytotoxicity of different NPs such as those formed by silica (SiO_2), nickel (Ni), and nickel(II) oxide (NiO). Each NP was predicted 41 times, and the results of the predictions converged with the reports of the literature. This work represents a great advance in the computational risk assessment of NPs, because, to the best of our knowledge, the present research is the first attempt to simultaneously predict the cytotoxicity of NPs under multiple experimental conditions by applying a single unified QSAR model.

The second work was based on a very similar QSAR-perturbation methodology, but with a different purpose. In this sense, the objective therein was to predict the ecotoxicity of NPs under dissimilar sets of experimental conditions (Kleandrova et al., 2014). Thus, 85 initial NPs were retrieved from the literature, and these have been tested by at least 1 out of 4 measures of ecotoxicity, against at least 1 out of 20 assay organisms (bio-indicators), and within 1 out of 9 intervals of time of exposure. The different NPs displayed at least 1 out of 7 labels related to their shapes, and their sizes were measured in at least 1 out of 5 conditions. The same physicochemical properties employed on the mentioned previous work

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were used. The QSAR-perturbation model was derived from a database containing 5520 cases, and its accuracy was *ca*. 99% in both training and prediction sets. Three different nickel-based (Ni) NPs with experimental values reported in the literature were predicted 85 times. As in the case of the first work referred to above, the predictions were also found to be in very good agreement with the experimental evidences, confirming that Ni NPs are not ecotoxic when compared with other NPs. The results of this study thus provided a valuable tool towards an efficient multi-tasking prediction of one of the most important aspects of risk assessment of NPs: *ecotoxicity*. Until now, this work constitutes the only report that really deals with the ecotoxicological profiles of nanomaterials.

The two QSAR-perturbation models reported here have a common detail that is of outstanding importance, since another major limitation of classical QSAR models is overcome. This is related with the fact that in classical models, each endpoint-case to be classified – if the dataset comprises only a single prediction to be made, as well as any future decision to classify a chemical species (external prediction) are based on the reliability of the predictions according to certain criteria belonging to the applicability domain (Dimitrov et al., 2005; Dragos et al., 2009; Fechner et al., 2010; Kulkarni & Zhu, 2008; Netzeva et al., 2005; Weaver & Gleeson, 2008). In the specific case of the QSAR-perturbation models, random pairs of NPs were generated, in which each NP was used many times in both conditions, that is, as one NP in the initial state (reference NP) and as one NP in the new (output or final) state. Therefore, each NP used as reference participated in many predictions of other NPs, and simultaneously, the very same NP was predicted many times using the others. This procedure breaks the boundaries of the classical QSAR models, allowing one to obtain more precise and realistic results. On the other hand, the decision to classify NPs using the QSAR-perturbation models is based on consensus predictions. For this reason, the new (external) NPs were predicted 41 times in the first work referred, and 85 times in the second one, since those are the number of NPs which were originally used to setup the QSAR-perturbation models. Thus, any NP is correctly predicted/classified according to its experimental value of toxicity (compared with the cutoff values) when the consensus percentage is higher than 50%. For a percentage lower than 50% the prediction is considered to fail, while a percentage equal to 50% is considered to be a random prediction. Notice that the consensus percentage is the ratio of the number of times that a NP was correctly classified with respect the total number of possible predictions (*i.e.*, the number of NPs originally used in the dataset).

A QSAR PERTURBATION-BASED MODEL FORWARD-TARGETING ECOTOXITICY OF METAL-BASED NANOPARTICLES

Here, one should emphasize that often no strong linear relationships between the descriptors of the chemical species and their biological activities/toxicities are found. One of the principal motives is that sometimes the process or phenomenon understudy is very complex. In such cases, machine learning methods like ANN can be used because they have become useful tools for virtual screening (Prado-Prado et al., 2010; Speck-Planche & Kleandrova, 2012; Tenorio-Borroto et al., 2012). Indeed, even when simple statistical methods yield good results, ANN can be employed in order to coverage more chemical space in terms of predictive capability. As a contribution for the advancement of our understanding on ecotoxicological profiles of NPs, we present here for the first time, a QSAR-perturbation model based on ANN aimed at predicting the ecotoxicity of metal-based NPs by taking into account the following

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varying factors: chemical composition, size, conditions under which the size is measured, shape, type of measures of the ecotoxicity, biological targets (bio-indicators), and time during which the biological targets are exposed to the NPs.

Dataset, Descriptors, and Set-Up of the QSAR-Perturbation Model

The dataset employed for setting the current QSAR model was the same as that discussed in the second report focused on establishing a QSAR-perturbation model (Kleandrova et al., 2014). So we will limit ourselves here to a brief outline highlighting only the most important aspects. Each of these 85 NPs were assayed by considering at least 1 out of 4 measures of ecotoxicity (m_e), against at least 1 out of 20 biological targets/bio-indicator species (b_t), where NPs exhibited at least 1 out of 7 types of shape labels (p_s). At the same time, the NP size was measured in at least 1 out of 5 conditions (d_m), and at least 1 out of 9 different times (t_e) during which biological targets were exposed to NPs in the ecotoxicity tests.

In the dataset, all the 85 cases/ NPs were associated to two possible categories related with its ecotoxicity in a specific experimental condition c_j. Each of the NPs was assigned to the category "non-ecotoxic" [$ETOX_i(c_j) = +1$] when it had a large value of the measure of ecotoxicity, otherwise, the NP was assigned to the category "ecotoxic" [$ETOX_i(c_j) = -1$]. We need to point out here that $ETOX_i(c_j)$ is a binary (categorical) variable which is employed to classify non-ecotoxic or ecotoxic NPs, according to certain cutoff values of ecotoxicity (Table 1). Another important element is the use of the experimental condition c_j, embodying the ontology with the form c_j => (m_e, b_t, p_s, d_m, t_e). That is, the combination of these five elements describes a specific experimental condition. Each NP was characterized by four descriptors. The first three were the molar volume (V), the electronegativity (E), and the polarizability (P), thus based on the chemical composition of the different NPs. These physicochemical descriptors were retrieved from the public source Chemicool Periodic Table (Hsu, 2013). In the case of the NPs composed by more than one element, the descriptors mentioned above were normalized; that is, the descriptors for such kind of NPs were computed as the sum of the descriptors of all the atoms forming the NPs, and the result of the sum was divided by total number of atoms. The fourth descriptor was the size L (expressed in nm) of each NP, which has been experimentally measured. All chemical and ecotoxicological data were deposited in an excel file, shown in Supporting Information (file **SM1**).

As explained before, Equation 1 was applied to calculate the descriptors of the type $DX_i(c_j)$, and $X_i(c_j)_{avg}$ for the entire set n_j was calculated as the average of all the X_i values for only those cases/NPs that were considered as non-ecotoxic cases (subset of n_j) in the same element of c_j. Then after, the 85 NPs

Table 1. Cutoff values used for the different measures of ecotoxicity

Measure of Ecotoxic Effect	Cutoff[c]	Meaning
EC_{50} (μM)[a]	≥ 168.45	Effective concentration of the nanoparticle which inhibits at 50% the growth of the assay organism.
IC_{50} (μM)[a]	≥ 177.62	Concentration of the nanoparticle which inhibits the root elongation of the assay organism (plants) at 50%.
TC_{50} (μM)[b]	≥ 225.81	Concentration which causes toxic effects in 50% of the assay organisms.
LC_{50} (μM)[b]	≥ 334.08	Lethal concentration which causes mortality in 50% of the assay organisms.

[a] Based on tests with bacteria, algae, and fungi; [b] Based on tests with crustaceans and fish; [c] Condition under which a nanoparticle was assigned to the category of non-ecotoxic.

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were used to setup 5939 random pairs of NPs. Notice now that Equation 2 expresses the perturbation approach when there is a linear dependence between the toxic effect of the NP in the new state and the toxic effect of the NP in the reference state together with the perturbation terms. A more general expression for any type of dependence would be:

$$ETOX_i(c_j)_{nw} = f[ETOX_i(c_j)_{rf}, DDX_i(c_j)] \quad (3)$$

In Equation 3, the ecotoxicity $ETOX_i(c_j)_{nw}$ of any NP in the new/final state can be obtained from the corresponding ecotoxicity $ETOX_i(c_j)_{rf}$ of another NP selected as reference, and the perturbations terms $DDX_i(c_j)$. In addition, *f* is a very complex function used to model the dependence of $ETOX_i(c_j)_{nw}$ with respect to $ETOX_i(c_j)_{rf}$ and $DDX_i(c_j)$. Notice that both $ETOX_i(c_j)_{nw}$ and $ETOX_i(c_j)_{rf}$ are binary variables, and the terms $DDX_i(c_j)$ are calculated according to the following formula:

$$DDX_i(c_j) = DX_i(c_j)_{nw} - DX_i(c_j)_{rf} \quad (4)$$

Thus, $DDX_i(c_j)$ describes the differences between any two NPs (new and references states) in terms of their variations in chemical composition, size, shape, conditions under which the sizes are measured, biological target that is tested, measures of ecotoxicity, and times of exposure of the biological targets to the NPs.

The dataset containing the 5939 cases (pairs of NPs) was randomly split into training and prediction sets. The training set was used to seek for the best QSAR-perturbation model and being formed by 4439 cases, in which 2081 cases correspond to NPs in the new state considered as non-ecotoxic and 2322 cases assigned ecotoxic. As to the prediction set, it contained 1500 cases with 715 cases considered as non-ecotoxic and 785 ecotoxic, and it was used to validate the model. The function *f* of high complexity appearing in Equation 3 was modeled by using different types of ANN. The program employed to perform this task was STATISTICA 6.0 (Statsoft-Team, 2001). In order to select the most appropriate descriptors to build up the QSAR-perturbation model, we performed a sensitivity analysis. In this sense, the neural network module of STATISTICA defined a missing value substitution procedure, which is used to allow predictions to be made in the absence of values for one or more input variables (Statsoft-Team, 2001). Thus, to define the sensitivity of a particular input variable (descriptor) *v*, in a first step, each ANN is run on a defined set of cases (training cases), where a network error is accumulated (Hill & Lewicki, 2006). Then, in a second step, the network is run again using the same cases, but this time replacing the observed values of *v* with the value estimated by the missing value procedure. So, a new network error is accumulated. Taking into account that some information that each network uses, has effectively been removed (*i.e.* one of the input variables), it is logical to expect some error' deterioration to occur (Hill & Lewicki, 2006). Then, the sensitivity of any input variable is calculated as the ratio of the error with missing value substitution to the original error. The more sensitive the network is to a particular input variable (descriptor), the greater the deterioration one can expect, and therefore the greater the ratio. The larger the value of sensitivity, the greater the importance/influence a descriptor has in the model. For this reason, only variables with the largest values of sensitivity were selected. Also, we attempted to avoid collinearity between the descriptors. The statistical quality of the QSAR-perturbation model was assessed by examining some statistical indices such as the percentages of correct classifications for both

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non-ecotoxic and ecotoxic cases, the overall correct classification in the whole dataset, the Matthews correlation coefficient (MCC) (González-Díaz et al., 2007), and the areas under the receiver operating characteristic (ROC) curves for the training and prediction sets (Hanczar et al., 2010).

In order to search for the best QSAR-perturbation model, we tested different kinds of architectures for the ANN, namely: linear neural networks (LNN), probabilistic neural networks (PNN), radial basis function (RBF), and multi-layer perceptron (MLP). The ANN with the highest quality identified had the profile MLP 8:8-12-1:1. The symbols and meanings of the descriptors of the final QSAR-perturbation model are shown in Table 2.

In the training set, our model correctly classified 2036 of 2081 (97.84%) cases in the category of non-ecotoxic, and 2322 of 2358 (98.47%) cases in the ecotoxic category. At the same time, the results in the prediction set showed that the model rightly classified 97.20% (695 of 715) of cases assigned as non-ecotoxic, and 97.45% (765 of 785) of cases considered as ecotoxic. The accuracies displayed by this QSAR-perturbation model were 98.18% and 97.33% for the training and prediction sets, respectively. The results of the classifications can be found in the Supporting Information (**SM1** file). On the other hand, we calculated the MCC values since these measure the strength in the correlations between the observed and predicted values of categorical variables. Thus, MCC values of 0.963 and 0.947 were found for the training and prediction sets, respectively. Accordingly, in both cases there was a very strong linear correlation between the observed and predicted values of the categorical variable $ETOX_i(c_j)_{nw}$, confirming the quality and predictive power of our model.

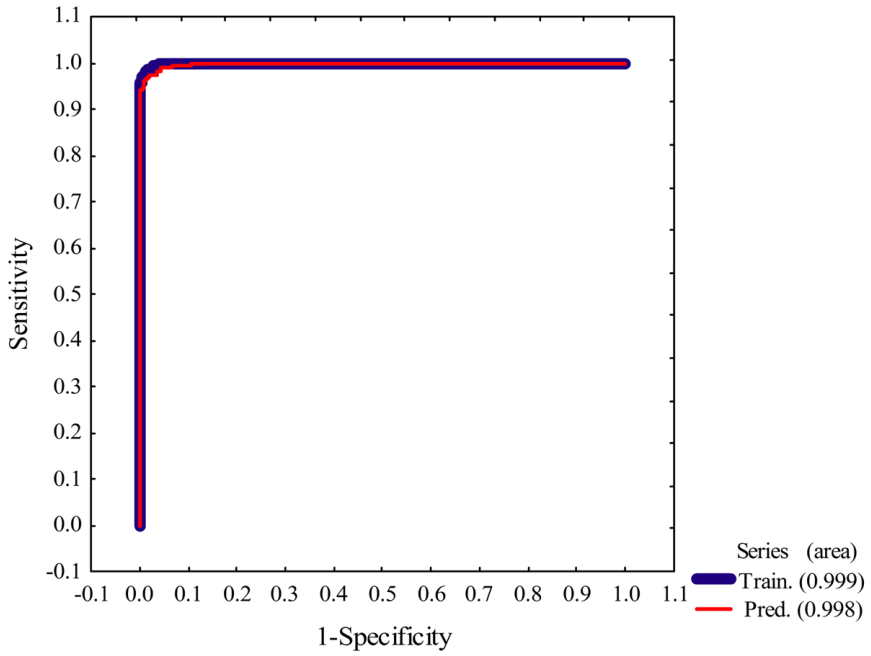
Finally, the areas under the ROC curves (see Figure 1) served as a final check-up of the very good performance of the derived QSAR-perturbation model. The values found were 0.999 and 0.998 for the training and prediction sets, respectively, indicating that the present model is not a random classifier because the areas under the ROC curves are very different from 0.5 – *i.e.* the value for a random classifier. The indices examined here demonstrate in terms of statistical quality and predictive power that

Table 2. Descriptors that entered into the final QSAR-perturbation model

Descriptor	Meaning
$\boldsymbol{ETOX_i(c_j)_{rf}}$	Categorical variable reflecting the ecotoxicity of the nanoparticle used as reference.
$\boldsymbol{DDV(m_e)}$	Perturbation term which accounts for the changes in the molar volume between the new (output or final state) nanoparticle and that used as reference, also depending on the measures of ecotoxicity.
$\boldsymbol{DDV(b_t)}$	Perturbation term based on the variations in the molar volume between the new (output or final state) nanoparticle and that used as reference, also depending on the biological targets.
$\boldsymbol{DDE(b_t)}$	Perturbation term focused on the changes in the electronegativity between the new (output or final state) nanoparticle and that used as reference, also depending on the biological targets.
$\boldsymbol{DDP(p_s)}$	Perturbation term which describes the changes in the polarizability between the new (output or final state) nanoparticle and that used as reference, also depending on the shapes of the nanoparticles.
$\boldsymbol{DDE(d_m)}$	Perturbation term which characterizes the variations in the electronegativity between the new (output or final state) nanoparticle and that used as reference, also depending on the conditions under which the sizes of the nanoparticles were measured.
$\boldsymbol{DDL(d_m)}$	Perturbation term which takes into consideration the changes in the size between the new (output or final state) nanoparticle and that used as reference, also depending on the condition under which the sizes of the nanoparticles were measured.
$\boldsymbol{DDE(t_e)}$	Perturbation term which describes the variations in the electronegativity between the new (output or final state) nanoparticle and that used as reference, also depending on the times during which the biological targets were exposed to nanoparticles.

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Figure 1. ROC curves obtained from the QSAR-perturbation model based on ANN



this QSAR-perturbation model is comparable with those reported in the literature (Concu et al., 2009a; Concu et al., 2009b; Garcia et al., 2011; Gonzalez-Diaz et al., 2013; Tenorio-Borroto et al., 2013). One should notice here that all the statistical indices reported for the QSAR-perturbation model based on ANN are very similar to those reported in the second mentioned work (Kleandrova et al., 2014). However, for the present QSAR-perturbation model a considerably larger number of pairs of NPs was generated and correctly classified. These simple but essential details confirm the idea that ANN can cover a wider "nanotoxicity space".

By definition, each descriptor present in any QSAR model has a degree of importance, which ensures its influence in the performance of that model. On the other hand, QSAR models should embody very useful interpretation in terms of structural and/or physicochemical properties, forward-giving an explanation regarding the changes in the activities/toxicities of the chemical species. In the case of our QSAR-perturbation model, we computed the corresponding sensitivity values of the descriptors (Figure 2). As commented before, sensitivities in ANN represent the relative importance of the variables (descriptors). Generally, for any ANN-based model built, no equation can be obtained and thus, it is very difficult to know how the descriptors should be varied to trigger particular changes in the activities/toxicities. Despite the lack of equations, one can interpret the variations of the different descriptors in such kinds of models following the procedure recently suggested by some of the present authors (Speck-Planche & Kleandrova, 2012). That is to say, if the mean values of the perturbation terms for non-ecotoxic cases are compared to the same values for ecotoxic cases, then, the analyst can have an idea about the tendency of variation of the descriptors in the model. Hence, by analyzing Figure 2 and Table 3, we can make this kind of deductions by considering how the different factors in the NP should be modified in order to decrease its ecotoxic effect.

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Figure 2. Relative influence of the descriptors in the QSAR-perturbation model

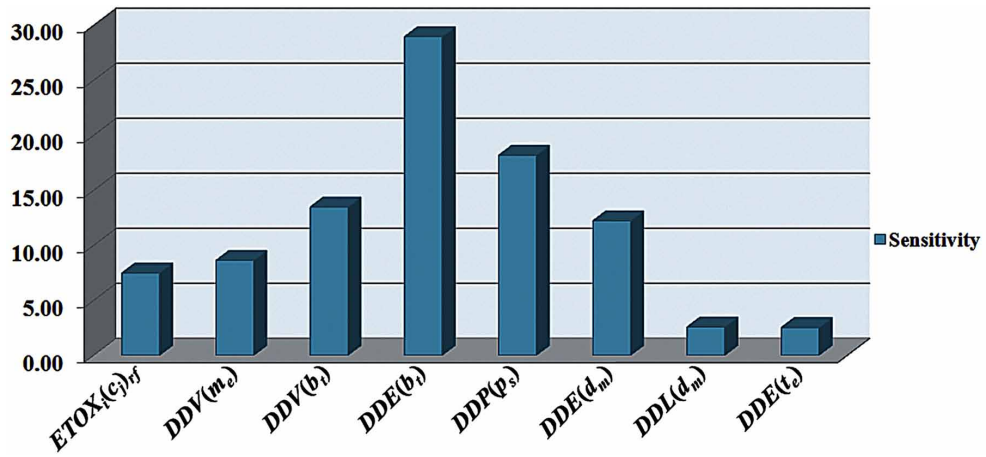


Table 3. Mean values of the perturbation terms

Descriptor	Mean (Non-Ecotoxic)	Mean (Ecotoxic)
$DDV(m_e)$	+0.742	−1.092
$DDV(b_t)$	−0.161	+0.460
$DDE(b_t)$	−0.213	+0.261
$DDP(p_s)$	−0.517	+1.090
$DDE(d_m)$	−0.154	+0.175
$DDL(d_m)$	+9.677	−7.286
$DDE(t_e)$	−0.043	−0.012

The descriptor $DDE(b_t)$ has the greatest influence in the QSAR-perturbation model based on ANN. This descriptor depends on the differences between the electronegativities of the NPs used as new and reference states, and the biological targets which were tested. Inspection of Table 3 tells us that the electronegativity of the elements that constitute the NPs should diminish, *i.e.* the presence of oxygen atoms should be avoided. Diminution of $DDE(b_t)$ will thus decrease the ecotoxic effect of the NPs. The same variation in the electronegativity characterized by $DDE(d_m)$ (4th in raking) will produce a similar effect in the ecotoxicity. This descriptor also depends on the conditions under which the sizes of the NPs have been measured. However, these changes in the electronegativity are constrained by $DDE(t_e)$ (having the smallest influence in the model) which additionally describes different times of exposure of the biological targets to the NPs.

The second most important descriptor is $DDP(p_s)$, which is based on the differences of polarizability between any two NPs, as well as in their shapes. Therefore, diminution in the polarizability of the NPs should make them less ecotoxic. With the aim of diminishing the ecotoxic effect of NPs, the values of $DDV(b_t)$ (3rd in raking) are also to be decreased. This descriptor has a similar interpretation as $\Delta\Delta E(b_t)$, with the differences between NPs involving molar volume instead of electronegativity. In any case, $DDV(b_t)$ is remarkably constrained by $DDV(m_e)$ (5th in raking) that depends on the diverse

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measures of ecotoxicity with which the NPs have been assessed. Notice that the combined interpretation of $\boldsymbol{DDV(b_t)}$ and $\boldsymbol{DDV(m_e)}$ indicates that there should be an equilibrium between the ability of the NPs to penetrate the cells of the biological targets and the possibility to undergo agglomeration. By considering the information provided in Table 3 for the case of the descriptor $\boldsymbol{DDL(d_m)}$ (7th in raking), one sees that the increment in the size of the NPs will diminish its potential ecotoxicity. This physical variable will depend on the difference in size of the NPs, and the conditions under which such sizes have been measured. As final aspect, we would like to emphasize that our approach depicted in Table 3 strongly converge to the physicochemical interpretation of the descriptors discussed in the previous report (Kleandrova et al., 2014).

Virtual Screening Using the QSAR-Perturbation Model

Even when any QSAR model is built on, its applicability should be demonstrated. Although the classical variants of such models restrict their use to try to understand the behavior of the chemical species, a more promising application can be realized, *i.e.* to perform virtual screening. For the specific case of our QSAR-perturbation model, we were able to give very simple physicochemical explanations about the characteristics of the NPs that can be desirable and/or detrimental to lessen the ecotoxicity. Following on, we will use our model to predict the ecotoxicity of different NPs extracted from external sources.

The first case of study is focused onto the prediction of ecotoxic effects of nickel (Ni) NPs, which have been reported experimentally a couple of years ago (Ispas et al., 2009). In such work, three different spherical Ni-NPs with sizes 30 nm, 60 nm, and 100nm (dry powders) were tested against zebra fish (*Danio rerio*) embryos during a period of 120 hrs. The acute toxicities (LC_{50}) were measured, exhibiting value of 328 mg/L (5588.40 μM), 361 mg/L (6150.65 μM), and 221 mg/L (3765.36 μM) for the sizes 30 nm, 60 nm, and 100 nm, respectively. The second case of study is devoted to predict the ecotoxicity of a CuO-NP with size 30 nm, which has been experimentally evaluated against *Pseudokirchneriella subcapitata* (Aruoja et al., 2009). This microalga was exposed to the CuO-NP (dry powder) during a period of 72 hrs, and the effective concentration leading to 50% reduction in the growth (EC_{50}) had a value of 0.71 mg Cu/L, which represents 0.889 mg/L (11.18 μM) for the CuO-NP. If we compare all these ecotoxicities with the corresponding cut off values, then, Ni-NPs should be classified as non-ecotoxic by our QSAR-perturbation model based on ANN, while the CuO-NP should be predicted as ecotoxic.

We need to emphasize that the decision of considering these new NPs as non-ecotoxic or ecotoxic by using our QSAR-perturbation model was not based on one single prediction. All the NPs were predicted 85 times, because the 85 NPs belonging to the original dataset were used as reference NPs. Accordingly, as commented in the previous section, the decision of considering NPs as non-ecotoxic (for the case of Ni-NPs) or ecotoxic (for the case of the CuO-NP) depends on the percentages of correct classification extracted from a consensus prediction, according to certain rules.

The particular results of predictions for each NP can be found in the Supporting Information (file **SM2**). Nevertheless, the summary of the results of the predictions are depicted in Table 4. The spherical Ni-NPs with sizes 30 nm, 60 nm, and 100 nm were correctly predicted as non-ecotoxic with very high consensus percentages. Of particular interest are the differences in the consensus percentages between the three Ni-NPs. Notice that the only condition which varies is the size of each NP, and the larger the size the higher the consensus percentage indicating the low ecotoxicity of the Ni-NP. These results have a strong convergence with our explanation regarding the physicochemical interpretations of the perturbation terms, where we suggested that the size of any NP should be increased in order to decrease its

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Table 4. Consensus predictions performed using the QSAR-perturbation model

Nanoparticle	m_e	b_t	p_s	d_m	t_e	$ETOX_i(c_j)_{nw}$	%CCT[a]
Ni-30 nm	LC_{50} (μM)	*Danio rerio* (embryos)	spherical	dry	120	+1	84.71
Ni-60 nm	LC_{50} (μM)	*Danio rerio* (embryos)	spherical	dry	120	+1	89.41
Ni-100 nm	LC_{50} (μM)	*Danio rerio* (embryos)	spherical	dry	120	+1	96.47
CuO-30 nm	EC_{50} (μM)	*Pseudokirchneriella subcapitata*	N/A	dry	72	−1	87.06
CuO-30 nm	EC_{50} (μM)	*Pseudokirchneriella subcapitata*	spherical	dry	72	−1	97.65
CuO-30 nm	EC_{50} (μM)	*Pseudokirchneriella subcapitata*	pyramidal	dry	72	−1	76.47
CuO-30 nm	EC_{50} (μM)	*Pseudokirchneriella subcapitata*	irregular	dry	72	−1	76.47
CuO-30 nm	EC_{50} (μM)	*Pseudokirchneriella subcapitata*	elliptical	dry	72	−1	65.88
CuO-30 nm	EC_{50} (μM)	*Pseudokirchneriella subcapitata*	needle	dry	72	−1	57.65
CuO-30 nm	EC_{50} (μM)	*Pseudokirchneriella subcapitata*	strip	dry	72	−1	100.00

[a] Consensus percentage, *i.e.* the percentage of times that the QSAR-perturbation model correctly predicted the nanoparticle according to its observed value of $\boldsymbol{ETOX_i(c_j)_{nw}}$.

ecotoxic effect. On the other hand, predictions performed for the CuO-NP were also in agreement with the experimental evidence. In this case, the authors of the research did not report the shape of the CuO-NP (Aruoja et al., 2009). Therefore, we decided to predict the ecotoxicity of the CuO-NP by considering six of the shape labels understudy, namely: spherical, pyramidal, irregular, elliptical, needle, and strip. We also took into account the category where no NP shape has been reported (N/A). Our predictions indicate us that CuO-NP is toxic against *Pseudokirchneriella subcapitata*, for all the possible shapes considered by the QSAR-perturbation model (Table 4). Therefore, the most important factor associated with the ecotoxicity of the CuO-NP is not the shape but its composition. That comes from the fact that the CuO-NP has one very electronegative element (oxygen), and as commented above, the presence of electronegative atoms can increase the ecotoxic effect of NPs. Both these suggestions and the prediction results demonstrate that our QSAR-perturbation model is very sensitive to the changes in both physico-chemical properties associated with the composition, and different experimental elements, being thus a promising computational tool for the efficient prediction of ecotoxicity of NPs under diverse sets of experimental conditions.

CONCLUDING REMARKS AND RECOMMENDATIONS

Several researchers have emphasized the use of promising guidelines for the computational modeling of toxic effects of nanomaterials (Kahru & Ivask, 2013; Westerhoff & Nowack, 2013), and there are also some reviews and chapters directed towards the development of reliable nano-QSAR models in recent years (Fourches et al., 2010; Kar et al., 2014a; Puzyn et al., 2011; Toropov et al., 2012). It is genuinely true that such task is a great challenge that may lead to new and exciting directions for QSAR modeling techniques. However, there are some problems one should face, namely:

- In the process of building reliable QSAR models, the ecotoxicological profiles of nanomaterials should be clear along with the development of toxicology studies.

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- There is a strong need to supplement the existing set of molecular descriptors by novel "nano-descriptors" that can represent size, shape, and so on-dependent properties of nanomaterials.
- More sophisticated methods such as the present QSAR-perturbation theory should be followed whenever developing QSAR models to tackle nanomaterials' endpoints.
- Most likely the current criteria of evaluating the models' quality (*i.e.* the five OECD rules) will have to be re-evaluated and adapted to nanomaterials. This is due to the specific properties of chemicals occurring at the "nano" level (for instance, electronic properties change with changing size), and the very limited number of available data (Notice that one has to overcome the problems of the "classical" method of validation that is biased towards small, low molecular weight molecules.).
- Regarding composition, nanoparticles in biological media are covered by a stabilizing layer and/or a protein corona, so that the NP surface may not be in direct contact with cells, so a change in the toxicity profile is to be expected and should be taken into account.

FUTURE RESEARCH DIRECTIONS

In this chapter, we have developed the first unified QSAR-perturbation model, demonstrating its ability to predict ecotoxicity of NPs under diverse experimental conditions. In so doing, the next promising horizons regarding the application of QSAR-perturbation models are to be devoted to the integration of different ecotoxicity profiles, with other biological effects such as cytotoxicities, and pharmacological activities (antibacterial, antiviral, anticancer). This will ensure the design and application of safer nanoparticles in very important fields like nanomedicine. On the other hand, further studies aiming at deriving QSAR-perturbation models to predict synthesis of NPs can contribute to the creation of more sustainable processes and/or alternatives in nanochemistry, helping to control the release of NPs into the environment. Finally, we would like to point out that the use of complex network theory may well be of great interest in order to gain a more thorough knowledge about the ecotoxic effects of NPs. Towards that goal, the design and study of the dynamics of NP-NP complex networks may help to understand the evolution of the ecotoxic behavior of NPs over time. All these ideas will contribute towards further developing and establishing one discipline that has just emerged as a need to study the 'nanoworld' in a more rational way: *nanoinformatics*.

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KEY TERMS AND DEFINITIONS

%CCT: Consensus percentage or percentage of times that the QSAR-perturbation model correctly predicts the NPs properties. This percentage is used to establish how promising is the QSAR model setup on the virtual screening of different NPs extracted from external sources (*i.e.* usually if it reaches %CCT greater than 50%).

ANN: Artificial neural network machine learning technique.

MAA: Moving average approach used in the QSAR-perturbation based models.

MNPs: Manufactured nanoparticles.

Nano-QSAR Model: Quantitative Structure–Activity Relationships model applied to target the properties of nanomaterials understudy.

Ontology: A set of factors that describe a specific experimental condition under which the properties of NPs have been measured.

Sensitivity of the Descriptors: Relative importance of the variables (descriptors) used in the final QSAR ANN-based model that allows estimating how those should be varied to trigger particular changes in the NPs' properties understudy.

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Chapter 52
Stratagems of Nanotechnology Augmenting the Bioavailability and Therapeutic Efficacy of Traditional Medicine to Formulate Smart Herbal Drugs Combating

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ABSTRACT

Mental illness is one of the most fundamental emotional states of conscious being which becomes unbalanced and leads to neurological disorders. It is a significant contributor to the global burden of disease and there is a strong desire to devise a remedy. Ayurveda represents a traditional medicine system of India that endorses antiquity than western medicine and relies on formulations rather than their active components. It has categorised a group of herbal medicines to improve mental abilities. Conversely, the mechanistic details of the therapy are not available in ayurvedic literature and there is a need to fortify this system with modern scientific analysis. The design of nanosystems encompasses promising characteristics in the field of drug delivery with a limited dosage thereby decreasing adverse effects. This chapter confers stratagems of devising polymeric nano herbal formulations as smart nano brain drugs to espouse mental health.

INTRODUCTION

The venture to maintain an optimal state of health includes both physical and mental wellbeing and a stable mind symbolizes an ideal physiology which is an imperative cause of existence. Mental health is an essential parameter that reflects the emotional, psychological and social wellbeing of an individual. Presently the populace stumble upon various emotional issues pertaining to their professional and

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personal life that evokes stress in their day today life. The unbalanced state of conscious being leads to mental disorders that erode subsistence. Trapped with pessimistic thoughts they feel hopeless and helpless thereby desire suicide as their only redeemer. Mental illnesses are a sober condition which can affect a person's thinking, mood, and behaviour. There are various types of mental illness and mental health problems, such as:

- Anxiety disorders,
- Mood disorders,
- Post-traumatic stress disorder (PTSD),
- Obsessive compulsive disorder(OCD),
- Impulse control and addiction disorders,
- Personality disorders.

Though medical field has advanced technologies, the threat of mental ailments intrudes the normal survival rate. There is a sturdy necessitate to treat these distressing conditions and fetch a remedy to stir up the universal welfare. There are several, medications available to combat mental illness but are accompanied with severe adverse effects that would lead to other physiological ailments. The advancement in modern science has to resolve the instinct of studying the context, nonclinical elements and alternative therapies required for enhancing mental health. Therefore, there is a strong desire to elevate research in the field of neuroscience with the resourceful mechanisms revealed, by manoeuvring the innovative technologies. Drug delivery is the most essential feature in drug administration facility and the design of nanosystems makes the action more consistent and appropriate. Polymeric nano particles bind efficiently to the drug and disperse the drug proficiently into the system. This mechanism significantly reduces the side effects of the synthetic drugs. Nanoscience focuses on the approaches of drug delivery in a nano scale, which needs a minimum dosage of drug than the conventional medicinal use. Nanomedicine is the medical application of nanotechnology which is considered as the future medicine. Biomedical nanotechnology, nanobiotechnology, and nanomedicine are the areas used to evolve this hybrid field of research (Figure 1).

The size of nanomaterials is similar to that of most biological molecules and structures. Therefore, nanomaterials can be useful for both *in vivo* and *in vitro* biomedical research and applications. Nanotechnology has provided the possibility of delivering drugs to specific cells using nanoparticles.

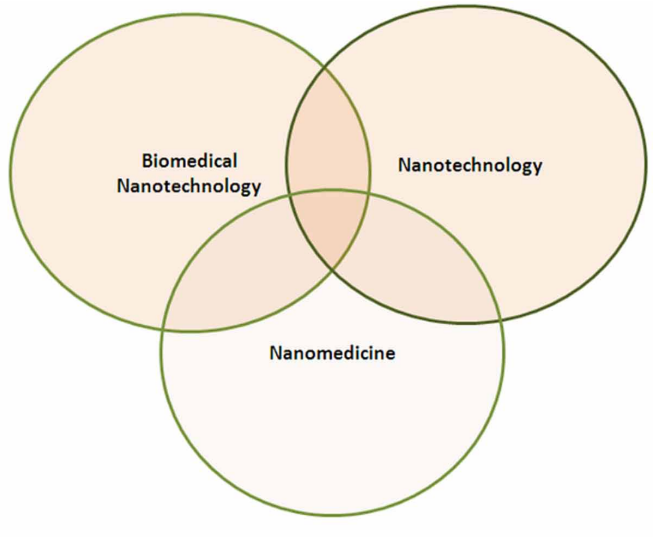
It has the following possibilities of facilitating:

- Lowered overall drug consumption and side-effects,
- Reduces expenses,
- Targeted action and develops personalized medicine,
- To improve drug bioavailability.

Drug delivery is the most essential feature in drug administration facility and the design of Nanosystems makes the action more consistent and appropriate. Drug delivery is the most essential feature in drug administration facility and the design of nanosystems makes the action more consistent and appropriate. Polymer nano particles bind efficiently to the drug and disperse the drug proficiently into the system. The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action. In most cases (conventional dosage forms), only a small amount of administered dose reaches the target

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Figure 1. Venn diagram indicating the integration of various research fields as a hybrid



site, while the majority of the drug distributes throughout the rest of the body in accordance with its physicochemical and biochemical properties. Therefore, developing a drug delivery system that optimizes the pharmaceutical action of a drug while reducing its toxic side effects *in vivo* is a challenging task.

Biocompatibility is an essential feature for drug administration and polymeric nanoparticles represent a significant efficiency and effectiveness, with unique physiochemical characteristics. There are various advantage of using polymers as encapsulating agents in drug delivery where, the foremost is the augmentation of stability and exact dispersion of drugs. The polymeric nanoparticles have been devised with specificity, which channelizes them to deliver a higher concentration of pharmaceutical agent to a desired location. Polymeric nanocomposites represent a new alternative to conventional polymers which exhibits markedly improved properties such as:

- Increased modulus and strength,
- Outstanding barrier properties,
- Improved solvent and heat resistance,
- Decreased flammability.

Thus polymers can abet imminent drugs with smart formulations to facilitate drug delivery and improve the pharmaceutical index. Alternative medicine is the use of a nonconventional approach instead of standard synthetic drugs. Traditional system of medicine has a deep rooted contextual derived from ancient communities that endorses antiquity than western medicine. Ayurveda, the Indian traditional system of medicine, has categorized a group of herbal medicines to improve mental abilities and restore metal health effectively. The treaties mention a number of single and compound drug formulations of plant origin that are used in the treatment of psychiatric disorders which have a less adverse effect profile, and exhibit efficacy in treating physical ailments. However, an ambiguity is reflected in the interpretation and mode of action pursued by the drug formulations. The current scenario has realized the significance of Ayurveda, where biomedical and ayurvedic scientists are making attempts to combine

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the best of different healing traditions to meet the challenge faced in healthcare requirements of modern era. Therefore, there is a robust requirement to promote ayurvedic medicine to reinstate mental illness with ingenious scientific technologies.

The considerable focus of this chapter is to reflect the advance strategies of nanotechnology on herbal medicine that can reduce adverse effects by improving the efficacy against mental illness.

The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action. In conventional dosage system only a small amount of administered dose reaches the target site, while the majority of drug distributes throughout the body in accordance with its physicochemical and biochemical properties. Polymers synchronized with the drug facilitates its delivery precisely across the targeted sites precisely. India is sanctified with a profound herbal resources whose attributes needs to be fortified with novel technologies. The deliverables discussed provides insights to hoist the implication of ayurvedic medicine on par with the western medicine.

BACKGROUND

Mental ailment is a serious physiological condition that affects the wellbeing of individuals. Globally, there is a periodic increase in psychological disorders which is a threat to the society, and depression ascertains a menace among mental illness. The World Health Organization (WHO) has ranked depression the 4th leading cause of disability worldwide and projects that by 2020, it will be the second leading cause (Murray et al., 1996). It is one of the major concerns in current scenario where, people are stumbled and is considered as a threat that intrudes the normal subsistence. Further it has reported that only 12.3% of the global population with this infirmity accept this condition and go for treatment. (Who Fact sheet N0.369, 2015). It is essential to investigate this existing issue and need to pay attention over the mental health worldwide. Fortunately, there is a wide range of synthetic drugs to treat these ailments but are accompanied with side effects which makes the treatment appalling. Commonly, alternative medicines incorporate the practice of herbal remedies which are effective with a low potential of toxic effects. Indian traditional medicine has formulated rich medical expertise knowledge of treatise which was handwritten in Sanskrit language as pre-European information. But after the establishment of British rule western medicine was looked upon as the leading system of medicine. This might be one of the reasons due to which ayurveda could not stand parallel to the western medicine. An ambiguity is reflected in the interpretation and mode of action in the formulations and drugs (Perinchery et al., 2013). The ancient Ayurvedic literature available today does not elaborate the effects of any therapy in terms of our current understanding of biology or physiology. The mode of action and the mechanism involved is not mentioned comprehensibly in the written treatise (Valiathan 2006). Although, there has been an increased interest in traditional and herbal medicine systems in recent times, most studies have used specific extracts or ‘‘active principles’’ derived from herbal or other traditional drugs and formulations. However current scenario has realised the significance of ayurveda, where biomedical and ayurvedic scientists are making attempts to combine the best of different healing traditions to meet the challenge faced in healthcare requirements of modern era and nanotechnology is one such advanced field.

Drug delivery is the most essential feature in drug administration facility and the design of nanosystems makes the action more consistent and appropriate. Delivering drugs across the physiological barrier is one of the most promising applications of nanotechnology in clinical neuroscience. Nanoparticles could potentially carry out multiple tasks in a predefined sequence, which is very important in the delivery of

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drugs across these barriers. Nanoscience is the study that involves the control of matter on an atomic and molecular scale which range usually below 100 nm. Initially nanomaterials were analyzed for its physical and electronic applications, but pharmaceutical appliance has been geared towards the area of drug delivery. Nanoparticles have the ability to defend the drugs from the degradation in the gastrointestinal tract, which channelizes the target delivery of drugs to various areas of the body. The oral bioavailability of drugs is profoundly increased due to absorptive endocytosis which is a cellular mechanism that sustains the drug for an extended time. This controls the release of drugs that minimizes the side-effects with increased effectiveness and safety that ultimately reducing health care costs. This technology also has the ability to enhance the performance of drugs that are unable to pass clinical trial phases. There is definite promise of nanodrugs to serve as drug delivery carrier of choice for the more challenging conventional drugs used for the treatment and management of various human ailments. Polymeric nano particles bind efficiently to the drug and disperse the drug proficiently into the system. The design of polymer-drug conjugates was propagated by Ringsdorf who used to deliver hydrophobic small molecules (Ringsdorf, 1975). The interpretation of his research described that, small molecule drugs, especially hydrophobic compounds, have a low aqueous solubility and a broad tissue distribution profile that may result in serious side effects. Consequently, conjugation of these compounds to hydrophilic, biocompatible polymers would significantly increase their aqueous solubility, modify their tissue distribution profile and enhance their plasma circulation half-life. The structural organization of a nanosystem is based on its composition and the presence of compartments within nanocapsules (Dong et al., 2013) leads to oily or aqueous cores surrounded by thin polymer membranes,(Arora et al.,2011) whereas, nanospheres provide a matrix-based organization of the polymeric chains.Nanoparticles have been prepared using several methods, including polymer polymerization(Chu, et al., 2013;Hu et al., 2011) ionic gelation or coacervation (emulsion solvent evaporation (Alshamsan, 2014), spontaneous emulsification or solvent diffusion, (Mainardes et al., 2012) nanoprecipitation, spray drying(Ali et al., 2014), supercritical fluid technology (Mazzarino et al., 2014), and (PRINT) particle replication in non-wetting templates(Galloway et al., 2013).Size is definitely a main advantage of these polymeric nanocomposites which enables them to penetrate capillaries (Brigger et al., 2002). Polymeric nanoparticles have been used brain drug targeting for neurodegenerative disorders such as Alzheimer's disease, to enhance penetration and distribution in and across the blood brain barrier (Medina et al., 2007). Nanoparticle based delivery methods are proved to be the best methods to transfer drugs across central nervous system (Jain, 2012). These strategies are proved to be the best methods to facilitate the delivery of drugs and biological therapeutics across the blood brain barrier (Gaillard et al., 2010). Due to their smaller size, nanoparticles penetrate into minute capillaries that distribute the drug after its delivery. Therefore, an efficient drug accumulation takes place at targeted sites in the body. However, to enhance the therapeutic action of drug its maximum absorption is required and polymers facilitate this aspect (Kreuter, 2014). The application of nanoscience in particular to drug delivery has extended immense accomplishments and has a hopeful elevation in the field of neuroscience. Subsequently, the chapter discusses the description and distinct features of nanotechnology in engendering smart herbal drugs to enhance mental health.

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APPROACHES TO CONTEST THE INTIMIDATION OF MENTAL ILLNESS

Synthetic Drugs as a Redeemer of Mental Infirmity: A Blessing Transformed as Desecrate

The preparatory phase for providing effective treatment against mental illness is to recognize the sort of problem and offer a proper diagnosis (NICE, 2010). There are fragmentary discussions regarding the effectiveness of the management of common mental health problems in general practice settings. Although evidence based clinical guidelines are available, initiation and adherence to effective treatment are usually poor (Richards, 2012). Untreated, depression can lead to serious complications that can lay life at risk. Fortunately, most clinical depression is treatable with the wide usage of antidepressant medications whose mode of action in the body occurs at the level of the synapse -the site in the nervous system where neurons communicate with each other or with other types of cells (e.g. muscle cell) by means of neurotransmitters. Some neurotransmitters (e.g. norepinephrine, serotonin and dopamine) are taken back into the nerve ending after release. This process is called uptake, reuptake or transport. This transport is a mechanism that prevents overstimulation of receptors in the receiving neuron. By blocking the (re-)uptake of these neurotransmitters, or inhibiting the mitochondrial enzyme monoamine oxidase, antidepressants alter the magnitude of the effects of neurotransmitter at these synapses (Richelson, 2001). The loss of sensitivity of the cell to the neurotransmitter is called desensitization, while the loss of the receptor protein from the cellular surface is called down-regulation. Though the specific mechanism of the therapeutic action of drugs remains unclear, it is known, that they adapt different mechanisms which can desensitize or down-regulate certain receptors for neurotransmitters by causing a local abundance of neurotransmitters. There are numerous synthetic drugs available to treat these psychological disturbances but are often prone to cause adverse effects, and the difficulty in tolerating these is the most common reason for discontinuing an effective medication.

The Major types of inhibitors are listed below:

- **Monoamine Oxidase Inhibitors:** MAOIs act by inhibiting the activity of the enzyme monoamine oxidase, thus preventing the breakdown of monoamine neurotransmitters and thereby increasing their availability. Well known examples of medicines in this class are phenelzine, isocarboxazid and trylcypromine. There are two isoforms of monoamine oxidase, MAO-A and MAO-B. MAO-A preferentially deaminates serotonin, melatonin, epinephrine, and norepinephrine. MAO-B preferentially deaminates phenylethylamine and trace amines. Dopamine is equally deaminated by both types. Due to the lethal dietary and drug interactions, monoamine oxidase inhibitors are usually reserved as a last line of treatment and used only when other classes of antidepressant drugs (for example selective serotonin reuptake inhibitors and tricyclic antidepressants) fail (Grady,2012).
- **Tricyclic Antidepressants:** TCAs were first discovered in the early 1950s and were subsequently registered later in the decade. The medicine is named after their chemical structure, which contains three rings of atoms. Examples of medicines from the TCA class are amitriptyline, imipramine and desipramine. The tetracyclic antidepressants, which contain four rings of atoms, are a closely related group of antidepressant compounds. The majority of the TCAs act primarily as serotonin-norepinephrine reuptake inhibitors (SNRIs) by blocking the serotonin transporter (SERT) and the norepinephrine transporter (NET), respectively, which results in an elevation of

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the synaptic concentrations of these neurotransmitters. In addition to their reuptake inhibition, many TCAs also have high affinity as antagonists at several receptors, which may contribute to their therapeutic efficacy, as well as their side effects (Gillman, 2007).

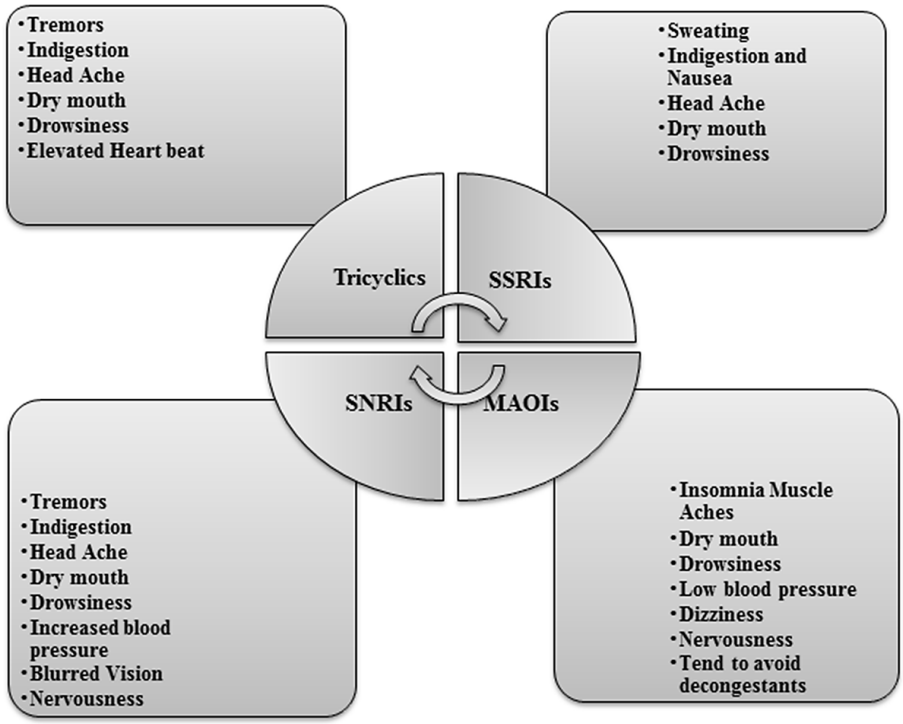
- **Selective Serotonin Reuptake Inhibitors:** SSRIs were first developed in the 1970s with the aim of targeting a specific site of the neuron, the reuptake protein, while not influencing other parts of the neuron such as the receptors. SSRIs are believed to increase the extracellular level of the neurotransmitter serotonin by inhibiting its reuptake into the presynaptic cell, increasing the level of serotonin in the synaptic cleft available to bind to the postsynaptic receptor. They have varying degrees of selectivity for the other monoamine transporters, with the more specific SSRIs having only weak affinity for the noradrenaline and dopamine transporter. Examples of SSRIs are escitalopram, fluoxetine, sertraline, paroxetine and fluvoxamine (Royal Pharmaceutical Society of Great Britain 2010).
- **Serotonin and Noradrenaline Reuptake Inhibitors:** SNRIs were first introduced in 1994. This type of antidepressant acts by inhibiting the reuptake of the neurotransmitters serotonin and norepinephrine. In relation to major depressive disorder, norepinephrine is thought to be related to alertness and energy as well as anxiety, attention, and interest in life; while (a lack of) serotonin is thought to be related to anxiety, obsessions, and compulsions. The inhibition of the reuptake of these neurotransmitters results in an increase in their extracellular concentrations and, therefore, an increase in neurotransmission. Venlafaxine and milnacipran are examples of SNRIs (Royal Pharmaceutical Society of Great Britain 2010).
- **Noradrenergic and Specific Serotonergic Antidepressants:** NaSSAs are a class of psychiatric drugs used that act by antagonizing the α2-adrenergic receptor and certain serotonin receptors. Antidepressants mianserin and its successor mirtazapine are examples of medicines in this class. By blocking α2-adrenergic autoreceptors and heteroreceptors, NaSSAs enhance adrenergic and serotonergic neurotransmission in the brain involved in mood regulation. In addition, due to their blockade of certain serotonin receptors, serotonin transmission is not facilitated in unwanted areas. This specificity is what is referred to in the name of this class of medicines (Julien, 2004).

In spite of the novel development for pharmacotherapy against mental illness, it is unfortunate that this disorder goes undiagnosed and untreated in many patients. It has been shown that less than 50% of patients experience a clinical response to treatment with the foremost drug prescribed. Yet, the presently prescribed molecules provide some improvement in the clinical condition of patients, it is at a cost of having to bear the burden of their adverse effects (Tripathi 2008, Hardman et.al, 2007). Many patients are not able to tolerate available (Figure 2) medications due to its deleterious side effects.

Studies show that as many as 50% of subjects may discontinue antidepressant treatments within a six month period, reporting adverse effects as a main reason for discontinuation (Cipriani et al., 2009). Along with the classical theory of decrease in the neurotransmitter levels in the brain leading to the pathogenesis of clinical disorders, recent studies have also shown the involvement of oxidative stress in the phenomenon (Sarandolet.al, 2007, Ibrahim, et.al. 2007).

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Figure 2. Signifies the common adverse effects caused by synthetic drugs



Alternative Herbal Remedies Formulated as Traditional Medicine to Combat Mental Illness: Plant Formulations as Potential Antidepressants

Although a number of synthetic drugs have been used as antidepressants they have adverse effects that compromise the treatment. These conditions create an opportunity for alternative treatment of depression by the use of medicinl plants or plant based antidepressant formulations. Several plants prove to contest depression but *Hypericum Perforatum* L. (St. John's Wort) is an established European medicine which is commercialized in large scale. The plant has profound medicinal properties and primarily, an extensive amount of clinical research shows that St. John's Wort extracts are effective in treating major depressive disorder. Botanical formulations are commercially available as herbal preparations over-the-counter in the forms of tea, tincture, tablet, and capsule usually containing its biologically active compounds such as hypericin (0.19 – 0.3%), hyperforin (1.3 – 3.9%) and flavonoids (calculated as rutin 4.8 – 11.4%.).The vital phytocompound Hypericin is a major psychoactive component of the plant is segregated with a phloroglucinol derivative compounds such as Hyperforin and Adhyperforin (Von Eggelkraut – Gottanka et al., 2002). A 300 mg - tablet of hypericum extract counterpart to about 15 mg hyperforin and clinical trials report a daily dose of 900 – 1500 mg (i.e., 0.3% hypericin) of the standardized extract (Biber et al., 1998). The European drug - monitoring study has reported that these commercial formulation associates certain adverse effects such as gastrointestinal irritation, allergic reactions, fatigue and restlessness. The blemish of this commercial herbal extract can be reinstated by the prominent ayurvedic medicinal plants.

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Indian Ayurvedic Medicine on Par with the Folk Medicine in Europe

Herbal medicine has the potential to decline the severity pertaining to the symptoms of mental illness and restore mental stability. There are various *ayurvedic* medicinal plants that have shown promise in reversing the neurological pathology. The vital phytochemical, biological, and cellular activities of these plants provides adequate baseline information that could be used in drug discovery campaigns and development processes.

- Medicinal plants as functional leads for mental illness classified as Medhya drugs:
  - **Ashwagandha (*Withania somnifera*):** Ashwagandha (*Withania somnifera*, family: *Solanaceae*) (Figure 3) is commonly known as "Indian Winter cherry" or "Indian Ginseng" which is widely spread in North western regions of India. It is one of the most important herb of *ayurveda* that has been used for ages as a Rasayana which is reputed to promote physical and mental health. The plant consists of vital phytochemical constituents that comprises of alkaloids (isopelletierine, anaferine, cuseohygrine, anahygrine), steroidal lactones (withanolides, withaferins) and saponins (Mishra, et al.,2000). Sitoindosides and acylsterylglucosides are considered as active ingredient of the herb that is established as anti-stress agents against acute models of experimental stress (Bhattacharya et al., 1987). As mentioned earlier in the text the plant belongs to a sub-group of Rasayanas known as Medhya rasayanas. Medhya is derived from Sanskrit language that refers to the mind and mental/intellectual capacity. The cognition intellectual effect of this ayurvedic drug enhances the learning skills in children, restores memory deficits during several circumstances such as head injury, protracted illness and old age (Singh and Udupa, 1993). Neuritic atrophy is a pathological condition that leads to synaptic loss which is the foremost cause of neurodegenerative diseases like Alzheimer's, Parkinson's, Huntington's and several other mental disorders. Numerous studies exhibit the

Figure 3. Pictorial representation of the medicinal plant Ashwagandha (Withania somnifera)



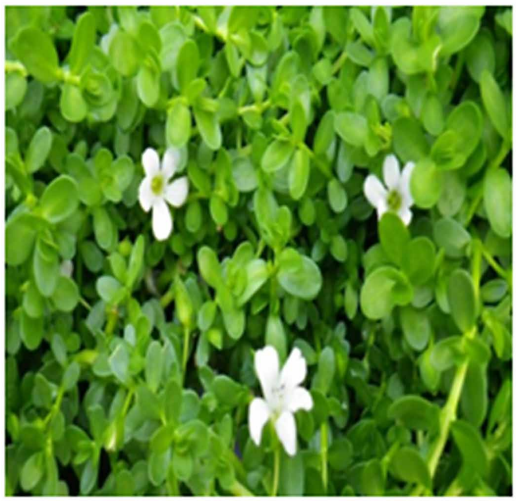
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realistic medication that can cease and reverse neuritic atrophy.Glyco withanolides withaferin- A and sitoindosides are the dynamic phyto compounds that have induced cognitive defects in Alzheimer's disease model (Bhattacharya et al., 1995).The furnished scientific details sustain the finale that Ashwagandha is a real potent herb that ingredients in regenerative tonic (Rasayana of *ayurveda*), due to its manifold pharmacological accomplishments like anti-stress and neuroprotective activity.

- **Brahmi (*Bacopa monnieri*)**: *Bacopa monnieri* (Brahmi) (Figure 4) belonging to the family *Schropulariaceae* is an ayurvedic herb which has a long primordial history of almost 3000 years and catalogued as an ingredient in medhya rasayana. The plant is habitat all over India particularly in wetlands and marshy tropical regions. The name of the plant is derived from Sanskrit language that reflects 'Brahma' who is considered as the god of creation according to Hindu religion. Brahmi has an alternative meaning "bringing knowledge of the supreme reality' that relates to brain which is the central organ of intellectual. In India this plant is utilized immensely as a revitalizing herb that fortifies the brain function and memory. The medicinal plant comprises of imperative phytocompounds among which saponins termed as "Bacosides" are considered to be a major bioactive marker that has therapeutic index (Deepak and Amit 2004). The whole plant extract of Brahmi has undergone various clinical trials that substantiate the reality to improve cognitive function in children and adults. A research conducted among school children by Sharma et al., 1987 improved their learning, memory and perception skills reaction without inducing significant side-effects. Brahmi has been commenced in the market as single or associated with other phytocompounds in treating attention deficient disorders. Pravina et al., 2007 devised a commercial formulation labeled as BacoMindR, which highlighted the non-noxious nature of the herbal extract reinforces ayurvedic medicine at the summit of healing mental illness. Hence there is a firm

Figure 4. Portrays the pictorial representation of the medicinal plant Bacopa monnieri (Brahmi)

Brahmi (*Bacopa monnieri*)



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requisite to elevate this herbal medicine with advent technologies that would stimulate it to be on par with the western medicines.

- **Shankhpushpi *Convolvulus pluricaulis*:** Shankhpushpi is a reputed drug constituted in medhya rasayana the name of the plant is derived from Sanskrit language that illustrates a plant with flowers shaped like a conch or shankha which is a sacred instrument used in ritual worships. According to the pharmacopoeia of India, *Convolvulus pluricaulis* (Convulvulaceae) (Figure 5a) as a whole plant rightfully claim the name of Shankapushpi but, Ayurvedic practitioners have used three other medicinal herbs such as *Canscora decussate* Schult. (Gentianaceae) (Figure 5b), *Evolvulus alsinoids* Linn. (Convulvulaceae) (Figure 5c), and (Figure 5d) *Clitoria ternatea* Linn. (Papilionaceae) (Nahata et al., 2008,2010) Indian Council of Medical Research(ICMR) has approved *C. pluricaulis as a herbal* drug with systematic potential as a brain drug, functioning as anxiolytic, tranquillizing, antidepressant, antistress, neurodegenerative, antiamensic, antioxidant agent. Phytochemical assays of the plant report to contain significant constituents like alkaloids, flavanoids, and coumarins (Sethiya et al., 2010).Though all the four plants are catalogued under the same class *Magnoliopsida*, *Clitoria ternatea* separates itself at the level of sub-class belonging to *Rosidae*, while the other three belong to *Asteridae*.

Figure 5. (a, b, c & d): Pictorial representation of the medicinal plants Convolvulus pluricaulis, Canscora decussate, Evolvulus alsinoides & Clitoria ternatea



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- ***Clitoria ternatea*:** *Clitoria ternatea,* is distinct among other herbs and has the property of being a good brain drug (Sivaranjan 1994). The plant has a rich source of vital phytoconstituents such as tri terpenoids, flavonol, glycosides, anthocyanins and steroids which are therapeutically significant. Phytochemical screening has yielded tannins, resins, taraxerol and ternatins.

Interestingly, the floral petals of the plant yielded three flavonol glycosides - kaempferol 3-O-(2″-O-α-rhamnosyl-6″-O-malonyl)-β-glucoside, quercetin 3-O-(2″-O-α-rhamnosyl-6″-O-malonyl)-β-glucoside, and myricetin 3-O-(2″,6″-di-O-α-rhamnosyl)-β-glucoside (Ramaswami et al., 2011). Banerjee and Chakravarthi (1963) reported the isolation and identification of pentacyclic triterpenoids, taraxerol and taraxerone from the roots. Yadava and Verma (2003) isolated antimicrobial flavonol glycoside3,5,4_-trihydroxy-7-methoxyflavonol-3-O-_-d-xylopyranosyl-(1,3)-O-d-galactopyranosyl (1,6)-O-d-glucopyranoside from the ethyl acetate soluble fraction of the defatted seeds of the plants.

The GC/MS profile of root extract extricated a major compound 9,17-Octadecadienal, (Z)- o with a elevated retention time of 32.99.The in silico docking analysis furnished a minimum binding affinity energy values of -6.5 / -7.71 kcal/mol against both the isoforms of Monoamine oxidase(MAO) A and B there by establishing the plant as MAO-inhibitors (Anita Margret et al.,2015)The roots, seeds and leaves of plant have been widely used as a brain tonic and is believed to promote memory and intelligence (Mukherjee et al., 2007). The whole plant of *Clitoria ternatea* has been widely screened for its various pharmacological activities. The aqueous root extract of the plant affects the brain structures concernedwith learning and memory regions of brain namely hippocampus and amygdala that can restore permanent changes in the brain. It may also affect the biosynthesis of neurotransmitter viz. acetylcholine, which has been implicated in the process of learning and memory. On the other hand, *Clitoria ternatea* may also induce long-term potentiation (Rai et al., 2002) that not only promotes neurogenesis in the amygdala, but also stimulates the release of hormones or neuromodulators that modulate the activity of neurotransmitters and neuromodulators involved in learning and memory, thereby contributing to enhanced learning and memory. After detailed clinical trials in adults, aqueous root extract treatment of the plant may be envisaged as a memory enhancer and also might be useful in the treatment of neuronal degenerative disorders involving the amygdala (Rai et al., 2005). *Clitoria ternatea*, a traditional ayurvedic medicinal plant elevates itself as an efficient botanical medicine combating neurological ailments. It can be used as a lead drug for developing new phytoceuticals for the treatment of neurological disorders and may be used effectively as a memory enhancer.

Though pharmacotherapy of modern medicine is well developed with chemical units it faces several challenges among which, adverse effects, lethal dosage levels and ability to surpass physiological barriers have been highlighted. Consequently, research features a novel facet of shifting from mono therapy (single active compound) to multi therapy that involves a collection of synchronized active compounds. Traditional herbs head this revolution in embracing phytochemicals which possess highly beneficial medicinal properties. Interestingly this novel attempt contests the western medicine and remain a challenge to the therapeutics. The advent of newer technologies like nano science can elevate herbal formulations as smart drugs that can fetch natural remedy against depression and mental illness.

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THE CONDUCT OF NANOTECHNOLOGY IN REFORMING THE MENACE OF MENTAL ILLNESS TO WELL-BEING

Nano particulate system is well devised to facilitate drug delivery that increases the therapeutic index of the drug. Nanoparticles are nanoscale sized polymeric particles which are made up of natural or artificial polymers that range in size between 10 and 1000nm (1 mm). Polymeric Nanoparticles have been shown to be promising carriers for central nervous system drug delivery due to their potential both in encapsulating drugs, and in delivering them across the blood-brain barrier without inflicting any damage to the barrier. The biodistribution of pharmaceuticals in a controlled manner and consequently protect them from excretion and metabolism. The capacity of the surfactants in the polymeric nanoparticles offer stability to the encased drugs and facilitates smart release and delivery. Studies have revealed ample number of mechanistic details pertaining to these particles such as site-targeted, controlled, and enhanced bioavailability of hydrophobic drugs (Panyam, et al., 2003).

Polymers Facilitating Bioavailability and Efficacy of Drugs

The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action. In most cases only a small amount of administered dose reaches the target site, while the majority of the drug distributes throughout the rest of the body in accordance with its physicochemical and biological properties. Therefore, developing a drug delivery system that optimizes the pharmaceutical action of drug while reducing its toxic side effects *in vivo* is a challenging risk. One of the approaches is the use of colloidal drug carriers that can provide site specific or targeted drug delivery combined with optimal drug release profiles. There are several drug delivery systems among which three efficient organizations are listed below:

- **Liposomes:** They are spherically shaped vesicles composed of natural phospholipids. The properties of liposomes can be varied with composition, size, and surface charge of lipids and also the method used for preparation. Liposomes can carry both hydrophobic and hydrophillic molecules inside them. The surface of liposomes can also be modified by attaching polyethylene glycol (PEG) chain to the lipid bilayer (stealth liposomes) to enhance their circulation time in the blood. Drugs can be actively targeted by conjugating liposomes with antibodies or ligands.
- **Dendrimers:** They are hyper branched structures that comprise of an inner core, a series of branches and outer surface with functional groups. Due to their nanometer size range, ease of preparation and functionalization they are attractive drug delivery systems. Due to the presence of internal cavities it is possible to encapsulate therapeutic agents in the inner core. The properties of dendrimers can be controlled by the functional groups on outer surface. Drug can either be encapsulated in the interior of the dendrimers or attached to surface functional groups.
- **Polymeric Micelles:** They consist of amphiphillic block copolymers, which can self-assemble to form micelles in aqueous solution. They have a narrow size distribution in the nanometer range and core-shell structure, in which hydrophobic segments are separated from the hydrophillic exterior (Table 1). Drugs can be partitioned in the hydrophobic core of micelles and the outer hydrophillic layer forms a stable dispersion in aqueous media.

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Table 1. Criteria for ideal polymeric carriers for nanoparticles and delivery systems

Polymeric Carriers	Nano Particle Delivery System
Easy to synthesize and characterize	Simple and inexpensive to manufacture and scale –up
Biocompatible	No heat high shear forces or organic solvents involved in the preparation process
Biodegradable	Reproducible and stable
Inexpensive	Applicable to a broad category of drugs, small molecules, proteins and polynucleotides
Non immunogenic	Ability to lyophilize
Non toxic	Non toxic
Water soluble	Stability after administration

Like liposome they can also be functionalized with PEG for stealth properties and with targeting ligands including antibodies to the micelle surface (Labhasetwar, 1993). Among these carriers, liposomes and nanoparticles have been the most extensively investigated. Liposomes present some technological limitations including poor reproducibility and stability, low drug entrapment efficiency. Polymeric nanoparticles which possesses a better reproducibility and stability profiles than liposomes have been proposed as alternative drug carriers that overcome many of these problems.

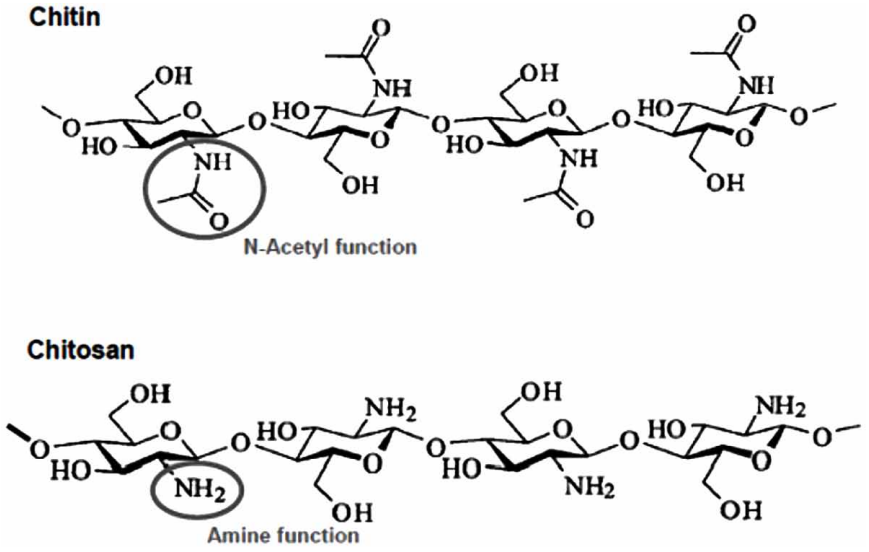
Chitosan: A Dextrous Biopolymer

Biopolymers are polymers produced by living organisms; in other words, they are polymeric biomolecules. Since they are polymers, biopolymers contain monomeric units that are covalently bonded to form larger structures. Chitosan is a natural polymer obtained by deacetylation of chitin. After cellulose chitin is the second most abundant polysaccharide in nature. It is biologically safe, non-toxic, biocompatible and biodegradable polysaccharide. Chitosan nanoparticles have gained more attention as drug delivery carriers because of their better stability, low toxicity, simple and mild preparation method and providing versatile routes of administration. Their sub-micron size is also suitable for mucosal routes of administration i.e. oral, nasal and ocular mucosa which is non-invasive route. Chitosan is a linear polysaccharide, composed of glucosamine and N-acetylglucosamine (Figure 6), produced by partial deacetylation of chitin by alkaline or enzymatic hydrolysis. Chitin is the major component of the exoskeleton of crustaceans, insects, cell wall of fungi and yeast.

The most remarkable difference between chitin and chitosan is their solubility characteristics. There are few solvents for chitin and most of them are toxic and therefore inappropriate for pharmaceutical applications. Chitosan is insoluble at neutral and alkaline pH, but forms positively charged salts with inorganic and organic acids (e.g., glutaminic acid, hyaluronic acid, lactic acid, and acetic acid), whereas the positive charge of the protonated amine group promotes solubility in aqueous solutions. Chitosan is available in a wide range of different molecular weights (50 kDa-2000 kDa) and degrees of deacetylation (40%-98%). The most commonly used forms of chitosan salts are chitosan glutamate and chitosan hydrochloride. Chitosan features different conformations of the molecule for high and low deacetylated chitosan. At a high degree of deacetylation (highly charged) chitosan has extended conformation with a more flexible chain, whereas at low degree of deacetylation the molecule adopts a more coiled shape due to lower charge. The amino group in chitosan has a pKa value of ~6.5, thus, chitosan is positively

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Figure 6. The chemical structures of Chitin and Chitosan



charged and soluble in acidic to neutral solutions. Chitosan with a relative low degree of deacetylation (40%) are soluble only at pH < 6.5 (Illum 2001).

Among water-soluble polymers available, chitosan is one of the most extensively studied. This is because chitosan possesses some ideal properties of polymeric carriers for nanoparticles such as biocompatible, biodegradable, nontoxic, and inexpensive. Furthermore, it possesses positively charge and exhibits absorption enhancing effect. These properties render chitosan a very attractive material as a drug delivery carrier. In the last two decades, chitosan nanoparticles (chitosan NP) have been extensively developed and explored for pharmaceutical applications.

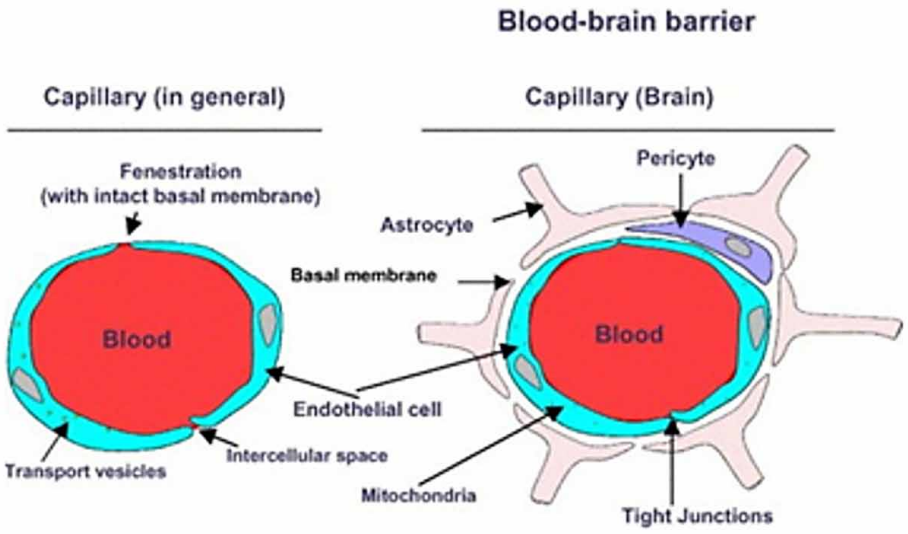
Physiological Barriers Hindering Therapeutic Molecules to the Targeted Sites and the Polymers Facilitating the Brain Drug

The Blood Brain Barrier (BBB) is the protective barrier that separates the brain from the circulatory system, which blocks harmful compounds from entering the brain tissue. It is a capillary network of tightly woven endothelial cells lining the blood vessels in the brain, regulating transport of essential molecules and maintaining a stable environment. It is a highly sophisticated structural design of the human body which determines what fluids are permitted in and near the brain and which would prove harmful. Capillaries (Figure 7) which surround the connective tissue and neuroglia known as astrocytes "stand guard" and select the substances which are permissible.

Glucose, water, lipid soluble compounds, carbon dioxide, and oxygen pass through the Blood Brain Barrier without difficulty. Inorganic compounds may pass but will be permitted more slowly and more hesitantly. The concentration of ions within the brain needs to vary from the concentration within the bloodstream. Antibiotics, most toxins, proteins, lipids, urea, insulin, and creatine are prohibited from passing through the barrier. This is the body's natural defence mechanism against toxins penetrating the brain, and is how we protect ourselves from cerebral invasion. The problem is, however, when serious diseases of the brain manifest, it has proven nearly impossible to transcend the BBB with therapeutic

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Figure 7. Morphological representation of the blood brain barrier



drugs in sufficient quantity to have an effect on the targeted malady without causing toxicity in the brain. The barrier provides such a protective shield to the brain that approximately 98% of small molecule drugs and 100% of large molecule drugs cannot cross it. This very tight regulation of the brain homeostasis results in the inability of some small and large therapeutic compounds to cross the blood–brain barrier (BBB). Therefore, various strategies are being developed to enhance the amount

Polymeric Nanoparticles as Drug Delivery Vehicles Encasing Brain Drug

Biodegradable polymers such as polylactic acid, polycaprolactone, poly(lactic-co-glycolic acid), the poly(fumaric-cosebacic) anhydride chitosan, and modified chitosan, as well as solid lipids, have revealed immense prospective in the delivery of proteins and peptidal drugs in nanao scale. Theses polymers channelize the drug *in vivo* is reported to mimic molecules that are easily surpassed through brain (example) polysorbate-coated nanoparticles are thought to mimic low density lipoprotein (LDL), allowing them to be transported across the capillary wall and into the brain by loading onthe LDL receptor (Kreuter, 2014) This mechanism adapted by the polymer improve the efficacy of the drug and allows it to pass through the Blood brain barrier. (Liu et al., 2012). It offers a sustained drug release at the targeted site after injection over a period of several days and weeks (. Nanoparticle based delivery methods are proved to be the best methods to transfer drugs across CNS (Jain, 2012). These strategies require the multifaceted attributes of nanoparticles which are combined and controlled to pass across the BBB. These are proved to be the best methods to facilitate the delivery of drugs and biological therapeutics for brain across the BBB. HAS (human serum albumin) is an illustrious example of a polymer that is used as nanoparticle and resists to the system by exhibiting no serious side effect. More exceptionally albumin functional groups can be utilized for surface modification of barrier that allows specific cell uptake (Vinogradov et al., 2002). It also acts like as a transforming growth factor in microbubble based drug delivery. (Kreuter, 2014). The efficacy of nanoparticulate drugs is, improved by these are coated biodegradable materials which make them more permeable to cross the blood brain barrier.

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Smart Nanoparticulate Herbal Formulations to Contribute Mental Health and Stability

The herbal formulation throws in immense of optimism in human health, and has advanced periodically in this modern era. Deplorably the active constituents of the plants require certain amendments that would lead to an unblemished effect. The vital phyoconstituents constituents such as flavonoids, tannins, and terpenoids, are highly soluble in water, but have low absorption, due to which they are unable to cross the lipid membranes of the cells. An additional snag pertaining to the phytocompounds that limits its usage is the intense high molecular size, which poorly absorbed, resulting in loss of bioavailability and efficacy. Nanotechnology provides solution to these deficits and hence has been widely proposed to synchronize with herbal medicine. Some prospective features of nanostructures in elevating herbal medicine are:

- Reduced dosage,
- Lessen adverse effects,
- Increase bioavailability and absorption,
- Control drug release and metabolism,
- Increased endurance of the drug.

Size is a blessing in nanotechnology where the therapeutic agent is distributed into the miniature blood capillaries (smaller than 5 mm) without forming aggregates, and ensure that the particles are not agglomerated. Polymers as nanocarriers provide more surface area and have the potential to increase solubility, enhance bioavailability, improve controlled release and enable precision targeting of the entrapped compounds to a greater extent. As a consequence, the expenditure intensity decreases widely, since isolated phytocompounds are expensive. Further, polymers are efficient for both hydrophilic and hydrophobic herbal drugs and can be channelized through the lymphatic system, blood brain barrier and arterial walls. Several clinical trials and experimentation has been performed by encasing herbal formulation into polymers to combat human infirmity. A wide number of plants are being used to as nanoformulations using various suspension modes against human infirmity Table 2 shows the details of the nano system incorporated.

Various research works has substantiated smart herbal formulations as nano drugs extensively. The proportion of research related to neuro drugs is comparatively low. Yet, few studies establish the proficiency of herbal drugs as nano formulations. Ginkgo biloba L. (Ginkgoaceae) is prominent medicinal plant that has been widely marketed for its brain cell activation properties

techniques) It was demonstrated (Shinji et al., 2011, Williams et al., 2004) that extract of G. biloba containing nanoparticles increase the release of acetylcholine from cerebral cortical synapses there by stimulating the response of hippocampal pyramidal cell. Thus, the nanosized G. biloba extract is expected to activate the brain cell and restore the habitual symptoms of Alzheimer's dementia (like loss of memory, thinking, language, judgement and behavior).

Several conditions such as oxidative stress, mitochondrial dysfunction, infections, hypoxia, apoptosis, and others, play important roles in the development and prevalence of these diseases (Cacciatore et al., 2012). It has been reported that oxidative stress (OS) is implicated in the development of neurodegeneration, mainly by the presence and attack of reactive oxygen species (ROS) that oxidize and injury neural cell components, such as lipids, proteins and DNA, leading to the appearance and prevalence of Alzheimer´s or Parkinson´s diseases, aging and many others neural disorders (Uttara et al., 2009). In this

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Table 2. List of potential Nano formulated medicinal plants targeting several human ailments

Name and Family of the Medicinal Plant	Nano System Employed	Purpose/Application	Reference
Panax ginseng(Araliaceae)	lipid-based systems	increase the absorption of the active components	Kesarwani and Gupta 2013
Artemisia arborescens L. (Asteraceae)	Liposomes	Facillitate the active compounds to penetrate the cytoplasmic viral barrier	Sinico C et al., 2005
Phytolacca decandra(Phytolaccaceae)	PLGA-encapsulate	lung cancer	Das et al 2012
Ocimum sanctum (Lamiaceae)	Sodium alginate chitosan nanoparticles(Cotton fabrics)	antimicrobial activity	Rajendran et al., 2013
Curcuma longa (Zingiberaceae)	copolymers of N-isopropylacrylamide, N-vinyl-2-pyrrolidone, and poly(ethylene glycol) monoacrylate.	pancreatic Cancer	Bisht et al 2007
Curcuma longa (Zingiberaceae)	PLGA	Prostate cancer	Mukerjee and Vishwanatha 2009
Magnolia officinalis (Magnoliaceae)	PEG/PLGA	anxiolytic, central nervous system depressant, and muscle relaxant activities	Zheng et al.,2010
Cuscuta chinensis Lam. (Convolvulaceae) nanoparticles	Eudragit (E) and PVA	Oxidation stress AND hepatoprotective effects	Yen et al., 2008
Camptotheca acuminata Decne (Cornaceae)	hydrophobically modified glycol chitosan (HGC)	Breast Cancer	Min et al., 2008
Ginkgo biloba (Ginkgoaceae)	polyvinyl alcohol (PVA)	anti-inflammatory, antitumor, antiviral, inhibitory of cataracts in diabetics, antihistamine (anti-allergic), cardiovascular, antioxidant, and hepatoprotective effects	Wu et al., 2008

way, flavonoids such as catechin, quercetin, rutin, have demonstrated that act as excellent free radical scavengers by rapid donation of a hydrogen atom to radicals (Seyoum et al., 2006). The work of Pool et al. (2012) was based in the development of polymeric nanoparticles (NPs) using Eudragit L30-D55 (Eud) polymers for the encapsulation, protection and target release within gastrointestinal tract of quercetin. In this study, the encapsulation of quercetin into Eud enhanced the protection of multi-lamellar vesicles of soybean phosphatidylcholine against lipid peroxidation of peroxyl and hydroxyl radicals. These results suggested that Eud NPs increased the lipid phase solubility of quercetin and also NPs act as protective agents against molecular oxygen, leading to a better free radical action that the free quercetin molecules used in the same experiments. Thus, these type or nanoparticulated systems might be suitable to across the BBB and deliver antioxidants to combat the OS in brain.

Studies have demonstrated that curcumin exert a net protective effect against oxidative damages initiated by free radicals, Despite, curcumin is a highly lipophilic compound and have the ability to cross the BBB, the poor bioavailability represent a limiting to its use in a functional product to combat neurological diseases. Accordingly, NPs decorated with appropriate ligands for curcuin brain delivery were developed lately (Mulik et al., 2010). These approaches were based in the preparation of curcumin-loaded PnBCA NPs decorated with ApoE3 ligand to exploit LDL-r- mediated transcytosis across the BBB and through SH-SY5Y cells. Results showed a reduction of Aβ1-42 related toxicity on cells treated with the function-

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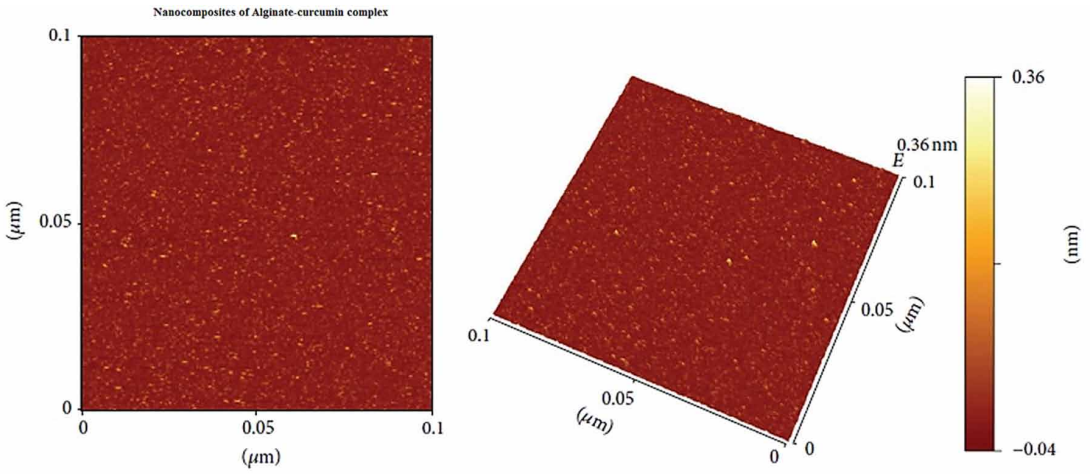
alized nanospheres along with a reduction of reactive oxygen species formation than the free curcumin used in this study. Another work demonstrated that curcumin-loaded PLGA nanoparticles obtained by an emulsion-diffusion evaporation method increase the neuroprotective effect on human SK-N-SH cells (neuroblastoma cell line) against the OS effect by reducing the elevation of ROS and the consumption of gluthatione induced by H_2O_2. Also the uptake of the encapsulated curcumin into SK-N-SH cells was significantly improved than the free curcumin, which impact on the effects found them by researchers. Overall, this study demonstrated that the PLGA NPs have the capacity to protect human neuronal cells against oxidative damages, having a great potential to be used as bioactive compounds carriers in the application to treat several neurodegenerative disorders (Doggui et al., 2012). Further alginate-curcumin nanocomposites (Yasir Hasan Siddique et al., 2013) reduced the oxidative stress as well as apoptosis in the brain of Parkinsons Disease model drosophila flies (Figure 8).

FUTURE RESEARCH DIRECTIONS

Neurodegenerative diseases a are characterized by a progressive and irreversible damage in the brain, which are the fourth leading cause of death in the developed world that precedes heart diseases, cancer, and stroke (Plassman et al., 2007). The most common neurodegenerative diseases are Alzheimer's disease, Parkinson's disease, Lewy body dementia, fronto temporal dementia, amyotrophic lateral sclerosis, Huntington's disease, and prion diseases (Bertram y Tanzi, 2005). However, the most widely recognized are Alzheimer's disease and Parkinson's disease, which are among the principal debilitating conditions of the current century. Approximately 24 million people worldwide suffer from dementia, 60% of cases being due to Alzheimer's disease, which occurs in 1% of individuals aged 50 to 70 and dramatically increases to 50% for those over 70 years (Cacciatore et al., 2012).

Several pharmaceutical and bioactive compounds have been used to treat neurodegenerative diseases, however, most of these components do not reach the brain completely and are, instead, metabolized totally or partially by the liver with a difficulty in solubility. Therefore, there is a necessity for the development of new technologies that help to protect and maintain the integrity of the drugs until they are released

Figure 8. Microscopic examination of Alginate: Curcumin nanocomposites



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at specific site of action in the brain (Nazem Mansoori, 2011). Nanosized drug delivery systems for herbal drugs can potentially enhance the biological activity and overcome problems associated with plant medicines (Pool et al., 2012;2013). However, significant challenges remain for implementation of clinically viable therapies in this field. Employing herbal drugs as nano formulations would aggravate the importance of ancient Ayurvedic medicine. The effectiveness of these potent phytocompounds as a synergetic effect with polymers can be further endorsed by performing clinical studies which would elevate the incredible competence of ayurvedic medicine on par with western medicine. The vital compounds present in the plant extract are further analysed towards the lysosomotrophic chronic effects along with the standard synthetic drugs by *in vitro* study using cell culture models. The regulation studies of the (neurotransmitters through receptors) β-Adrenoceptors and G protein would endorse the efficacy of the herbal drugs. Receptors stimulate the protein AC (Adenylate Cyclase) which there by induces CAMP (Secondary messenger) signalling the neurotransmitters which can authenticate the efficacy of drug released by the polymeric carriers. Experimental modes such as *in silico, in vitro* and *in vivo* studies with Cell lines and animal models can validate the therapeutic index of the herbal formulation. The cytotoxicity assay is the fore most parameter of in the process of drug development and in order to understand the *in vivo* mechanisms of ayurvedic nano formulations there is a need to propose good model system. This would propagate the therapeutic value of neurological diseases and disorders.

CONCLUSION

Herbal medicines are asserting importance in the current global scenario as the severity of pathogenesis is ascending. The advancement of modern science is elucidating the capacity several medicinal plant products. In order to compete with the rate of diseases and disorders there has to be an extensive rise in the therapeutic index. Multiple and synchronized therapy of combining the active ingredients of herbal extract would be an ideal stoppage solution. Polymeric nanocomposites augment the efficacy of these herbal formulations thereby causing less side effects. Nanosystems can deliver the active constituent at a sufficient concentration during the entire treatment period, directing it to the desired site of action which is failed by the conventional treatments. Brain drug delivery is a challenge as the rate of mental disorders are increasing hence there is stern constraint to exert more research to develop versatile drugs. An efficient herbal remedy without adverse effects is the need of the hour and smart herbal formulations can do this deed in a immaculately. This would rejuvenate the ancient riches present in the ayurvedic treatise.

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Section 6
Toxicology

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Chapter 53
Toxic Effects of Engineered Nanoparticles on Living Cells

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ABSTRACT

Measuring toxic effects of engineered nanoparticles on living cells would require a deep understanding of themselves by the mean of their composition, physical and chemical properties and exposure concentrations. Actually, high exposure concentrations are needed to generate quantifiable effects and to perceive accumulation above background. This chapter presents an overview on the assessment about the toxic effects of engineered nanoparticles on living cells. It consists of three main sections starting with a brief introduction, the current state of engineered nanoparticles in the environment, physical and chemical properties of some important engineered nanoparticles such as "Ag, Au, ZnO, TiO2" and the target organ toxicity of the engineered nanoparticles in several biological organisms.

INTRODUCTION

Because of their small-scale technology which means in scientific terms, 'Nano' where one nanometer is equivalent to one billionth of a meter, the nanoparticles (NPs) have the ability to interpose at a cellular level of living organs. They easily enter into the living cells, which may cause hostile effects on the environment and human health at their entry. Meanwhile, the potential development of nanotechnologies is expected to become new source of human or environmental risks through inhalation, ingestion, skin applications, or injection of engineered nanoparticles in factories, drugs or the use of customer foodstuffs.

In order to improve a natural development, new technologies always need an ecological balance between the benefit and risk. Understanding the toxic effects of nanoparticles on the environment is the biggest obstacle to the safe development of nanotechnology. A major challenge in understanding the environmental implications of nanotechnology lies in studying nanoparticles acceptance in organisms at environmentally truthful exposure concentrations.

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Nevertheless, little is known about how NPs affect human health and the environment. This chapter provides recent information on harmful effects of NPs particularly including their toxicity on living cells.

The objectives of this chapter are to:

1. Investigate the current state of engineered nanoparticles in the environment.
2. Provide recent information about the main properties of some innovated nanoparticles.
3. Estimate whether this data are sufficient to facilitate their comprehensive and effective influence on living cells and human health.

BACKGROUND

During the last decade, the use of NPs has successfully increased in several areas such as drug delivery systems (Jin & Ye, 2007; Brannon-Peppas & Blanchette; 2012) food technology (Chaudhry et al., 2008; Tapia-Hernández et al., 2015), photovoltaic devices (Stratakis & Kymakis, 2013; Huang, De Valle, Kana Kana, Simmons-Potter, & Potter Jr, 2015), modern chemistry (Hosseinimotlagh, 2014), biomedicine (Pankhurst, Connolly, Jones, & Dobson, 2003; Canfarotta & Piletsky, 2014; Cabuzu, Cirja, Puiu, & Grumezescu, 2015) and cosmetics (Perugini et al., 2002; Wiechers & Musee, 2010; Bolzinger, Briançon, & Chevalier, 2011; Kathawala, Ng, & Joachim Loo, 2015).

However there is a crucial necessity to verify their harmlessn ss relative to human health and the environment. This important issue was raised in the last few years when many International Organizations (Asia Pacific Nanotechnology Forum, 2005), governmental bodies within the European Union (European commission, 2004), United States Institutions (IEEE, 2004; US National Institute of Environmental Health Sciences, 2004), learned institutions and societies (Institute of Nanotechnology, 2005; Australian Academy of Sciences, 2005; UK Royal Society & Royal Academy of Engineering, 2004) and scientists (G.Oberdörster, E. Oberdörster, & J. Oberdörster, 2005; Donaldson & Stone, 2003) reported several assessments on the current state of nanotechnology, and most attracted attention to this requirement for an exhaustive risk investigation. Nevertheless, the influence of these nanomaterials (NMs) on human and environmental health remained unclear (Maynard, 2006; Nel, 2006; Helmus, 2007).

Although a rich literature is now available, we are still surprisingly uncertain as to whether engineered nanoparticles affect the human health and the environment or not!.

The organism has several semi-open interfaces for exchange some elements with the environment. The same interfaces are the principal routes of exposure of nanoparticles such as inhalation, dermal absorption and ingestion. An increasing number of researches have appeared in the last decade with an interesting goal of understanding the interactions between different types of nanoparticles and living cells of organisms as functions of size, shape, and surface chemistry of the nanomaterial (Lewinski, Colvin, & Drezek, 2008; Prinz, 2015). Unfortunately, no simple conclusions have emerged from the available studies due to the variability of parameters such as the physical and chemical properties of the particle, cell type, dosing parameters, and the biochemical assays used. Moreover, the majority of the scientific reports that investigate the cellular impact of NMs are in vitro, with far less effort to understand the real situation in vivo (Fischer & Chan, 2007).

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CURRENT STATE OF ENGINEERED NANOPARTICLES IN THE ENVIRONMENT

Sources of Free Nanoparticles

Free nanoparticles are formed over either the breaking down of larger particles or by controlled gathering processes.

Recently, engineered nanoparticles for innovative technologies and consumer products have become an intentional source of exposure. Actually, till now, it is unrevealed how significantly human exposure to these engineered nanoparticles has increased, is it in the workplace, or over the use of nanotechnology-based products.

There are two methods to the synthesis of nanoparticles and the fabrication of nanostructures; top-down and bottom-up approaches.

1. Top-Down Approach

The "Top-down" approach involves the breaking down of the bulk material into nano sized particles.

Many approaches are widely used in nanotechnology process such as:

1. **High Energy Wet Ball Milling:** This method is basically appropriate for making interconnected and integrated nanostructures such as in electronic circuits. By this route, nanocrystalline high-temperature phases can be obtained without going through extreme heat treatment for long times, which would promote grain growth and surface area loss (He et al., 2014).
2. **Heat-Up Method:** The synthesis of colloidal nanocrystals using a heat-up (non-injection) method is a reliable means to realize high quality NMs on large scale with little or no batch-to-batch variation (Vaughn, Bondi, & Schaak, 2010).
3. **Laser Ablation Synthesis in Solution (LASiS):** It is a green method that does not require chemicals or stabilizers, because NPs are directly obtained in water as a stable colloidal system (Amendola, Litti, & Meneghetti, 2013).

2. Bottom-Up Approach

The Bottom-up approach refers to the buildup of a material from the bottom: single atoms and molecules are assembled into larger nanostructures. This method is very powerful for creating identical structures with high atomic precision.

1. **High-Temperature Flame Process:** This method is basically applied in chemical product and engineering process to produce well-defined nanostructures with the desired phase without the need for post calcination (Poliak et al., 2015).
2. **Chemical Vapor Deposition (CVD):** It consists in activating a chemical reaction between the substrate surface and a gaseous precursor. Activation can be realized either with temperature or with plasma. CVD process is widely used to produce various types of carbon nanostructures (Xu et al., 2015).

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3. **Template Synthesis:** Any substance containing regular nanosized holes can be used as a template to synthesize NPs. Protein cages assembled from a defined number of subunits present a powerful example of such templates. Because of their extreme homogeneity "in size and structure", their interior cavities can serve as ideal template to encapsulate and synthesize well-defined NPs (Uchida, Qazi, Edwards, & Douglas, 2015).

How Engineered Nanoparticles Get into the Environment

The main factors determining human and environmental exposure to nanoparticles are their sources, the pathway they take from the source to the ecosystems and environment, and their fate and behavior during transport.

A number of potential sources of exposure for nanoparticles have been well-known. These involve the workplace, both deliberate and unintentional environmental statements, and straight application from users and medical products.

Nanoparticles exist in the environment naturally; nevertheless, natural nanoparticles are present at very low concentrations and have insignificant effect (Klaine et al., 2008). Recently, manufactured NPs have arisen and have been integrated into an increasable number of commercial products and human activities. The effort, both scientific and socioeconomic, to well understand the hazard posed by nanoparticles and to control their release into the environment has become very important. Although, in many cases, the statement of nanoparticles is compulsory, the main task should be to reduce those statements of nanoparticles that could pose a serious hazard in the environment and human health.

1. Human Activities

There are a number of human activities that discharge nanoparticles. These include burning fossil fuels, large scale mining, demolition, and automobile traffic. Traffic emissions and fossil fuel combustion typically produce ultrafine (nano) particles of soot or carbon black. Fossil fuel combustion (including natural gas burning) has also been exposed to produce more legally recognized types of carbon nanoparticles containing carbon nanotubes and fullerenes. Mining and metal factory operations have recently been presented to produce metal and metal oxide nanoparticles. One particularly harmful aspect of nanoparticle release from incineration reactions in which:

1. Airborne pollutants are difficult to comprise and can briskly extent to other ecosystems.
2. Airborne particle contaminants are being involved in many pulmonary conditions "including chronic frustrating pulmonary infection, pulmonary inflammation, and pulmonary fibrosis", because their reduced size makes it easy for them to pass deep into cellular lungs, where they may cause several damages in lung tissues.

2. Consumer Products

The other most expected source of nanomaterial pollutants is nanoparticle allowed consumer products. More than several thousand consumer products, including many items of clothing, personal care products, next-generation batteries, and sporting goods now contain NMs. In the next ten years, nanoparticles will likely be incorporated into many more sophisticated products, including pharmaceuticals and next-

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generation solar cells or batteries. While the inclusion of NMs in these products can enhance their performance, the breakdown of these products at the end of their useful life also provides several key points of entry for man-made nanoparticles into the environment. When nanoparticles are incorporated into products intended for domestic use (like anti-microbial fabrics or UV-blocking clothing), nanoparticles can end up in landfills or washed down the drain when clothes have been laundered. Nanoparticles that end up in drain water or in landfills can then enter the environment by many different routes.

3. Nanowastes and Living Cells

Once nanoparticles have been disposed of in household garbage or down the drain, their journey through the environment is just beginning. Nanoparticles that escape with water down the drain will eventually enter a wastewater treatment facility. Here, nanoparticle-contaminated water will undergo several purification processes, including mechanical filtering and settling treatments (designed to remove large particles [larger than a grain of sand]), followed by digestion with microbes, and ultimately chemical disinfection. Unfortunately, none of these treatment stages are specifically designed to eliminate nanoparticles from the waste stream. As a result, following waste water treatment, nanoparticles may remain in the purified water that is released back into the environment, or nanoparticles may remain trapped in the microbe-bearing sludge left over from the purification process. Often, the bio-sludge left over from wastewater purification is repurposed as fertilizer for farm land, which can potentially allow man-made nanoparticles to enter soils or small rivers. Alternatively, when nanoparticle-enabled products end up in a landfill, the original product can break down, allowing man-made nanoparticles to leach into soil in and around the landfill area, providing a route for man-made NMs to enter new environments via soils or even ground water sources.

Nanowastes are discharged from many different human activities, such as research and development, industrial production, transport and storage, and primarily after disposal of consumer products that contain NMs (Novack, 2007, 2012).

A little bit is known now about how nanoparticle contaminants may escape into the environment. Unfortunately, we still know far less about the specific environmental or human health hazards that some man-made nanoparticles may pose. Understanding the health implications of man-made nanoparticles is just one of many challenges still remaining if we want to understand the environmental behavior of these nanoparticles in great detail. For instance, we don't understand how the various components of a given environment (like available ions, minerals, local bacteria, and organic matter) may interact with different nanoparticles to change their size, shape, composition, and bioavailability. Furthermore, we are still unable to easily detect and monitor man-made nanoparticles that have been released into the environment. As a result, nanoparticles are difficult to track through real ecosystems and many different time-intensive assays are required to even determine whether humans or animals have been exposed to nanoparticles.

Nanotechnology is still a young field. While this means there are many things we still don't know about both the applications and implications of these new materials, this also gives us a new and unique opportunity to develop a chemical industry that could be truly sustainable from its inception. Hopefully, in the near future, we can overcome many of the barriers described above and help nanotechnology grow into a sustainable industry–one in which we can prevent environmental contamination by nanoparticles, rather than just clean up chemical waste after the fact. Look for the next post in this series soon, where

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we discuss the specific issues of nanoscale titanium dioxide (a very common industrially produced nanoparticle) as an environmental contaminant.

Figure 1 presents an outline of the major possible paths through which fabricated nanoparticles may enter into human life and environment to potentially cause toxicity to living cells.

MAIN CHARACTERISTICS OF SOME INOVATED ENGINEERED NANOPARTICLES

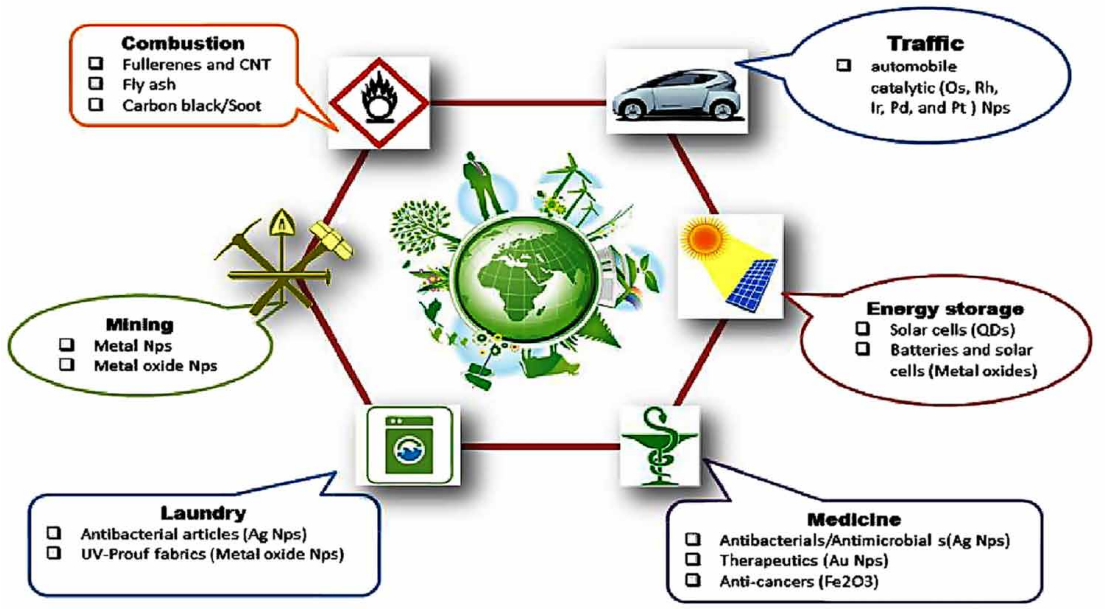
When performing a toxicity study, characterization of the test substance is of crucial importance in order to understand the parameters that can be used to predict the hazard of NPs. The consequent changes in physicochemical, optical and reactive properties can all influence NPs toxicity.

Metallic Nanoparticles

1. Silver Nanoparticles

Noble NMs have been synthesized using a variety of methods, including hard template, bio-reduction and solution phase synthesis. Among noble metal NMs, Silver nanoparticles have received considerable attention owing to their attractive physicochemical properties (*e.g.,* size and shape depending optical, electrical, and magnetic properties) which can be incorporated into antimicrobial applications, biosensor materials, composite fibers, cryogenic superconducting materials, cosmetic products, and electronic components. The surface plasmon resonance and large effective scattering cross section of individual Silver nanoparticles make them ideal candidates for molecular labeling where phenomena such as surface

Figure 1. Immersion of engineered nanoparticles (NPs) into human life and environment



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enhanced Raman scattering (SERS) can be exploited. In addition, they exhibit strong toxicity in various chemical forms to a wide range of microorganism is well known and have shown to be a promising antimicrobial material.

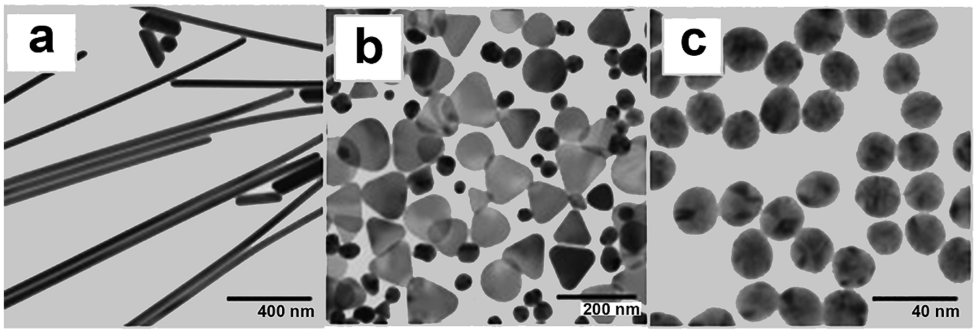
Several physical and chemical methods have been used for synthesizing and stabilizing silver nanoparticles (Senapati, 2005; Klaus-Joerger, 2001). There are many consumer products and applications utilizing nanosilver in consumer products; nanosilver-related applications currently have the highest degree of commercialization. A wide range of nanosilver applications has emerged in consumer products ranging from disinfecting medical devices and home appliances to water treatments.

a. Shape and Sizes

Silver nanoparticles can be produced with various sizes and shapes depending on the fabrication method. Silver spheres, rods, wires and plates can be produced by varying the manufacturing method. Typically anisotropic shapes are formed in the presence of a stabilizing polymer that preferentially binds to one crystal face and results in one crystal direction growing faster than others. However, even "spherical" silver nanoparticles can have a range of shapes and sizes (Figure 2).

Superior physical and chemical properties were observed for the metal nanostructures with complex shapes. A typical example is the shape and size dependent optical properties exhibited by triangular silver nanoplates. Triangular plates show three surface plasmon resonance (SPR) bands corresponding to dipole and quadruple plasmon resonance, but only one SPR band is observed for spherical silver nanoparticles. A large number of aqueous and non-aqueous methods were reported in the past decade for the synthesis of silver nanoparticles having different size and shapes such as rods, prisms, cubes, wires and disks (Maillard, 2002; Gu, 2006; Bastys, 2006). For some photochemical syntheses, reaction times can be longer ranging from several hours to days. Stability of nanoparticles and use of environmental friendly chemicals are other considerations for real life applications. Recently a two-step seed mediated methods are reported for the synthesis of triangular silver nanoparticles, which involve the synthesis of spherical silver seeds and their growth into triangular nanoplates using polyvinylpyrrolidone (PVP) as a stabilizing agent (Ledwith, Whelan, & Kelly, 2007). Reliability of this procedure is highly dependent on the structure of silver seeds and a range of morphologies were observed in the final sol. Extreme care was required while storing the silver seeds.

Figure 2. TEM image of a) Ag Nanowires, b) Ag- nanoplates, c) Ag- nanospheres
Source: (http://nanocomposix.com/pages/silver-nanoparticles-physical-properties)



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b. Surface Chemistry

It is important to remember that the surface of most nanoparticles is dynamic and is strongly influenced by the local environment. Different conditions will affect the particle in different ways. High salt environments will collapse the double layer and cause nanoparticle aggregation. Proteins and other biomolecules will often associate with and stabilize particles. When working with nanoparticles you must anticipate changes to the surface and plan accordingly. For example, when nanoparticles are to be added to solutions used in-vitro and in-vivo experiments, it is best to expose the particles to a low-salt solution that contains the protein components of the media and then add salt to bring the solution up to isotonic conditions. The protein binds to the particle surface and the suspension remains stable. If the nanoparticles are added directly to a high salt buffer, the particles may aggregate before protein stabilization can occur.

In solution, molecules associate with the nanoparticle surface and these surface bound molecules establish a double layer of charge that prevents nanoparticle aggregation.

Tannic acid is a multi-dentate capping ligand that can be displaced with many thiol containing molecules. Tannic acid is often used as a capping agent in applications where high particle concentrations are required.

Polyvinyl pyrollidone (PVP) is a polymer that binds strongly to the silver nanoparticle surface. It provides greater stability than citrate or tannic acid capping agents, but is more difficult to displace.

c. Surface Plasmon Resonance

There is increasing importance in developing the optical properties of silver nanoparticles as the functional component in various products and sensors. Silver nanoparticles are amazingly efficient at absorbing and scattering light and, unlike many dyes and pigments, have a color that depends upon the size and the shape of the particle. The strong interaction of the silver nanoparticles with light occurs because the conduction electrons on the metal surface undergo a collective oscillation when excited by light at specific wavelengths.

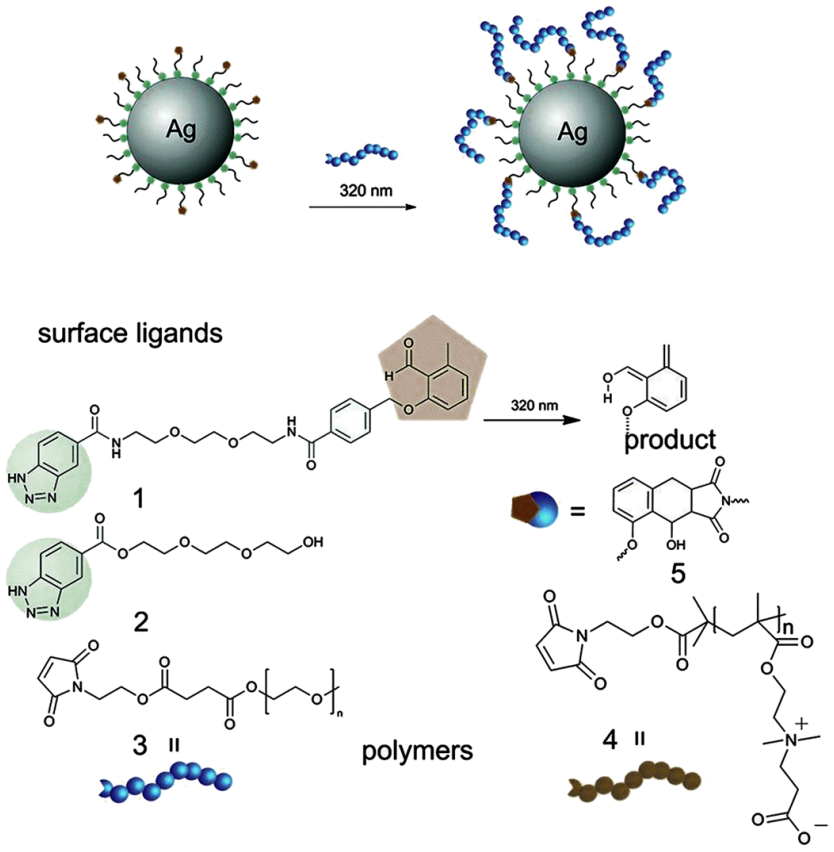
d. Surface Functionalization

Silver nanoparticles can be functionalized with a wide range of materials. Polymers such as polyvinyl pyrrolidone (PVP) and tannic acid are commonly used capping agents for silver nanoparticle materials applications, a zwitterionic polymer as well as polyethylene glycol are used to modify optical properties of Ag NPs (Stolzer et al., 2014). Silver nanoparticles used in biological applications are commonly coated with polyethylene glycol (PEG), bovine serum albumin (BSA), or numerous other proteins, peptides, and oligonucleotides. Particles can be functionalized with molecules that 'flip' the surface charge of the negatively charged silver nanoparticles to a positively charged surface. Particles can also be functionalized to provide reactive groups (for example, amine- or carboxy-terminated surfaces) for subsequent conjugation by the customer. Dielectric shells (for example, silica, aluminium oxide, and TiO_2) with a precisely controlled thickness can be used to encapsulate the particles, change the optical properties, or incorporate a fluorescent layer. Figure 3 illustrates the Light-induced modification of silver nanoparticles with functional polymers.

The approach of combining different mechanisms of antibacterial action by designing hybrid NMs provides a new paradigm in the fight against resistant bacteria.

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Figure 3. Light-induced modification of silver nanoparticles with functional polymers
Source: (Stolzer et al., 2014)



The green synthesis of Ag-NPs using dextrose as reducing agent have recently revealed powerful characteristics in biomedical field. The as-synthesised Ag-NPs showed better efficacy than the antibiotics; ciproflaxin and imipenem, the minimum inhibition concentration (MIC) was 6 µg/mL against Pseudomonas aeruginosa (Mohan, et al., 2014).

It has been demonstrated that a material comprised of chitosan-stabilized Ag NPs is a very promising candidate to assemble voltammetric sensors for practical applications in the field of analytical chemistry and biomedicine (de Lima et al., 2014; Potara et al., 2011).

A facile method was developed to prepare a magnetic Ag nanocomposite. The 3-D nanofibrous structure of bacterial cellulose (BC) was homogenized with a ferric and ferrous mixture by a high speed blender. Magnetite NPs were precipitated and incorporated into BC nanostructure by adjusting the homogenate to alkaline pH. The magnetic Ag nanocomposite possesses a high antimicrobial activity against the model microbes Escherichia coli and Bacillus subtilis. It also has potential as a mild fermentation medium sterilizing agent that a freshly prepared LB medium shows no appreciable contamination after incubating with Ag nanocomposite for 4 h and removed by an external magnet (Sureshkumar et al., 2010).

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2. Gold Nanoparticles

The potential of gold nanoparticles (Au NPs) in therapeutic and diagnostic cancer applications is becoming increasingly recognized. These biologically compatible particles can be easily synthesized, tuned to different sizes, and functionalized by conjugation to various biologically useful materials. Efficient and specific delivery to tumor tissue can then be accomplished either by passive accumulation in leaky tumor vessels and tissue, or by directly targeting tumor-specific biomarkers.

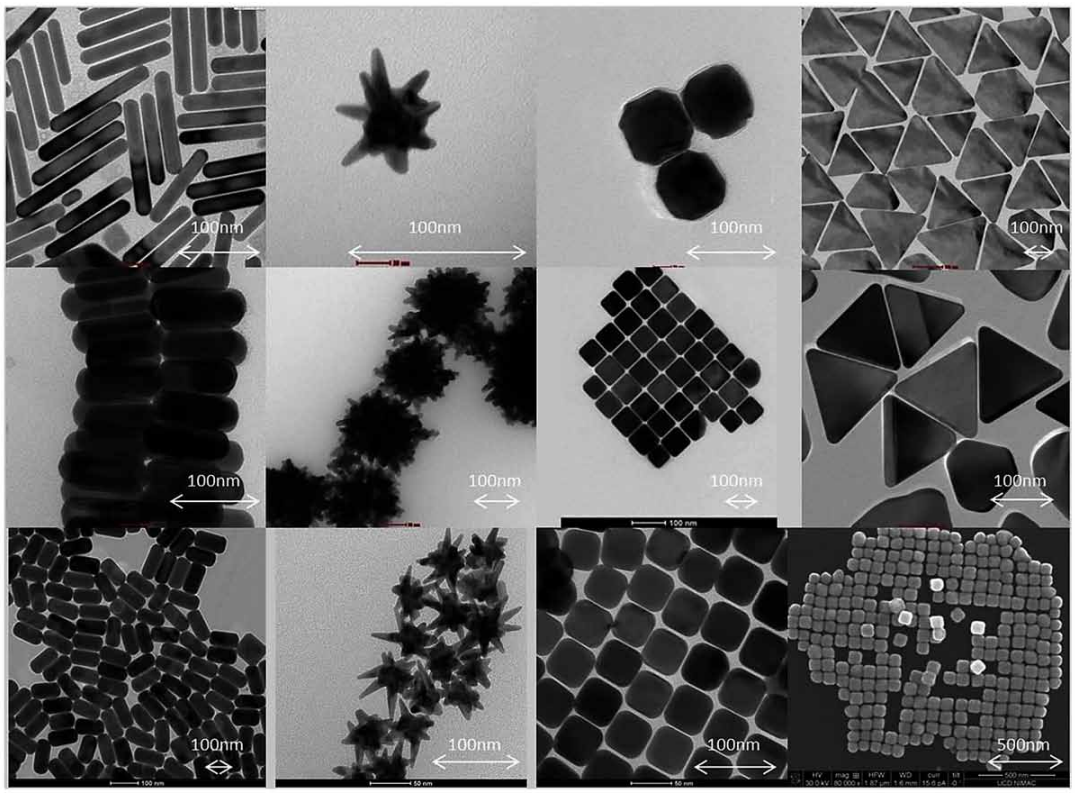
Depending on the shape, size, and degree of aggregation and nature of the protecting organic shells on their surface, Au NPs can appear blue and other colors and emit bright resonance light of various wavelengths, which falls under visible region. Because of this property, Au NPs have been extensively used as probes for sensing/imaging a wide range of analysts/targets such as proteins, cells and nucleic acids.

a. Shape and Sizes

Gold Nano-particles come in a wide variety of shapes, including, sphere, rod, star and triangle, just to name some of them. Researchers are using several different shapes and sizes in this technology (Figure 4). It is still being determined what shape works best for killing cancerous cells.

However, although Gold Nano-Rods, Stars and Triangles behave similarly to the gold nano-Spheres, their shapes optimize their light scattering characteristics.

Figure 4. Library of nanoparticle shapes: TEM and SEM micrographs gold nanoparticles by Dr Željka Krpetić (CBNI, UCD)



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In addition, Gold Nano-Rod have also been proven to enter a tumor more readily, than spheres.

Gold Nano-Particles can penetrate many cells throughout the body, they accumulating at tumor sites due to their EPR Effect "Enhanced Permeability and Retention Effect" (Maeda, 2010). It is the property by which certain sizes of molecules tend to accumulate in tumor tissue much more than they do in normal tissues.

b. Surface Plasmon Resonance (SPR)

Gold nanoparticles with unique optical properties may be useful as biosensors in living whole cells. They absorb and scatter light orders of magnitudes stronger than other materials (I. H. El-Sayed, Huang, & M. A. El-Sayed, 2005). The improved scattering property has been used in imaging and allows for detecting single cancer cell once nanoparticles are conjugated to cancer cell antibodies (I. H. El-Sayed, Xiaohua, & M.A. El-Sayed, 2006).The physical origin of the strong light absorption by noble metal nanoparticles is the coherent oscillation of the conduction band electrons (Surface Plasmon Resonance) induced by interaction with an electromagnetic field.

Recent methods to enhance SPR detection capabilities using gold nanoparticles are reviewed, as well as device fabrication and the results of incorporation (Bedford, Spadavecchia, Pradier, & Gu, 2012). As illustrated in Figure 5, SPR detection is a highly versatile method for the detection of biomolecules and, with the incorporation of Au NPs, shows promise in extending it to a number of new applications.

c. Functionalization of Gold Nanoparticles

Au NPs can be functionalized with a wide range of materials. The use of polymers such as cellulose, starch, alginate, dextran, and chitosan among others, gain great relevance not only due to their renewable nature and biodegradability, but also because a variety of formulations can be exploited depending on the envisaged functionality (Barhate, Gautam, Gairola, Jadhav, & Pokharkar, 2014; Chen, Cao, Quinlan, Berry,& Tam, 2015; Anh, Phu, Duy, Du, & Hien, 2010; Jang, Ryoo, Kostarelos, Han, & Min, 2013).

Metal Oxide Nanoparticles

1. Titanium Dioxide

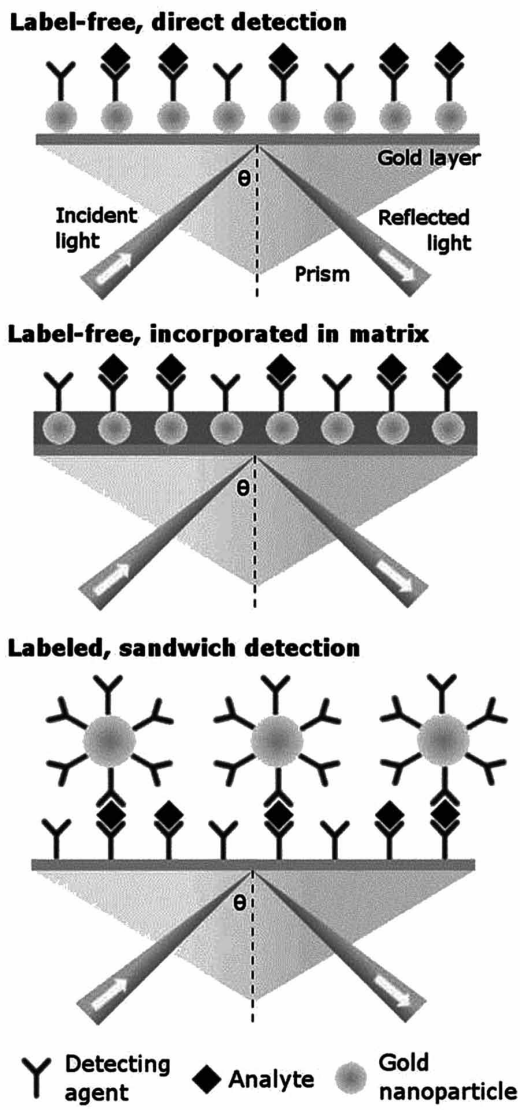
Titanium dioxide (TiO_2) has become part of our everyday lives. It is found in various consumer goods and products of daily use such as cosmetics, paints, dyes and varnishes, textiles, paper and plastics, food and drugs, and even paving stones.

a. Shape and Sizes

Titania (TiO_2) is the most widely used photocatalyst (Fujishima, 2008; Henderson, 2011) for decomposition of organic pollutants because it is chemically stable and biologically benign. The band gap of TiO_2 is larger than 3 eV (~3.0 for rutile and ~3.2 for anatase), thus making pure TiO_2 primarily active for UV light. The most common commercial photocatalyst is the Degussa P-25, a powder consisting of both rutile and anatase crystallites (Ohno, Sarukawa, Tokieda,& Matsumura, 2001). The great versatility of titanium dioxide is owing to its various forms and sizes. Titanium dioxides may be used in the form of microscale pigments or as nano-objects. Their crystal structures may vary: Depending on the arrangement of TiO_2 atoms, one differentiates between rutile and anatase modifications.

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Figure 5. SPR detection capabilities using gold nanoparticles
Source: Bedford et al., 2012



b. Surface Chemistry

In a recent research, the effects of surface chemistry on the morphology and phase stability of titanium dioxide nanoparticles have been investigated using a thermodynamic model based on surface free energies and surface tensions obtained from first principles calculations. It has been found that surfaces representing acidic and alkaline conditions have a significant influence on both the shape of the nanocrystals and the anatase-to-rutile transition size (Bernard & Curtiss, 2005). The latter introduces the possibility of inducing phase transitions by changing the surface chemistry.

Surface modification such as coating, influences the activity of TiO_2 NPs. For example, diminished cytotoxicity was observed when the surface of TiO_2 NPs was modified by a grafting-to polymer technique combining catalytic chain transfer and thiolene click chemistry (Tedja et al., 2012).

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c. The Photocatalytic Activity

The photocatalytic activity for the degradation of phenol was improved under both visible and UV light by codoping. In 2009, Lai and Lee (Lai & Lee, 2009) revealed the ability and mechanism of photoexcited TiO_2 nanoparticles modified with folic acid to kill cells. Folic acid was bound to TiO_2 nanoparticles to provide cell targeting specificity. These nanoparticles exhibited higher cytotoxicity under photoexcitation. Mizukoshi et al. (Mizukoshi, Ohtsu, & Masahashi, 2009) prepared rutile TiO_2 by anodic oxidation in a sulfuric acid electrolyte. Sulfur doping played an important role in the response of the product to visible light by narrowing the band gap of TiO_2, allowing the photocatalyst to effectively bleach an aqueous solution of methylene blue under irradiation with visible light of >413 nm. Wang et al. (Wang, Ao, Wang, Hou, & Qian, 2010) synthesized TiO_2-coated magnetic silica that showed enhanced photocatalytic activity for the degradation of reactive brilliant red X-3B compared with Degussa P25 under both UV and visible light irradiation. Furthermore, the prepared composite photocatalyst had tunable magnetic properties.

2. Zinc Oxide

ZnO NPs have unique properties including small size and correspondingly large-specific surface area to volume ratio. ZnO has been increasingly employed in a variety of industrial applications including production of wave filters, UV detectors, catalysts, paint, transparent conductive film, cosmetics, gas sensor and catalytic processes, solar cells and microelectronics (Nel, 2006; Reijnders, 2006; Zhu, 2005), personal care products (toothpaste, beauty products, and sunscreens (Serpone, Dondi, & Albini, 2007), and textiles (Becheri, Dürr, Lo Nostro, & Baglioni, 2008). Increased applications in industry will increase chances of exposure of the nanoparticles to humans. Thus, zinc oxide nanoparticles are the subject of much research because of their high probability exposure to human and environment (Kahru & Dubourguier, 2010).

a. Shape and Sizes

The variety of structures of nanometric zinc oxide means that ZnO can be classified among new materials with potential applications in many fields of nanotechnology. ZnO provides one of the greatest collections of varied NPs microstructures among all known materials (Figure 6).

b. Surface Coatings of ZnO Nps

Surface coatings are added to ZnO nanoparticles for ease of handling and to modulate their properties. For example, coating facilitates their dispersability in the oil phase of sunscreen formulations, as well as improving the texture of the sunscreens on skin (Wang & Tooley, 2011).

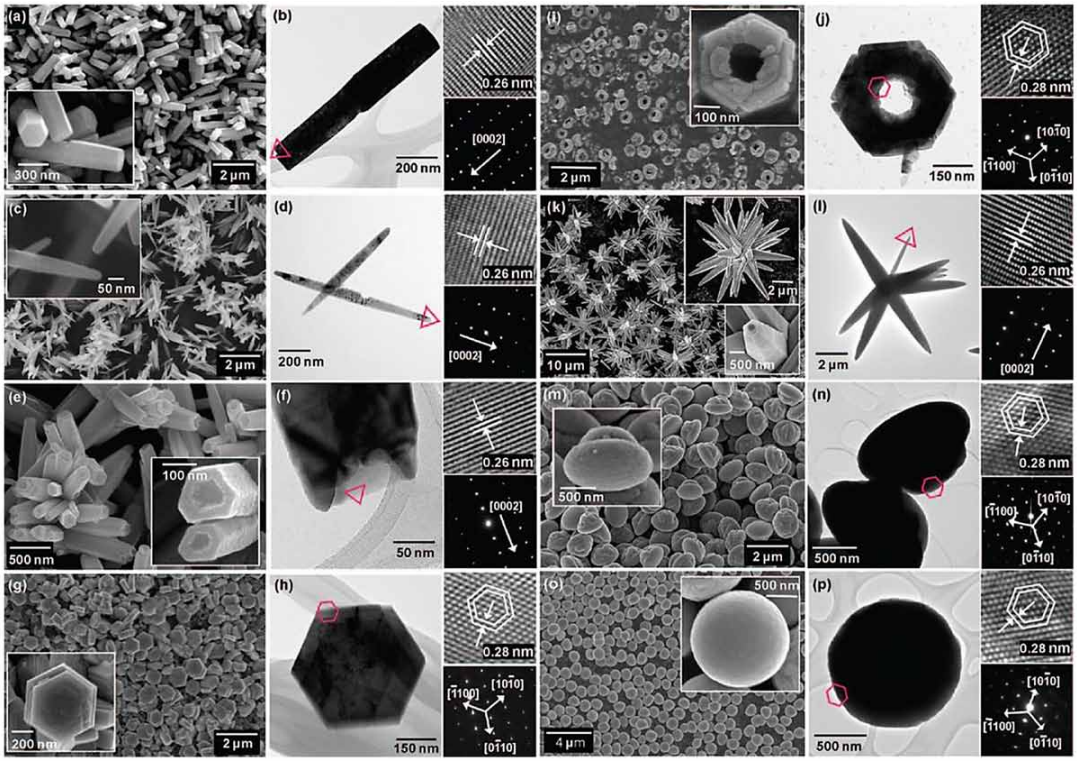
From a nanotoxicological perspective, stable surface coatings have been reported to suppress the generation of reactive oxygen species (ROS) by ZnO nanoparticles (Yin, 2010, 2010) and may also decrease the propensity for ZnO nanoparticles to dissolve in biological environments.

c. Catalytic Activity

In recent years, in biological field, the potential utility of ZnO NPs in the treatment of cancer has been reported by many researchers. Owing to numerous advantages associated with this ecofriendly nature,

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Figure 6. Nanorods (a & b), nanoneedles (c & d), nanocandles (e & f), nanodisks (g & h), nanonuts (i & j), microstars (k & l), microUFOs (m & n), microballs (o & p)
Source: Cho et al., 2008



it has been explored as a powerful catalyst for several organic transformations (Moghaddam, 2009; Kumar, 2011; & Alinezhad, 2012) such as Mannich reaction, and the Knoevenagel condensation reaction, in the synthesis of coumarins, quinolines, polyhydroquinoline, 2,3-disubstituted quinalolin-4(1H)-ones, and benzimidazole.

3. Iron Oxide

Iron oxide NPs are the most popular magnetic NPs used in biomedical applications due to their low cost and unique magnetic properties. Magnetic iron oxide NPs have two main forms, magnetite (Fe_3O_4) and its oxidized form maghemite (γ-Fe_2O_3). They have attracted extensive interest due to their superparamagnetic behavior and their potential applications in many fields (drug delivery, diagnosis, therapy, and etc) and have been recently employed as supporting materials for enzymes, exhibiting striking characteristics, such as large surface area, mobility and high mass transference (Netto, Toma, & Andrade, 2013).

Applications of iron oxide NPs include terabit magnetic storage devices, catalysis, sensors, and high-sensitivity biomolecular magnetic resonance imaging (MRI) for medical diagnosis and therapeutics. These applications require coating of NPs by agents such as long-chain fatty acids, alkyl-substituted amines and diols.

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a. Shape and Size

Many successful challenges has been made to synthesis shaped and nanosized iron oxides using several mechanochemical and chemical methods (Wilcoxon & Provencio, 1999; Seip & Connor, 1999; Hyeon, Lee, Park, Chung, & Hyon, 2001; Jiang et al., 2004). By employing solvothermal techniques, various morphologies of iron oxides like nanorods, rings, cubes, spindles, hollow particles etc. have been prepared (Sun et al., 2004; Vayssieres et al., 2005; Huang, et al., 2011; Li et al., 2015.).

b. Functionalization of Iron oxide NPs

Magnetic iron oxide NPs with a bare surface tend to agglomerate because of strong magnetic attractions among the particles. Stabilizers such as carboxylates, inorganic compounds and polymeric compounds have functional groups to modify these particles and enhance its stability (Laurent et al., 2008; Zhang, He, & Gu, 2006). Among the various polymers, chitosan an alkaline product of chitin has been widely examined due to its hydrophilicity, biocompatibility and biodegradability (Unsoy, Yalcin, Khodadust, Gunduz, & Gunduz, 2012). Chemically chitosan is insoluble in water and behaves as a weak base making it inappropriate for biological and environmental applications. On the other hand, chitosan oligosaccharides, which can be produced by degradation of chitosan polymer chain, are water soluble making it suitable for biological and environmental applications (Mourya & Inamdar, 2008).

INTERACTION ETHICS OF NANOPARTICLES WITHIN LIVING CELLS

Some questions about the safety of such engineered NPs are very important and the most critical subject with this regard is the penetration of biological barriers allowing particle distribution throughout the living cells. Such translocation studies are technically challenging and many issues have been considered to obtain meaningful and comparable results.

A number of studies have demonstrated that NPs toxicity is extremely complex, and that their biological activity will depend on a variety of physicochemical properties such as particle shape and size, agglomeration state, crystal structure, chemical composition, surface area and surface properties.

Impact of Physicochemical Properties

The unusual physicochemical properties of engineered NPs are attributable to their small size (surface area and size distribution), chemical composition (purity, crystallinity, electronic properties, etc.), surface structure (surface reactivity, surface groups, inorganic or organic coatings, etc.), solubility, shape, and aggregation.

Although impressive from a physicochemical viewpoint, the novel properties of Nps raise concerns about hostile effects on living systems, which at the cellular level include structural arrangements that resemble NPs in terms of their function. Indeed, some studies suggest that Nps are not inherently benign and that they affect biological behaviors at the cellular, subcellular, and protein levels (Service, 2004; Colvin, 2003; Donaldson, 2001; Oberdörster, 2005; Ivask et al., 2015). Moreover, some nanoparticles readily travel throughout the body, deposit in target organs, penetrate cell membranes, and may trigger injurious responses (Barbasz, Oćwieja, & Barbasz, 2015).

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1. The Surface Effects

All NPs, upon exposure to tissues and fluids of the body, will immediately adsorb onto their surface some of the macromolecules that they encounter at their portal of entry. The specific features of this adsorption process will depend on the surface characteristics of the particles, including surface chemistry and surface energy, and may be modulated by intentional modification or functionalisation of the surfaces (Sperling & Parak, 2010). This is well demonstrated through the use of specific biomolecular linkers that are anchored on the surface of nanoparticles or within vesicles and liposomes (Nardin, Thoeni, Widmer, Winterhalter, & Meier, 2000). In this way the affinity of a NP can be shaped to fit to a particular protein, and thus target a specific biomolecular assembly on a membrane, or within a specific organelle or cell surface. Both the existence of passive surface layers and surface active agents compromise the risk evaluation of nanoparticles by mere chemical composition. In agreement with bulk surface chemistry, metallic nanoparticles are of considerable chemical reactivity and it is well established that metal NPs will often form passive surface layers on exposer to oxygen, just as do their bulk counterparts. (EE, International Handbook on Regulating Nanotechnologies, 2010).

2. Size, Shape, and Composition

The interaction of NPs with living cells is also affected by the characteristic dimensions. As noted above, NPs, of a few nm in size, may reach well inside biomolecules situation not possible for larger particles. It has been reported that inhaled nanoparticles reach the blood and may reach other target sites such as the liver, heart or blood cells (Oberdörster, 2002; MacNee, 2000; & Kreyling, 2000).

Nanoparticles may translocate through membranes. There is little evidence for an intact cellular or sub-cellular protection mechanism. For humans, inhalation is the most frequent route of access, and therefore the process of aggregation of NPs in the inhaled air has to be taken into account.

3. Solubility

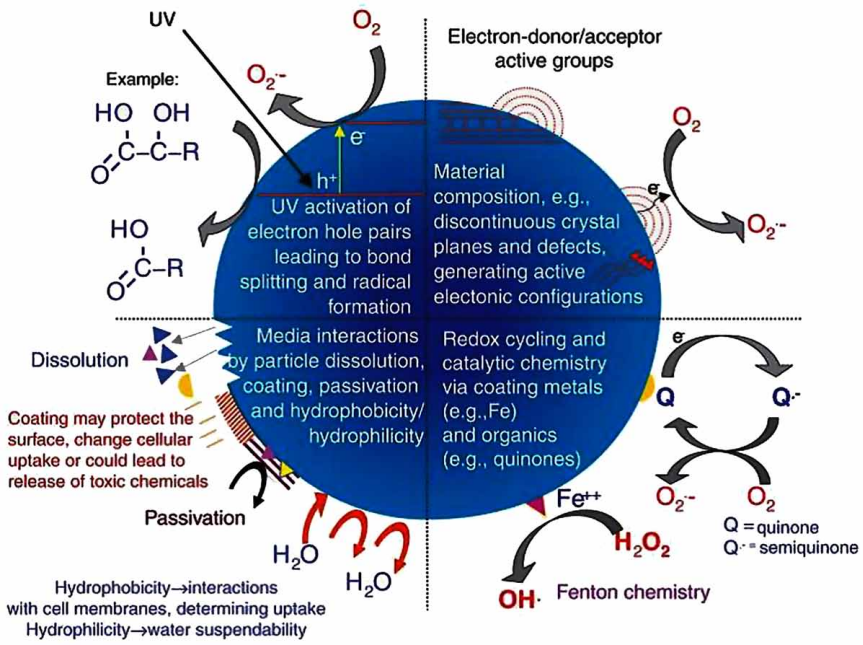
In view of the active functionalization and the possible interaction of NPs with bio-molecular structures, it is important to consider the dose and dose rate of the particulate agent, its ability to spread within the body and ecosystem, the decay of number concentration and the erosion of individual particles. Many nanoparticles will have considerable solubility.

For these materials the interaction with living systems remains close enough to the bulk chemical agent to justify the use of well-established toxicological testing procedures and approaches. For biodegradable particles, the particle composition and degradation products will influence their biological effects. On the other hand, materials with very low solubility or degradability could accumulate within living cells and persist there for long durations. It is with nanoparticles of this character that the greatest concerns must arise, and attention will have to be paid to the comparison of the persistence of the particles and the time constants of the metabolic and cellular activities within the target host.

Figure 7 illustrates the importance of material composition, electronic structure, bonded surface species, surface coatings (active or passive), and solubility, including the contribution of surface species and coatings and interactions with other environmental factors (e.g., UV activation).

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Figure 7. Possible mechanisms by which nanomaterials interact with biological tissue
Source: Nel et al., 2006.



STUDIES SHOWING HARMFUL EFFECTS OF NANOPARTICLES ON LIVING CELLS

Uses of engineered Nps in sunscreens (e.g., TiO_2 and zinc oxide ZnO) and cosmetics and as bioimaging probes (e.g., superparamagnetic iron oxides) or tumor-specific photothermal therapy agents, antiangiogenic agents, and molecular reporters (Au NPs, Ag NPs) have not lead to reports of clinical toxicity in humans.

Nowadays, some answers related to fundamental aspects of the interactions of NPs with living cells, have been investigated. Studies reported in the literature have delivered only limited principles about the nano-bio interfaces and thus the biological behavior of NPs is yet far from being completely understood.

The in-vitro cytotoxicity findings and the potential for exposures to ZnO NPs from different sources via different routes of entry into the body have raised public health concerns.

However, although inhalation of ultrafine ZnO particles at relatively high dose (500 mg/m3) for 2 hours did not induce acute systemic effects in humans, inhalation of ZnO fumes in an occupational setting can cause metal fume fever (fatigue, chills, fever, myalgias, cough, dyspnea, leukocytosis, metallic taste, and salivation) (Beckett et al., 2005). Only a limited number of NPs have so far been shown to exert toxicity in tissue culture and animal experiments, usually at high doses (Service, 2004; Oberdörster, 2005).

Although recent studies have shown the cytotoxic effects of these NPs, including oxidative stress, apoptosis and necrosis induction, genotoxicity, and others, irradiation-induced cytotoxicity has not been systematically studied. This was the aim of Yang & Ma study which focused on whether irradiation in the forms of visible light (approximately 400-600 nm), ultraviolet (UV) A (366 nm), and UVC (254 nm) would affect ZnO NPs-induced cytotoxicity. The results of their tests demonstrated that the cytotoxicity of 60 to 80 nm ZnO NPs to A549 cells is dosage, time, and wavelength dependent. Nuclear

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decomposition by ZnO NPs, prior to membrane deformation, was found to be enhanced when exposed to irradiation. This study suggested that this phenomenon may be attributed to the irradiation-induced formation of positively charged sites on ZnO NPs, which enhances nuclear affinity and generation of reactive oxygen species (ROS). Finally, the data demonstrated that while ZnO NPs act preferentially toward nuclear regions, destructions of cell membrane and the cytosol have also been observed. The photocatalytic properties of ZnO NPs play a critical role during the early stages of cell death, and their effects were reduced through the use of an antioxidant, N-acetylcysteine. In conclusion, they released that both visible light and UV irradiations have been found to enhance the cytotoxic effect of ZnO NPs on A549 cell line. This finding supports the need for further in-vivo exposure studies relevant to actual conditions to confirm whether combined irradiation and ZnO NPs exposure could enhance the nanotoxicity of ZnO NPs (Yang & Ma, 2014).

On the other side, not much work is available on the bio-interaction of ZnO NPs with astrocytes, the major cells involved in brain homeostasis. Therefore, S. Sruthi and P.V. Mohanan have focused on the interaction of ZnO NPs with rat C6 glial cells. The results of their findings reveal that NPs are taken up by the astrocytes and induce time and dose dependent toxicological response which is indicated by NPs uptake studies and cell viability assays. Also the results of DCFH-DA (2',7'-dichlorofluorescein diacetate) assay showed that ZnO NPs induce strong oxidative stress in cells at 3 and 6 h. However, at 24 h the reactive oxygen species (ROS) detected in NPs treated groups were same as that of control. The mode of cell death induced by ZnO NPs was apoptosis as revealed by the nuclear condensation studies, live dead assay using acridine orange/ethidium bromide and apoptosis detection kit. This bio-test, which explores the interaction of ZnO NPs with astrocytes, concludes that the persistence of particle can continue to have a damaging effect on the astrocytes. Finally they concluded that the time of exposure and particle clearance by the system should be evaluated more thoroughly to prevent the health hazards due to nanoparticles (Sruthi & Mohanan, 2015).

Figure 8 reports toxic effect of polyethyleneimine (PEI) functionalized zinc oxide (ZnO) Nps against different human cells.

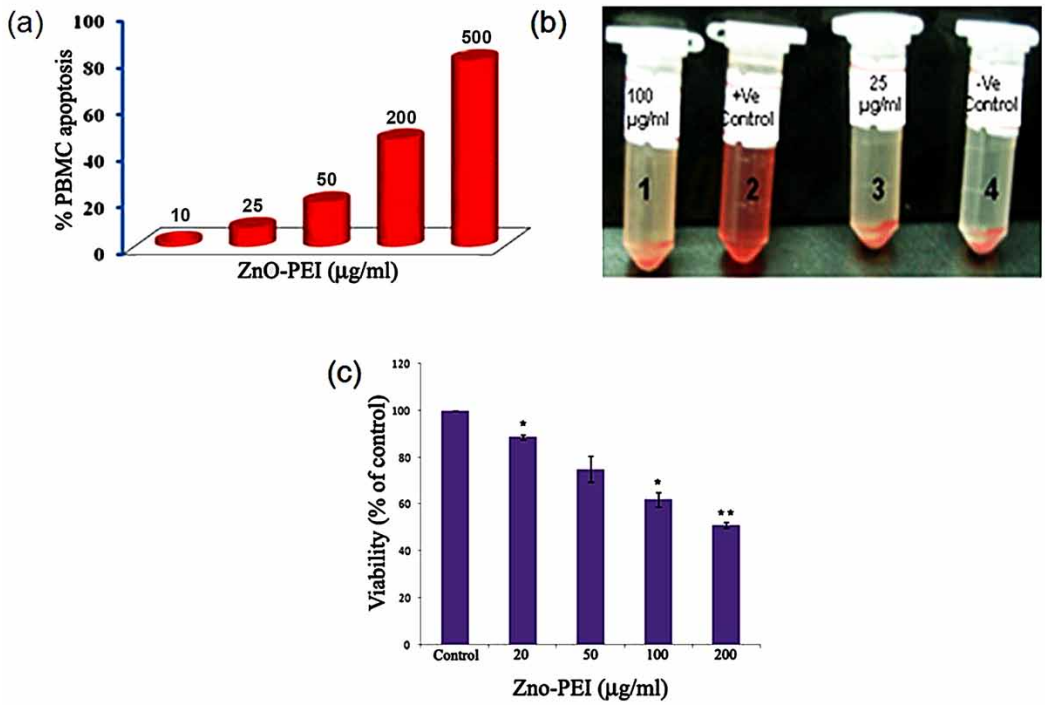
Titanium dioxide (TiO_2) is one of the most widely manufactured and consumed engineered and utilized NPs in numerous industries such as cosmetics, pharmaceuticals, water treatment and foods. Recent studies indicate that TiO_2 NPs have adverse effects on human health but there are limited studies that correlate the effects observed to specific biological pathways.

Intratracheal instillation of TiO_2 particles in rodents demonstrates that these nanoparticles induce bigger inflammatory responses than larger particles of an equivalent mass dose (Oberdörster, 2005). However, when the instilled dose is expressed as particle surface area, the inflammatory response fits the same dose-response curve (Colvin, 2003; Donaldson, 2002). This supports the concept that the surface area is the dose measurement that best predicts pulmonary toxicity (Oberdörster, 2005). In addition to the generation of pro-inflammatory effects, nanoparticles of various sizes and chemical composition are able to lodge in mitochondria (Oberdörster, 2005; Li, 2003). This can lead to disruption of the mitochondrial electron transduction chain, which leads to additional O_2^- production (Li, 2003). Further, ambient ultrafine particles perturb the mitochondrial permeability transition pore, which leads to the release of pro-apoptotic factors and programmed cell death (Li, 2003).

On the other hand, the toxicological effects of TiO_2 on liver which is the major accumulation site of TiO_2 NPs, directly through intentional ingestion or indirectly via NPs dissolution from food containers, have been concisely investigated. In the work reported by V. Natarajan et al., the concentration and phase dependent effect elicited by TiO_2 NPs on primary hepatocytes, has been investigated. Hepatocytes were

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*Figure 8. Toxicity of ZnO-PEI NPs against different human cells: (a) PBMC, (b) RBC, (c) AGS cells. In (b) tube ''2'' is the positive control (100% hemolysis); tube ''4'' is the control, where NP is absent and tubes ''3'' and ''1'' represent RBC at different concentrations of NP. * P,0.05, ** P,0.01.*
Source: Chakraborti et al., 2013



the most abundant cell type of the liver and perform numerous vital hepatic functions related to synthesis, catabolism and clearance. In this study, they exposed the primary hepatocytes to three different forms of commercially available TiO_2 NPs; Degussa P25, pure Anatase (50 nm) and pure Rutile (50 nm). TiO_2 exposure was resulted in a concentration dependent loss of cell viability and alteration in cell morphology. It was also observed an increase in production of Reactive Oxygen Species (ROS). Furthermore, liver-specific functions (urea and albumin synthesis) were also affected by TiO_2, P25 treated cells exhibiting the most significant loss in the functions. Currently, they were investigating the molecular mechanistic aspects of the observed change in cell phenotype, focusing primarily on the effect of oxidative stress and mitochondrial dysfunction induced by TiO_2 NPs (Natarajan et al., 2014).

Recently, increasing concern has been raised about the neurotoxicity of TiO_2 NPs. But the mechanisms underlying such toxic effects are still unknown. Z. Mao et al., have employed a human neuroblastoma cell line (SH-SY5Y) to study the effects of TiO_2 NPs on neurological systems. The results showed that TiO_2 NPs did not affect cell viability but induced noticeable morphological changes until 100 µg ml (-1). Immunofluorescence detection showed disorder, disruption, retraction, and decreased intensity of the microtubules after TiO_2 NPs treatment. Both α and β tubule expressions did not change in TiO_2 NPs treated group, but the percentage of soluble tubules was increased. A microtubule dynamic study in living cells indicated that TiO_2 NPs caused a lower growth rate and a higher shortening rate of microtubules as well as shortened lifetimes of de novo microtubules. TiO_2 NPs did not cause changes in the expression and phosphorylation state of tau proteins, but a tau-TiO_2 NPs interaction was observed. TiO_2 NPs

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could interact with tubule heterodimers, microtubules and tau proteins, which led to the instability of microtubules, thus contributing to the neurotoxicity of TiO_2 NPs (Mao et al., 2015).

A. Katsumiti et al. have investigated the effects of a set of TiO_2 NPs on the viability of mussel hemocytes and gill cells using neutral red and thiazolyl tetrazolium bromide assays. For this, they have compared the cytotoxicity of TiO_2 NPs (0.1-100 mg Ti/L) produced by different methods: rutile NPs (60 nm) produced by milling and containing disodium laureth sulfosuccinate (DSLS), rutile NPs (10, 40 and 60 nm) produced by wet chemistry and anatase/rutile NPs (~100 nm) produced by plasma synthesis. The commercially available P25 anatase/rutile NPs (10-20 nm) were also checked in their essays tested. Exposures were performed in parallel with their respective bulk forms and the cytotoxicity of the additive DSLS was also tested. Z potential values in distilled water indicated different stabilities depending on the NPs type and all NPs tested formed agglomerates/aggregates in cell culture media. In general, TiO_2 NPs showed a relatively low and dose-dependent toxicity for both cell models with the two assays tested. NPs produced by milling showed the highest effects, probably due to the toxicity of DSLS. Size-dependent toxicity was found for NPs produced by wet chemistry (10 nm > 40 nm and 60 nm). All TiO_2 NPs tested were more toxic than bulk forms excepting for plasma produced ones, which were the least toxic TiO_2 tested. The mixture bulk anatase/rutile TiO_2 was more toxic than bulk rutile TiO_2. The authors concluded, that the toxicity of TiO_2 NPs varied with the mode of synthesis, crystalline structure and size of NPs and can also be influenced by the presence of additives in the suspensions (Katsumiti et al., 2014).

In the last decade, numerous in vitro studies have shown that Ag NPs have potential to induce toxicity in cells derived from a variety of organs. The use of Ag NPs in cosmetics and textiles has substantially increased the potential for human skin exposure. Recently, a study was carried out by using an artificial human skin exposure, which demonstrated that silver could be released from antibacterial fabric products into sweat (Kulthong, Srisung, Boonpavanitchakul, Kangwansupamonkon, & Maniratanachote, 2010). Ag nanoparticles are found to induce cell death and oxidative stress in human fibrosarcoma and skin carcinoma cells (Arora, Jain, Rajwade, & Paknikar, 2008) and it was demonstrated that Ag NPs could enter cells and cause DNA damage and apoptosis in fibroblasts and liver cells (Arora, Jain, Rajwade, & Paknikar, 2009).

W. Liu et al. investigated the cytotoxicities of three types of Ag NPs; SNP-5, SNP-20 and SNP-50 with different sizes (approximately 5, 20 and 50 nm) using four human cell models (A549, SGC-7901, HepG2 and MCF-7). Endpoints included cell morphology, cell viability, cellular membrane integrity, oxidative stress and cell cycle progression. Observable deleterious effects on the cell morphologies and membrane integrity were induced by SNP-5 and SNP-20. SNPs elevated the ROS levels in cells and arrested the cells at Ag phase. Apoptosis occurred for 4-9% of the exposed cells. All these cellular responses as well as EC50 values were found to be size-dependent for the tested SNPs. Ultrastructural observations confirmed the presence of Ag NPs inside cells. Elemental analysis of Ag in cells by ICP-MS showed that smaller NPs enter cells more easily than larger ones, which may be the cause of higher toxic effects (Liu et al., 2010).

C. Arulvasu et al., (2014) revealed the toxic effect of Ag NPs in Artemia nauplii and evaluated the mortality rate, the hatching percentage, and the genotoxic effect in Artemia nauplii/cysts. The AgNPs were commercially purchased and characterized using field emission scanning electron microscope with energy dispersive X-ray spectroscopy. NPs were spherical in nature and with size range of 30-40 nm. In their assessment, Artemia nauplii (II instar) were treated using Ag NPs of various nanomolar concentrations and LC50 value (10 nM) and mortality rate (24 and 48 hours) was evaluated. This study

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showed that as the concentration of Ag NPs increased, the mortality rate, aggregation in gut region, apoptotic cells, and DNA damage increased in nauplii, whereas the percentage of hatching in Artemia cysts decreased. Thus this study revealed that the nanomolar concentrations of Ag NPs have toxic effect on both Artemia nauplii and cysts (C. Arulvasu, et al., 2014).

The toxic risk of Ag NPs usage, liver, as a detoxifying organ, is particularly important. This was the purpose study reported by I. V.Vrček et al. The authors explored the toxicity mechanisms of nano and ionic forms of Ag on human hepatoblastoma (HepG2) cells. The results showed that Ag ions and citrate-coated Ag NPs reduced cell viability in a dose-dependent manner. The IC50 values of Ag ions and citrate-coated Ag NPs were 0.5 and 50 mg L−1, respectively. The LDH leakage and inhibition of albumin synthesis, along with decreased ALT activity, indicated that treatment with either Ag NPs or Ag ions resulted in membrane damage and reduced the cell function of human liver cells. Evaluation of oxidative stress markers demonstrating depletion of GSH, increased ROS production, and increased SOD activity, indicated that oxidative stress might contribute to the toxicity effects of nano and ionic forms of Ag. The observed toxic effect of Ag NPs on HepG2 cells was substantially weaker than that caused by ionic Ag (Vrčekstudy et al., 2014).

Ag NPs are expected to end up in the aquatic environment. Mussels have been proposed by Katsumiti et al., (2015) as marine model species to evaluate NPs toxicity in-vitro. In this work, the mechanisms of toxicity of Ag NPs were studied on mussel hemocytes and gill cells, in comparison to ionic and bulk Ag. The cytotoxicity of commercial and maltose stabilized Ag NPs was screened in parallel with the ionic and bulk forms at a wide range of concentrations in isolated mussel hemocytes and gill cells using cell viability assays. Maltose-stabilized Ag NPs showed size-dependent cytotoxicity, smaller (20 nm) NPs being more toxic than larger (40 and 100 nm) NPs. Maltose alone provoked minor effects on cell viability. Ionic Ag was the most cytotoxic Ag form tested whereas bulk Ag showed similar cytotoxicity to the commercial Ag NPs. The main mechanisms of action of Ag NPs involved oxidative stress and genotoxicity in the two cell types, activation of lysosomal AcP activity, disruption of actin cytoskeleton and stimulation of phagocytosis in hemocytes and increase of MXR transport activity and inhibition of Na-K-ATPase in gill cells. Similar effects were observed after exposure to ionic and bulk Ag in the two cell types, although generally effects were more marked for the ionic form. In conclusion, results suggest that most of the observed responses were due at least in part to dissolved Ag (Katsumiti et al., 2015).

With the advance in materials science and the need to diversify market applications, Ag NPs are widely modified by different surface coatings. However, how these surface functionalization's influence the effects of Ag NPs on living cells and human health is still largely unknown. Pang et al., (2015) have evaluated the uptake, toxicity and pharmacokinetics of Ag NPs coated with citrate, polyethylene glycol, polyvinyl pyrolidone and ranched polyethyleneimine (Citrate Ag NPs, PEG Ag NPs, PVP Ag NPs and BPEI Ag NPs, respectively). It was demonstrated that the toxicity of Ag NPs depends on the intracellular localization that was highly dependent on the surface charge. BPEI Ag NPs (ζ potential = +46.5 mV) induced the highest cytotoxicity and DNA fragmentation in Hepa1c1c7. In addition, it showed the highest damage to the nucleus of liver cells in the exposed mice, which is associated with a high accumulation in liver tissues. The PEG Ag NPs (ζ potential = -16.2 mV) showed cytotoxicity, a long blood circulation, as well as bioaccumulation in spleen (34.33 μg/g), which suggest better biocompatibility compared to the other chemically modified Ag NPs. Moreover, the adsorption ability with bovine serum albumin revealed that the PEG surface of Ag NPs has an optimal biological inertia and can effectively resist opsonization or non-specific binding to protein in mice. The overall results of this assessment indicated that the biodistribution of Ag NPs was significantly dependent on surface chemistry: BPEI Ag NPs >

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Citrate Ag NPs = PVP Ag NPs > PEG Ag NPs. The authors finally concluded that this toxicological data could be useful in supporting the development of safe Ag NPs for consumer products and drug delivery applications (Pang et al., 2015).

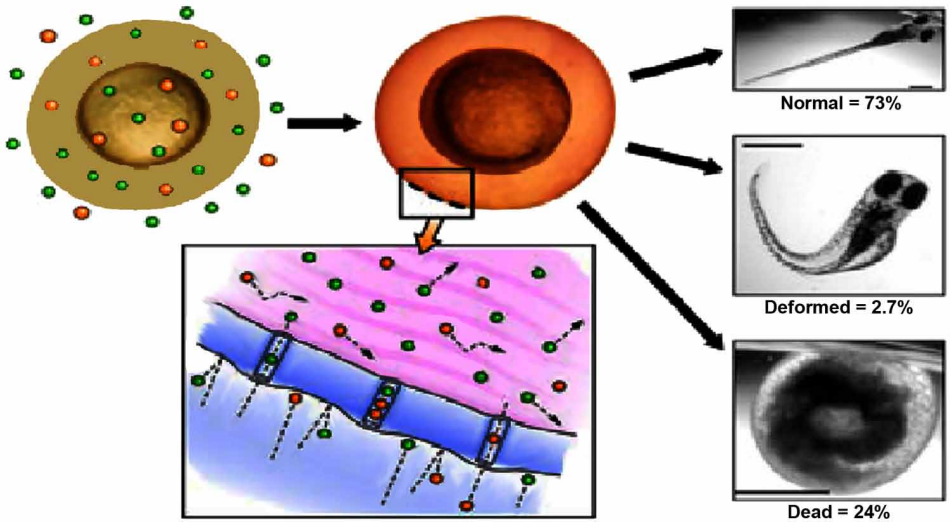
Several research groups have investigated the cellular toxicity of Au NPs with regard to particle shape, size, and surface group. Rotello et al. have investigated the toxicity of 2 nm Au NPs functionalized with both cationic and anionic surface groups in three different cell types. The results suggested that cationic particles are generally toxic at much lower concentrations than anionic particles, which can be related to the electrostatic interaction between the cationic NPs and the negatively charged cell membranes (Goodman, McCusker, Yilmaz & Rotello, 2004). Chithrani group have examined the uptake of Au NPs of various shapes and sizes into HeLa cells, a well-known line of human cervical cancer cells (Chithrani, Ghazani, & Chan, 2006).

Au NPs are materials with unique optical properties that have made them very attractive for numerous biomedical applications. With the increasing discovery of techniques to synthesize novel NPs such as star-shaped Au nanostars for biomedical applications, the safety and performance of these new NMs must be systematically assessed before their practical use. In the study conducted by Pelagie Marlene Favi et al., Au nanostars (AuNSTs) with multi-branched surface structures were synthesized and their influence on the cytotoxicity of human skin fibroblasts and rat fat pad endothelial cells (RFPECs) were assessed and compared to gold nanospheres (AuNSPs) with un-branched surfaces. The results indicated that AuNSPs with diameters of approximately 61.46 nm showed greater toxicity with fibroblast cells and RFPECs compared to the synthesized AuNSTs with diameters of approximately 33.69 nm. AuNSPs were lethal at concentrations of 40 μg/mL for both cell lines, while AuNSTs were less toxic at higher concentrations (400 μg/mL). The calculated IC50 (50% inhibitory concentration) values of Au NSPs exposed to fibroblast cells were greater at 1 and 4 days of culture (26.4 and 27.7 μg/mL, respectively) compared to the RFPECs (13.6 and 13.8 μg/mL, respectively), indicating that AuNSPs have a greater toxicity to endothelial cells. It was proposed that the possible factors that could be promoting the reduced toxicity effects of AuNSTs to fibroblast cells and RFPECs, compared to AuNSPs may be size, surface chemistry, and shape of the Au NPs. The reduced cell toxicity observed with AuNSTs suggests that the latter may be a very promising material for use in biomedical applications (Favi et al., 2015).

Combining computational tools to experimental approaches helps to precisely probe the nano-bio interface and allows the development of predictive and descriptive relationships between the structure and the activity of NMs. In this regard Bigdeli group has statistically established a nano-quantitative structure-activity relationship (nano-QSAR) model, using Partial Least Squares Regression (PLSR) model. Also, variable importance on PLS projections (VIP) has been used to find the most responsible factors on NPs exocytosis. Physicochemical properties of a set of different sized Au NPs with different surface coatings were greatly correlated to their exocytosis in macrophages. The results of this study suggest that among the pool of physicochemical properties defined as nano-descriptors, charge density and surface charge seem to be the paramount factors leading to higher exocytosis values. Furthermore, charge accumulation and circularity of NPs are in the next level of priority among other nano-descriptors. The regression based nano-QSAR model reported in this work is satisfactory in both statistical quality and interpretability. The authors concluded finally that the results could serve as a quantitative framework for better understanding the mechanisms that govern the interactions at the nano-bio interface (Bigdeli et al., 2015). Figure 9 presents an illustration of the accumulation mechanism of Gold NPs in the embryos of zebrafish.

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Figure 9. Schematic of the research findings. Gold nanoparticles accumulate in the embryos. Optical images of zebrafish at a given concentration of gold nanoparticles.
Source: Image - Lauren M. Browning, X. Nancy Xu, Old Dominion University



Superparamagnetic iron oxide NPs (IONPs) have attracted a great interest for use in several biomedical fields. In general, they are considered biocompatible, however little is known about their effects on the human nervous system. C. Costa et al. evaluated the cytotoxicity of two IONPs (magnetite), coated with silica and oleic acid, previously determining the possible interference of the IONPs with the methodological procedures to assure the reliability of the results obtained. Human neuroblastoma SHSY5Y and glioblastoma A172 cells were exposed to different concentrations of IONPs (5-300 µg ml(-1)), prepared in complete and serum-free cell culture medium for three exposure times (3, 6 and 24 h). Cytotoxicity was evaluated by means of MTT, neutral red uptake and alamar blue assays. Characterizations of the main physical-chemical properties of IONPs were also performed. The results of this assessment have demonstrated that both Fe_2O_3 NPs could significantly alter absorbance readings. To reduce these interferences, protocols were modified by introducing additional washing steps and cell-free systems. Significant decreases in cell viability were observed for both cell lines in specific conditions by all assays. In general, oleic acid-coated IONPs were less cytotoxic than silica-coated IONPs; besides, a serum-protective effect was observed for both IONPs studied and cell lines. These results contribute to increase the knowledge of the potential harmful effects of IONPs on the human nervous system (Costa et al., 2015).

Ge group has evaluated the cytotoxicity effect of dimercaptosuccinic acid-coated iron oxide (DMSA-Fe_2O_3) using cultured human aortic endothelial cells (HAECs). Their results showed that DMSA- Fe_2O_3 in the culture medium could be absorbed into HAECs, and dispersed in the cytoplasm. The cytotoxicity effect of DMSA- Fe_2O_3 on HAECs was dose-dependent, and the concentrations no more than 0.02 mg/ml had little toxic effect which were revealed by tetrazolium dye assay. Meanwhile, the cell injury biomarker, lactate dehydrogenase, was not significantly higher than that from control cells (without DMSA- Fe_2O_3). However, the endocrine function for endothelin-1 and prostacyclin I-2, as well as the urea transporter function, was altered even without obvious evidence of cell injury in this context. It was also shown by real-time PCR analysis that DMSA- Fe_2O_3 exposure was resulted in differential effects on the expressions of pro- and anti-apoptosis genes of HAECs. It was noted that DMSA- Fe_2O_3 exposure could activate the

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expression of genes related to oxidative stress and adhesion molecules, which suggested that inflammatory response might be evoked. Moreover, it was demonstrated by in-vitro endothelial tube formation that even a small amount of DMSA- Fe_2O_3 (0.01 and 0.02 mg/ml) could inhibit angiogenesis by the HAECs. Altogether, the results indicate that DMSA- Fe_2O_3 have some cytotoxicity that may cause side effects on normal endothelial cells (Ge et al., 2013).

Zavisova et al., (2014) have studied the toxicity of magnetite NPs modified with bioavailable materials such as dextran, bovine serum albumin, polyethylene glycol, and polyvinylpyrrolidone in normal and cancer cells. The size distribution and magnetic properties of the modified magnetic NPs were characterized by different techniques. Transmission electron microscopy showed a nearly spherical shape of the magnetite core with diameters ranging from 4 to 11 nm. Dynamic light scattering was employed to monitor the hydrodynamic size and colloidal stability of magnetic NPs: Z-average hydrodynamic diameter was between 53 and 69 nm and zeta potential in the range from −35 to −48 mV. Saturation magnetization of the modified NPs was 55-64 emu/g Fe_3O_4. The authors found that the prepared biocompatible NPs had no significant toxic effect on Chinese hamster lung fibroblast cell line V79, however they substantially affected mouse melanoma B16 cell line (Zavisova et al., 2014).

CONCLUSION

With growing public awareness about the potential harmful effects of novel technologies, the world today is viewed with more doubt than before. Thus, scientists aiming to introduce new technology must be more assured of the benignity of their contribution.

Understanding the toxic effects of NPs on the environment and human health is the biggest obstacle to the safe development of nanotechnology. However, little is known about their toxic mechanisms. The main important question is whether the indefinite hazard of engineered NPs particularly their environmental impact are compensating their recognized benefits for human daily life.

To fully understand and predict possible health effects following exposure to NPs, information about their physicochemical properties and NPs -living cell interactions are indispensable.

In this chapter, we have tried to collect, present and summarize recent studies from the literature on the effect of engineered NPs on living cells which may ultimately establish exposure standards and recommended handling practices to avert significant human health risks in the future. Better understanding these effects is essential to establish satisfactory regulatory policies on the safe use of NPs by human.

Currently successful application of NPs for therapeutic objects needs their effective uptake by the living cell. Hence, studying of the interaction between NPs and cell membrane for effective cellular uptake seems to be vigorous and imperative. Transferring of lipids, proteins, glucose, and other biomaterials into living cells is possible from two major exocytic and endocytic pathways. The penetration ability of NPs into the living cell must be considered in manufacturing nanotechnology of these particles. Enormous in vivo and in vitro experiments in the field of nanotechnology have confirmed the effect of physiochemistry properties in state of NPs /living cell interactions. Thus, the parameters directly related to physicochemical characteristics (e.g., size and shape) and biopersistence, or to specific interaction with biomolecules through their surface properties (e.g., surface chemistry and reactivity) or release of toxic ions have to be optimized through the preparation process or during the bio-tests for improving therapeutic effects of nanocarriers.

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Besides, living cells exposed to NPs may undertake repairable oxidative stress and DNA damage or be induced into apoptosis. Exposure to NPs may cause the cells to alter their reproduction or differentiation or their cell-cell signaling with neighboring cells in a tissue. NPs are usually more toxic to some cell subpopulations than others, and toxicity often varies with cell cycle. All of these facts dictate that any nanotoxicity assay must be applied at the single-living cell level and try whenever viable to include one or many of other factors. Thus, focusing on one type of quantitative measure of nanotoxicity, is very promising to obtain more focused information about interaction between NPs and living cells.

Finally, it is our trust and recommendation that every nanoparticle-based product must be subjected to appropriate nanotoxicity tests and associated assessments before being used.

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Chapter 54
Cyanobacterial Toxins in Water Sources and Their Impacts on Human Health

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ABSTRACT

Cyanobacteria are a group of phytoplankton of marine and freshwaters. The accelerated eutrophication of water sources by agricultural and industrial run-off has increased the occurrence and intensity of cyanobacterial blooms. They are of particular concern because of their production for potent hepato-, neuro-, and dermatoxins, being hazardous to human health. Dissemination of knowledge about cyanobacteria and their cyanotoxins assists water supply authorities in developing monitoring and management plans, and provides the public with appropriate information to avoid exposure to these toxins. This chapter provides a broad overview and up-to-date information on cyanobacteria and their toxins in terms of their occurrence, chemical and toxicological characteristics, fate in the environment, guideline limits, and effective treatment techniques to remove these toxins from drinking water. Future research directions were also suggested to fill knowledge and research gaps, and advance the abilities of utilities and water treatment plant designers to deal with these toxins.

INTRODUCTION

Cyanobacteria are ancient prokaryotic microorganisms that have been in existence for at least 2.7 billion years and the first organisms to have released oxygen into the primitive atmosphere (Zanchett & Oliveira-Filho, 2013). Cyanobacteria were formerly regarded as blue-green algae because of the presence of chlorophyll a and performing oxygen-evolving photosynthesis in a manner very similar to algae. They occupy a broad range of habitats including freshwater, marine, terrestrial and even extreme niches such as hot springs. In aquatic ecosystems, cyanobacteria constitute the most important compartment in the food web, as they are one of the main primary producers of organic matter and serve as a food source for aquatic organisms. However, cyanobacteria become a problem only when they increase to excessive numbers forming water blooms under eutrophication conditions (high nutrient concentrations) along

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with suitable light and temperature. These blooms can also consume all of the oxygen in the water when they die and decompose, leaving a dead zone (hypoxia) where fish and other animals cannot survive (Diaz & Rosenberg, 2008). The hypoxic dead zone is typically found in the central basin of Lake Erie for decades (Zhu, Michalak, Beletsk, Rao, & Richards, 2015). Additionally, because many species of cyanobacteria are capable of producing potent toxins called cyanotoxins, these blooms are harmful and may lead to many negative environmental impacts such as mortality of domestic and wild animals, and unsustainability of the overall ecosystem. Cyanotoxins vary in their chemical structure and mode of toxicity in human including hepatotoxins, neurotoxins, and irritants and gastrointestinal toxins (Codd, Morrison, & Metcalf, 2005). These cyanotoxins may lead to a wide array of biological impacts on aquatic organisms, including: allelopathic effects on other phytoplankton (Suikkanen, Fistarol, & Granéli, 2004); suppression of zooplankton grazing, leading to reduced growth and reproductive rates and changes in dominance, toxic effects on fish and accumulation of toxins in tissues of invertebrates (Lehtiniemi et al., 2002) and fish (Mohamed, Carmichael, & Hussein, 2003). Furthermore, cyanotoxins are known to affect a number of processes in plants, and their presence in water used for irrigation may have considerable impact on the growth and development of crop plants (Wiegand & Pflugmacher, 2005). The exposure of edible crop and vegetable plants to cyanotoxins is also a concern for human health, as the toxins can accumulate in plant tissues and might be transferred into the food chain (Mohamed & Al-Shehri, 2010). Cyanotoxins are not easily removed by conventional treatment techniques as they are resistant to heat and most physico-chemical factors. Consequently, the presence of such potent toxins in water sources poses a risk to human health upon exposure to them. Additionally, *In vitro* and *in vivo* studies conducted by Zhou, Yu, & Chen (2002) and Svircev, Krstic, Miladinov-Mikov, Baltic, & Vidovic (2009) provided evidences that chronic exposures to low concentrations of the cyanotoxins contribute to the increased the risk for primary liver in China and Serbia and colon cancer development (Zegura, Volcic, Lah, & Filipic, 2008), as microcystin is mutagenic tumor promoter and inhibit DNA repair (Zegura, Straser, & Filipic, 2011). This chapter provides information on cyanotoxins and their human health risks in water sources because many water suppliers may have little understanding of how to manage them, particularly in rural communities and developing countries. This chapter covers six parts: section 1 introduces the occurrence of toxin-producing cyanobacteria in different water sources; section 2 presents the environmental factors promoting cyanobacterial blooms; section3 describes the human health impacts of cyanotoxins; section 4 presents the exposure routes to cyanotoxins; section 5 highlights management strategies and guideline levels of cyanobacteria and their cyanotoxins in the water sources; and finally section 6 sheds some light on the sustainability of cyanotoxins in the aquatic environment.

BACKGROUND ON OCCURRENCE OF TOXIN-PRODUCING CYANOBACTERIA

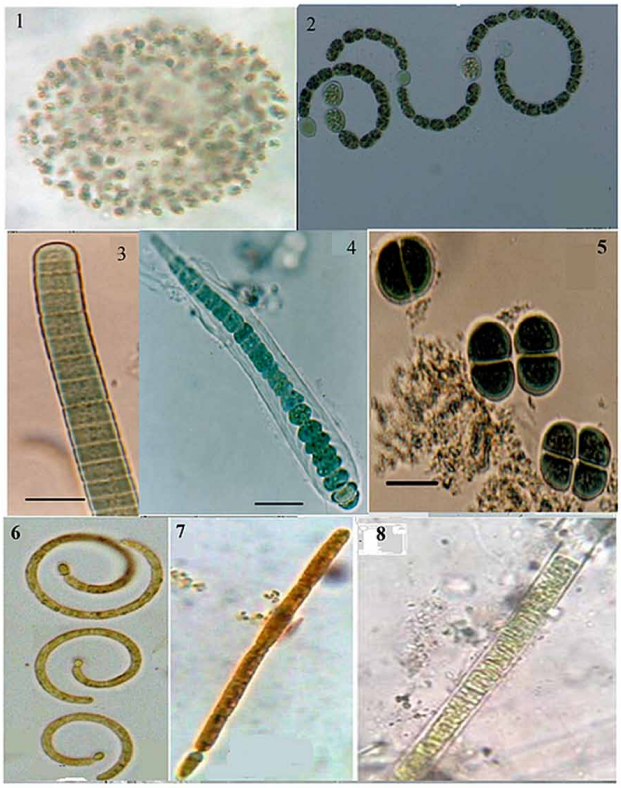
Cyanobacteria can be found in diverse environments including freshwater bodies, oceans and estuaries, extreme environments such as cold and hot springs, and Antarctic lakes (Taton et al., 2006; Mohamed, 2008). The ability of cyanobacteria to tolerate huge changes in salinity and temperature and survive low light intensity gives them an extremely large competitive advantage in these environments (Funari & Testai, 2008). Cyanobacteria can multiply rapidly in surface waters and form blooms when favorable conditions prevail, such as high temperature, intense light, high pH, and increased availability of nutrients, especially phosphorous and nitrogen, artificially released by anthropogenic activities (Funari & Testai, 2008). Blooms will persist in waters with temperatures between 15 and 30°C and pH levels

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between 6 and 9, with maximum growth rates occurring at temperatures in excess of 25°C (Robarts & Zohary, 1987). Most, if not all, bloom-forming cyanobacteria in freshwater bodies are planktonic such as *Microcystis* spp., *Cylindrospermopsis raciborskii*, *Planktothrix rubescens*, *P. agardhii*, *Gloeotrichia* spp., *Anabaena* spp, *Aphanizomenon* spp. (Figure 1). Planktonic cyanobacteria can also be found in marine environments including *Trichodesmium* and *Nodularia*, which produce heavy toxic blooms during summer months (Charpy, Casareto, Langlade, & Suzuki, 2012).

In addition to planktonic cyanobacteria, benthic cyanobacteria can proliferate at the bottom of lakes, rivers and coastal waters forming dense mats near the shore. The presence of benthic cyanobacterial mats in water sources is of public concern as many studies reported that benthic cyanobacteria could produce hepatotoxins and neurotoxins (Catherine et al., 2013). Despite they have been linked to numerous animal deaths worldwide, there is a little information regarding the distribution, toxin production and species composition of toxic benthic cyanobacterial communities, compared to planktonic species. Cyanotoxins have been assigned for many benthic cyanobacterial species in both marine and freshwater ecosystems (Zanchett & Oliveira-Filho, 2013; Mohamed & Al-Shehri, 2015, Table 1). Toxic benthic species of cyanobacterial mats have been found in hot springs, a habitat characterized by harshness and extreme conditions. As most hot springs are accessible to the public worldwide, as recreational places for citizens and tourists and some are also used as cooking resources by rural communities, human exposure

Figure 1. Most common toxic cyanobacterial species: (1) Microcystis aeruginosa; (2) Anabaena sp.; (3) Oscillatoria sp.; (4) Calothrix sp.; (5) Chroococcus sp.; (6, 7) Cylindrospermopsis raciborskii; (8) Lyngbya majuscula.



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Table 1. Toxicological properties of main cyanotoxins produced by cyanobacteria (Adapted from Funari & Testai, 2008, Carey et al., 2012, Catherine et al., 2013, and Zanchett & Oliveira-Filho, 2013).

Cyanotoxin	Chemical Nature	Producer	*I.P. LD_{50} (µg/kg b.w)	Target Organ	Mode of Action	Genotoxicity/ Carcinogenicity
Hepatotoxins						
Microcystins	Cylclic peptide	*Microcystis spp., Anabaena* spp. *Nostoc sp., Planktothrix, Snowella, woronichinia, Anabeanopsis,Oscillatoria* spp., *Gloeotrichia, Synechococcus, Synchocystis sp., Rivularia Phormidium, Leptolyngbya*	50-1200	Liver	PP1 and PP2A phosphatases inhibition-Tumor promotion	Genotoxic, tumor promoters- DNA damage and interfere with DNA damage repair pathways
Nodularins	Cylclic peptide	*Nodularia spumigena*	50	Liver	PP1 and PP2A phosphatases inhibition-Tumor promotion	Genotoxic, tumor promoters-DNA damage and interfere with DNA repair pathways
Cylindrospermopsin	Alkaloids	*Cylindrospermopsis raciborskii, Umezakia natans, Aphanizomenon spp., Rhaphidiopsis curvata, Anabaena, Lyngbya*	200-2100	Liver/ Kidney	irreversible inhibition of protein synthesis	pro-genotoxic, induces DNA strand breaks, potential carcinogen
Neurotoxins						
Anatoxin-a	Alkaloids	*Anabaena spp., Aphanizomenon spp., Oscillatoria, Cylindrospermum,* Phormidium, *Planktothrix, Raphidiopsis mediterranea*	375	Neuro-muscular system (Nerve synapse)	Depolarizing effect: Irreversible bind to nicotinic acetylcholine receptor	No data on genotoxic or carcinogenic effects
Anatoxin-a (s)	Alkaloids, organo-phosphate	*Anabaena spp., Aphanizomenon*	20-40	Peripheral nervous system	Irreversible inhibitor of acetyl-cholinesterase e, nerve hyper-excitability	No genotoxic or carcinogenic effects
Saxitoxins	Carbamate alkaloids	*Anabaena, Aphanizomenon spp., Cylindrospermopsis raciborskii, Lyngbya wollei, Scytonema sp., Tricodesmium erythraeum*	10-20	Nerve axons	block membrane sodium channels	No data on genotoxic or carcinogenic effects
BMAA	Amino acid (Alkaloid precursor)	Most Cyanobacteria, *e.g. Microcystis, Anabaena, Nostoc, Planktothrix spp*	10–30 nM	Neurons	interference with cystine/glutamate receptor, neurodegenerative diseases, e.g. Alzheimer, Parkinson and Amyotrophic Lateral Sclerosis	No data on geno-toxic or carcinogenic effects

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Table 1. Continued

Cyanotoxin	Chemical Nature	Producer	*I.P. LD_{50} (µg/kg b.w)	Target Organ	Mode of Action	Genotoxicity/ Carcinogenicity
Dermatotoxins						
Aplysiatoxins Debromoaplysiatoxin	Alkaloid	*Lyngbya, Oscillatoria, Planktothrix*	**107-117**	Skin	potent tumor promoters, acting through potentiation of protein kinase C (PKC)	Tumor promoter
Lyngbyatoxins	Alkaloid	*Lyngbya majuscula*	**300**	Skin, gastro-intestinal tract	potent tumor promoters, acting through potentiation of protein kinase C (PKC)	Tumor promoter
Gastrointestinal irritant toxins						
Lipopolysaccharide endotoxins	Lipopolysacch-aride	All cyanobacteria	**40-190**	Skin/ gastro-intestinal tract	Inflammatory agents,, gastroenteritis	Possible pro-carcinogenic

*I.P. LD50 = Intraperitoneal median lethal dose in microgram per kilogram of animal body weight

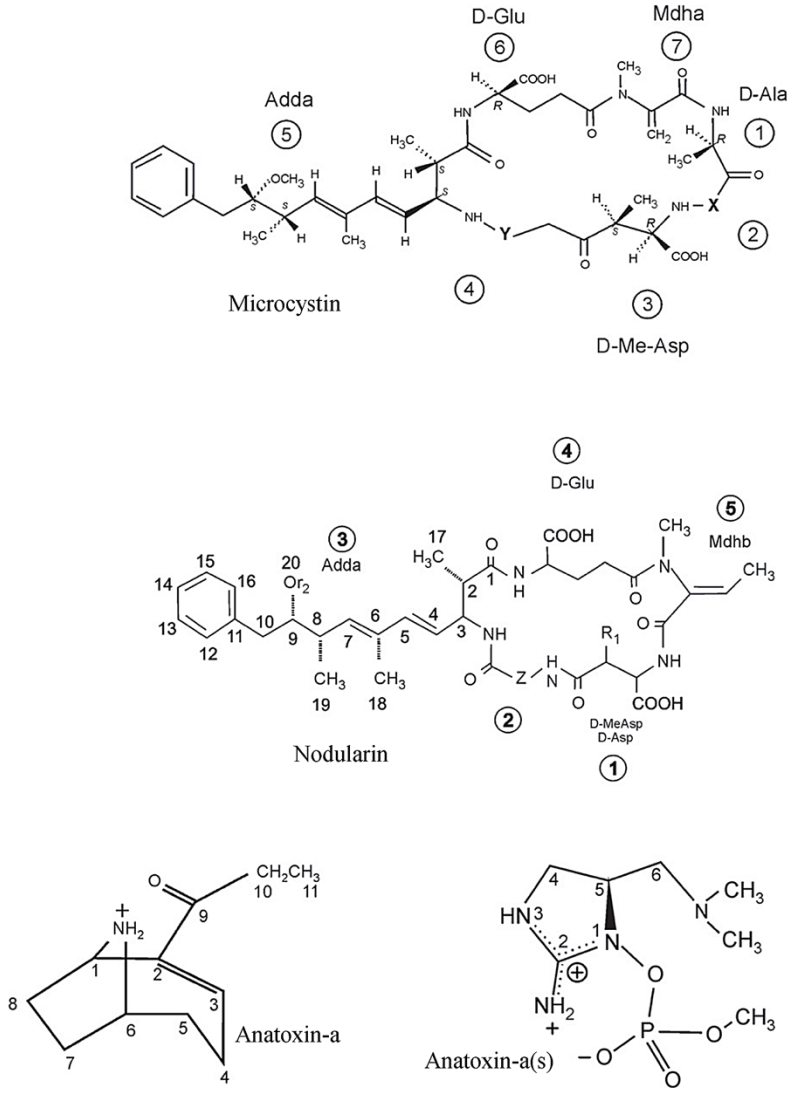
can easily occur (Mohamed, 2008). Toxin production has also been reported for epiphytic cyanobacteria on macrophytes in the aquatic environment, and caused the avian vacuolar myelinopathy (Wilde et al., 2014). These aquatic macrophytes provide ideal substrate for attachment of epiphytic toxic cyanobacteria, which may pose toxicological risk to marine organisms upon feeding on such toxic cyanobacteria or by the exposure to their potent cyanotoxins. Furthermore the invertebrates feeding on toxic cyanobacteria can act as vectors transferring cyanobacterial toxins to organisms at higher trophic levels in the food web. This leads to a potential poisoning of human populations that eat contaminated seafood from these areas. Therefore, epiphytic cyanobacteria should be considered when evaluating risk of harmful algae in fresh and marine water bodies in order to prevent possible adverse effects of their occurrence.

Depending on the human organ affected, the known cyanotoxins are categorized into hepatotoxins, neurotoxins, dermatotoxins, and gastrointestinal irritant toxins (Codd et al., 205; Figure 2, 3).

The hepatotoxins produced by cyanobacteria include cyclic peptides with 7 amino acids, microcystins (MCs) or with five amino acids, nodularin (NOD), and the alkaloid cylindrospermopsin (CYN). The cyanobacterial neurotoxins include the alkaloid anatoxins (ANTXs) and saxitoxins (STXs) and the non-protein amino acid β-methylamino-L-alanine (BMAA). Dermatotoxins produced by cyanobacteria include the indole alkaloid lyngbyatoxins (LTXs) and the bromophenol aplysiatoxins (APTXs) (Rzymski & Poniedziałek, 2012). The gastrointestinal irritant cyanotoxins are a structural constituent of the outer layer of gram negative prokaryotes including enteric bacteria and cyanobacteria, and composed of lipopolysaccharide (LPS) (Jann & Jann, 1984).

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Figure 2. Chemical structure of some cyanotoxins produced by cyanobacteria

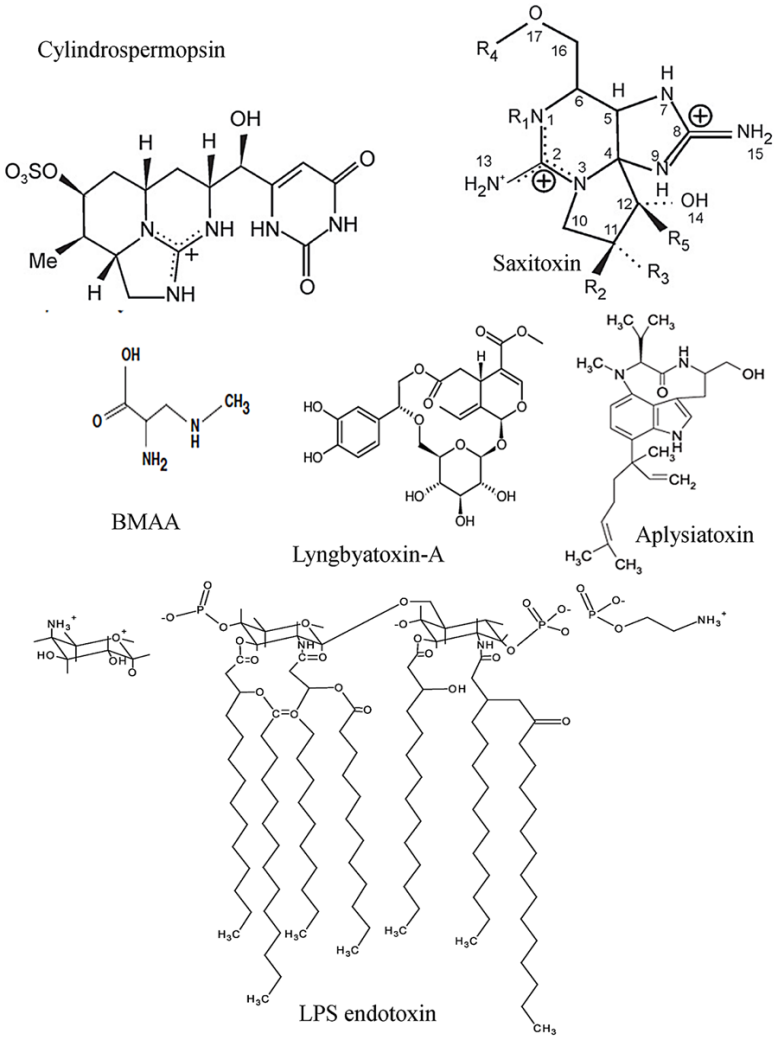


ENVIRONMENTAL FACTORS PROMOTING CYANOBACTERIAL BLOOMS

The growth and toxin production of cyanobacteria can be influenced by some environmental factors such as temperature, light intensity, pH, nutrients, and environmental pollutants (Neilan, Pearson, Muenchhoff, Moffitt, & Dittmann, 2013). However, the empirical modeling has shown that phosphorus, nitrogen and temperature are the most important drivers for cyanobacterial proliferation and harmful bloom formation (Beaulieu, Pick, & Gregory-Eaves, 2013). Over-enrichment of freshwater and marine ecosystems by nitrogen (N) and phosphorus (P) from anthropogenic activities (urban, agricultural, and industrial) promotes cyanobacterial growth and harmful bloom formation (Paerl, Hall, & Calandrino, 2011). However, it seems that the cyanobacterial growth depends largely on N:P ratio rather than concentrations of absolute N and P in the aquatic environment. Cyanobacteria successfully proliferate and form blooms

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Figure 3. Chemical structure of some cyanotoxins produced by cyanobacteria



at high P concentrations and low N:P ratio. Lakes with cyanobacteria dominance have total nitrogen: total phosphorus (TN:TP) ratio <29:1 by mass, and lakes without such dominance have TN:TP>29:1 (Smith,1983). Eutrophic and hypertrophic lakes are usually characterized by lower N:P ratio, and thus dominated by cyanobacteria (Liu, Lu, & Chen, 2011). In addition to nutrients, high temperatures of surface water (> 20 ºC) favor cyanobacterial growth and bloom formation (Pearl & Paul, 2012). This implies that climate change, in particular global warming may promote the growth and the dominance of harmful cyanobacterial blooms (Paerl et al., 2011). This may occur through a variety of mechanisms including: warming of surface water, which exhibits optimal growth of cyanobacteria and lengthens the period of stratification leading to long-time buoyancy of cyanobacteria; intense precipitation and heavy rainfall increase enrichment of water bodies with land-derived nutrients through enhanced erosion surface runoff and groundwater discharge; and high evaporation leading to rising in salinity of freshwaters systems, which allows for the expansion of salinity-tolerant cyanobacteria into brackish waters, and causes

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leakage of cells of non-tolerant cyanobacteria and the release of their cyanotoxins (Paerl et al., 2011). Besides their effects on cyanobacterial growth, some environmental factors such as nutrient concentration, light intensity, and temperature could also affect cyanotoxins production (Paerl & Paul, 2012; Holland & Kinnear, 2013). These effects vary among environmental parameters and types of cyanotoxins. For instance, anatoxins production was found to increase with nutrient limitation, light, temperature, and not related with cell growth, but microcystin production is associated with growth rate (Boopathi & Ki, 2014). Furthermore, environmental factors could regulate cyanotoxin production at molecular level (Neilan et al., 2013; Boopathi & Ki, 2014), as many strains have the capability to turn certain genes on or off depending upon environmental conditions (Merel et al., 2013). This means that strains with and without the genetic potential for cyanotoxin production may co-occur in natural systems at the same time.

HUMAN HEALTH IMPACTS OF CYANOTOXINS

Cyanotoxins vary widely in their chemical nature and structure, toxicity, mode of action and organ affected (Codd et al., 2005). Most of the hepatotoxins, MCs and NOD are water-soluble and stable molecules (Sivonen & Jones1999), bind to and inhibit protein phosphatases 1 and 2A (MacKintosh, Beattie, Klumpp, Cohen, & Codd, 1990), leading to the accumulation of phosphorylated proteins in the liver, cell necrosis, massive hemorrhage and death, depending on the dose. Human intoxication by MCs has been demonstrated by many authors in different countries. For instance, over 50 dialysis patients died after exposure to dialysate prepared from microcystin-contaminated drinking water derived from surface sources in Brazil (Carmichael et al., 2001). Lévesque et al. (2014) documented a relationship between gastrointestinal symptoms and contact of residents who receive their water from a plant equipped with a full treatment system whose source is highly contaminated by cyanobacteria and microcystins in Canada. High incidence of primary liver cancer was associated with the consumption drinking water contaminated with cyanobacterial toxins in China (Svircev et al., 2009). However, no human intoxication of NOD has been reported so far, and no guidelines have been proposed for drinking water due to the lack of suitable toxicological data. The alkaloid hepatotoxin, CYN is highly polar compound and mainly impacts the liver via the irreversible inhibition of protein synthesis with LD_{50} of 2100 µg/kg in mice, 24 h after i.p. injection (van Apeldoorn, van Egmond, Speijers, & Bakker, 2007). However, CYN exposure can also lead to fetal toxicity and tumor initiation (Falconer & Humpage, 2001). The hepatotoxicity of CYN to human has been shown in Australian and Brazilian (Carmichael et al., 2001; Griffiths & Saker, 2003). In addition to acute toxicity, cyanobacterial hepatotoxins may also bring genotoxicity and carcinogenicity upon exposure to low toxin levels (Table 1). This should be taken into consideration in toxicity assessment, especially in case of the toxins of long persistence in water, such as MCs. As MCs and NOD are inhibitors of protein phosphatases, they may affect activity of DNA-dependent protein kinase that is the key enzyme involved in the non-homologous end joining (NHEJ) of radiation induced DNA strand breaks (Lankoff, Wojcik, Fessard, & Meriluoto, 2006). This means that MCs are genotoxic and can act as tumor initiators. Genotoxicity of cyanobacterial extract and MCs has been studied *in vitro* and *in vivo* in different mammalian test systems (Zegura et al., 2011). Most studies have reported that the genotoxic activity of MCs is mediated by induction of reactive oxygen species (ROS) formation, leading to the formation of DNA strand breaks and mutagenic oxidative DNA lesions. MCs have also been shown to inhibit DNA repair pathways, which is a very important factor involved in carcinogenesis (Douglas, Moorhead, Ye, & Lees-Miller, 2001). NOD also can induce oxidative DNA

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damage and apoptosis in HepG2 cells, leading to genetic alterations and carcinogenesis (Lankoff et al. 2006). CYN induced micronuclei formation in human liver-derived HepG2 cells along with nuclear bud and nucleoplasmic bridge formation, with the cytokinesis block micronucleus assay after 24 h exposure to 0.05 and 0.5 μg CYN ml^{-1} (Straser, Filipič, & Žegura, 2011). It can also induce DNA strand breaks in human HepG2 cell line (Straser et al., 2011), and in human peripheral blood lymphocytes (Zegura et al., 2011) after exposure to lower CYN concentrations. Furthermore, the carcinogenic potential of CYN was investigated *in vitro* using Syrian hamster embryo (SHE) assay at very low concentrations (Maire et al., 2010). However, evidence of carcinogenic potential of CYN *in vivo* is limited, with only one study reporting tumors in 5 mice out of 53 who were orally dosed with cell-free extracts of cylindrospermopsin producing *C. raciborskii* (Falconer & Humpage 2001).

Cyanobacterial neurotoxins affect the nervous systems in different mechanisms. ANTX-a binds to acetylcholine receptors and remains available to overstimulate muscle, being degraded by acetylcholinesterase (Osswald, Rellán, Gago, &Vasconcelos, 2007). Consequently, death can occur by respiratory arrest when muscles involved in breathing activity are affected. ANTX-a is very toxic with LD_{50} of 375 μg/kg leading to death within 24 hours after i.p. injection in mice (Table 1). Anatoxin-a (S) inhibits acetylcholinesterase and induces muscular paralysis with potential death by respiratory arrest (Molica et al., 2005). It is a highly toxic with LD_{50} of 20 μg kg^{-1} body weight (mouse ip) (Table 1). ANTXs have caused poisoning for various animals e.g. sheep, birds dogs and cattle (Krienitz et al., 2003; Wood et al., 2007), but no human poisonings have yet been reported. STXs block sodium ion channels in nerve axon membrane and induce nerve dysfunction, paralysis then death due to respiratory failure (van Apeldoorn et al., 2007). The LD_{50} of the most toxic variant in mice is 10 μg/kg after i.p. injection (Table 1). Cases of human poisoning were mostly associated with consumption of STX-positive shellfish (van Apeldoorn et al., 2007). However, no intoxication through drinking water has been documented so far. BMAA is structurally similar to glutamate and binds to glutamate receptors, causing toxicity to neurons *in vitro* at concentrations as low as 10–30 nM (Lobner, Piana, Salous, & Peoples, 2007). BMAA has been linked to neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis (ALS) (Pablo et al., 2009). The implication of BMAA in these diseases was supported by the detection of this toxin in the brains of Chamorro patients with ALS/PDC (Pablo et al. 2009), and in brains from North American ALS, Parkinson's disease, and Alzheimer's disease (Murch, Cox, Banack, Steele, & Sacks, 2004). Recently, Banack et al. (2015) have demonstrated the presence of BMAA in aquatic food web and in aerosol samples from a lake adjacent to an area of previously documented high ALS incidence. This indicates that BMAA toxin may transfer through aquatic organisms and reaches to human via food intake. Despite the acute toxic effects of cyanobacterial neurotoxins have been studied extensively, there is nearly no information regarding the genotoxicity or carcinogenicity of these toxins to human. However, some *in vitro* studies reported the possible genotoxic effects of ANTX-a, and STXs (Sieroslawska & Rymuszka, 2010; da silva et al., 2014). No data are available yet for the genotoxicity and carcinogenicity of BMAA toxin.

LTXs are slightly lipophilic, penetration through human skin after 1 h of exposure to 26 μg cm^{-2}. They are potent skin irritants and cause a condition known as Swimmer's Itch off Oahu, Hawaii. They exert this activity through potent activation of protein kinase C (PKC). Direct contact with APTXs contaminated water caused acute skin irritation, rashes and blisters (Osborne, Webb, & Shaw, 2001). LTXs and APTXs can also be carried by wind in an aerosolized form and cause a health risk for people not exposed to water (Rzymski & Poniedziałek, 2012). LTXs can also accumulate in tissues of aquatic organisms upon feeding on *L. majuscula* being a source of food as sea turtles *Chelonia mydas* (Ysaumoto,

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1998). Consumption of such products, as well as ingestion of water containing lyngbyatoxin leads to inflammation of the esophagus and digestive tract (Ysaumoto, 1998). APTXs and LTXs are also potent tumor promoters through the activation of protein kinase C (van Apeldoorn et al., 2007).

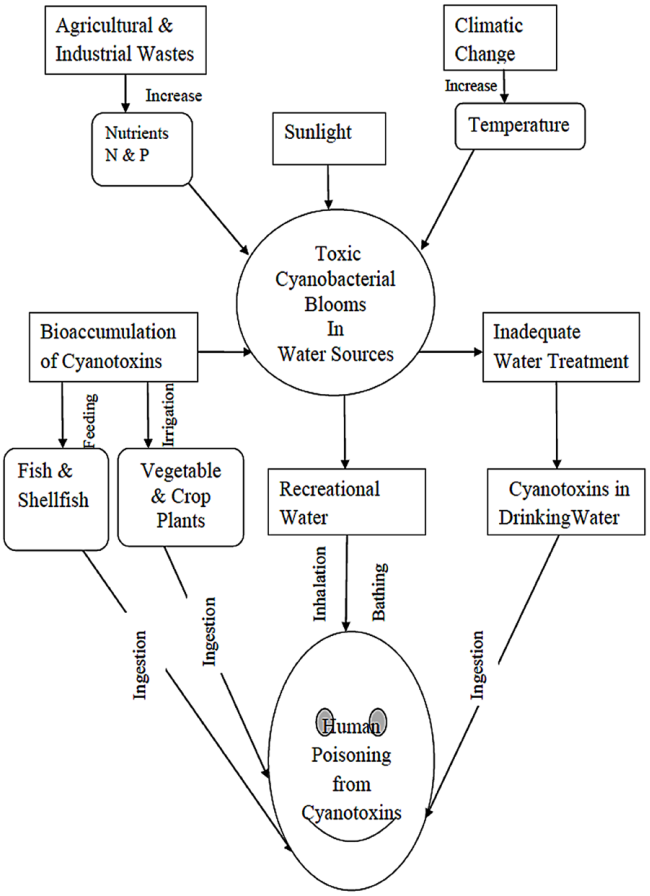
Cyanobacterial LPS endotoxins are heat stable and toxic to mammals. LPS endotoxins exposure has been associated with local effects due to direct contact, such as skin or eye irritation, gastrointestinal problems or allergic reactions (Rzymski and Poniedziałek, 2012). No genotoxic effects have been assigned yet with cyanobacterial lipopolysaccahride endotoxins.

EXPOSURE ROUTES TO CYANOTOXINS

Generally, humans may be exposed to cyanotoxins through different routes: drinking water, recreational water and food consumption (Figure 4).

Humans are exposed to cyanotoxins through drinking water, depending on their concentrations in raw surface water and the effectiveness of treatment methods for removing cyanobacteria and cyanotoxins. However, rural communities in developing countries using untreated surface waters will be exposed to

Figure 4. Schematic representation for bloom formation and possible exposure routes to cyanotoxins



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both cell-bound and dissolved cyanotoxins. Depending on cyanotoxin levels in drinking water, both acute/short-term and chronic effects in humans may occur (Chorus & Bartram, 1999). Cyanotoxin concentrations in surface waters vary in a very wide range of values, from trace to several milligrams per liter, being strongly influenced by the occurrence and amount of biomass (Funari & Testai, 2008). For instance, MC concentrations of 10–350μg L^{-1} have been reported in surface waters in Germany (Fastner et al., 1999), but much higher levels (up to 25,000μg L^{-1}) were recorded elsewhere (Sivonen & Jones, 1999). Total CYN and anatoxin-a(s) concentrations up to 12.1 and 3300μg L^{-1} have been determined in surface waters (Sivonen & Jones, 1999; Rucker et al., 2007). STXs have been detected in high concentrations in Australia (2,040–3,400 μg g^{-1}) (Negri, Jones, Blackburn, Oshima, & Onodera, 1997). Recreational activities can also represent a source of exposure to cyanobacteria and their cyanotoxins through direct contact, inhalation, and/or ingestion (Funari & Testai, 2008).

Besides their presence in drinking water, cyanotoxins may accumulate in some aquatic organisms such as fish, bivalves, snails, and other macroinvertebrates, via ingestion of cyanobacteria cells or via the transdermal route for dissolved toxins (Li, Chung, Kim, & Lee, 2004). Crop and vegetable plants can also accumulate cyanotoxins in their edible parts such as leaves, roots, fruits, when surface water bodies containing cyanobacteria are used for irrigation (Mohamed & Al-Shehri, 2009). Cyanotoxin accumulation in such food organisms and edible vegetable plants may pose a risk to human health, upon consumption of such contaminated food. Therefore, Information on cyanotoxin concentrations accumulated in these animals and plants is very important for risk assessment and derivation of safe guideline levels. In this respect, significant levels of MCs have been detected in aquatic organisms at maximum concentrations of 370, 2700 and 16 000 µg kg^{-1} have been reported in the edible parts of fish, crustaceans, and mussels, respectively (Table 2). CYN can also accumulate in edible tissues of aquatic organisms, including invertebrates and vertebrates at concentrations ranging from 0.8-103 ng/g (Berry, Jaja-Chimedza, Davalos-Lind, & Lind, 2012; Table 2). Bioaccumulation of cyanobacterial neurotoxins (ANX-a, STXs, BMAA) has also been reported in marine and freshwater seafood animals at considerable concentrations (Sephton, Haya, Martin, Legresley, & Page, 2007; Mondo et al., 2012; Table 2).

The available data on the accumulation of cyanotoxins in crop and vegetable plants revealed that MCs are, by far, the most commonly reported (Table 3). MCs were detected in several plants destined for human and animal consumption such as ryegrass (*Lolium perenne*), clover (*Trifolium repens*), rape (*Brassica napa*), lettuce (*Latuca. sativa*), radish (*Raphanus sativus*), arugula (*Eruca sativa*), dill (*Anethum graveolens*), parsley (*Petroselinum hortense*), cabbage (*Brassica oleracea*) and fruits of *S. lycopersicum* and *C. annuum plants* (Crush, Briggs, Sprossen, & Nichols, 2008; Mohamed & Al Shehri, 2009; Romero-Oliva, Contardo-Jara, Block, & Pflugmacher, 2014). MCs concentrations accumulated in these plants ranged from 70 to 1450 ng g^{-1} (Table 3), which may exceed the WHO limit proposed for this toxin (µg L^{-1}) (WHO, 1998) depending on the amount consumed. MCs were also detected in water chestnut and rice grains but at low concentrations ((Xiao et al., 2009; Chen, Han, Wang, Zhang, & Shi, 2012). For other cyanotoxins, there is only one study on CYN accumulation (maximum, 49 µg g^{-1}) in the leafy components of some cruciferous vegetables (e.g. *Brassica* spp., *Sinapsis alba*) (Kittler et al., 2012), but no available data on the accumulation of ANTXs, STXs, LTXs and LPS toxins in vegetable or crop plants.

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Table 2. Cyanotoxin concentrations accumulated in edible tissues of aquatic animals (Adapted with some modification from Berry, 2013)

Toxins	Fish/Shellfish	Concentrations (ng g^{-1})	References
Microcystins	Fish: Silverside, Silver Carp, Carp, *Goodea,* Redbreast Tilapia, Nile Tilapia, Trout, Yellow Perch. Mussels: *Anodonta woodiania, Hyriopsis cumingii, Cristaria plicata, Cristaria plicata, Lamprotula leai* Crayfish*: Procambarus clarkia,* Unidentified crab species Freshwater shrimp: *Palaemon* *Modestus*, *Macrobrachium nipponensis*	*0.8-340* 23-58 10-103 6-12	Berry 2013
Nodularin	Fish: Flounder, Roach Mussels:Unidentified mussel species (Whole animal) Freshwater shrimp: Unidentified prawn species	100-200 2500 5-22	Berry 2013
Cylindrospermopsin	Fish: Blue Tilapia, Topote Mussels: Unidentified mussel species (Whole animal) Crayfish: Red Claw Crayfish (*Cheraxquadrica rinatus* Freshwater shrimp: Unidentified prawn species	0.09-0.8 247 180 205	Berry 2013
Anatoxin-a	Fish: Prussian carp, roach and perch	5.9-36	Pawlik-Skowrońska et al., 2012.
BMAA	Blue crab, Mojarra, Pink shrimp Panaeus duorarum, Paper pond shell mussel, Oyster, Least puffer' Blue striped grun, Scrawled cowfish, Sea bream, Sailors choice grunt	55-7000 µg g^{-1}	Brand et al., 2010

Table 3. Microcystin concentrations accumulated in edible tissues of crop and vegetable plants

Toxin/Plant	Concentrations (ng g^{-1})	References
Green Pea, Sugar Pea, Chick Pea, Green Mung bean, Red Mung Bean, French Bean, Soya Bean, Alfalfa, Lentil, Maize, Wheat, Lettuce	10-125	Peuthert et al., 2007; Crush et al., 2008.
Tomato (root, leaves, green, and mature tomato)	10.8-12,298.2	Gutiérrez-Praena et al., 2014
Clover, rape, and lettuce	200–1450	Crush et al., 2008
Apple (Malus pumila) shoot cultures	510.23	Chen et al., 2004.
Broccoli and mustard (roots)	0.9 -2.6	Jarvenpa¨a et al., 2007
Broccoli	14500-145000	Maejima et al., 2014
Crops growing in the field Radish, arugula (rocket), lettuce, dill, parsley and cabbage (roots & leaves)	70 -1200	Mohamed & Al-Shehri, 2009.
Vegetable products Chestnuts	7	Xiao et al., 2009
Rice grains	1.01-3.19	Chen et al., 2012
Lycopersicon esculentum and Chilli Pepper Capsicum annuum (fruits)	1.16 &1.03, resp.	Romero-Oliva et al 2014

SOLUTIONS AND RECOMMENDATIONS: MANAGEMENT STRAEGIES AND GUIDELINE LEVELS

Water suppliers and authorities in different countries have developed monitoring and management strategies to mitigate problems presented by cyanobacteria and their cyanotoxins in drinking and recre-

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ational waters (Chorus, 2012). Generally, the management of cyanobacterial blooms and cyanotoxins in surface water includes three levels: bloom prevention, management of the source water or reservoir, and treatment to remove cyanobacteria or their toxins. The best approach for prevention of cyanobacterial bloom formation is decreasing the inputs of nutrients in agricultural and industrial wastes into surface water (Paerl et al., 2011). The concentration of these nutrients, particularly nitrogen and phosphorus is a key factor in promoting bloom formation and eutrophication of surface waters worldwide (Merel et al., 2013). Bloom monitoring and management in the source water is another option to reduce the levels of cyanobacteria and their cyantoxins. For drinking water sources, the most common monitoring and management action is the development of an Alert Levels Framework (ALF). These alert levels are defined by the determination of cyanobacterial cell numbers, cell biovolume or chlorophyll-a concentration. Although some countries have developed their own ALFs, most countries adopted the ALF released by WHO (Chorus & Bartram, 1999). The WHO alert level framework consists of three levels to be followed when cyanobacteria are detected in the water source during routine monitoring. During vigilance level (low cyanobacterial cells), regular surveillance and monitoring occurs weekly. Alert level 1 is activated, when greater than 2000 cyanobacteria cells mL^{-1} or 1 μg/L chlorophyll a is detected. At this alert level, an increase in monitoring frequency (daily), toxin analysis and consultation with health authorities occurs. Alert Level 2 is triggered when cyanobacterial cell concentration are more than 100,000 cells mL^{-1} or 50 μg L^{-1} chlorophyll a, and toxin level is higher than 0.8 μg L^{-1}. At this level the WHO suggests that an alternative water supply may be needed. But other alert level frameworks such as the ALF of South Africa (Du Preez &Van Baalen, 2006) suggested treatment optimizations, continuous monitoring of cyanobacteria and cyanotoxins, mouse test bioassay, and response committee meetings at alert level 2. The sequence can then move to a higher alert level, Alert level 3 when mouse bioassay is positive and toxin concentration is higher than 2.5 μg L^{-1}. The main actions at alert level 3 are the daily monitoring of cyanobacteria and cyanotoxins, optimization of treatment process, and communication with governmental departments. Finally, alternative drinking water should be supplied when the microcystin concentration in the drinking water is between 2.5 and 5 μg L^{-1} for eight consecutive days or exceeds 5 μg L^{-1} for two consecutive days.

For managing cyanobacteria in recreational water, WHO (2003) suggested 3 guideline levels: low level of 20 000 cyanobacterial cells ml^{-1} (10 μg L^{-1} chlorophyll-a, with dominance of cyanobacteria and MC < 10 μg L^{-1}) which corresponds to a low probability of adverse effects; moderate level of level of 100 000 cyanobacterial cells ml^{-1} (50 μg L^{-1} chlorophyll-a, with cyanobacteria dominance and MC =10-20 μg L^{-1}) which corresponds to a moderate probability of adverse effects ; and high level of more than 100,000 of total cyanobacterial cells mL^{-1} OR > 50 μg L^{-1} chlorophyll-*a* with dominance of cyanobacteria and MC > 20 μg L^{-1}) which corresponds to highest risk of adverse effects and immediate action should be taken to prevent totally swimming and other water contact activities.

In addition to alert level frameworks for managing toxic cyanobacteria, some countries established guidelines values for the presence of cyanotoxins in drinking water supplies for human health protection (Table 4). These guideline values were derived from the available toxicological data of these toxins based on animal studies, particularly, the identification of an acute NOEL, which is the highest dose at which no effects are observed. For instance, WHO (1998) established a provisional guideline of 1 μg L^{-1} for MC- LR in drinking water (WHO 1998) using a subchronic NOEL of 40μg/kg bw/day obtained upon orally administration of mice with MC-LR (Fawell, Hart, James, & Parr, 1993). Guideline levels for other cyanotoxins were also released by authorities in different countries including Australia, Canada and New Zealand (Table4).

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Table 4. Guideline values estimated for cyanotoxins in drinking and recreational waters

Cyanotoxin	Drinking Water ($\mu g\ L^{-1}$)	Recreational Water ($\mu g\ L^{-1}$)
Cyanobacterial cells (Australia)	6500 cells ml^{-1}	(WHO) 20000 cells ml^{-1}
MCs (WHO)	1	20 (Canada)
NOD (NewZealand)	1	ND
CYN (Australia)	1	4.5 (Washington)
ANTX-a (NewZealand)	6	1 (Washington)
ANTX-a(s) (NewZealand)	1	ND
STX (NewZealand)	3	7.5 (Washington)
MMBA	ND	ND
Aplysiatoxins	ND	ND
Lyngbyatoxins	ND	ND
LPS	ND	ND

ND, Not determined; the word in parentheses refers to the country who derived the guideline
(Adapted from Chorus, 2012)

For cyanotoxins in food, some countries calculated guideline values (GV) for the maximum tolerable concentration of most common cyanotoxins in aquatic animals and edible plants by using NOEL from the available literature data and on the basis of assumptions for body weight (bw), the amount of food ingested (C) and a factor for the relative allocation of toxin intake to that food (Allocation Factor, AF) (Ibelings & Chorus, 2007). However, it is not prudent to generalize and use these guidelines worldwide, as the major factors contributing to human exposure to cyanotoxins through food consumption are variable. For instance, it has been found that different species of fish accumulate different amounts of cyanotoxins, and the same fish species can accumulate higher amount of toxins when they are found in heavy toxic cyanobacterial blooms. Additionally, the tolerable daily intake (TDI) of these toxins depends on the relative of importance of food as a source of exposure and the amount consumed. Finally, the allocation factor (AF) depends also on the share of this food in the diet of a given country population. Therefore, different countries are advised to derive their own guideline values of cyanotoxins in edible food (animals or plants) according to the local conditions.

Cyanotoxin Removal and Treatment Efficiency

As prevention of cyanobacterial bloom formation in drinking water resources is a long term solution, drinking water treatment option should be adopted by companies and authorities to protect consumers from exposure to such potent cyanotoxins. Drinking water treatment plants usually apply different processes to remove both cyanobacterial cells and dissolved cyanotoxins (Pantelic, Svircev, Simeunovic, Vidovic, &Trajkovic, 2013; Table 5). The effectiveness of a water treatment system differs according to many factors including the types and properties of cyanotoxins (e.i. hydrophobicity/ hydrophilicity and intracellular/extracellular), and species of cyanobacteria dominated in the source water (Westrick, Szlag, Southwell, & Sinclair, 2010). Applying the wrong treatment process at a specific treatment stage could lyse cells and result in the release rather than removal of cyanotoxins. Generally a treatment system removing intact cells without lysis and toxin release is the most efficient treatment approach (Westrick et al., 2010). There are conventional and advanced oxidation treatment options available for the removal of cyanobacteria and their cyanotoxins during drinking water treatment. Conventional technologies in-

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Table 5. Effectiveness of most common treatment methods for removal of intact cyanobacteria cells and dissolved toxins (Adapted from Pantelic et al. 2013)

Treatment Method	Cells/ Intracellular Toxins	Extracellular Toxins	Remarks
Pre-treatment oxidation	Cell lysis	Not effective	Enhance coagulation, but lead to toxin release
coagulation/ sedimentation	>90%	Not effective	Efficient for cell removal but may lead to cyanotoxin release.
Slow sand filtration	< 86%	Low effective	Effective for cell removal, but its removal for dissolved toxins depends on bacteria found on the filter, sometimes lead to toxin release
Rapid sand filtration	low	Not effective	Not effective for cell removal or extracellular toxins, and may lead to toxin release
UV Radiation	Cell lysis	Effective	Able to degrade MCs, ANTX-a, and CYN, but at impractically high doses.
Chlorination	Cell lysis	Effective	Effective for oxidation MCs, CYN and STXs, free chlorine is >0.5 mg L^{-1} after >30 min at pH < 8 and low DOC, but not effective for ANTX-a oxidation
Ozonation	Cell lysis	Effective (> 98%)	Effective for degradation of MCs, ANTX-a, CYN and STXs. Effectiveness decreases at lower temperatures and high organic matter., may lead to cell lysis and toxin release
Potassium Permanganate	Cell lysis	Effective (95%)	Effective for oxidizing MCs and ANTX-a, but in absence of the whole cells.
Activated Carbon	>60%	Effective (85-95%)	Powdered activated carbon of wood origin is the most effective type for removal of MCs, ANTX-a and CYN. Granular activated carbon is effective for MCs but less effective for ANTX-a and CYN
Membranes	>96%	Effective.	Micro- and ultrafiltration are effective in removing intact cyanobacterial cells. Dissolved toxins MCs, CYN, NOD, ANTX-a are removed by nanofiltration and reverse osmosis. Membranes may be clogged leading to cell lysis and toxin release

clude coagulation, sand filtration, chlorination and activated carbon adsorption; and advanced oxidation technologies are ozonation, UV photolysis, Fenton oxidation and sonolysis (Xagoraraki, 2007). In this section, we will shed the light on the effectiveness and drawbacks of most common technologies used for removing both cyanobacterial cells and cyanotoxins (Table 5).

Pre-Oxidation

The pre-oxidation process by chlorine or ozone aims to improve and enhance the efficiency of next treatment steps, particularly coagulation. Although pre-oxidation may reduce taste, odor compounds, zebra mussels and other contaminants, it damages the membrane of cyanobacteria and induces cell lysis leading to the release of intracellular toxins (Miao & Tao, 2009). Furthermore, pre-oxidation result in the production of harmful by-products e.g. trihalomethanes (Merel et al., 2013). Therefore pre-oxidation should be avoided when a bloom occurs in drinking water resources.

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Coagulation–Flocculation–Sedimentation

Coagulation is the aggregation of cells using chemicals (i.e. ferric chloride or aluminum sulfate) into larger particles flocs) subsequently removed by sedimentation. This is the first step in conventional drinking water treatment. This process is only efficient for eliminating cyanobacterial cells from water, but dissolved cyanotoxins (i.e. extracelllar toxins) are not efficiently removed by this method (Teixeira & Rosa, 2007). Efficiency of coagulation process is dependent on pH values and chemical doses (Mouchet & Bonnelye, 1998). The presence of natural organic matter (NOM) in surface waters may result in lower cyanobacterial removal efficiencies (Ma &Liu, 2002). Coagulation can remove up to 90% of cyanobacterial cells and intracellular MCs (Xagoraraki, 2007). But, sludge containing toxic cyanobacterial cells can break down rapidly (within 24 h) and release dissolved MCs into the aqueous phase toxin (Drikas, Chow, House, & Burch, 2001). Therefore, sludge should be quickly removed from the treatment process.

Sand Filtration

Rapid sand filtration is usually used after coagulation to remove the particles. Rapid filtration is not effective method for removal of cyanobacterial cells and extracellular cyanotoxins (Ho et al., 2006). Conversely, slow sand filtration and riverbank filtration were shown to remove both cyanobacteria and their toxins (85% to 99%) during water treatment (Mohamed, El-Sharouny, & Ali, 2007). However, this process requires regular and careful backwashing of the filters to avoid lysis of cyanobacterial cells and release of cyanotoxins into the water (Chorus & Bartram, 1999). Moreover, microorganisms growing on the upper layer of sand filters efficiently degrade cyanotoxins (Edwards, Graham, Fowler, & Lawton, 2008). Biodegradation has now been suggested as a promising alternative for the removal of cyanotoxins in drinking water treatment (Merel et al., 2013).

Membrane Filtration

Membrane filtration processes can efficiently remove cyanobacteria and their toxins, depending on the membrane employed (i.e. microfiltration (0.1–10 μm), ultrafiltration (1–100 nm), nanofiltration (1 nm) and reverse osmosis (0.1 nm). Microfiltration and ultrafiltration membranes were effective for cell removal, but they may not be able to retain dissolved cyanotoxins, particularly toxins with small molecular weight (Westrick et al., 2010). As any filtration technique, microfiltration and ultrafiltration membranes may be clogged, and cell lysis and toxin release will occur. On the other hand, extracellular cynaotoxins such as MCs, CYN, NOD, ANTA-a have been shown to be removed by nanofiltration and reverse osmosis at a range of 90-100% (Pantelic et al., 2013). However, use of membrane filtration is expensive and filtered water may need subsequent re-mineralization due to the high retention potential of nanofiltration and reverse osmosis.

Activated Carbon

Activated carbon is usually used in two forms in drinking water treatment: powdered (PAC) to perform adsorption simultaneously with clarification, or granulated (GAC) to perform adsorption in percolation units (Westrick et al., 2010). Activated carbon adsorption was found to be effective in removing extracellular MCs, CYL, ANTX-a and STXs from drinking water (Pantelic et al., 2013; Table 5). The removal

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of cyanotoxins by activated carbon mostly depends on the kind of adsorbent employed and the type of cyanotoxin (Newcombe & Nicholson, 2004). For instance, MCs can be efficiently adsorbed on mesporous adsorbents (pore diameter = 2–50 nm), but other cyanotoxins such as STXs are well adsorbed on microporous instead of mesoporous carbon, being smaller than MCs (Newcombe & Nicholson, 2004). PAC is effective in cyanotoxin retention providing up to 85% removal of extracellular MCs and about to 98% removal of extracellular ANTX-a (Alvarez, Rose, & Bellamy, 2010). GAC was found to remove up to 95% of extracellular MCs and ANTX-a (Xagoraraki, 2007), but it takes place slowly and needs more than 12 h exposure for complete removal of MCs (Lee, Kim, Cho, Kim, & Kim, 2004). Although its effectiveness for cyanotoxin removal, activated carbon is not always a very efficient method, due to its short lifetime of adsorption and the need to be changed frequently, increasing the treatment costs (Haider, Naithani, Viswanathan, & Kakkar, 2003).

Chemical Inactivation and Degradation of Cyanotoxins

Some advanced oxidation technologies such as oxidants, catalysts and radiation have been adopted for the destruction of cyanotoxins. The efficiency of the oxidative agent in cyanotoxins oxidation depends on the type of toxins present, the concentration of released toxins, the water quality and the contact time (Zamyadi et al., 2010). UV irradiation is effective in destroying MCs, ANTX-a and CYL from drinking water (He et al., 2012; Table 5). However, this degradation occurs at higher doses (1,530 and 20,000 mJ/cm2) than practicable required (10 and 40 mJ cm^{-2}) for disinfection, making it a not viable treatment for these cyanotoxins (Westrick et al., 2010). Chlorination has been shown as an effective mechanism for the cyanotoxins MCs and NOD as well as CYL and STXs (Merel, Clément, & Thomas, 2010). However, Inactivation of cyanotoxins by chlorine is usually pH-dependent because the pKa of hypochlorous acid is 7.6. For instance, chlorination is efficient for inactivation of MCs at pH <8.0, and CYN at pH 6-9, (Merel et al., 2010). SXTs inactivation by chlorination has improved from 20% to 98% when pH increases from 4 to 9 (Nicholson et al., 2003).The inactivation of ANTX-a by chlorine is not effective (Rodriguez et al., 2007). Ozonation is very effective for destruction of intracellular and extracellular cyanotoxins. Ozone can degrade MCs, even at extremely high concentrations of the toxin (Miao et al., 2010) at pH <7, but not effective at pH7 (Alvarez et al., 2010). Inactivation of ANTX-a and CYN by ozone is pH-dependent and is effective at pH 7-10, and pH 4-10, respectively (Westrick et al., 2010). Both chlorination and ozonation of cyanotoxins may be affected by the presence of organic matter. Additionally, oxidant dose or the residual oxidant level required for complete toxin oxidation is a key factor for water quality and treatment issues. KMnO4 is effective in oxidizing microcystin and anatoxin-a (from pH 6 to 8), but is not very reactive with CYN or STXs (Pantelic et al. 2013). Although The use of permanganate does not release intracellular cyanotoxins (Ho, Tanis-Plant, Kayal, Slyman, & Newcombe, 2009), it leads to the formation of particulate manganese dioxide in solution which can cause potential problems in treated drinking water (Lahti et al., 2001).

SUSTAINABLITY OF CYANOTOXINS IN THE AQUATIC ENVIROMENT

Cyanotoxins are produced within cyanobacterial cells (i.e. intracellular), and released into the ambient environment during cell senescence, death and lysis (Chorus &Bartram, 1999). The release of cyanotoxins from the cells is enhanced by chemical treatment for eradication of cyanobacteria, particularly

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pre-chlorination and algicide application (Jones & Orr, 1994). Once released into the water, cyanotoxins can persist for a relatively long period before being removed through various processes naturally occurring in the environment such as photochemical degradation by UV, adsorption in particles in suspension or onto sediments, and biodegradation (Corbel, Mougin, & Bouaicha, 2014). However, cyanotoxins differ greatly in their chemical stabilities in the environment. MCs and NOD, being cyclic peptides are extremely stable and persistent to chemical breakdown. These toxins can persist in the aquatic environment for weeks (Edwards et al., 2008) and sometimes several months (Harada, Tsuji, Watanabe, & Kondo, 1996). The photodegradation of MCs in full sunlight occurs slowly and is enhanced by the by the presence of cell pigments (Welker & Steinberg, 2000). It can last for 2- 6 weeks, depending on the pigment concentration. The photodegradation of MCs and NOD is high in the presence of humic substance (Thirumavalavan, Hu, & Lee, 2012), and low in deep and muddy waters of lakes due to low radiation penetration (Wormer, Huerta-Fontela, Cires, Carrasco, & Quesada, 2010). CYN is also relatively stable in the dark, but it is rapidly degraded under sunlight and in the presence of cell pigments (Chiswell et al., 1999). ANTX-a is stable in the dark, but it undergoes rapid photochemical degradation ($t_{1/2}$ = 1-2 h) in sunlight under alkaline conditions, even in the absence of cell pigments (Smith & Sutton, 1993) in the laboratory. STXs undergo slow chemical degradation in the dark at room temperature with a half-life of 1-3 months (Chorus & Bartram, 1999). Cyanotoxins can also be removed by adsorption on suspended particles or sediments in aquatic environment (Corbel et al., 2014). Some studies reported that about 20% of toxins can be adsorbed on sediments (Lahti, Kilponen, & Kivimaeki, 1996). Moreover, it was suggested that the removal of cyanotoxins by this process is not only due to adsorption but also to biodegradation by bacteria present in sediments (Lahti et al., 1996). Therefore, sediments may play a crucial role in cyanotoxin biodegradation by supplying toxin-degrading bacteria to the water column (Chen et al., 2010).

Many studies reported the degradation of MCs by different species of aquatic bacteria e.g. *Sphingomonas, Paucibacter, Burkholderia* sp., *Methylobacillus, Arthrobacter, Bacillus*, and *Lactobacillus (*Kormas & Lymperopoulou, 2013). MC degradation is regulated by *mlr* gene cluster consisting of four genes (*mlrA, mlrB, mlrC*, and *mlrD)* which codes for at least three intracellular enzymes (Bourne, Riddles, Jones, Smith, & Blakeley, 2001). This gene cluster was identified and sequenced in all MC-degrading bacteria. MC removal by biodegradation can be >90% within 2–10 days depending on the water body, initial MC concentration and water temperature (Park et al., 2001). Some MC-degrading bacteria can also degrade NOD, because of the similar mode of action of mlrA that opens the ring of its cyclic structure at the Adda-Arg peptide bond (Ho, Sawade, & Newcombe, 2012). Biodegradation has also been reported for the alkaloid heptaotoxin, CYN, by indigenous microorganisms (Smith, Boyer, & Zimba, 2008) and isolated *Bacillus* strain (Mohamed & Al-Amri, 2012). Regarding to cyanobacterial neurotoxins, there is scarcity of literature regarding their biodegradation compared to heptotoxins, despite of their increasing occurrence in surface waters. Kayal, Newcombe, & Ho (2008) revealed bacterial degradation of STXs during passage through bioactive treatment plant, but this led to structural modification of the predominant C-toxins variants to more toxic variants (G-toxins). ANTX-a biodegradation was assigned for *Pseudomonas* sp strain isolated from *Anabaena* culture, with a rate of 6–30 mg mL^{-1} per 3 d (Kiviranta, Sivonen, Luukkainen, Lahti, & Niemela, 1991). To date, no published data exist on the biodegradation of other cyanotoxins including ANTX-a(s), BMAA, LTXs and LPS endotoxins.

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FUTURE RESEARCH DIRECTIONS

From inspection of the available literature on cyanotoxins, some other specific issues have received very limited attention or not explored yet. Some of which are listed in this section to be addressed in the future.

Regarding the risk assessment of cyanotoxin to human health, the available toxicological data are limited to MCs, but scant for other cyanotoxins. For example, there is no toxicological data for BMAA toxin and its relationships with neurodegenerative illnesses, though it has been investigated in free-living and symbiotic cyanobacteria in most ecosystems. For the accumulation of cyanotoxins in crop plants irrigated with contaminated water, most studies have evidenced the accumulation of MCs in some plants in laboratory experiments. Therefore, more studies on the presence of MCs and other cyanotoxins are needed, especially in real crop conditions, to evaluate the risks for humans more accurately. Since cyanobacterial blooms contain a mixture of different kinds of cyanotoxins, combined exposure and interaction between different cyanotoxins can occur. Hence, further research is needed on possible additive, synergistic or antagonistic effects of these cyanotoxins. As removal of cyanobacteria and cyanotoxins from water bodies by physical or chemical treatment may lead to cell lysis and toxin release causing a great problem for the aquatic ecosystem, more efficient and cost effective technology needs to be developed. For instance, the efficiency of aquatic macrophytes might be tested for cyanotoxin elimination from raw water before entering drinking water treatment plants.

CONCLUSION

Cyanobacteria are a group of primary producers, present naturally in marine and freshwater environments. However, eutrophication (i.e. high nutrient concentrations) of water bodies along with high temperatures and intense sunlight provide optimum conditions for the growth and proliferation of cyanobacteria and bloom formation. The presence of cyanobacteria are of particular concern for human health, because their ability to produce potent cyanotoxins, which affect human in different ways including hepatotoxicity, neurotoxicity, dermatotoxicity, genotoxicity, and carcinogenicity. These cyanotoxins are synthesized and remain within the cells and only release into the ambient water upon cell lysis during senescence or chemical treatment. Consequently, they can be found in inadequately treated drinking water, accumulate in seafood and fish upon feeding on toxic cyanobacteria, and accumulate in vegetable and crop plants irrigated with contaminated waters. This definitely poses human health at a risk upon consumption of drinking water and/or food products contaminated with cyanotoxins. In response to such health hazards of cyanotoxins, WHO and other agencies in some countries have estimated guideline values for these toxins. These guideline limits could be used during monitoring of cyanobacteria and their toxins in drinking and recreational waters in other countries. Nevertheless, each country has to derive its own guideline value for the presence of cyanotoxins in food products (i.e. fish, seafood & edible plants), as these guidelines differ among countries according to local conditions. For example, the amount of food ingested and the frequency of food consumption (i.e. food preference) affect on the value of allocation factor (AF) for the relative allocation of toxin intake to that food. Once cyanobacterial blooms are detected in the water, water source has to be monitored and managed to prevent or reduce the growth of these organisms. The best way to prevent bloom formation is decreasing the input of agricultural and industrial wastes into the water bodies. Eradication of cyanobacterial bloom by algaecides should be avoided because of the subsequent release of toxins. On the other hand, to protect consumer's health,

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cyanobacteria and their cyanotoxins have to be removed during drinking water treatment processes by efficient and effective techniques. In this respect, water operators and companies have to increase and update their knowledge about the effective methods used for removal of cyanobacteria and cyanotoxins in drinking water treatment plants. This is because some conventional treatment methods are effective for removing cyanobacterial cells rather than cyanotoxins. Some methods can lead to breakdown of cyanobacterial cells and release of cyanotoxins into water. Other methods are efficient for removing one type of toxins, but cannot simultaneously remove all cyanotoxins.

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KEY TERMS AND DEFINITIONS

Bloom: A large population or extremely high cell densities of phytoplankton (20,000 to 100,000 cells per milliliter) found on the water surface.

Cyanobacteria: Cyanobacteria are gram negative bacteria with a prokaryotic cell structure containing chlorophyll-*a*, and phycobilin pigments. Cyanobacteria were previously called blue-green algae.

Cyanotoxins: Secondary metabolites produced cyanobacteria including dermatoxins, hepatotoxins, and neurotoxins. They have a serious impact on plant, animal and human health.

Dermatoxins: The toxins that affect the skin and membrane tissues.

Extracellular Toxins: The toxins released from the cells into the ambient environment upon cell lysis.

Hepatotoxins: The toxins that affect the liver.

Intracellular Toxins: The toxins produced and remained within the cells.

Neurotoxins: The toxins that affect the central nervous system.

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Chapter 55
Pharmaceuticals and Personal Care Products as Emerging Water Contaminants

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ABSTRACT

With the advent of better detection, more micro-contaminants are being found in water. Many of these micro-contaminants come from medical therapies and personal care products. These chemicals are comprised of a wide-range of substances including pharmaceuticals, dietary supplements, veterinary drugs, fragrances, hair care products, body lotions, oral care, and cosmetics. Many of these products enhance our quality of life and in some cases, provide life-saving therapies. But, they come with an environmental cost. Scientific research has found sub-therapeutic levels of many of these chemicals in our waterways and in our finished drinking water, causing concern about the potential environmental and public health impacts associated with very low, chronic exposure. As tailored therapies and personal care products are developed, it is crucial to consider how to control emerging contaminants from medical therapies and personal care products. Specific actions and policies can be implemented now by adopting upstream approaches to prevent waste and decrease environmental exposures.

INTRODUCTION

The 1950's adage, *better living through chemistry*, still resonates today through our use of pharmaceuticals and personal care products. America is the prescription nation, with more than four billion prescriptions dispensed annually, and with the average citizen using not only prescribed medications, but over-the-counter drugs, homeopathic treatments, and herbal remedies as well (Lindsley, 2015). The average female consumer uses at least 12 personal care products, while males use at least six products (Leonard, 2010). Humans are not alone in their use of pharmaceuticals. The increased use of pharmaceu-

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ticals among animals, especially antibiotics, is well documented. And the use of all of these chemicals comes with an environmental cost: much of what we use ends up in our water and little can be removed through water treatment processes.

The purpose of this chapter is to:

1. Describe how pharmaceuticals, and personal care products (PPCP) as well as some medical therapies end up in various water sources, and identify some pharmacological, chemical, and toxicological properties of PPCP.
2. Identify how medical therapies and PPCP are detected and evaluated in various water sources, and identify some of the issues associated with determining levels of exposure and risks associated with exposure.
3. Define the problems associated with emerging contaminants (EC) from medical therapies and personal care products (PCP), using two general categories of EC (antibacterials and endocrine-disruptors) and two specific examples (Iodine 131 and nanotechnology) to illustrate these problems, by:
    a. Identifying the chemicals associated with the medical therapy or PCP and their potential and actual human health hazards;
    b. Characterizing potential policy implications as a result of these examples.
4. Examine the life-cycle of emerging contaminants from design through disposal to consider the following questions:
    a. How can the design and production of these contaminants be improved to decrease bioactivity, increase absorption, reduce waste, and lesson their carbon footprint?
    b. What incentives may be helpful in the development of new products and/or the redesign of existing products that utilize green chemistry and decrease the impact upon the environment?
    c. What production methods would decrease chemical waste, use less or least harmful materials, and reduce the carbon footprint?
    d. How can policies and practices be changed to reduce the waste and decrease pollution from emerging contaminants?
5. Explore potential recommendations to decrease the effects of emerging contaminants and future contaminants based upon existing research and some new areas for consideration.

With thousands of pharmaceuticals and chemicals that make up different PPCP, there is no way a chapter like this can be all-inclusive. By discussing key components of emerging contaminants in water, this chapter is an exploration of what needs to be considered now and in the future to mitigate exposure and safeguard our drinking water.

BACKGROUND

Pharmaceuticals and medical therapies not only save lives by preventing and treating diseases, but also by enhancing the quality of life for those with chronic diseases. Personal care products also improve our daily living by providing products that ease discomfort, improve our perceptions of appearance, and make

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us feel good about ourselves. Both pharmaceuticals and personal care products (PPCP) contain chemicals that are biologically active, toxic, and hormone-disrupting. Both are found in drinking water sources and wastewater effluents worldwide. Their presence in water has been documented and publicly reported by government experts, academic researchers, and the media for nearly two decades. While this is not a new discovery, the level of detection at trace amounts of non-therapeutic levels of pharmaceuticals or non-harmful levels of personal care products has changed and created a heightened awareness due to media coverage (Donn, Mendoza, & Pritchard, 2008; Donn, et al., 2009). Detection of trace amounts of chemicals will continue to improve, documenting the effects of low-level, chronic exposures of PPCP, linking these exposures to biological harm for aquatic and human life.

Pharmaceuticals enter the environment as a result of human activity: 1) unintentionally through human excretion; 2) intentionally through flushing unwanted, unused, or expired medications (Daughton & Ruhoy, 2008); 3) design and large-scale production of medications through wastewater effluent (Fick, et al., 2009); 4) animal excretion through large-scale livestock and aquaculture farming practices (Gunnarsson, et al., 2009; Jones, Voulvoulis, & Lester, 2002).Currently there is no single method to remove pharmaceuticals from wastewater, and the majority of water treatment plants cannot address removal of trace amounts (Bolonga, Ismail, Salim, & Matsuur, 2009).

The route of entry for personal care products into various water sources is a little different. Instead of excretion, most personal care products enter the water exclusively through human interventions like showering, hand washing, cleaning, or laundry (Grassi, Rizzo, & Farina, 2013). However, through the application of a personal care product, the skin may absorb some chemicals that are then excreted through the body in trace amounts into the sewage system.

When metabolized by humans or animals, many PPCP undergo structural changes resulting in metabolites, which may differ in pharmacological, chemical, and toxicological properties from the original products (Ortiz de Garcia, et al., 2013; Boxall, et al., 2012). The methods of administration may also impact how PPCP are metabolized. For example, if a substance is ingested versus administered dermally, it will have an effect on how it gets metabolized. Two key factors affect the metabolism: its pharmacodynamics and pharmacokinetics. Pharmacodynamics describe what influences the drug has on the body including therapeutically, side effects, concentrations, and mechanism of drug interactions. The influence the being has on the drug is pharmacokinetics, and considers areas such as absorption, distribution, metabolism, elimination, and the time it takes to process the chemical (Khetan & Collins, 2007). Since pharmaceuticals are made up of chemicals, and personal care products are also made up of chemicals, similar principles may be applied to these products as well.

Europeans have taken a different approach to PPCP. For example, since the 1990s, guidelines for human and veterinary medicinal products have existed. These guidelines provide a framework to predict environmental concentrations, assess the effects of pharmaceuticals in the environment, and provide some baseline ecotoxicity data (Koschorreck & Hickmann, 2008). Although it is not in wide use throughout Europe, other countries are considering various aspects of these guidelines. Commonly used chemicals found in PCP have been discontinued or banned in Europe, including phthalates and parabens. Manufacturers who sell in multiple international markets already have found ways to reformulate products to exclude banned substances for the European market. Extending the discontinuation of these products to all personal care products might be a consideration in the future.

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DETECTION AND EVALUATION

PPCP were first detected in water systems through aquatic life during a national survey of organic wastewater contaminants in U.S. waterways conducted by the U.S. Geological Survey (USGS) in 1999-2000 (Kolpin, et al., 2002). This survey found the following in very low concentrations: human and veterinary drugs (including antibiotics), natural and synthetic hormones, detergent metabolites, plasticizers, insecticides, and fire retardants. These substances were found in residential, industrial, and agricultural wastewater, sampled from 139 streams representing 30 states, with half the streams containing one or more of these chemicals (Kolpin, et al., 2002). Subsequently, other studies have been done that demonstrate the pervasiveness of these chemicals and other emerging contaminants that influence water quality. As detection technology improves, more contaminants from PPCP will be found at lower concentrations but at sufficiently high enough levels to harm aquatic organisms (Ferrari, Paxeus, Lo Giudice, Pollio, & Garric, 2003).

While there is little evidence that PPCP exposure affects human health, evaluating this exposure is very difficult for several reasons. First, most of the detectable levels of PPCP are at sub-therapeutic concentrations. Second, PPCP are not detected as single compounds but as part of a group of environmental contaminants, and it is difficult to tease out what is causing the problem, and/or the chemicals may mix to form other contaminants. Next, very little is known about the efficacy or health effects of low-level, chronic, non-therapeutic exposure to PPCP. Lastly, the environmental persistence of PPCP varies widely (Löffler, Römbke, Meller, & Ternes, 2005; Scheytt, Mersmann, Leidig, Pekdeger, & Heberer, 2004; Scheytt, Mersmann, & Herberer, 2006; Tixier, Singer, Oellers, & Müller, 2003). Some of the most widely prescribed drugs or frequently used personal care products may not show up in drinking water because their persistence in the environment is relatively short, and therefore may be undetected.

ANTIBACTERIALS AND ENDOCRINE-DISRUPTING DRUGS

Chemicals of Concern and Health Effects

Two key contaminants in water are of particular concern: antibacterials and endocrine-disrupting drugs. Half the antibiotics used in livestock are in the same classes as those used in humans, raising concerns about contributing to growing antibiotic resistance (World Health Organization, 2002; Centers for Disease Control and Prevention, 2009). Specific antibiotics have been used historically in the feed or drinking water of livestock such as poultry, hogs, and cattle to decrease the amount food that is used and for the animals to gain weight quickly. Many of these antibiotics also are used to treat human infections, creating genuine concern about bacteria, which become resistant to antimicrobial drugs. Once resistance occurs, a drug is no longer effective in treating various infections or illnesses.

Endocrine-disrupting pharmaceuticals are excreted as waste by-products from the use of birth-control pills (ethinylestradiol), menopause treatments, thyroid replacements, and cancer therapies. Ethinylestradiol is extremely potent at very low concentrations and bioaccumulates within an organism; a concentration of 0.1 ng/L is sufficient to induce production of the female egg protein vitellogenin in male rainbow trout (Purdom & Randall, 1998; Larsson, et al.,1999).

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Policy Implications

The Food and Drug Administration (FDA) has encouraged eliminating the use of these antibiotics for growth production (FDA, 2009; Pew Trusts, 2014), but FDA's guidance policy still permits about a quarter of antibiotics to be used for growth purposes or to treat bacteria. By not banning the use of antibiotics in the feed of farm animals altogether, antibiotic resistance will continue to be an ongoing problem that will hinder the ability of medical personnel to provide adequate treatments for infections and illnesses.

The policy implications for limiting or banning endocrine disrupters are not as easy to assess. Since many PPCP contain chemicals that act as endocrine disrupters in the environment, eliminating these chemicals will prove to be much harder. In some cases, which chemicals act as endocrine disrupters in the environment are known and in other cases they are unknown. For example, certain pharmaceuticals, like thyroid medications, are endocrine disrupters, so a mitigation strategy is needed to reduce the chemical and biologic activities associated with the drug once it enters the environment. But in some PCP, it is unclear which chemicals act like endocrine disrupters, but the effect is similar on aquatic life. Additional research should be required to prove if PPCP may act like an endocrine disrupter and then other alternatives should be considered for reformulation when possible.

IODINE 131

Chemical of Concern and Health Effects

Medical treatments are another source of emerging contaminants in water. For example, Iodine 131(^{131}I) is a frequently used radioactive isotope that can be found in human and veterinary medical treatments, released from nuclear power plants (unplanned releases or from their laundry facilities), or as a tracer into the environment as part of hydraulic fracturing fluid to determine the location of fissures created in this process.

Most commonly, ^{131}I is used frequently for the treatment of thyroid cancer in humans and animals, primarily cats. Yet the major health effect caused by over exposure to ^{131}I is thyroid cancer. Low dose therapy of ^{131}I is also used to treat an overactive thyroid. Health care providers know that this type of medical treatment must be keenly balanced in order to provide treatment not harm, and therefore exposure to ^{131}I treatment is strictly monitored (Institute of Medicine Committee on Thyroid Screening Related to I-131 Exposure, 1999).

When patients are treated with ^{131}I, they are told to drink a great deal of fluids to flush their system. As a result, patients excrete waste directly into the sewage collection systems, which pass through the wastewater treatment facilities. The radioactive half-life for ^{131}I is eight days but patients do not store their bodily waste until the half-life is completed. While ^{131}I does decrease in activity over time, it is introduced to surface waters through wastewater plant effluents. The extent to which ^{131}I is present in detectable concentrations is unknown. The U.S. national environmental radiation monitoring program, RadNet, is not designed to help water departments assess the amount of ^{131}I in drinking water. While drinking water utilities participate in the RadNet program, it is hard to assess the amount of ^{131}I that occurs in water due to how sampling occurs and the locations of the samples (source water, finished water, and tap), corresponding to different ages of water and therefore different levels of dilution and decay of ^{131}I. Since the half-life is relatively short, if targeted monitoring is not occurring on a very regular basis,

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water utilities may miss an increase in detectable ^{131}I in source water used for drinking. The majority of utilities do not monitor for ^{131}I on a regular basis, so the chances of missing that elevation are highly likely. Additionally, some analytic methods do not account for radioiodine in all the chemical forms that may be present in environmental samples, leading to difficulty in evaluating this exposure in drinking water throughout the United States.

Policy Implications

In 2011, an earthquake and tsunami caused the Japanese Fukushima Daichi Nuclear Plant to emit large amounts of radiation into the atmosphere and water near the plant. As a result of these emissions, U.S. water departments increased monitoring for radioactive iodine. One water department noticed an uptick in ^{131}I. After extensive monitoring and intergovernmental/non-profit cooperation, all parties concluded that the likely source of ^{131}I in this location's source waters is not from the Diachi Nuclear Plant melt down. Rather, it is from patients receiving medical treatments (most likely for thyroid cancer), since this region has a large number of medical facilities that treat patients for thyroid cancer. More intensive monitoring would help other water departments identify detectable ^{131}I in drinking source water, especially in areas where people are treated for thyroid cancer. Since this kind of monitoring for ^{131}I had not been done before, and most water departments would not voluntarily increase the targeted monitoring for this contaminant, it would be hard to determine if this was a one-time anomaly or is more common than previously suggested. In addition to more monitoring, the need to develop analytic techniques for more sensitive and less expensive ways to detect ^{131}I should be considered, as well as better equipment that can remove and process ^{131}I in drinking water through the wastewater treatment process.

With this kind of medical treatment emerging as a contaminant to water, the need to engage the healthcare community and patients will become necessary and essential. Outreach to the healthcare community and their patients needs to be done with the realization that medical practitioners work within their regulations, as well as time and economic constraints.

NANOTECHNOLOGY

Chemical of Concern and Health Effects

Another example of an emerging contaminant comes from personal care products. A large number of personal care products contain nanotechnology. Nanotechnology is the manipulation of atoms and molecules on a subminute scale – "80,000 times smaller than the width of a human hair," (Silpa, Jose, Sumod, & Sabitha, 2012). Nanotechnology is being incorporated by cosmetic manufacturers as ingredients for UV protection, moisturizing, increased and more intense cosmetic colors, and improving the finishes of makeup. Due to their very small size, and their variety in terms of shapes and availability in many different products, nanoparticles can enter the environment during the manufacture, use, or disposal of these materials. For example, the antibacterial nature of nanoparticles might inhibit helpful bacteria in wastewater treatment or sewage plants. Additionally, nanoparticles may bind to other substances like cadmium or petrochemicals to contaminate finished water. Fish who have been exposed to nanoparticles may experience brain damage (large mouth bass) or toxicity (rainbow trout) (Chen, Dong, Zhao, & Tang,

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2009; Ernie, 2004). Nanoparticles may be able to biomagnify through the microbial food chain (Jonathan, Jason, Jason, & Paul, 2011) and are considered biopersistent, meaning they do not dissipate quickly.

Not only can nanoparticles get into water as an emerging contaminant, but also these particles can affect humans and produce profound effects. Entry can occur through three routes: inhalation, ingestion, and absorption through the skin. According to the National Institute of Occupational Health and Safety, workers may inhale nanomaterials as part of the production of these products and consumers may inhale particles when they spray on materials that contain nanos. This exposure may result in a wide variety of respiratory issues, as well as potential toxicity for the brain, blood and other organs (Silpa, et al., 2012). Unintentional ingestion occurs when products that contain nanoparticles are on people's hands and transfer from hand-to-mouth occurs. In addition to inhalation, the exposure to nanotechnology occurs through absorption, which is how the products are supposed to be used. Products containing UV nanoprotection have been shown to enter the blood stream in animal models in 24 hours, posing concerns about their effects in humans in the same time frame (Ryman Rasmussen, Riviere, & Monteiro Riviere, 2006).

Policy Implications

Both environmental and health organizations have raised safety concerns about products containing nanotechnology. Consumers and regulatory bodies are voicing similar concerns. Since limited safety testing is done with cosmetics or personal care products, it is hard to assess what are the safety concerns with these products. The U.S. FDA is being asked about oversight for these products. Yet, few studies exist about the potential health and safety concerns, and more and more products are containing these particles. The few studies that have been conducted have shown links between exposures to nanotechnology and some form of toxicity, to both aquatic organisms and laboratory animals. The European Union has updated its cosmetics directive to include information about nanoparticles and requires safety compliance and information about the materials. However, much like other chemicals such as phthalates or parabens, without a consistent global approach to nanotechnology, these materials will continue to be sold with limited safety and monitoring information. Additionally, instead of the cosmetic industry providing oversight about safety, it might be more efficient and effective if a third party group provided that information. Research of this nature, which is globally funded through a mixture of government and industry funds to assess the safety and monitoring of emerging products such as nanotechnology, could provide oversight and data for future decision-making about products.

LIFECYCLE

There are four areas for potentially reducing or eliminating waste from pharmaceuticals and personal care products in the environment: design and production; approval and regulation; use; and discharge and disposal.

Design and Production

The design, development, and production of PPCP should consider ways to increase effectiveness. For pharmaceuticals, this means that the bioactivity and absorption within the intended target is increased based upon individualized traits such as body weight or genetics. With PCP, the amount of residual chemi-

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cals that are not absorbed through the skin or hair should be decreased. To accomplish these goals, the use of green chemistry should be applied to PPCP. Green chemistry is defined as the invention, design, and application of chemical products and processes that reduce or eliminate the use and generation of hazardous substances (Khetan & Collins, 2007). Green chemistry would address two key challenges: improved absorption of PPCP in or on the body and better formulation to facilitate rapid removal upon release into the environment, decreasing their persistence, and creating a much shorter half-life for these products when they enter the environment through processing.

Producing PPCP is resource-intensive and generates significant amounts of waste worldwide. To produce an active ingredient used in PPCP, the waste generated can vary drastically, from 200 kilograms to 30,000 kilograms. The manufactured wastes may include biological compounds like fermentation wastes, or leftover solvents when active ingredients are extracted from natural sources. Sometimes pharmacologically active reagents such as anticoagulants and chemotherapeutic agents are created as byproducts of the production of PPCP. Additionally, cleaning agents, disinfectants, and extraction solvents are used to clean, sterilize, or purify active compounds (Henderson, Kindervater & Manley, 2007). For example, locations where the production of pharmaceuticals occurs discharge effluents that contain much higher levels of chemicals and active drug compounds (Langford & Thomas, 2009).

To reduce design and production costs, green chemistry offers great potential for savings. By reducing the use of water and hazardous solvents in the production process, green chemists have identified methods that provide PPCP with the same therapeutic effect using less active pharmaceutical ingredients. This method should help decrease the amount of drug that must be manufactured, decreasing production waste and the amount of drug in each dose, and ultimately resulting in reduced costs (Daughton, 2003; Becker, 2009). The downside to reformulation of pharmaceuticals is that it may take more than a decade to test and receive approval for any reformulation of a product in the U.S., costing the manufacturers millions or billions of dollars (Becker, 2009). Reformulating personal care products is costly, so it needs to be incentivized through reducing production costs or chemical bans before manufacturers will take the initiative. It needs to be done on a global scale since products are sold globally, and then the highest environmental and safety standards should set the industry bar for design and production.

With the design and production of PPCP, another issue should be considered besides green chemistry: the carbon footprint of design and production. The National Health Service of England (NHS) studied the carbon footprint of pharmaceuticals through procurement, defined as the manufacture and transportation of NHS goods and services used by humans (NHS, 2009). The NHS found that procurement constituted 60% of carbon emissions, with pharmaceuticals and medical equipment making up half of these emissions. Pharmaceuticals alone accounted for about a fifth of total emissions in the healthcare system. This is comparable to the emissions of entire sectors of the economy, including energy usage of buildings or travel.

Approval and Regulation

In the U.S., the development, collection, and discharge of pharmaceuticals are regulated by a number of federal laws and by at least three different federal agencies – 1) Federal Drug Administration (FDA - approval and regulation), 2) Environmental Protection Agency (EPA - environmental impact and waste disposal, but EPA does not have the authority to review pharmaceuticals for environmental consequences because drugs are exempted from the Toxic Substances Control Act [TSCA]), and 3) Drug Enforcement Administration (DEA – diversion and regulation of controlled substances). Pharmaceuticals used

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prophylactically in animal feed for livestock are overseen through direction from the FDA for the types of medication and quantities, while the U.S. Department of Agriculture provides guidance for animal waste management (quantity and storage), yet the regulation of environmental impacts from animal waste is deferred to EPA. With so many agencies involved in different aspects of pharmaceutical regulation, there is little coordination among these agencies, creating both conflict and confusion about how to handle many aspects of this life cycle.

Personal care products do not have the same regulations as pharmaceuticals when it comes to their development but are often treated similarly when it comes to waste disposal. The U.S. EPA groups PCP with pharmaceuticals for their environmental impacts, because of their likeness to drugs' environmental impacts. PCP may be disposed in household trash without special precautions, which is different from disposing of pharmaceuticals at home.

In the U.S., the Federal Food, Drug and Cosmetic Act of 1980 empowers the FDA to regulate pharmaceuticals. When a drug seeks approval to come to market, it must meet the standard of "safe and effective," (FDA, 2009). Through the guidance of this Act, FDA is accountable for reviewing the potential environmental impact from human and veterinary medicines and their intended uses. An Environmental Assessment (EA) (42 U.S.C. §4371, et seq.) is required to be submitted to the FDA to assess the potential effects of a proposed compound, yet there are a number of exclusions to this requirement. For example, if the production of drugs is predicted to occur at less than one parts per billion (ppb) in the aquatic environment or 100 ppb in soil (*Id.* at § 25.31(b).), then the compound is excluded, which means that many drugs are included in this exemption (21 C.F.R. § 25.10, 2008). However, effects on aquatic life have been seen at very low, sub-therapeutic concentrations, suggesting that these benchmarks should be reconsidered and need to be coordinated with the EPA and the U.S. Geological Survey, since the latter group studies the effects of these compounds on fish and amphibians.

Use

There is no doubt about it, pharmaceutical use has increased over the past several decades, leading to more pharmaceutical discharge and waste into the environment. Every month in the U.S., 135 million people use prescription medicines, for a total of over four billion prescriptions filled each year (Henry J. Kaiser Family Foundation, 2008). In one study, almost 70% of Americans take one prescription drug, with more than 50% taking two or more, and older people and women representing the largest percentage of people using medications (CDC, 2009).

Prescription drug use will increase because of several factors: 1) an aging population who are living longer with at least one or more chronic diseases; 2) younger people diagnosed with treatable chronic diseases, including neurobehavioral developmental disorders; 3) the medicalization of obesity; and 4) personalized and tailored medicine (Becker, 2007; Zhong, et al., 2013). All of these factors contribute to the rise of prescription drugs, not only in the U.S. but also, worldwide. As this increase occurs, stakeholders such as the government, the industry, and the pharmaceutical industry will need to provide better public health and environmental protections. Increased regulatory guidance will be needed to improve environmental protection, and this includes how to dispose of unused, unwanted, or expired medications. The current shift from twice-yearly U.S. DEA drug disposal drop-offs at law enforcement agencies or community group-designated locations to pharmacy-based, year-round drop-off sites should help to decrease the amount of unwanted drugs in the home medicine cabinet. Additionally, a system to

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encourage short-cycling of new prescriptions and prescribing smaller quantities of medications should be encouraged.

The Environmental Working Group found that the average person used at least nine personal care products each day (EWG, 2011). This may include, but is not limited to: soaps, body washes, perfumes, deodorants, moisturizers, shampoos, conditioners, toothpaste, and other products. Each of these products is made up of many chemicals that ultimately are washed off our bodies and into the water system. With little regulation or oversight on cosmetics and personal care products, much of what is known about these products is limited to the ingredient list posted on the packaging. And none of these products' manufacturers are required to demonstrate an Environmental Assessment to ascertain what potential impact it may have when it enters the environment. Since many of the chemicals used in these products may have similar properties to the ones used in drugs, the question arises if similar testing should be considered for cosmetics and PCP.

Discharge and Disposal

How PPCP enter the waste stream can be described as either unintentional or intentional. Unintentional releases suggest excretion of pharmaceuticals (metabolized and unmetabolized) from animals or humans. When we bath or wash, PCP, as part of our grooming habits, enter into the waste stream. Intentional releases refer to the disposal of unused, unwanted, or expired PPCP by flushing them down the toilet, or by throwing them into the trash. Intentional releases also include disposing of pharmaceuticals purposely as part of the manufacturing process (Boxall, 2004).

It is difficult to assess how much PPCP are disposed of on an annual basis. Several factors contribute to this. Some pharmaceuticals are prescribed but never filled, some prescriptions are filled and not used or used inappropriately, and some are prescribed, filled and used appropriately. In other cases, due to adverse reactions, people need to switch medications, leaving them with unused drugs from previous prescriptions. Similar factors apply to personal care products; people buy products and may or may not use the products to completion. A rough estimate can be made about the intentional releases of pharmaceuticals into the waste stream. In some studies, as much as 50% of many prescriptions and 80% of antibiotics are approximated to go unused, although pharmaceutical reports suggest that number is about three percent (Bound & Voulvoulis, 2004; National Association of Clean Water Agencies, 2005). Most unused or unwanted medications are put in the trash, flushed down the toilet, or rinsed down the sink (Kuspis & Krenzelok, 1996). Another factor that contributes to waste is the disposal of deceased persons' unused medications. Much of that medication is often trashed or put into the sewage system via flushing or rinsing. Lastly, hospitals, long-term care facilities, and other healthcare institutions contribute pharmaceuticals to the waste stream through large quantities of unused pharmaceuticals as part of patient treatment. The contribution from these sources to the waste stream is estimated to be between 20%-65% (Ruhoy & Daughton, 2007; Daughton & Ruhoy, 2009).

To estimate the amount of unintentional release from human and animal excretion, scientists are creating models. These models forecast the amount of active pharmaceutical ingredient (API) that potentially enters the environment by analyzing information about usage, rates of excretion of API, and effectiveness of treatment techniques used by wastewater and drinking-water facilities (Kostich & Lazorchek, 2008). However, the accuracy of these models have yet to be tested.

Two factors largely determine which PPCP enter the environment: the nature of the wastewater treatment plant (WWTP) where they are discharged and the type of contaminant. WWTPs can successfully

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remove a few specific compounds; but overall, most removal rates are highly variable (Tauxe-Wuersch, De Alencastro, Grandjean, & Tarradellas, 2005; Kimura, Hara, & Watanabe, 2005; Lee, Sarafin, Peart, & Svoboda, 2004; Lindqvist, Tuhkanen, & Kronberg, 2005). Activated carbon, oxidation by chlorination or ozonation, and membrane filtration are advanced sewage-treatment techniques that can increase pharmaceutical removal rates. However, even with advanced techniques, many of the drugs may not be completely removed from wastewater. Unfortunately, with the diversity of pharmaceuticals, no single technique will effectively treat all the different kinds. Because of this variability and the increase of personalized medicine, the recommendation is to focus on upstream ideas through the use of green chemistry to decrease bioactivity, its persistence in the environment, and absorptivity. With personal care products, many of the same issues also are considered as pharmaceuticals.

Activated carbon, oxidation, activated sludge, nanofiltration, and reverse osmosis membranes are other technologies that have been used to remove pharmaceuticals from water (Bolonga, et al., 2009). None of these technologies eliminate pharmaceuticals from the water or consistently render them both biologically and chemically inert. Future technological developments need to be advanced to address this waste.

One detrimental byproduct of wastewater treatment is sludge. Some pharmaceuticals and their metabolites are hydrophobic and end up in this sludge. Even if removal from wastewater can occur, this process creates the potential for groundwater or surface water contamination when the resulting sludge, which contains hydrophobic pharmaceuticals/metabolites, is spread on fields as an agricultural fertilizer (Dizer, et al., 2002). Additionally, accumulation of pharmaceuticals in the soil may occur with repeated spreading of this sludge on fields (Golet, Xifra, Siegrist, Alder, & Giger, 2003).

To avoid flushing or disposal in the trash, collection programs for unused, unwanted, or expired pharmaceuticals have been developed. In countries such Australia, Canada, Portugal, and Spain, community pharmacies are used to collect medications for final destruction via incineration, funded by these governments (Northwest Product Stewardship Council, 2009). In France, the pharmaceutical industry funds the nationwide collection program. In all cases, a massive public education program accompanies the collection programs as well as education geared toward providers on proper disposal practices.

In the U.S., PPCP collection programs are at best, very local and participation is hit-or-miss, due to a lack of consistent funding and timing. Different programs such as Take Back" or Mail-In programs, where unused or outdated medications are returned to a hospital, pharmacy, reverse distributor, or a police station, have been conducted in various locales. Twice a year (Fall and Spring), the DEA has sponsored collections at many police stations in the country. But the availability of information about these efforts for the public has been highly variable, and achieving a desirable level of public participation requires much notification and outreach. Unlike what occurs in Europe, the pharmaceutical manufacturers have not offered alternatives, incentives, or funding to address this issue. While there have been several product stewardship bills introduced at the state level to encourage pharmaceutical manufacturers to fund disposal programs, little movement on this issue has occurred. The DEA has recently authorized U.S. pharmacies to begin to take back prescription, over-the-counter, and homeopathic medications, but it's unclear when pharmacies will begin to accept this waste and how this will happen. Patient education will be crucial in the success of having consumers use pharmacies to dispose of unused, unwanted or expired medications.

Incineration is used to dispose of pharmaceutical waste because the materials are permanently destroyed. Concerns about efficiency, efficacy, and environmental impacts need to be considered. These concerns include: air emissions and ash residue from the incinerators, the variations in temperature and

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burn time to destroy pharmaceuticals, halogenated dioxins from burning of halogenated pharmaceuticals or containers containing polyvinyl chloride, and the costs and impacts of transportation to and from disposal sites for communities.

Solutions and Recommendations

With new technology or medical treatments, new emerging contaminants in water will continue to be an ongoing issue, and potentially an escalating issue. Specific ideas may help in decreasing the amount and types of emerging contaminants. These include:

1. Using green chemistry as the means to designing and producing PPCP. Green chemistry offers a number of advantages such as using less resources in the design and production of PPCP, increasing the absorptivity of a product, decreasing waste, decreasing the amount of time in the environment, increasing efficiency of production, and reducing the amount and toxicity of chemicals needed in this process. U.S. government policies should encourage the use of green chemistry in all parts of the PPCP lifecycle. Green chemistry use will become crucial not only in addressing bioactivity issues, but also in decreasing water usage in the design and production of PPCP products, as the scarcity of potable water sources increases..
2. Revamping U.S. federal policies that govern approval and regulation of pharmaceuticals, while expanding regulation to cover personal care products, needs to be considered. With new delivery systems (such as nanotechnology) and individualized medicine, the need to review which agencies have a role in the approval and regulatory process should be considered and streamlined to ensure better monitoring and oversight, decreasing the complexity of who does what. Since little interagency coordination exists among federal groups, simplifying and streamlining the process for development, distribution, access, and disposal of PPCP is imperative.
3. Shifting the focus to upstream approaches such as short-cycling prescriptions, decreasing selling large quantities of over-the-counter medications through chain stores, and educating people who prescribe and dispense medications about environmentally preferred ways to dispose of unused, unwanted, or expired PPCP. The need to eliminate the use of non-therapeutic antibiotics in farming practices altogether is essential for ensuring the future efficacy of this class of drugs.

FUTURE RESEARCH DIRECTIONS

When considering the gaps in knowledge on PPCP, a number of key questions should be considered for potential research: 1) What is the volume of PPCP produced within the U.S. and worldwide? 2) How much water is used in that production? 3) What are the potential adverse human and animal health effects at sub-therapeutic levels? 4) What are the synergistic effects (if any) when different PPCP are present in the same water samples? 5) How do these different PPCP affect the waste from the production of PPCP and how can this waste be processed? 6) Can we measure the efficacy of pharmaceuticals after long-term, sub-therapeutic, chronic exposure? These are a small fraction of the potential questions that can be considered when contemplating PPCP as emerging contaminants in drinking water.

Requiring new research to improve disposal practices and decrease discharges, including investment capital that can help discover ways to reduce chemical and biologic activity through non-incineration,

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is critical. Current wastewater treatment processes cannot remove PPCP from finished water. Decreasing the waste associated with PPCP needs to be addressed from an upstream perspective by prescribing less pharmaceuticals and being more selective in purchasing of personal care products. Going forward, implementing green chemistry, short-cycling, and other waste reduction efforts may reduce the quantity of waste, but it will not reduce the need for new methods of waste treatment. Research in these areas is essential to providing the needed data to drive policy decisions at the institutional and governmental levels.

CONCLUSION

Society is facing a confluence of factors: 1) an aging population that is living longer and relying on PPCP to address multiple chronic diseases and signs of aging; 2) a younger population that relies on pharmaceuticals to tackle long-term conditions such as obesity and Attention Deficit/Hyperactivity Disorder, and uses a multitude of personal care products; 3) the growth of personalized and tailored medicine; and 4) the increasing challenge of providing potable water to support human and animal populations. The combination of these factors will create an unprecedented use and development of PPCP as well as new medical therapies not only in the U.S., but also throughout the world. The challenge will be to find ways to decrease the chemical and biological activity, increase the use of green chemistry in all phases of the product lifecycle, eliminate non-therapeutic use of antibiotics for animals, mitigate prescribing of pharmaceuticals and purchasing of personal care products to promote less waste, and promote policies that foster practices to address these factors.

Our reliance on pharmaceuticals and personal care products including medical therapies to maintain our health and improve our quality of life will increase over time. Identifying these chemicals as emerging contaminants that may impact our drinking water provides us with opportunities to pursue new strategies and policies that can address current challenges and provide solutions into the future.

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KEY TERMS AND DEFINITIONS

Active Pharmaceutical Ingredient (API): The primary chemical of action that a pharmaceutical is comprised of.

Antibiotic-Resistance: The phenomenon of pathogenic agents of disease building immunity against antibacterial agents that they were once susceptible to because of overuse and over exposure.

Bioaccumulation: An organism's retention of an API, chemical, or substance that it is exposed to in various concentrations.

Bioactivity: The biological effect(s) of an API, chemical, or substance on various organisms.

Ecotoxicity: The harmful effect(s) of an API, chemical, or substance on the environment, or more specifically an organism's ecosystem.

Effluent: The waste or sewage that is resultant of various industrial and agricultural processes. In can, more specifically, refer to the byproduct of wastewater treatment.

Endocrine-Disruptors: APIs, PPCP, or chemicals that can inhibit or alter the normal function of an organism's hormones or endocrine system to the point of causing adverse health effects on the organism or its offspring.

Green Chemistry: The development of PPCP that is considerate of and mitigates the effects of PPCP throughout its lifecycle, from design and production through discharge and disposal.

Half-Life: The amount of time it takes an API, chemical, or substance to decrease its concentration in an organism or ecosystem by half. In reference to radioactive isotopes, it is the amount of time it takes the radioactivity of an isotope to decrease to half of its original value.

Iodine 131 (^{131}I): A frequently used radioactive isotope that can be found in human and veterinary medical treatments, released from nuclear power plants (unplanned releases or from their laundry facilities), or as a tracer into the environment as part of hydraulic fracturing fluid to determine the location of fissures created in this process.

Nanotechnology: The manipulation of atoms and molecules on a subminute scale. This technology has been increasingly utilized in the production of a variety of personal care products (PCP).

Parabens/Phthalates: Common ingredients in PCP that are known to exhibit endocrine-disrupting properties.

Short-Cycling: The concept of encouraging consumers of PPCP to purchase smaller quantities, especially in regard to newly prescribed/first-time purchases, to prevent excess waste of product in the event of adverse reaction or disapproval of a product.

Wastewater Treatment Plant (WWTP): A municipal facility where water that has been utilized for residential use is filtered and sanitized to be redistributed for residential use.

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Chapter 56
Toxicological Effects of Carbon Nanotubes

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ABSTRACT

The rapidly evolving field of nanotechnology offers many potential societal and economic benefits. Carbon Nanotubes (CNTs) are one of the most widely produced engineered nanomaterials and have diverse applications in engineering, electronics, and medicine. They have also been extensively investigated for their toxicological properties. Studies with rodents indicate that CNTs can cause lung fibrosis or granuloma formation, exacerbate pre-existing respiratory disease, cause injury to the sensitive pleural lining of the lungs, and have systemic immunosuppressive effects. CNTs have also been reported to cause genotoxic effects on cultured cells. The fiber-like structure of CNTs has led to comparisons with asbestos fibers; yet the debate over whether CNTs cause mesothelioma remains highly controversial, and evidence thus far is lacking. The aim of this chapter is to overview the evidence in rodent models that CNTs cause lung disease and to discuss the potential of CNTs to cause adverse immune, fibrogenic, or carcinogenic effects in humans as a result of occupational, consumer, or environmental exposure.

INTRODUCTION

Carbon nanotubes (CNTs) are proto-typical engineered nanomaterials that possess superior strength with minimal weight. CNTs also can be easily functionalized at the atomic level to enhance their properties for use in electronics, engineering, and biomedicine. While promising for many uses, the length and high tensile strength of CNTs also make them a potential risk for lung diseases and as well as other systemic sites of injury [Bonner 2010a]. The most likely route for human exposure is inhalation. A growing number of studies using rats or mice show that CNTs delivered to the lungs cause interstitial lung fibrosis, granuloma formation, or fibrogenic reactions at the pleura [Bonner 2011]. CNTs also exacerbate pre-existing respiratory disease and cause systemic effects in other organ systems such as heart and spleen [Thompson et al., 2013]. While rodent studies indicate that CNTs cause adverse pulmonary and systemic effects, there is currently no epidemiologic data demonstrating that CNTs cause disease

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in humans. This is likely due to the infancy of the nanotechnology industry and the long latency time for chronic lung diseases in humans. A major goal for investigators in the field of nanotoxicology is to better understand the adverse effects and mode of action for engineered nanomaterials in rodent models of disease in order to predict the risk for human disease.

BACKGROUND

Lung Exposures in Rodents

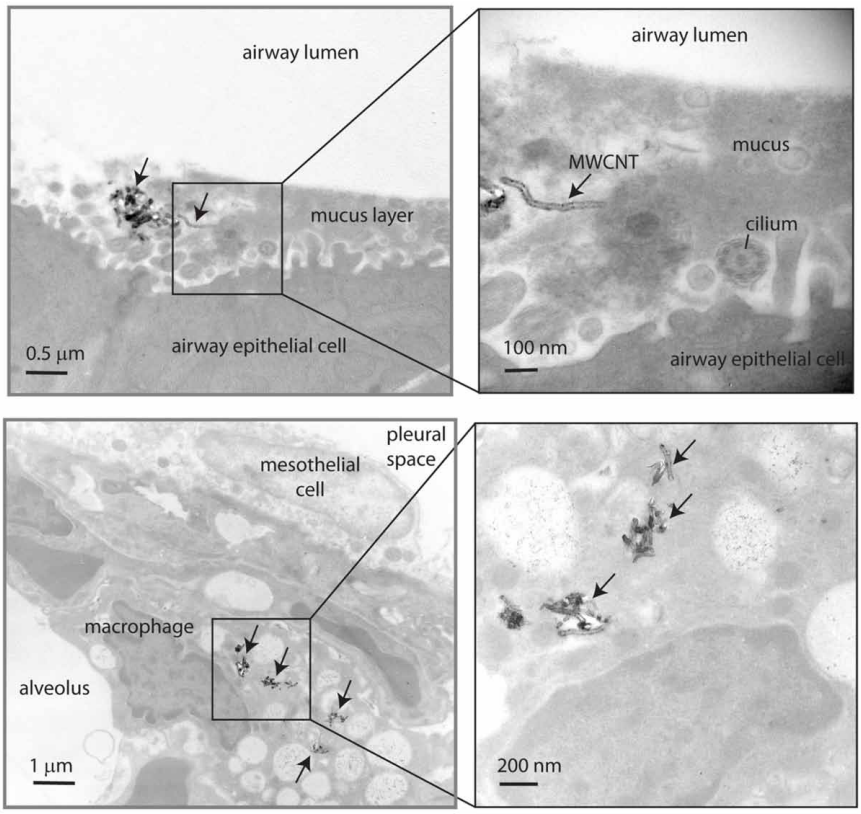
Inhalation studies are most relevant because they provide information on "real world" exposures and therefore more accurately model the true nature of deposition patterns for particles and fibers. Rodent studies show that inhaled CNTs are deposited in the distal regions of the lung, including alveolar duct bifurcations and alveolar epithelial surfaces [Ma-Hock et al. 2009; Pauluhn 2010; Mitchell et al., 2007; Shvedova et al., 2008; Ryman-Rasmussen et al. 2009 a,b]. CNTs are then mainly taken up by alveolar macrophages, which then remove shorter nanotubes (i.e., <10 μm) through airway mucociliary clearance or through the pleural lymphatic drainage. However, some individual CNTs evade phagocytosis by macrophages uptake and are internalized within epithelial cells or mesenchymal cells, or within the extracellular matrix [Ryman-Rasmussen et al. 2009a].

The site of deposition for CNTs following an inhalation exposure is determined by a variety of factors such as size, shape, electrostatic charge, and aggregation. Changing the surface features of CNTs through functionalization (e.g., carboxylation) can alter aggregation status and influence the toxicological outcome in the lung [Wang et al., ACS Nano 2011; Bonner et al., 2013]. In general, studies with mice show that inhaled, well-dispersed CNTs are deposited throughout lower airways of the lung and alveolar compartments [Figure 1]. The majority of published studies on CNT exposure in rodents employ intratracheal instillation (IT) or oropharyngeal aspiration (OPA) techniques rather than inhalation, due mainly to the relative ease and low cost of performing IT or OPA compared to inhalation. IT or OPA techniques deliver an aqueous bolus of CNT and therefore do not precisely reproduce the deposition patterns achieved by inhalation exposures with dry aerosolized or nebulized suspensions of CNTs. Many of the early reports of granulmona in the lungs of rodents caused by CNTs could be due to poor dispersion and non-specific reactions; i.e., highly agglomerated CNTs attract focal accumulations of mononuclear cells that stimulate the formation of fibrotic scar to "wall off" the CNT agglomerate. However, significant improvements have been made in dispersing CNTs in aqueous suspension by using surfactant-containing media to improve dispersal of CNTs for IT or OPA delivery to the lungs of rats and mice [Mercer et al. 2008].

CNTs are cleared from the lung by macrophage-dependent mechanisms; either via the airway mucociliary apparatus or via the pleural lymphatic drainage [Figure 2]. The mucociliary apparatus is comprised of a coating of mucus on the surface of the airways that is constantly moving up the airways by the coordinated movement of cilia on the airway epithelium [Bonner 2008]. Macrophages with engulfed particles or fibers migrate to the distal portion of small airways where they are transported by the escalator to larger airways and ultimately out of the trachea where they are swallowed or expelled through coughing. Migration of macrophages containing CNTs across the pleura, through stomata in the mesothelium that covers the pleura, could cause adverse effects (e.g. DNA damage to mesothelial cells) as CNTs could possess some physical characteristics (e.g., high aspect ratio, durability) that resemble asbestos fibers [Bonner et al., 2010a; Donaldson 2010]. However, the relative carcinogenicity of CNTs

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Figure 1. Electron microscopic images of multi-walled carbon nanotubes in the lungs of mice 1 day following a single inhalation exposure at a dose of 30 mg/m3 for 6 hours. The upper panels show nanotubes deposited on the surface of airway epithelium. Lower panels show nanotubes within a macrophage that has migrated to the subpleural region of the lung.



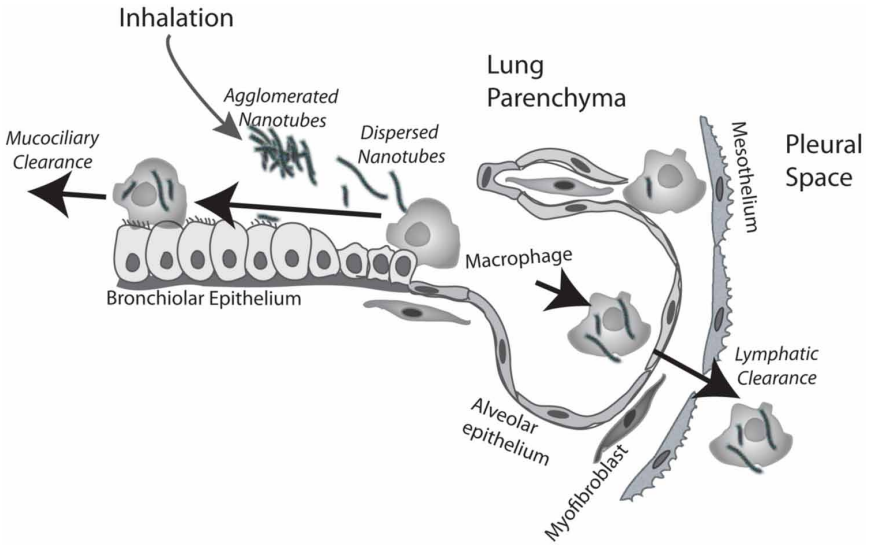
compared to asbestos fibers remains a highly controversial topic and no reports of human lung disease have yet to be reported. Nevertheless, some CNTs, particularly long CNTs, remain embedded within the subpleural tissue of the lung for up to 3 months [Ryman-Rasmussen et al., 2009b]. Also, macrophages carrying CNTs that exit the lung via the pleural lymphatic system can be found in lung-associated lymph nodes [Ma-Hock et al. 2009; Pauluhn et al., 2010]. The clearance of inhaled MWCNTs from the lungs and lung associated lymph nodes (LALNs) of rats following a nose-only inhalation exposure was reported to be more than a year [Pauluhn et al., 2010]. These findings indicate that CNTs are relatively persistent in the lungs following inhalation.

Lung Inflammatory and Fibroproliferative Responses to CNTs

The acute response of inhaled CNTs, both SWCNTs and MWCNTs, is characterized by neutrophilic inflammation of the lower lungs within the alveolar regions and alveolar duct bifurcations [Bonner et al., 2013 EHP]. Macrophages provide a central role as the primary immune defense against particles CNTs, partly through clearance as mentioned above, but also the production of chemokines that drive neutrophilic inflammation. Both inhalation and instillation studies in rodents demonstrate that CNTs

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Figure 2. Illustration showing the two major routes of carbon nanotube clearance, mucociliary clearance and lymphatic clearance, following inhalation exposure



cause acute lung inflammation within 1 day following exposure [Li et al., 2007; Shvedova et al., 2007; Ryman-Rasmussen et al., 2009a, b; Ma-Hock et al., 2009, Pauluhn et al., 2010]. Of greater concern are the chronic fibroproliferative effects of CNTs in the lung that may take weeks or months to manifest. Pulmonary fibrosis is a fatal disease in humans associated with occupational exposure to asbestos or certain metals such as cobalt [Bonner et al., 2010]. Fibrosis has also been reported in rodents exposed to CNTs [Mangum et al., 2006; Li et al., 2007; Shvedova et al., 2007; Ryman-Rasmussen et al., 2009a, b; Pauluhn et al., 2010]. As mentioned above, highly aggregated CNTs produce focal granulomatous lesions [Mangum et al., 2006; Lam et al., 2004; Muller et al., 2005; Shvedova et al., 2005; Warheit et al., 2004; Murray et al., 2012]. In contrast, well-dispersed CNTs, achieved by agents such as bovine serum albumin (BSA) and dipalmitoylphosphatidylcholine (DPPC), cause more diffuse fibrosis and alveolar wall thickening that is perhaps more destructive in terms of reducing lung function. Dispersed CNTs also stimulate increased growth factor (PDGF-AA, TGF-β1) production by lung cells compared to non-dispersed CNTs, supporting the idea that dispersal state determines profibrogenic cellular responses that correlate with severity of fibrosis [Wang et al., 2011].

The fibrogenic potential of CNTs is also determined by tube length; longer CNTs produce greater inflammation and fibrogenic reactions that shorter CNTs. Longer CNTs pose a problem for macrophage-mediate clearance and tubes longer than ten to fifteen micrometers (the approximate width of an alveolar macrophage) are difficult to clear from lung tissues as they impede macrophage migration and cause 'frustrated phagocytosis' that involves cell membrane damage and leakage of cytoplasmic components. This is important only with regards to certain types of rigid MWCNTs and other high aspect ratio nanomaterials and fibers, and likely does not apply to long SWCNTs which are more likely to fold upon themselves and are ingested by macrophages. Therefore, both rigidity and length are important factors in determining whether CNTs will cause frustruated phagocytosis by macrophages.

The presence of residual metal catalyst plays an important role in mediating CNT toxicity and disease potential. Nickel, cobalt, and iron are commonly used as catalysts in the manufacture of CNTs. These same

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metals have also been reported to cause pulmonary diseases in humans, including pulmonary fibrosis and asthma [Kelleher et al., 2000]. For example, cobalt has been shown to cause 'hard metal disease' in welders and nickel is a known cause of occupational asthma and contact dermatitis. Iron has been linked to interstitial pulmonary fibrosis in occupational settings related to mining and metallurgy. Metal catalysts can be removed to some extent from CNTs by acid washing, although this process has yet to be shown completely effective in removing the metal catalyst. Nevertheless, reduction in Ni content has been shown to significantly reduce CNT-induced inflammation in the lungs of mice [Bonner et al. 2013]

Genotoxicity and Carcinogenesis

Because CNTs are cleared from the lungs in part through macrophage phagocytosis and trafficking across the pleural lining through lymphatic ducts, they have the opportunity to interact with the mesothelial lining that makes up the pleura. The durable nature of CNTs along with their fiber-like shape could pose a problem of long term lung persistence, which could result in carcinogencity and the formation of mesothelioma. CNTs have been demonstrated to migrate to the subpleural tissue in mice after a single inhalation exposure and produce aggregates of mononuclear cells on the pleural surface [Ryman-Rasmussen et al., 2009a]. MWCNT inhalation exposure has also been reported to cause subpleural fibrosis [Ryman-Rasmussen et al., 2009b]. These observations are directly relevant to early inflammatory or immune reactions that could preceed carcinogenic effects at the visceral or parietal pleura but do not directly provide evidence of tumor formation. However, a surrogate assay wherein long MWCNTs are injected into the peritoneal cavity of mice has shown granulmoma formation that suggests asbestos-like pathogenicity of CNTs [Poland et al., 2008]. Using this same intraperitoneal technique, transgenic p53 heterozygous null mice, which are susceptible to the development of mesothelioma by asbestos, were reported to have an increased incident of mesothelioma formation in the abdominal cavity after intraperitoneal injection of MWCNTs [Takagi et al., 2008].

Studies in mice have shown that MWCNTs delivered to the lungs of mice by nose-only inhalation exposure or by oropharyngeal aspiration migrate to the pleura [Ryman-Rasmussen et al., 2009a; Porter et al., 2010]. The biological response to inhaled MWCNTs was characterized by mononuclear cell accumulation at the pleural surface within 1 day after exposure and subpleural fibrotic lesions within 2 weeks, which resolved by 6 and 14 weeks, respectively [Ryman-Rasmussen et al., 2009a]. MWCNTs delivered to mice by oropharyngeal aspiration penetrate the pleural lining, suggesting direct mesothelial injury similar to asbestos fibers [Mercer et al., 2010]. However, these studies showed no evidence of mesothelioma. This may be because wild type mice are a relatively poor model for induction of mesothelioma, even when exposed to asbestos, a known inducer of mesothelioma in humans. Mesothelioma has been reported after exposure to MWCNTs in transgenic p53-deficient mice, which are susceptible to a variety of cancers [Takagi et al., 2008; Takagi et al., 2012]. Importantly, it has been demonstrated that MWCNTs or crocidolite asbestos delivered to the lungs of rats by intratracheal instillation both exhibit pleural mesothelial cell proliferation [Xu et al., 2013]. While no human data exist for cancer or other diseases as a result of CNT exposure, the issue of whether CNTs are capable of causing mesothelioma in humans remains a key topic of research that is relevant to disease prevention and an important issue linked to the regulation of engineered nanomaterials. For example, NIOSH set a recommended occupational exposure limit for MWCNT that was based on rodent studies [Castranova et al., 2012].

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Cellular and Molecular Mode of Action

CNTs likely mediate toxicity similar to other fiber-like particles (e.g., ROS generation), but also might operate through unique and unexpected mechanisms since they interact at the molecular levels with cellular organelles, proteins, phospholipids, and nucleic acids. CNTs stimulate the release of a variety of cell-derived mediators such as growth factors, cytokines, chemokines, proteases, and lipid mediators that play important roles in inflammation, fibrosis, asthma, and cancer [Bonner 2010b; Thompson et al. 2013].

As mentioned above, lung macrophages play a central role in the inflammatory response to inhaled particles and fibers. CNTs have been shown to cause macrophage inflammasome activation [Palomäki et al. 2011; Meunier et al. 2012; Sun et al. 2012]. Inflammasomes are multi-scaffold cytoplasmic structures that cleave pro-IL-1β and pro-IL-18 to mature forms of these cytokines, which are then secreted by macrophages to mediate an inflammatory response. Inflammasome activation is initiated by CNT-induced lysosomal disruption and ROS production. Also, nickel catalyst in MWCNTs has been demonstrated to mediate inflammasome activation in macrophages [Hamilton et al., 2012]. Inflammasome activation is an important innate immune response and IL-1β release is important for recruiting neutrophils to the injured lung, which then play an important role the resolution of inflammation and tissue repair [Martinon 2009]. On the other hand, inflammasome activation could play a role in certain disease states, including fibrosis and asthma [Strowig et al., 2012]. Chemokines are also induced by CNT exposure and are important in orchestrating the inflammatory response in the lung. CXCL8 (IL-8), a potent neutrophil chemoattractant, is produced by a human bronchial epithelial cell line *in vitro* after exposure to MWCNT [Hirano et al., 2010]. The monocyte chemoattractantCCL2 is elevated in the lavage fluid of mice exposed to MWCNT inhalation [Ryman-Rasmussen et al., 2009a]. CCL2 is produced by a variety of cell types, including pleural mesothelial cells [Visser et al., 1998], and could play a role in mediating pleural inflammation in mice after inhalation of MWCNTs [Ryman-Rasmussen et al., 2009a].

Macrophages also play a role in lung fibrosis caused by CNTs, largely by producing soluble factors that activate lung myofibroblasts to proliferate, migrate, or produce extracellular matrix. Classically activated "M1" macrophages exposed to IFN-γ and LPS are important for microbial killing and innate immune responses. Alternatively activated "M2" macrophages exposed to Th2 cytokines IL-4 or IL-13 to produce growth factors (e.g., PDGF, TGF-β1) that are involved in the pathogenesis of fibrosis [Gordon and Martinez 2010]. CNTs, either single- or multiwalled-, delivered to the lungs of rats or mice increase PDGF and TGF-β1 [Mangum et al., 2006; Ryman-Rasmussen et al., 2009a; Cesta et al., 2010, Shvedova et al., 2005]. PDGF is a key mediator of lung myofibroblast survival, growth, and chemotaxis that serves to promote fibrogenesis [Bonner et al., 2004]. Mice exposed to SWCNTs have elevated levels of TGF-β1, which is a potent stimulator of collagen production by myofibroblasts [Shvedova et al., 2005; 2008]. Other factors in the lungs of rodents induced by SWCNT, such as osteopontin (OPN), could also serve to stimulate collagen deposition [Mangum et al., 2006]. Investigations of cell-cell interactions using co-culture systems could be very useful elucidating mechanisms through which CNTs mediate inflammation, fibrosis, or pleural disease. For example, because macrophages transport inhaled CNTs to the subpleural region of the lung, it is important to understand how CNTs influence the interaction between macrophages and pleural mesothelial cells. It has been reported that human Met5A pleural mesothelial cells do not produce inflammatory cytokines when directly challenged with MWCNTs, yet the supernatants from MWCNT-stimulated human THP-1 macrophages stimulate significant cytokine release from Met5A cells.

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Like other toxic fibers, CNTs mediate toxic effects on cells through the generation of reactive oxygen species (ROS). CNTs activate the antioxidant mediator Nrf2 in cultured human THP-1 cells [Brown et al., 2010]. CNTs also increase levels of the COX-2 enzyme through a MAP kinase-dependent signaling pathway in mouse RAW267 cells *in vitro* [Lee et al., 2012]. Also, COX-2 knock-out mice are susceptible to airway remodeling induced MWCNT exposure [Sayers et al., 2013]. COX-2 and Nrf2 are both protective factors against fibroproliferative lung disease that are increased to counteract ROS-induced injury initiated by CNTs.

A number of studies have addressed the possible genotoxic effects of CNTs using lung cell types and in rodents *in vivo*. SWCNTs have been shown to cause fragmented centrosomes, mitotic spindle disruption, anaphase bridges, and aneuploid chromosome number in cultured primary or immortalized human airway epithelial cell types [Sargent 2009; Sargent et al. 2012]. CNTs were also shown to disrupt mitotic spindle formation and it has been speculated that the similar size and geometry of SWCNT bundles might account for physical interaction of SWCNT with the microtubules that form the mitotic spindle. MWCNTs have been reported to cause DNA damage in mouse embryonic stem (ES) cells [Zhu 2007]. Moreover, MWCNTs accumulated and induced apoptosis in mouse ES cells and activate the tumor suppressor protein p53, a marker of DNA damage. In addition MWCNTs have been shown to cause dose-dependent genotoxic effects of structural chromosome aberrations, DNA damage, and micronuclei formulation in mice when administered by intraperitoneal injection [Patiolla et al., 2010]. Persistent DNA damage was reported in rat lung cells one month post-inhalation exposure to MWCNTs [Kim et al., 2012]. Therefore, it appears that both SWCNT and MWCNT have genotoxic effects in cultured cells and in the lungs of rodents *in vivo*.

EMERGING ISSUES AND CONTROVERSIES

Human Exposure and Potential Risk of Disease

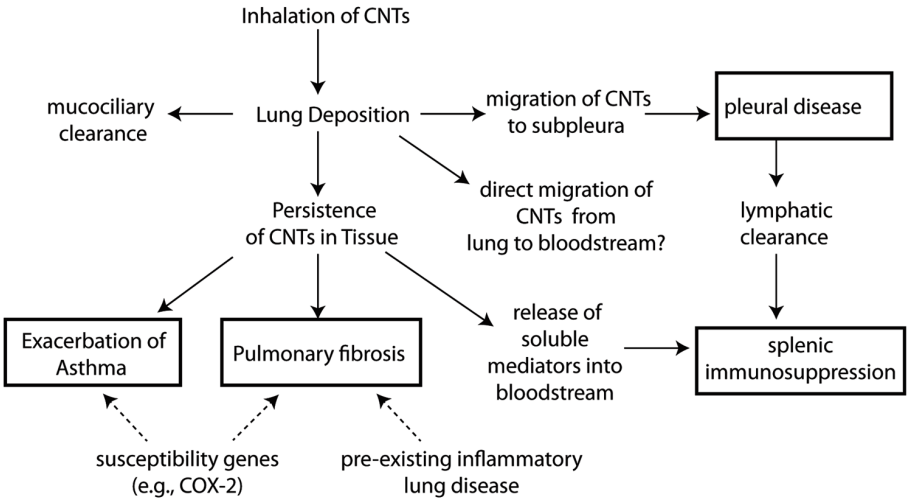
It is currently unknown whether CNTs will cause fibroproliferative or carcinogenic effects in humans. However, human exposure to CNTs is inevitable, especially in occupational settings where relatively high amounts of CNTs are synthesized, refined, or functionalized. The lack of human exposure data is due to the infancy of the nanotechnology field in general. Moreover, it could be years before any epidemiologic data are available, as lung fibrosis or cancer caused by inhaled toxic particles and fibers can take years to manifest in humans. Evidence from rodent studies indicates that some adverse effect will be observed in humans exposed to some types of CNTs, particularly long, rigid tubes that are more difficult to clear from the lungs.

Functionalization of Carbon Nanotubes

Nearly all of the published work on CNTs have used ‘pristine’ or unmodified nanotubes that are generated by processes such as carbon vapor deposition. These studies are probably most relevant to human occupational exposures where individuals will be exposed to CNTs directly after synthesis. However, most CNTs will likely undergo some sort of post-synthesis processing or ‘functionalization’ to modify the surface properties of the CNT. This means that most consumer and environmental exposures will be related to some type of functionalized CNT. For example, aggregation of CNTs due to electrostatic

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Figure 3. Diagram showing potential adverse effects of carbon nanotubes on lung and other organs systems following inhalation exposure. Adverse disease outcomes are in boxes. Dotted arrows indicate factors that influence disease severity.



forces presents a problem for the purposes of many engineering designs and a variety of dispersal agents (e.g., surfactants) have been employed to improve dispersion by reducing electrostatic attraction. More dispersed CNTs delivered to mice cause a more severe chronic interstitial fibrosis in the lungs along with elevated levels of growth factors (PDGF and TGF-β1) that play important roles in fibrogenesis [Wang et al., 2011]. However, some functionalizations can reduce the fibrogenic response of CNTs, even though they remain dispersed. For example, carboxylated (COOH)-CNTs cause less neutrophilic lung inflammation as compared to unmodified CNTs [Bonner et al., 2013], and cause less pulmonary fibrosis [Wang et al., 2011]. In contrast to COOH-CNTs, polyetherimide (PEI) functionalized CNTs increased collagen deposition and fibrosis in the lungs of mice [Li et al., 2013]. Therefore, some functionalization processes reduce toxicity and lung injury while others increase toxicity and the potential for disease. Therefore, in order to carefully assess human health effects, consideration should be given to both acute and chronic effects of functionalized CNTs in particular and to engineered nanomaterials in general. A increasing variety of functionalizations are employed to modify specific characteristics of CNTs, which makes assessing the toxicity each type of functionized CNT in rodents impractical. In response to this growing challenge, high through-put screening techniques will be necessary to test the vast majority of engineered nanomaterials for toxic signatures [Nel et al., 2013].

Environmental and Genetic Susceptibility Factors

It is likely that CNTs might have the most profound adverse health effects on individuals with pre-existing respiratory diseases such as asthma, bronchitis, or chronic obstructive pulmonary disease (COPD) [Bonner 2010a, Bonner 2011]. A variety of environmental and genetic susceptibility factors will likely influence CNT toxicity [Figure 3]. Allergic airway remodeling in mice induced by ovalbumin allergen challenge is exacerbated by SWCNTs or MWCNTs, suggesting that CNTs have the potential to exacerbate asthma in humans [Ryman-Rasmussen et al., 2009b; Inoue et al. 2009; Inoue et al., 2010; Nygaard et al. 2009].

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In addition to asthma exacerbation, repeated exposure to MWCNTs induces a Th2 allergic response in the absence of any allergen pre-exposure, suggesting that CNTs possess adjuvant-like properties [Park et al., 2009; Beamer et al. 2013]. However, it remains unknown whether CNTs will cause or exacerbate asthma in humans.

Bacterial or viral infection could also be important environmental susceptibility factors in determining CNT toxicity in the lung. Lipopolysaccharide (LPS) derived from gram-negative bacteria mediates a variety of occupational and environmental lung diseases in humans. SWCNTs or MWCNTs exacerbate LPS-induced lung inflammation, pulmonary vascular permeability, and production of pro-inflammatory cytokines in the lungs of mice [Inoue et al., 2008]. LPS pre-exposure also enhances MWCNT-induced pulmonary fibrosis in rats and synergistically enhances MWCNT-induced production of PDGF by rat alveolar macrophages and lung epithelial cells [Cesta et al., 2010]. These studies indicate that pre-existing neutrophilic inflammation caused by LPS is a major determinant in CNT lung toxicity and suggests that individuals with pre-existing neutrophilic lung inflammation would be at increased risk for CNT-induced lung disease.

In addition to environmental susceptibility factors, a variety of genes play important roles in determining susceptibility to CNTs and other engineered nanomaterials. For example, mice deficient in the COX-2 enzyme are susceptible to exacerbation of allergen-induced airway remodeling by CNTs [Sayers et al., 2013]. While COX-2 mediates pro-inflammatory effects, it has also been implicated in the pathogenesis of asthma and pulmonary fibrosis [Bonner 2010]. Mice deficient in myeloperoxidase (MPO) have fewer CNTs in their lungs compared to wild type counterparts, suggesting that MPO mediates the degradation of some CNTs in the lungs [Shvedova et al., 2012]. It is likely that many genes will influence the disease potential of CNTs and therefore relative expression of these genes will determine individual susceptibility in humans.

Systemic Effects of Inhaled CNTs

Growing evidence indicates that inhaled CNTs move from the lungs into the circulation or lymphatic system to reach other organs [Figure 3]. Some of the CNTs delivered to the lungs of rodents are removed via the lymphatic system and localize in lymphoid tissues [Ma-Hock et al. 2009, Pauluhn 2010]. In addition, inhaled CNTs can cause systemic effects by stimulating resident lung cells to produce soluble mediators that enter the circulation and cause splenic immunosuppression [Mitchell 2007]. This particular mechanism involved the release of TGF-β1 from the lung which enters the bloodstream to signal COX-2-mediated increases in prostaglandin-E2 and IL-10 in the spleen, which in turn suppress T cell proliferation [Mitchell 2009]. CNTs delivered to the lungs of mice have been reported to exacerbate cardiovascular disease, although it is unclear whether this effect is due to direct translocation of CNTs to heart tissue or the release of soluble inflammatory mediators from the lungs [Erdely et al., 2009]. Recent evidence shows that CNTs delivered to the lungs of mice are capable of reaching multiple organ systems, including kidney, liver, and brain [Mercer et al., 2013]. Nevertheless, there is little information available on whether CNTs adversely affect the physiology of these organ systems and this is an important area for future research.

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Transcriptomics and Proteomics Related to CNT Toxicity

The majority of studies on CNT toxicology have focused on selected mediators of tissue injury or disease progression. However, it will be increasingly important to consider how a rapidly growing number of engineered nanomaterials, including CNTs, affect large sets of genes or protein expression in cells or tissues. It is anticipated that such investigations will lead to gene or protein expression profiles that can be used to assess CNT risk for disease using cultured cells, thereby reducing the need for animal studies. For example, a recent transcriptomics study comparing lung tissue from CNT exposure of mice *in vivo* to cultured lung epithelial cells exposed to CNTs *in vitro* showed some similarities observed between the two models at the pathway-level, but many of the specific genes altered under these pathways were different [Søs Poulsen et al., 2013]. These findings indicated that the underlying mechanisms of responses are different in cells in culture as compared to lung tissue and the authors suggested that careful consideration should be given to the selection of relevant endpoints when substituting animal with *in vitro* testing. Proteomics have also been valuable in comparing CNTs to carcinogenic and fibrogenic fibers. For example, proteomic analysis of lung tissue from mice exposed to SWCNTs, crocidolite asbestos, or carbon black nanoparticles supported the conclusion that lung tissue responses to SWCNTs and crocidolite asbestos were similar, but the response was generally greater on a mass dose basis for SWCNTs than for asbestos [Teeguardin et al., 2011]. These examples highlight the utility of transcriptomic and proteomic analysis for predicting risk of CNTs in human disease. Such studies will be valuable to assessing the toxicity of a growing number of functionalized CNTs.

CONCLUSION AND FUTURE DIRECTIONS

Carbon nanotubes have enormous potential for technological innovation in engineering, electronics, and medicine. However, careful consideration should be given to the potential human health risks that CNTs pose in occupational, consumer, or environmental exposure scenarios. Predictions that CNTs will have the potential to cause fibrosis and/or cancer in humans are based on physical similarities to toxic fibers and growing evidence that CNTs cause fibrosis and perhaps cancer in rodents. As with other engineered nanomaterials, the nanoscale of CNTs make it difficult to predict how these structures will interact with intracellular structures such as DNA, cell membranes, and cytoskeletal proteins. Also, CNTs contain residual metals (e.g., nickel, cobalt, iron) introduced from the manufacturing process and many of these metals are known to cause fibrosis or cancer in humans. Finally, a variety of post-synthesis functionalization modifications will be applied to CNTs to enhance their novel properties and it is anticipated that high throughput screening techniques will be required to assess the toxicity of a growing number of CNT types.

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KEY TERMS AND DEFINITIONS

Engineered Nanomaterial: An intentionally manufactured nanoparticle with precise physical and chemical characteristics.

Functionalized Carbon Nanotube: A carbon nanotube with intentional post-synthesis modifications to alter surface properties and functionality.

Immunosuppression: Reduced activity and function of immune cells resulting in impaired host defense.

Mesothelioma: Rare cancer affecting the mesothelial linings of the lungs and chest cavity and most often associated with asbestos exposure.

Multi-Walled Carbon Nanotube: A carbon nanotube composed of multiple concentric stacks of graphene.

Pulmonary Fibrosis: Tissue scarring in the lung alveolar region usually associated with environmental exposure to inhaled particles or fibers.

Single-Walled Carbon Nanotube: An engineered nanomaterial consisting of a cylindrical, hollow tube of graphene.

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Chapter 57
Understanding Toxicity of Nanomaterials in Biological Systems

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ABSTRACT

Nanotechnology is a growing applied science having considerable global socioeconomic value. Nanoscale materials are casting their impact on almost all industries and all areas of society. A wide range of engineered nanoscale products has emerged with widespread applications in fields such as energy, medicine, electronics, plastics, energy and aerospace etc. While the market for nanotechnology products will have grown over one trillion US dollars by 2015, the presence of these material is likely to increase leading to increasing likelihood of exposure. The direct use of nanomaterials in humans for medical and cosmetic purposes dictates vigorous safety assessment of toxicity. Therefore this book chapter provides the detailed toxicity assessment of various types of nanomaterials.

INTRODUCTION

The field of nanotechnology is a fast-growing research niche (Ostiguy et al., 2006). Nanoparticles are particles that have at least one dimension in nanoregime, i.e., less than 100 nm. Nanotechnology is a comprensive term that involves the ability to work with materials at a nanometre scale. Nanotechnology has a potential applications in a range of sectors including energy (production, catalysis, storage), materials (lubricants, abrasives, paints, tires, and sportsware), electronics (chips and screens), optics, and remediation (pollution absorption, water filtering and disinfection), cosmetics (skin lotions and sun screens), and medicine (diagnostics and drug delivery) to food (additives and packaging) (Joner, Hartnik, & Amundsen, 2007; Rana & Kalaichelvan, 2013). The word ”nano,” is derived from the Greek “nanos” meaning “dwarf”, and is becoming increasingly widespread in scientific literature. “Nano” word has

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become so popular in modern science that many "nano-"derived words have recently find their places in dictionaries, including: nanometer, nanoscale, nanoscience, nanotechnology, nanostructure, nanotube, nanorod nanowire, and nanorobot and many more. Many new words like nanoelectronics, nanocrystal, nanovalve, nanoantenna, nanocavity, nanoscaffolds, nanofibers, nanomagnet, nanoporous, nanoarrays, nanolithography, nanopatterning, nanoencapsulation, etc. are used in respected and reputed publications, such as Science and Nature that are not yet widely recognized. The nanometer denotes one billionth of a meter or 10^{-9} meters and is a metric unit of length. The word 'nano' is popularly used as an adjective to describe objects, systems, or phenomena with characteristics arising from nanometer-scale structure and emphasize the atomic granularity that produces the unique phenomena observed in nanoscience. Most of the exciting properties begin to be apparent in systems smaller than 1000 nm, or 1 micrometer, 1 μm. Birth of the concept of nanotechnology is usually linked to a speech by Richard Feynman at the December 1959 meeting of the American Physical Society (Feyman, 1959) where he asked a very funny and strange question to the scientific audiences, "What would happen if we could arrange the atoms one by one the way we want them?"

Nanomaterials are materials possessing structural components smaller than 1 micrometer in at least one dimension. Many authors, however, limit the size of nanomaterials to 50 nm (Kittelson, 2001) or 100 nm (Borm et al., 2006), the choice of this upper limit being justified by the fact that some physical properties of nanoparticles approach those of bulk when their size reaches these values. However, a legitimate definition extends this upper size limit to 1 micron, the sub-micron range being classified as nano(Buzea, Pacheco, & Robbie, 2007b).

Nanoparticles are particles potentially as small as atomic and molecular length scales (~0.2 nm) or with at least one dimension falling in nanoregime. Nanoparticles can be amorphous or crystalline and their highly active surfaces can act as carriers for gases or liquid droplets. Nanoparticulate matter should be considered as a distinct state of apart from the solid, liquid, gaseous, and plasma states owing to their distinct properties like large surface area and quantum size effects.

Nanomaterials being versatile in nature has various applications in biology and medicine such as Fluorescent biological labels(Fu et al., 2007), drug and gene delivery(Yan & Chen, 2013)(Malmsten, 2013), bio detection of pathogens (Yang, Li, & Jiang, 2008), detection of proteins(Yi Zhang et al., 2013), probing of DNA structure (Mahtab & Murphy, 2005), Tissue engineering (Cipriano & Liu, 2013), tumour destruction via heating (hyperthermia) (Dutz & Hergt, 2014), MRI contrast enhancement (Na, Song, & Hyeon, 2009) etc

NANO VS. BULK MATERIALS AND NANOTOXICOLOGY PUBLICATION STATISTICS

Nanomaterials behave significantly different from bulk materials due to two primary factors which are surface effects and quantum effects (Roduner, 2006). These two factors affect the mechanical, electrical, optical and magnetic as well as chemical reactivity of the materials. Compared to the microparticles, nanoparticles have a very large surface area and high particle number per unit volume due to the high fraction of atoms at the surface. For example the ratio of surface area to volume (or mass) for a carbon particle with a diameter of 60 nm is 1000 times larger than a particle with a diameter of 60 μm. Since the material in nanoparticulate form presents a much large surface area, due to which chemical reactivity of the material is greatly enhanced roughly 1000 folds. The atoms situated at the surface has lower

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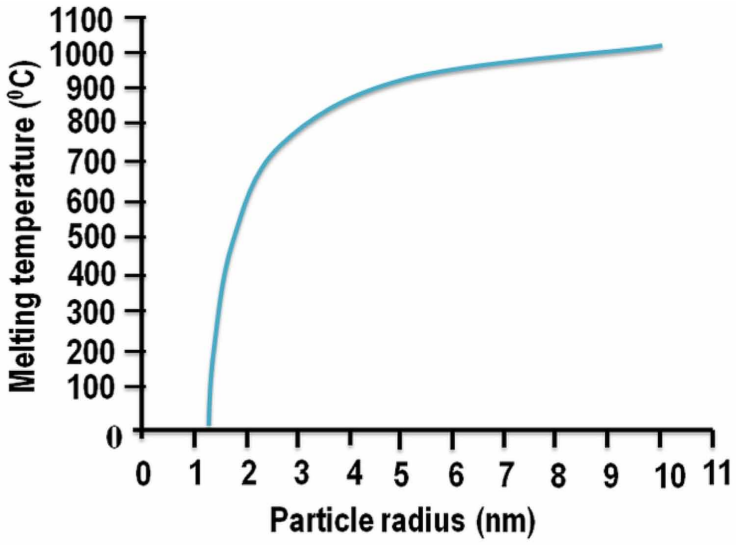
binding energy per atom due to the lesser no of neighbors than bulk atoms (Roduner, 2006). As a result, melting point reduces with reduction in particle size due to lesser binding energy at smaller particles sizes as followed from the Gibbs-Thomson equation. For example, the melting temperature of 3 nm gold nanoparticles is more than 300 degrees lower than the melting temperature of bulk gold (Roduner, 2006) (Figure 1).

There has been an exponential increase in the number of publications on the synthesis and properties of nanoparticles since 1090s (Figure 2) as indicated by the advanced search on Google Scholar Database. There is also an increase in number of publications discussing their toxicity for the past few years (Figure 2). However, the total no of papers on toxicity remains low in comparison to the total no of papers published on nanomaterials. Since Nanoscience and Nanotechnology encompasses a wide range of fields ranging from chemistry to physics, materials science and engineering, biology, medicine and electronics, which explains the reason of the vast no of papers published in this field. However, several reviews have been published addressing the nanotoxicology aspects of nanomaterials. Several are comparatively general(Borm et al., 2006; Nel, Xia, Mädler, & Li, 2006; X. Zhang, Hu, Li, Tao, & Wei, 2012) while others address selected aspects of nanoparticles toxicology such as occupational aspects of nanoparticle(Pfau, Sentissi, Weller, & Putnam, 2005); particle inhalation, retention, and clearance (Buzea, Pacheco, & Robbie, 2007a); pulmonary effects of inhaled particles (Oberdörster, 2000); inhalation and lung cancer(Borm, Schins, & Albrecht, 2004), oxidative mechanisms(Brown, Lockwood, & Sonawane, 2005; González-Flecha, 2004; Peters, 2005) and gastro-intestinal uptake of particles (Fubini & Hubbard, 2003).

Nanoparticle Synthesis

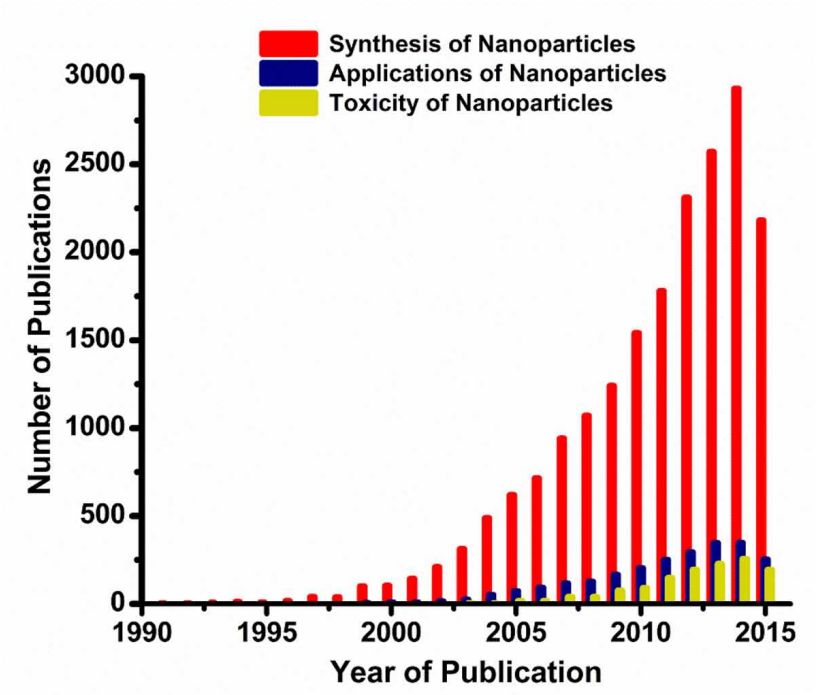
Various excellent review have been published on the synthesis and characterization of nanomaterials (Saber M Hussain et al., 2009; Rodriguez-Hernandez, Chécot, Gnanou, & Lecommandoux, 2005; Teow, Asharani, Hande, & Valiyaveettil, 2011; Y. Wang & Cao, 2006). Two different but complementary

Figure 1. A plot of melting temperature of gold as a function of size



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Figure 2. Evolution of no of papers on Synthesis, Applications and Toxicity on nanoparticles since 1990



approaches have been used for synthesizing nanomaterials (Figure 3). First one known as "top-down" approach where bulk materials are broken down into particles in nanorange by physical processes such as milling, grinding, etching and pyrolysis. In this approach particle size of the starting bulk material is reduced to nanorange using mechanical or chemical methods. Photolithography and silicon microfabrication are two processes often employed or prepared using the top-down approach (Mishra, Patel, & Tiwari, 2010). This approach has the advantage of the possibility of mass production for industrial purposes. However, this approach involves some disadvantages such as lengthy time consuming processes, generation of a broad feature size distribution, and imperfection or defects of the surface morphology generated. Since surface deformations can have a significant impact on the physical and chemical properties of the nanomaterials(Thakkar, Mhatre, & Parikh, 2010).e.g., pyrolysis involves the burning of organic precursor after forcing through an orfice at high pressures. The resulting ash produced is processed to recover the oxidized nanoparticles having wide particles size distribution. (Thakkar et al., 2010), thus consuming an enormous amount of energy so as to sustain high pressures. Bottom-up approach, involves creation of nano-objects by combining atomic scale materials. A variety of bottom up approaches exist involving comples coacervation of two oppositively charged polyelectrolyted, co-precipitation, salting out from aquous-organic mixture or solvent displacement, solvent emulsification, diffusion using oil-water microemulsions or using supercritical fluids. The process operated under ambient conditions leading to high energy saving. A wide variety of nanomaterials can be generated using this approach including nanoemulsions, liposomes, polymeric NPs, polymeric micelles, solid lipid NPs, protein NPs and carbohydrate such as chitosan, alginate, hyaluronic acid, dextran nanoparticles(Zonghua Liu, Jiao, Wang, Zhou, & Zhang, 2008; Szarpak et al., 2010). The most commonly employed method involves the

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chemical reduction of metal ions by a suitable reducing agent in the presence of a capping or stabilizing agent. The metal atoms obtained after reduction of the metal ions aggregate to form metal nanoparticles. The further growth of these metal nanoparticles is inhibited by the addition of the suitable capping agent which works either by conferring a suitable charge to the surface which repels neighboring naoparticles or by sterically preventing the agglomeration of two NPs (Thanh & Green, 2010). Examples of such capping agents include carboxylic acids or amines. Capping agent also serves important function such as controlling particle size, shape, charge and surface functionality which can lead to special properties. There has been a growing interest in synthesis of nanoparticles using biological approaches using bacteria (Reddy et al., 2010), yeast (Kowshik et al., 2003) fungi, plants and algae (Thakkar et al., 2010). This method offers advantage over conventional chemical methods as avoid the use of toxic organic solvents and reagents, generation of hazardous side-products, high cost and energy consumption. The nanoparticles produced by this approach relatively non-toxic and biocompatible. Nanoparticles of different materials ranging from from Ag, Au, CdS, magnetite and uranium were produced using biological approach (Nanda & Saravanan, 2009). A similar method has been used in the synthesis of Ag NPs using fungal cell filtrate (Gajbhiye, Kesharwani, Ingle, Gade, & Rai, 2009).

Toxicity of Different Nanoparticles

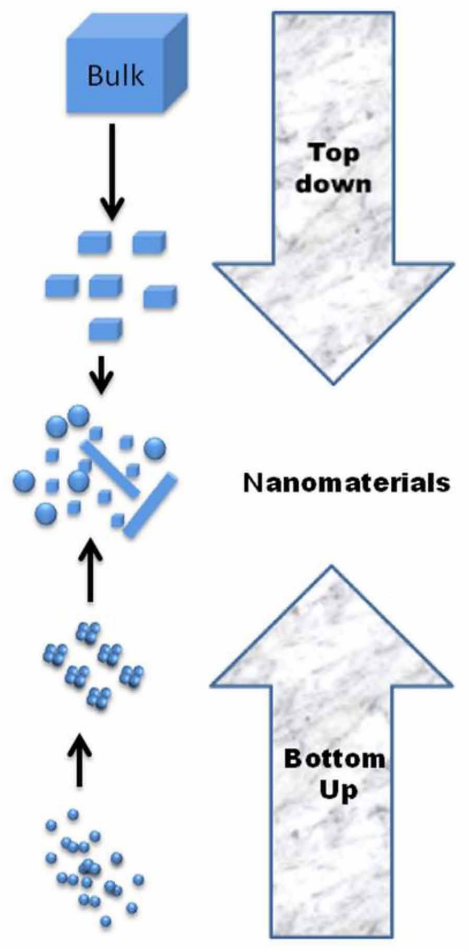
There has been tremendous expansion in research on potential application of nanotechnology in medicine ranging from exploiting nanomaterials as carriers of active pharmaceutical drugs in delivery and targeting applications to medical imaging purposes (Moghimi, Hunter, & Murray, 2005). These nanosized materials can affect biological functions by interaction with the biological tissues. Recent studies on potential nanotoxicity to human health and the eco-environment have drawn attention from both government agencies and the general public (Crist et al., 2013; Monteiro-Riviere, Inman, & Zhang, 2009). With the increasing commercialization and increasing human exposure of animals to nanomaterials, a safety assessment of these materials is needed. To facilitate such efforts, we herein present a brief survey the toxic effects of several organic and inorganic several nanocatalysts, mainly used in environmental remediation.

Carbon-Based Nanomaterials

There are three well-known carbon-based nanomaterials like fullerenes (Da Ros & Prato, 1999), carbon nanotubes (CNTs) (Ajayan & Zhou, 2001), and graphene (Sun et al., 2008). Regarding the cytotoxicity of C60 nanoparticles on various cell lines, controversial results have been published (Lewinski, Colvin, & Drezek, 2008). C60 nanoparticles have been found to be cytotoxic to some cell lines such as such as human dermal fibroblasts (HDFs)(Sayes et al., 2004, 2005) human liver carcinoma cells (HepG2) (Sayes et al., 2004, 2005), human epidermal keratinocytes (HEK)(Saathoff, Inman, Xia, Riviere, & Monteiro-Riviere, 2011), rat C6 glioma cells(Isakovic et al., 2006), and human U251 glioma cells(Isakovic et al., 2006), whereas no significant cytotoxic responses to pristine C60 nanoparticles were observed in murine macrophages(Fiorito, Serafino, Andreola, & Bernier, 2006), human monocyte-derived macrophages (Fiorito et al., 2006; Porter, Muller, Skepper, Midgley, & Welland, 2006), and Guinea pig alveolar macrophages(G. Jia et al., 2005). However it has been observed that surface functionalization of C60 particles may partially alleviate their cytotoxicity and reduce reactive oxygen species (ROS) generation(Sayes et al., 2004), but not in all the cases, since polyhydroxylated fullerenes can still in-

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Figure 3. Two different approaches viz., Top down and Bottom up approaches for synthesis of nanomaterials



duce photooxidative stress in human cells(Vileno et al., 2006; Wielgus, Zhao, Chignell, Hu, & Roberts, 2010). In comparison to C60 nanoparticles, both single walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs) exhibit cytotoxic effects at high concentrations. Cytotoxicity of both types of carbon nanotubes has been observed in HEK(Monteiro-Riviere & Inman, 2006), human embryo kidney cells (HEK293)(Cui, Tian, Ozkan, Wang, & Gao, 2005), rat alveolar macrophage cells (NR8383)(Pulskamp, Diabaté, & Krug, 2007), human alveolar epithelial cells (A549) (Pulskamp et al., 2007), human skin fibroblasts (HSF42)(Ding et al., 2005), and human embryonic lung fibroblasts (IMR-90) (Ding et al., 2005). MWCNTs and SWCNTs induced pulmonary inflammatory responses and granuloma formation after intratracheal instillation in animal models (Chou et al., 2008; Lam, James, McCluskey, & Hunter, 2004; Muller et al., 2005). However literature reports have proven that SWCNTs are more toxic than MWCNTs and C60 nanoparticles (G. Jia et al., 2005; Pulskamp et al., 2007; Tian, Cui, Schwarz, Estrada, & Kobayashi, 2006). With surface functionalization the cytotoxicity effects of pristine SWCNTs can be lessened and the extent of cytotoxicity depends upon the degree of sidewall functionalization (Sayes et al., 2006).

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Likewise graphene and its derivative like graphene oxide have been shown to have cytotoxicity effects in a dose dependant manner in various cells including A549, Henrietta Lacks cells (HeLa), National Institute of Health 3T3 mouse fibroblast cells (NIH-3T3), Sloan Kettering breast cancer cells (SKBR3), Michigan cancer foundation-7 breast cancer cells (MCF7), human neuroblastoma SH-SY5Y cells and even normal human lung cells (BEAS-2B)(Chang et al., 2011; Lv et al., 2012; Vallabani et al., 2011). Size and surface coating have been observed to influence the cytotoxicity of graphene and its derivatives. Reduced graphene oxide nanoplatelets (rGONPs) with average lateral dimensions (ALDs) of 11 ± 4 nm caused considerable cell destruction at the concentration of 1.0 μg/mL, while the rGONPs with ALDs of 3.8 ± 0.4 μm exhibited significant cytotoxic effects only at high concentrations of 100 μg/mL(Akhavan, Ghaderi, & Akhavan, 2012). In addition, surfaces coated with chitosan significantly reduce the haemolytic activity of GONP to human erythrocytes (RBCs) (Liao, Lin, Macosko, & Haynes, 2011).

Metal Nanoparticles

Although great progress have been made towards production of metal based nanoparticles worldwide both at the academic as well as at the industrial level, but still there is a serious lack of information about the impact of NPs on human health and environment, especially the potential for NP-induced toxicity(Canesi et al., 2008). Preliminary reports of the inherent toxicity of some NPs are available and indicate that they can affect biological behavior at the organ, tissue, cellular, subcellular, and protein levels (Schrand et al., 2010).

Aluminum Nanoparticles

Cellular interaction of aluminum oxide and aluminum nanoparticles including their effect on cell viability and cell phagocytosis, with reference to particle size and chemical composition has been investigated by Wagner et al (Wagner et al., 2007). Experiments were conducted to know the in vitro cellular effects of aluminium nanoparticles in rat alveolar macrophages (NR8383). Characterization techniques employed showed agglomeration in cell exposure media and Aluminium (Al) NP in the dose range of 100 to 250 μg/mL produced significantly reduced viability after 24 h of continuous exposure. No significant effect on the cellular viability was observed at 50, 80, or 120 nm Al NPs at 25 μg/mL for 24 h but cell phagocytotic ability was significantly hindered by exposure to same concentration (25 μg/mL) (Braydich-Stolle, Hussain, Schlager, & Hofmann, 2005).

Copper Nanoparticles

Copper nanoparticles (Cu NPs) have been recently investigated as a novel antimicrobial material, i.e. antiviral, antibacterial, antifouling, and antifungal and used in applications as biocides, antibiotic treatment alternatives, and nanocomposite coatings(Anyaogu, Fedorov, & Neckers, 2008; Cioffi et al., 2005). However, severe toxicity of Cu NPs have been demonstrated including heavy injuries in the kidney, liver, and spleen of mice after ingestion, as evident via histological analysis(Z. Chen et al., 2006). The Cu nanoparticles (23.5 nm) with oral LD50 of 413 mg/kg, is considered to be moderate toxic similar to Zn powder(Z. Chen et al., 2006; Suzuki, Toyooka, & Ibuki, 2007) in contrast to Cu microparticles (17 μm) which did not produce similar effects and were classified as nontoxic with LD50 values of >5000 mg/kg. Moreover, mice exposed to Cu NPs showed glomerulitis degeneration, and necrobiosis of renal

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tubules, but not in the mice exposed to Cu microparticles indicating that particle size and surface area are important material characteristics from a toxicological perspective. Meng et al gave the supporting evidence for the more efficient deposition of Cu NPs compared to micro-sized particles in renal tissues. They proposed that once inside the kidney, Cu NPs reacted with gastric juices and were converted to more toxic cupric ions (Meng et al., 2007).

Toxicity of Cu nanoparticles (80 nm) (1.5 mg/L; LC50) was also investigated and reported in zebrafish showing a decrease in gill Na^+/K^+ ATPase activity(Griffitt et al., 2007). Cu NP treatment also decreased blood urea nitrogen (BUN) levels and increased plasma alanine amino transferase (ALAT) levels. Additionally, dose-dependent damage of gill lamellae characterized by proliferation of epithelial cells, as well as edema of primary and secondary gill filaments, was observed after Cu NP treatment, indicating that the gill was the primary target organ for CuNP in zebrafish (Griffitt et al., 2007). In zebrafish exposed to Cu NPs, RT–PCR results showed higher levels of gene expression changes compared to $CuSO_4$ exposed fish. Furthermore, cluster analysis of these gene microarrays demonstrated that the transcriptional response induced by Cu NP was highly divergent (Griffitt et al., 2007).

Gold Nanoparticles

Several factors play important roles in determining the cytotoxicity of GNP, most important being the surface properties which have been shown to affect the biological interactions of GNPs (S. T. Kim, Saha, Kim, & Rotello, 2013). In one of the studies, polyethylenimine-modified GNPs has been shown to cause decrease in cell viability of in monkey kidney cells (COS-7) (Thomas & Klibanov, 2003). However, surface modified GNPs with different surface charges exhibited different cell uptake (Su et al., 2012) and toxic effects (Goodman, McCusker, Yilmaz, & Rotello, 2004) in cells. For instance, during cellular uptake, positively charged GNPs were reported to modulate the cell membrane potential and cause cell membrane perturbations. However, peptide functinalized GNPs were found to enter cells and targeted the nucleus without decreasing cell viability (Tkachenko A G, Xie H, Coleman D, Glomm W, Ryan J, Anderson M F, Frazen S, 2003). Apart from the surface properties, the particle size is another important physical parameter that determines the cytotoxicity of GNPs. Triphenylphosphine derivative-stabilized GNPs of 1.4 nm diameter exert most prominent cytotoxic effect when added to HeLa cells, SK-Mel-28 melanoma cells, L929 mouse fibroblasts, and mouse monocytic/macrophage cells (Pan Y, Neuss S, Leifert A, Fischer M, Wen F, Simon U, Schmid G, Brandau W, 2007). GNP-induced cytotoxicity was also found to depend on the cell type (Patra, Banerjee, Chaudhuri, Lahiri, & Dasgupta, 2007). In addition to cytotoxicity, GNPs cause an immunological response in cells. For instance, the surface hydrophobicity of GNPs dictates the immune response of splenocytes (Moyano et al., 2012).

The GNP dose is important for the toxicity of the nanomaterial *in vivo*. Intraperitoneal administration of 13 nm GNPs at the dose up to 0.4 mg/Kg/day was found to cause no acute or subacute physiological damage (Lasagna-Reeves et al., 2010), daily intraperitoneal administration of GNPs at the rate of 1.15 mg/Kg/day for fourteen days caused severe sickness in mice (Y. S. Chen, Hung, Liau, & Huang, 2009). Keeping the particle size of GNP in consideration as an important factor in vivo, GNPs of 3, 5, 50, or 100 nm at a dose of 8 mg/kg/week did not show harmful effects in mice. However, GNPs with a size ranging from 8 to 37 nm at the same dose caused a loss of structural integrity in the liver with an increase in Kupffer cells and the diffusion of white pulp in the spleen (Y. S. Chen et al., 2009). There are also some reports showing size dependant toxicity of gold nanoparticles with gold nanorods showing higher toxicity than gold nanospheres (Yinan Zhang, Xu, Li, Yu, & Chen, 2012)

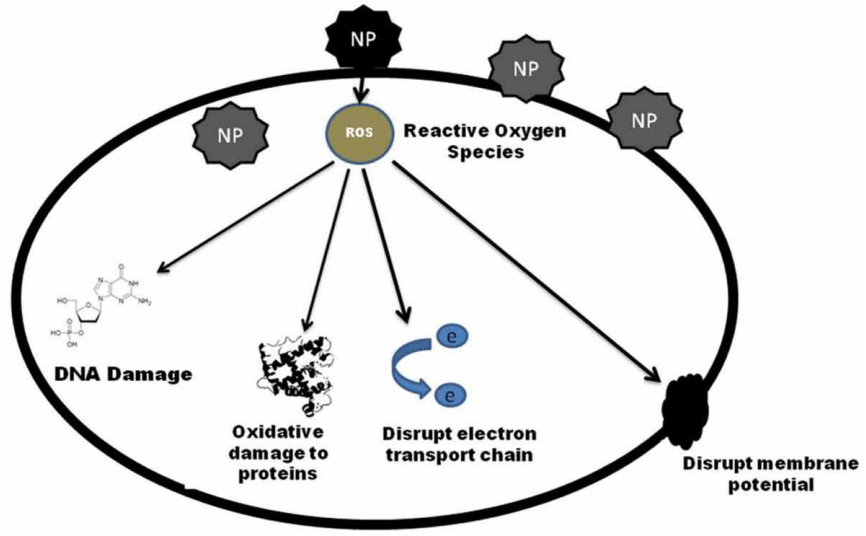
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The generation of ROS and reactive nitrogen species (RNS) is considered to be responsible for GNP-induced toxicity. Oxidative stress-related autophagy or necrosis in HeLa cells and MRC-5 human fetal lung fibroblasts were found to be induced by GNPs (J. J. Li, Hartono, Ong, Bay, & Yung, 2010; Pan et al., 2009). GNPs were also found to be responsible for the release of nitrogen oxide (NO) from endogenous *S*-nitroso adducts with thiol groups in blood serum (H. Y. Jia et al., 2008). GNPs also inhibited vascular endothelial growth factor (VEGF)-induced cell proliferation by affecting related signaling pathways (Bhattacharya et al., 2004; Kalishwaralal et al., 2011). Furthermore, respiratory activity of macrophages and the activity of macrophage mitochondrial enzymes (Staroverov et al., 2009) was found to be stimulated by both citrate-coated and antigen-conjugated GNPs (Staroverov et al., 2009). Figure 4 shows the schematic representation of the possible mechanistic pathway of nanoparticles on cell toxicity.

Silver Nanoparticles

Ag NPs have been previously seen wide use as antimicrobial agents and are have recently received considerable attention for use in possible defense and engineering applications (Arora, Jain, Rajwade, & Paknikar, 2008; Chadeau, Oulahal, Dubost, Favergeon, & Degraeve, 2010) Therefore, exposure to Ag nanoparticles underlines the underline the importance of assessing new uses of NPs for potential cytotoxicity. Such an evaluation was undertaken for Ag NP coated cotton fabric and the treated fabric caused no skin irritation and hence was found to be to be safe to guinea pigs (YeonáLee, KunáPark, MiáLee, & BumáPark, 2007). Ag NPs were has been reported to be safe to human cell carcinoma and human fibrosarcoma cell lines (Arora et al., 2008). However, Ag NPs have been found to agglomerate in the cytoplasm and in nuclei and induce oxidative stress-mediated cytotoxicity in human hepatoma cells Kim et al. (S. Kim et al., 2009). Interestingly, the mRNA level of oxidative stress-related genes was discovered to be regulated variably (S. Kim et al., 2009). Ag NPs due to their small size may also interact with the cellular genetic material but little is known about their genotoxicity. Surface chemistry based genotoxicty effects of Ag NPs on mouse embryonic fibroblasts, and on stem cells was reported

Figure 4. Possible mechanism of nanoparticle toxicity on cells



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(Ahamed et al., 2008). It was found that polysaccharide functionalized Ag NPs exhibited more apoptosis than did uncoated Ag NPs (Ahamed et al., 2008). Ag NPs of 15 and 100 nm diameters reduced GSH levels, compromised cellular antioxidant defenses that led to ROS accumulation and induced toxicity in cells of a rat liver-derived BRL 3A cell line by generating oxidative stress. However, the mechanism of depletion of GSH by the action of Ag NPs is not yet known (S M Hussain, Hess, Gearhart, Geiss, & Schlager, 2005). Low and high concentrations of Ag NPs induced vasoconstriction and vasodilatation in isolated rat aortic rings, respectively when mediated by the endothelial cells of the aortic rings (Rosas-Hernández et al., 2009). Neurotoxic effects on rat hippocampal CA1 neurons were induced by Ag NPs, ranging from 32 to 380 nm in diameter. The resulting alterations in sodium current that probably led to the observed neuronal dysfunctioning were long lasting (Zhaowei Liu, Ren, Zhang, & Yang, 2009). Ag NPs (15 nm) though reported to be cytotoxic to a PC-12 Rattus norvegicus cell line and caused cell shrinkage but didn't affected cellular morphology (Saber M Hussain et al., 2006). Dose dependant cytotoxic effects were reported in some in vivo studies of Ag NPs (Sung et al., 2009). Free radical induced oxidative stress, alteration in gene expression, and apoptosis in brain tissue was reported due to the intraperitoneal administration of Ag NPs (Rahman et al., 2009).

METAL OXIDE NANOPARTICLES

Toxicity of some of the important metal oxide nanoparticles are briefly discussed below

Titanium Dioxide Nanoparticles

Both micro and nano structured Titanium dioxide (TiO_2) nanoparticles can break down environmental pollutants and hence have been widely used in environmental remediation due to their photocatalytic activity (Daghrir, Drogui, & El Khakani, 2013; Ingale et al., 2011; Tominaga, Kubo, & Hosoya, 2011). TiO_2 nanocatalysts combined with Ultraviolet A (UVA) radiations have been used in food processing industry to eliminate pathogenic microorganisms in food-contacting surfaces. Although considered to be non toxic, but with increased commercialization of TNP related products there are concerns about the possible toxicity which has motivated further research efforts in this direction (Griffitt, Hyndman, Denslow, & Barber, 2009).

Cytotoxicity assessments of TNPs have been performed in various cells, and the toxicity depends upon the cell type. TNPs up to the concentrations of 100 μg/mL have shown very little cytotoxicity in primary human peripheral blood mononuclear cells (PBMCs) (Andersson-Willman et al., 2012), monocyte-derived dendritic cells (MDDCs) (Andersson-Willman et al., 2012), rat peripheral blood neutrophils (Huang Tao, Nallathamby Prakash D, 2008) and primary cultures of human hematopoietic progenitor cells(Bregoli et al., 2009). However, in other cell lines a dose-dependent cytotoxicity was observed such as human skin fibroblasts (Dechsakulthorn, Hayes, Bakand, Joeng, & Winder, 2007), mouse fibroblasts (L929) (Jin, Zhu, Wang, & Lu, 2008), rabbit erythrocytes(S.-Q. Li et al., 2008), and human B-cell lymphoblastoid cells (J. J. Wang, Sanderson, & Wang, 2007). Moreover, genotoxicity studies confirms the DNA damage and increase the mutation frequency (Barillet et al., 2010; J. J. Wang et al., 2007; Wilhelmi et al., 2012) caused by TNP, but with a more serious DNA damage detected under ultraviolet radiation (Vevers & Jha, 2008). Therefore, the cell type appears to be a vital factor influencing the cytotoxicity of TNPs, which was also confirmed in a study of the responses of several eukaryotic cells to TNP exposure (Barillet et al., 2010).

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The in vivo toxicity of TNPs has been investigated in various aquatic organisms and in animal models. A general growth inhibition was observed with variable EC50 values, ranging from 5.83(Aruoja, Dubourguier, Kasemets, & Kahru, 2009) to 241 mg/L (Vallabani et al., 2011) in freshwater algae, and in Pseudokirchneriella subcapitata. Biodistribution and toxicity of TNPs was also evaluated in rats and mice. It was observed that TNPs predominantly accumulated in the liver, in addition to the spleen, lung, and kidney after a single intravenous injection (5 mg/kg body weight) in wistar rats (Fabian et al., 2008). However no obvious toxic effect, immune response, or change in organ function was observed. TNP accumulation in CD-1 (Imprinting Control Region, ICR) mice after an abdominal cavity injection was found to be highest in Liver and lowest in heart with the order: liver > kidneys > spleen > lung > brain > heart(H. Liu et al., 2009).

Iron Oxide Nanoparticles

In vivo mouse studies have shown that tumor-targeted superparamagnetic iron oxide nanoparticles (SPION) may act as antitumor agents. Entrapment of these NPs, in the growing intravascular thrombi, led to cell death, hence, tumor-targeted SPION can be used to inhibit tumor growth (Simberg et al., 2009). Intratracheal administration of Fe_2O_3 NPs to male Sprague Dawley rats induced oxidative stress in the lungs (M.-T. Zhu et al., 2008). Such oxidative stress produced follicular hyperplasia, protein effusion, pulmonary capillary vessel hyperaemia, and alveolar lipoproteinosis.

SPIONS after entering into cells are probabily degraded to iron ions within the lysosomes under the influence of hydrolytic enzymes. After crossing the nuclear and mitochondrial membrane these iron ions may generate reactive hydroxyl radicals via the Fenton reaction. These hydroxyl radicals then cause lipid peroxidation, DNA damage, and protein oxidation. In brief, cytotoxicity of SPIONP has been linked to cellular uptake and ROS generation. Although iron nanoparticles were shown to be biocompatible *in vivo* at a low concentration, these particles were found to be cytotoxic *in vitro*. Polyvinyl alcohol-coated superparamagnetic iron oxide NPs have caused shape and sizedependent toxicity to primary mouse connective tissue cells. Moreover, it has been observed that toxicity increases for NP forms in the following order: nanobeads > nanoworms > nanospheres. Moreover, with increase in hydrodynamic size toxicity decreased. Easy internalization of certain comparatively small particles into cells has been suggested as a possible reason for the size-dependent toxic effects (Mahmoudi, Simchi, Milani, & Stroeve, 2009).

Zinc Oxide (ZnO) Nanoparticles

Zinc oxide (ZnO) NPs have been widely used as ingredients in cosmetics, and in other dermatological preparations. Due to small size, these NPs always have some possibility to interact with DNA and cause damage. In fact, such cases have been reported on a human epidermal cell line (A431). Similarly, ZnO NPs have been reported to induce cytotoxicity in L2 cells (Hillegass et al., 2010). Lipid peroxidation (LPO) and oxidative stress mechanism are thought to be the causes of such genotoxic effects. Cytotoxicity has been found to be dependent on concentration and time of exposure. ZNO NPs showing higher cytotxicity at higher concentrations and higher exposure times (Sharma et al., 2009). Concentration and time-dependent increase in oxidative stress, intracellular [Ca^{2+}] levels, and cell membrane damage has been reported to occur in a cultured BEAS-2B cell line. In such cases alteration in gene expression from oxidative stress and apoptosis was considered to be the main reason behind the induced cytotoxicity (Huang, Aronstam, Chen, & Huang, 2010). ZnO NPs in 8–10 nm size range have induced more

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toxicity to human colon cancer cells (RKO) than has the micrometersized ZnO of less than 44 mm size. Depolarization-induced neuronal injury in rats by the activation of voltage-gated Na^+ channels, after exposure to ZnO NPs has been reported thereby giving a clue that these NPs can probably cause neuronal apoptosis (Zhao, Xu, Zhang, Ren, & Yang, 2009). In rats, the intratracheal instillation and inhalation exposure of ZnO NPs for 1-3 h induced metal-fume-fever-like responses in lungs that was characterized by short term lung inflammatory or cytotoxic responses(Warheit et al., 2007). Invivo pulmonary exposure of both nano and fine sized ZnO in rats demonstrated potent, but reversible, inflammation. (Hillegass et al., 2010). The effect of ZnO NPs has also been tested on aquatic species. Dose-dependent reduction in hatching rate and induction of pericardial edema in developing zebrafish embryos, and in larvae has been reported. The Zn^{2+} dependent mechanism was responsible for the embryonic toxicity that resulted from ROS generation (X. Zhu, Wang, Zhang, Chang, & Chen, 2009).

Cerium Oxide Nanoparticles

There has been an increased use of nanosized cerium oxide (CeO_2) in polishing and computer chip manufacturing (Limbach et al., 2005; Mädler, Stark, & Pratsinis, 2002) and as an additive to decrease diesel emissions (Jung, Kittelson, & Zachariah, 2005). However, CeO_2 nanoparticles of sizes in range of 15nm, to 45 nm NPs caused toxicity, ROS increase, GSH decrease, and induced oxidative stress-related genes such as heme oxygenase-1, catalase, glutathione-Stransferase, and thiorexoxin reductase in cultured human lung epithelial cells (BEAS-2B). It was found that increased ROS by CeO_2 NPs triggered the activation of cytosolic Caspase and chromatin condensation, causing toxicity via the apoptotic process (Park, Choi, Park, & Park, 2008). On the contrary, other reports showed high biocompatibility of ceria nanostructures, prevented renal degeneration induced by intracellular peroxidases and conferred radioprotection to normal cells compared to no protection for tumor cells(J. Chen, Patil, Seal, & McGinnis, 2006; Tarnuzzer, Colon, Patil, & Seal, 2005). This apparent incongruity might be due to the surface oxidation state of nanoceria to scavenge superoxides or act in a catalytic manner. on the other hand, no significant change in LDH leakage or cell morphology is observed in A549 lung cells exposed directly to flamespray synthesized CeO NPs for 10–30 min (Perez JM, Asati A, Nath S, 2008) but resulted in a decreases in the mean total lamellar body volume per cell, reduction of cell-cell contacts and a significant increase in 8-oxoguanine positive cells, however NP induced oxidative stress resulting in altered gene expression (Rothen-Rutishauser et al., 2009).

CONCLUSION

Nanotechnology is a fast-growing field of activity that will allow development of materials with brand-new properties. Various scientific groups are keen about this technology and are devoting themselves to the development of more, new, and better nanomaterials. In the near future, expectations are that no field will be left untouched by the magical benefits available through application of nanotechnology. However, in this pursuit the number of Quebec workers exposed to nanoparticles should increase over the next few years. Presently, there is only limited knowledge concerning the toxicological effects of NPs. Before, NPs are commercially used it is most important that they be subjected to appropriate toxicity evaluation. Among the parameters of NPs that must be evaluated for their effect on toxicity are surface charges, types of coating material, and reactivity of NPs. Because of their very small size, these particles offer a large contact surface per mass unit.

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Since Nanoparticles (NPs) have already found a wide range of applications around the world. In vitro and in vivo studies on NPs have revealed that most are toxic to animals. The types of effects that NPs have produced are those on the pulmonary, cardiac, reproductive, renal and cutaneous systems, as well as on various cell lines. After exposures, significant accumulations of NPs have been found in the lungs, brain, liver, spleen, and bones of test species. However, their toxic behavior varies with their size, shape, surface charge, type of coating material and reactivity. Dose, route of administration, and exposure are critical factors that affect the degree of toxicity produced by any particular type of NP. Since, toxicity of nanoparticles has been reviewed in several reports and the most severe problem is related to the carcinogenic potential of NPs that has been associated both to the chemistry as well as to the physical properties. In particular, chemistry was considered to be relevant for the oxidative DNA damage and the formation of radical oxygen species (ROS) involved *via* direct mechanisms, whereas size, morphology and surface seem to be more important in all the other indirect mechanisms that underlie of cancer. Nevertheless, the majority of studies on genotoxicity of NPs have been so far performed on cell cultures. Thus in order to obtain data more relevant to the knowledge on human exposure, more trials on animal and long-term exposure experiments should be carried out. We believe a careful and rigorous toxicity testing is necessary before any NP is declared to be safe for broad use. We also believe that an agreed upon testing system is needed that can be used to suitably, accurately, and economically assess the toxicity of NPs. Moreover, there is a timely need to pay attention on how to control the in vivo transport and toxicity of different type of nanoparticles as only few reports have been published in this direction (Y.-S. Chen et al., 2012).

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Chapter 58
Quantitative Structure–Activity/Property/Toxicity Relationships through Conceptual Density Functional Theory–Based Reactivity Descriptors

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ABSTRACT

Developing effective structure-activity/property/toxicity relationships (QSAR/QSPR/QSTR) is very helpful in predicting biological activity, property, and toxicity of a given set of molecules. Regular change in these properties with the structural alteration is the main reason to obtain QSAR/QSPR/QSTR models. The advancement in making different QSAR/QSPR/QSTR models to describe activity, property, and toxicity of various groups of molecules is reviewed in this chapter. The successful implementation of Conceptual Density Functional Theory (CDFT)-based global as well as local reactivity descriptors in modeling effective QSAR/QSPR/QSTR is highlighted.

1. INTRODUCTION

The properties of molecules mainly depend on the distribution of electron density within the individual molecules. It is, therefore, believed that molecules having similar structures will exhibit similar properties. On this hypothesis, the idea of structure-activity relationship (SAR) exists (Nantasenamat et al., 2009). Accordingly, it is assumed that molecules with similar structures exhibit comparable activities (properties), and, so on the basis of known properties of a set of molecules, prediction of the properties of unknown molecules having similar structures could be made, provided appropriate models are de-

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veloped. Models which are able to predict the properties of an unknown molecule on a qualitative basis are termed as Qualitative Structure-Activity Relationship models (Arning et al., 2008), and, the ones which can quantify such relationship are termed as Quantitative Structure-Activity Relationship (QSAR) models (Gramatica, 2007). Such QSAR models are very useful in the design of molecules which can serve as a better drug or in the manufacturing of materials which could serve as better substance in terms of utility, economy, durability and environmental effects in totality. The quest for such molecules begins with SAR studies. On the basis of activities of a set of molecules of similar structures or parameters, different SAR models are designed. Thereafter, those molecules which are found to have a potential to show a desired characteristic (property/activity) are filtered with the aid of such models, and, then the systematic efforts towards their syntheses begin. Thus, such an approach helps in the reduction of the cost effectively. Since a molecule can have different kinds of properties, a large number of QSAR models could be developed for the same set of molecules depending on their properties. Such properties could be physico-chemical properties, quantum-chemical properties or even toxicity properties. It clearly implies that not only the unknown toxicity parameter of a molecule of whose different parameters are yet to be assessed, could be determined, but its other set of properties could also be determined provided effective QSAR models are generated. When the attempt is to determine the toxicological effect of a molecule based on a model having correlation between structure and toxicity of already determined molecules, then such an approach is defined as Quantitative Structure-Toxicity Relationship (QSTR) model (Roy and Ghosh, 2004). Such models are very effective in the prediction of toxicity of molecules which have potential to act as a toxic substance. Thus, QSTR helps in building up of an effective regression model that helps in predicting the reactivity of an unknown molecule on the basis of behavior of an analogous set of chemically or structurally similar compounds.

QSTR models are proposed based on different parameters. Most often these parameters are defined in terms of descriptors. These descriptors could be based on different experimental and theoretical properties such as physico-chemical and quantum-chemical properties, respectively. One of the most popular and cheap methods in the field of quantum-chemical prediction of the properties of molecules is Density Functional Theory (DFT) (Parr & Yang, 1989). It predicts the properties of a molecule based on its molecular electron density. A closely related method describing the chemical properties such as electronegativity, ionization potential, hardness etc with the aid of DFT method is known as Conceptual Density Functional Theory method (CDFT) (Chattaraj, 2009; Chakraborty et al., 2010; Chattaraj & Giri, 2009; Geerlings et al., 2003). This method has been successfully and exhaustively employed in the generation of different descriptors, which have been able to produce QSAR and QSTR models of very high efficiency. Thus, CDFT has helped in the assessment of activity and toxicity of different molecules within the ambit of QSAR and QSTR respectively. It has been found that CDFT is quite cheaper in comparison to other expensive quantum-chemical methods such as *ab initio* methods. CDFT has been proven to develop effective and efficient QSTR models.

In this chapter, at first we have provided a general introduction highlighting the necessity of structure-activity/toxicity based regression models. Various QSAR/QSPR/QSTR based methods are outlined in section 2. Section 3 describes the conceptual density functional theory and the related global and local reactivity descriptors. Section 4 deals with the utility of electrophilicity index and net electrophilicity index and their local variants in predicting toxicity of various classes of molecules. Section 5 mentions about the importance of the group philicity towards modeling an effective QSAR. Section 6 highlights the effect of studied levels and / or basis sets in constructing QSAR/QSPR/QSTR models. Section 7 concludes the chapter by mentioning the effectiveness of various CDFT based descriptors in building QSAR/QSPR/QSTR models.

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2. QUANTITATIVE STRUCTURE-ACTIVITY/ PROPERTY/TOXICITY RELATIONSHIPS

Quantitative Structure Activity Relationship (QSAR) acts as a function which connects structure of a molecule to different properties. Those properties which show medicinal applications are considered as an activity of a molecule, and, are thus studied within the purview of QSAR. In the present world of global recession, such a technique is able to save money and time. Much of the efforts are now directed towards development of models which predict accurately the properties of a molecule on the basis of their structures. Thus, QSAR models are able to predict biological activities and physico-chemical properties and rationalize the mechanism of action for different chemical reactions. Normally, in order to develop a QSAR, five to ten compounds are necessary for each descriptor as argued by Topliss and Costello (1972) and further mentioned by Schultz et al. (2003c). However, in order to develop robust QSAR, many more compounds are required. With the advent of sophisticated techniques, new software, and, hardware which works on novel algorithms, has made the task of generation of an accurate model within the purview of QSAR simplier. QSAR has its genesis in the pioneering work of Hansch and coworkers (1962, 1964) wherein they used linear multiple regression models to describe the biological property of the compounds, which were not synthesized till then. Different parameters such as hydrophobicity, electronic effects and steric properties were utilized to generate a relationship among them and the concentration of the molecule. Free and Wilson (1964) and Fujita et al. (1971) developed non-parameteric methods to define biological activity. Later Linear Solvation Free Energy Relationship (LSFER) was proposed which predicted the properties of a molecule on solute-solvent interactions (Kamlet et al., 1983; Taft et al., 1985). It consists of different solvatochromic descriptors such as cavity/bulk term, polarizability term, and H-bond terms which are used to derive a relationship between overall property of a molecule and different parameters. A good account of the role of QSAR in chemical toxicology was elaborated by Schultz et al. (2003a, 2003b). Other articles also highlight the development of the QSAR methodology and their role in the prediction of bio-toxicity (Gombar et al., 2006; Kar & Roy, 2010; Nantasenamat et al., 2009; Puzyn et al., 2010; Selassie et al., 2010). Hammett (1935, 1937) by employing correlation principles, in 1930s had efficiently shown the effect of structural changes on reaction mechanism of substituted benzoic acid derivatives. It led subsequently to the birth of well known linear free-energy relationship essentially by connecting Gibbs free energy of reaction and Gibbs free energy of activation which in a broader sense established a connection between thermodynamics and kinetics. The concept was further extended by Taft (1956) for aliphatic compounds. This paved the way of strong QSAR models as was evident from the further work carried out by Hansch and coworkers (1993, 1996) wherein they discussed the role of QSAR. The role of QSAR in the study of chemico-biological interactions (Blaney & Hansch, 1990; Debnath et al., 1994; Gao et al., 1999; Hansch et al., 1986, 1989, 1995, 1997, 1998, 2001; Hansen et al., 1995; Langridge & Klein, 1990; Lien et al., 1968;) and also for the drug design (Bajot, 2006; Garg et al., 1999; Hansch et al., 1973, 1995; Kubinyi, 1997; Maddalena, 1998; Norinder, 1998; Voskresensky & Levitsky, 2002; Yang & Huang, 2006;) was also established.

In order to generate an effective QSAR model, the first and foremost requirement is the collection of data and generation of database. This helps in defining training sets which generally consist of known data obtained from various sources. With the aid of such data, different kinds of descriptors are developed on the basis of molecular formula (1-D descriptor), the 2-dimensional structure (2-D descriptor), and the 3-dimensional conformation (3-D descriptor) of a molecule. Generally, molecular descriptors generated from some special types of atoms or fragments counting or based on bulk and physico-chemical proper-

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ties like molecular weight, atomic number, number of OH groups, number of non-hydrogenic atoms and so on correspond to 1-D descriptors.2-D descriptors are usually obtained by applying algorithms to the topology based representation. 3-D descriptors, also called geometrical descriptors are generated from the spatial coordinates (x,y,z) of a molecular system whereas the interaction energy values between an embedded molecule in a grid and a probe will provide 4-D descriptors. In general, molecular descriptors should satisfy some basic requirements as suggested by Randić (1991). Different other articles also showed the role of QSAR in the generation of different algorithms (Karcher & Devillers, 1990; Roy & Mitra, 2009; Selassie et al., 2002).

Molecular descriptors are mathematical representations of various properties of a molecule which are generally derived after the application of a reliable algorithm to the molecular representation or are determined from experiment. Being related to the molecular Hamiltonian, the molecular descriptors like electron density, electrostatic potential, etc. would represent all the properties of a molecule (Bonaccorsi et al., 1970; Murray & Politzer, 2003; Pathak & Gadre, 1990; Politzer, 1988). These descriptors help in the development of models for chemistry, toxicology, health research, pharmaceutical sciences, etc. They are generated by the application of different principles from different fields and theory, such as organic chemistry, graph theory, quantum-chemistry, information theory etc. The electron localization and delocalization functions obtained from the quantum theory of atoms in molecules (QTAIM) are also very effective in modeling QSAR (Buttingsrud et al., 2007a, 2007b, 2007c; Chaudry & Popelier, 2003, 2004; O'Brien & Popelier, 1999; Popelier et al., 1999, 2004; Smith & Popelier, 2005). Popelier et al. (1999) showed that a QTAIM based approach called quantum topological molecular similarity (QTMS) could predict the toxicity of health-hazardous lipid-soluble environmental pollutants as efficient as that of comparative molecular field analysis (CoMFA). Very recently, Matta et al. (2014) reviewed the applicability of the electron density, electron localization and delocalization functions, and the electrostatic potential in developing effective QSAR models.

Bohari et al. (2011) adopted so-called analogue-based approach to predict the anti-cancer activity of 266 different compounds against 29 various cancer cell lines. Various models were constructed through 1 to 10 different descriptors. They found that in general three-parameters regression models could predict the anti-cancer property satisfactorily. Filho et al. (2012) performed QSAR studies on 32 Morita–Baylis–Hillman adducts against Leishmania amazonensis. Energy of the lowest unoccupied Kohn–Sham orbital, number of $o-NO_2$ group, number of $-CH_2-$ group and an increment in log P parameters were used to build the regression model. A reasonably good predictivity was found for this model. QSAR studies were also performed in two sets of antitumour drugs 2-(4-aminophenyl)benzothiazoles by using different indices encompassing constitutional, geometrical, topological, and electronic descriptors (Hilal & Elroby, 2011). Good correlation having a range of r^2 as 0.867-0.954 was attained between computed and experimental activities with these models. A number of molecular descriptors like polar surface area, Randic shape index, number of phenolic groups and energy of the highest occupied molecular orbital were used to predict the estrogenic activity of different terpenoid ester derivatives obtained from *Ferula* plants (Rasulev et al., 2007). An r^2 value of 0.892 implied the reliability of the regression model to predict the activity of these compounds. Li et al. (2010) carried out the QSAR studies on 27 diacyl-hydrazine derivatives by using DFT based method and semi-empirical AM1-MOPAC method. They found higher predictivity at the DFT level than that at the semi-empirical level. 3D-QSAR models were also generated based on the geometries obtained at the DFT level and electrostatic potential fitting charges. The results of the QSAR and 3D-QSAR were found to be comparable. For these compounds, it was argued that the electrostatic and hydrophobic properties are important in deciding their overall biological activities.

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Zhang et al. (2009) combined DFT-based conformation analysis with QSAR to develop a simple new DFT/QSAR approach to predict the nature of bioactive conformation in the side chain of cyclic imide derivatives as protoporphyrinogen oxidase inhibitors. The potential energy surface scan, molecular docking and molecular dynamic simulation supported the reliability of this new approach. Damme and Bultinck (2010) employed conceptual DFT bsed quantum chemical molecular field in order to construct 3D-QSAR models to describe the antituberculotic activity of salicylamide derivatives. QSAR studies on the interactions between the terpenoid mosquito repellents and lactic acid were made by Song et al. (2013). They found that in addition to the structures of the repellents, the repellent–lactic acid complexes also have a role in deciding their activities. Use of ordered predictors selection algorithm and partial least squares regression was also made to select variables and to construct QSAR models of various 4,5-dihydroxypyrimidine carboxamide derivatives as HIV-1 integrase inhibitors (de Melo et al., 2009). The electronic distribution of the studied compounds appeared to have deciding role in their inhibition activities. The antifungal activity of N-nitrosopiperidone semicarbazone derivatives against the plant pathogens viz., *Fusarium oxysporum* and *Rhizoctonia solani* can also be predicted by the regression models devepoled by the descriptors like molecular electrostatic potential, charge on N center, dipole moment, minium surface area and log P (Hemalatha et al., 2012). The QSAR studies on the imidazole and benz-imidazole derivatives as corrosion inhibitors revealed that HOMO energy, polarizability, dipole, frontier orbital charge density, the interaction mode between inhibitors and metal surface and steric hindrance of molecules are deciding descriptors to represent their inhibiting potency (Zhang et al., 2005). Various DFT based descriptors like HOMO-LUMO energy gap, HOMO and LUMO energies, mean molecular polarizability, atomic charge, weighted nucleophilic atomic frontier electron density were employed in order to build regression models for the various cyclic imide derivatives as protoporphyrinogen oxidase (Protox) inhibitors (Wan et al., 2004). The DFT-based QSAR and 3D-QSAR approaches on the activity of aurone derivatives against chloroquine resistant parasite by Adhikari et al. (2013) are also notworthy. A review summarizing various quantum-chemical descriptors in developing QSAR and QSPR written by Karelson and Lobanov (1996) is highly recommended to get more details about these approaches.

Geometrical descriptors were designed and included in the QSAR during the development of 3D-QSAR models in mid 1980s. These included gravitational indices (Katritzky et al., 1996), shadow indices (Rohrbaugh & Jurs, 1987), EVA descriptors (Ferguson et al., 1997), GETAWAY descriptors (Consonni et al., 2002), 3D-Morse descriptors (Schuur et al., 1996), charged partial surface area descriptors (Stanton & Jurs, 1990), and WHIM descriptors (Todeschini et al., 1994).

The descriptors such as topological descriptors utilize molecular graph. Different topological indices such as those developed by Weiner (1947), Randić (1975), Balaban (1998), Kier and Hall (1995) were reported in literature to generate QSAR models. Other descriptors such as steric (Camenisch et al., 1998; Mezey, 1992) viz., volume, shape and surface, were found to be useful in representing the global structural characteristics of a molecule and different software such as VolSurf software (Cruciani et al., 2000) were employed to create effective QSAR models. In order to generate 2D and 3D descriptors for the prediction of physicochemical properties, the methods provided by Rekker (1992) were used.

CoMFA (Cramer et al., 1988) is an effective method in building effective 3D-QSAR, which was successfully applied in drug designing and other related applications (Aboye et al., 2004; Akamatsu, 2002; Amin & Welsh, 2006; Avery et al., 2002; Bhongade & Gadad, 2004; Hannongbua etal., 2001; Jayatilleke et al., 2000; Sippl & Holtje, 2000). History behind the idea of CoMFA in modeling QSAR is that in the classical QSAR, the different biological acitivies of drugs or other compounds of interest are correlated with various physico-chemical properties or quantum-chemical descriptors, provided

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they could represent certain structural characteristics. Therefore, they did not take care of the information regarding their 3-D geometries and chirality. On the other hand, at longer distance an active site of a drug can only experience the electrostatic field of a target molecule and at shorter distance its hard body including the charge distribution (Kubinyi, 1998). Other two important observations were that the most of the molecular interactions which generate biological activity, are of non-covalent type and the most of the molecular mechanics force fields, wich describe the non-covalent interaction as steric and electrostatic forces, could explain a large variety of molecular properties. Therefore, it appeared that an appropriate sampling of the steric and electrostatic forces surrounding the considered molecules could give all the necessary information regarding their observed biological properties. Cramer and Milne (1979) compared the molecules for the first time by arranging them in space and by molecular field mapping in a 3-D grid. The same approach was then implemented in dynamic lattice-oriented molecular modeling system (DYLOMMS) method (Kubinyi, 1993). However, to obtain success in broader applications of this approach, certain factors like the use of partial least square analysis instead of principal component analysis and use of appropriate software are important (Kubinyi, 1998). In 1988, Cramer et al. (1988) termed this approach as CoMFA. The major precondition for this approach is that all the selected molecules under study must interact with the receptors of same kind and in same mechanistic way. Then in order to develop a CoMFA model, a traning set is chosen from a certain subgroup of the considered molecules and the remaining part is treated as a test set. This test set is used to verify the efficacy of this model to describe certain properties. The final output of this analysis provides a regression equation having several coefficients and they are very often depicted as a set of contour plots. From these plots, the favorable and unfavorable steric regions and favorable and unfavorable regions for electropositive or electronegative substituents could be identified. Then one could predict the desired properties of the test set or other related samples by qualitative examination of these plots or by computing the fields of these molecules quantitatively. Cramer et al. (1988) first employed this method to predict the binding affinities of steroids towards the human corticosteroid. The squared correlation coefficient (r^2) for the affinity values was found to be very high (close to 0.9). Since 1988, a lot of articles including reviews are reported in the literature on this topic (Adhikari et al., 2014; Blankley, 1996; Kim, 1995; Martin et al., 1996; Martin & Lin, 1996).

In addition to the development of the QSAR, quantitative structure-property relationship (QSPR) was also used to predict a certain property of the molecules. The hypothesis, that structural alteration changes the observed macroscopic properties of systems is the base of QSPR methods. Several research groups contributed significantly in the development of QSPR models (Abraham, 1994; Abraham et al., 1995; Balaban, 1997; Hilal et al., 1994; Katritzky et al., 1995; Kier & Hall, 1986; Lucic & Trinajstic, 1999; Murray & Politzer, 1994; Randic & Razinger, 1996; Stuper et al., 1979). The efficacy of QSPR as a useful tool in connecting molecular structure with the various physical, chemical, biological and technological properties was further demonstrated by Katritzky et al. (2000). The successful implication of QSPR in medicinal chemistry to predict different property was also available in the literature (Katritzky et al., 2002). A fair correletion between structure and property was obtained by using QSPR models made on an empirical basis even for large data sets. The importance of data reduction methods like principal component analysis (PCA) of a matrix generated by the different properties of the systems was also found in providing insight into these properties quantitatively (Leo & Hansch, 1999). The reviews summarizing the construction of QSPR models in predicting diverse properties of materials are highly recommended. Variuos approachs were also made to model effective QSPR for anti HIV drugs.

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The applicability of QSPR in pharmaceutical arena including different pharmacokinetic aspects towards drug designing was reviewed by Grover et al. (2000a, 2000b).

Further, quantitative structure-toxicity relationship (QSTR) is essential in order to have proper knowledge about the toxicity of various essential systems; hence it is an issue of environmental safety and security. The approach of QSTR modeling even becomes more popular since experimental determination of toxicity of each and every essential compound is hard with respect to time, cost, and the availability of resources. Different theoretical approaches have been made to connect the structural features (electronic, geometric, topological, quantum chemical, etc.) with the toxicity. An effective QSTR model could predict the relative toxicity/safety of different systems in a reliable way saving the experimental effort. Cronin and Schultz (1998) developed useful QSTR model for v*ibrio pscheri* toxicity including all probable mechanisms. Schultz et al. (1999, 2002) evaluated toxicity of numerous aliphatic chemicals and substituted benzenes towards *Tetrahymena pyriformis*.

The databases regarding toxicity of a number of fish species like the guppy (*Poecilia reticulata*) and zebrafish (*Brachydanio rerio*) are also available. The fish data sets for the fathead minnow (*Pimephales promelas*) to predict the acute toxicity and related modes of toxic action was built by studying the establishment of toxicodynamic profiles, and behavioral and dose-response interpretation of 96-h LC_{50} tests (Russom et al., 1997). Good correlation between fish toxicity and toxic potency measures in *T. pyriformis* was also found in few studies (Bearden & Schultz, 1998; Schultz, 1997). McFarland (1970) proposed quite simple toxicokinetic- and toxicodynamic-based approach in which chemical toxicity was expressed as a combination of uptake into or through biological membranes and the interaction of the toxicant with the site of action to predict toxicity. Different approaches in modeling QSTR were also reported in the literature (Chakraborty et al., 2013; Cronin & Schultz, 1996; Karabunarliev et al., 1996; Veith & Mekenyan, 1993). QSTR could also be applied to measure toxicity of disinfection by-products (DBPs) generated during disinfection of water (Moudgal et al., 2000).

Cronin et al. (1998) developed QSTR model by using LUMO energy and maximum acceptor superdelocalizability as descriptors to predict toxicity of various alkyl- and halogen-substituted nitro- and dinitrobenzenes towards *T. Pyriformis*. Mishra et al. (2014) employed various quantum-chemical descriptors like HOMO energy, electronegativity, electron affinity (EA), ionization potential (IP), total energy and log P to predict the toxicity of nitrobenzene derivatives towards *T. Pyriformis*. The topological descriptors were also found to be effective to describe the toxicity of nitrobenzene derivatives towards the same (Tripathi & Mishra, 2014). Further based on several energy descriptors, heat of formation, atomic charge, dipole moment and polarizability values, Bao et al. (2012) predicted the toxicity of several nitrobenzene derivatives towards the *Scenedesmus obliguus*. Very recently, Tawari et al. (2014) attempted to build QSTR model to describe the mutagenicity of nitroaromatic compounds. Pandith and Islam (2013) further built QSTR models by using log P, electrophilicity (ω) and LUMO energy to predict the acute toxicity of aliphatic compounds towards *V. fischeri*. HOMO energy, molecular weight, LUMO energy, chemical potential, zero point energy, and hardness were also used to model QSTR in order to predict 50% effective inhibition concentration of various substituted benzene derivatives towards the *algae Scenedesmus obliquus* (Haghdadi & Fatemi, 2010). Quite high correlation ($r = 0.91$) revealed that such properties can be properly described by using such molecular indices. Eroglu et al. (2007) used quantum-chemical descriptors like translational entropy, principal moment of inertia, the highest and the lowest vibrational wave numbers to predict the acute toxicity of several organic compounds towards *Fathead Minnow*. Further, toxicity of organophosphorus compounds was assessed using the regression models mady by different quantum-chemical and topological descriptors (Senior et al., 2011). Wiener

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index, Harary index, modified Randic index, Kupchik modified connectivity index (Todeschini & Consonni, 2000), electronegative Randic index, heteroatom corrected extended connectivity Randic index as different topological descriptors and HOMO-LUMO gap, maximum positive charge, total negative charge, charge polarization, local dipole index, electronic topological index, etc. as quantum-chemical descriptors were used for this purpose. Attempt to predict the lethal toxicity of organophosphorous pesticides occurred in the tadpoles was also reported in the literature (Yan et al., 2008). Antifungal and insecticidal activities of perfluorophenyl antimony(III) and antimony(V) chlorides were predicted by several energy and CDFT based descriptors by Singhal et al. (2014). Regression model based on total energy and LUMO energy was also found effective in describing the toxicity of several substituted benzenes against *freshwater photobacteria* (Mo et al., 2011). The hardness, electronegativity and total energy descriptors were found to be useful descriptors for QSTR modeling on a series of aliphatic alcohol derivatives (Kumar et al., 2011). Trohalaki and Pachter (2003) showed that the use of reciprocal of LUMO energy could improve the correlation in predicting the toxicity of halogenated aliphatic hydrocarbons than that of LUMO energy. The acute fish toxicity of organic solvents was also described by using the octanol-water partition coefficient, LUMO energy, dielectric constant and surface tension as descriptors (Levet et al., 2013). Gironés and Carbó-Dorca (2006) reported the molecular quantum similarity measures approach in develping QSTR.

3. CONCEPTUAL DENSITY FUNCTIONAL THEORY

The conceptual density functional theory (CDFT) (Chattaraj, 2009; Chattaraj & Giri, 2009; Chakraborty et al., 2010; Geerlings et al., 2003) has become a powerful theoretical tool to shed light into the chemical reactivity of a molecule. Within the framework of the CDFT, several response functions were introduced in order to describe chemical reactivity, which are also called as reactivity descriptors. CDFT along with its different global reactivity descriptors viz., hardness (η) (Parr & Pearson, 1983; Pearson, 1997), electronegativity (χ) (Chattaraj, 1992; Parr et al., 1978) and electrophilicity (ω) (Parr et al., 1999; Chattaraj et al., 2006, 2007, 2011) and different local reactivity descriptors like Fukui functions (Parr & Yang, 1984) $f(\mathbf{r})$ and their condensed-to-atom variants (Yang & Mortier, 1986) (f_k), local softness ($s(\mathbf{r})$) (Wang & Parr, 1985; Berkowitz & Parr, 1988), local hardness ($\eta(\mathbf{r})$) (Wang & Parr, 1985; Ghosh & Berkowitz, 1985) and philicity ($\omega(\mathbf{r})$) (Chattaraj et al., 2003; Roy et al., 2006a) predict the reactivity behavior of a molecule as a whole and / or at a particular site.

The concept of electronegativity (χ) was first introduced by Pauling (1932, 1960), which represents the "power" of an atom in a molecule to attract the bonded electrons towards itself. Later, Mulliken (1934, 1935) described χ as the average of the first ionization potential (*IP*) and the electron affinity (*EA*), two experimentally obtainable parameters of a molecule. He showed that χ could be determined by an arithmetic mean of the corresponding *IP* and *EA* values as given in Equation 1.

$$\chi = \frac{IP + EA}{2}. \tag{1}$$

The chemical potential (μ) (Parr & Yang, 1989; Hohenberg & Kohn, 1964) of an atom/molecule may be quantitatively assessed by the theory of statistical ensembles. Suppose, an atom or a molecule is a part

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of a grand canonical ensemble in which energy (E) and the number of electrons (N) are two independent parameters and continuous functions as well, then μ of the ensemble can be expressed as follows:

$$\mu = \frac{\partial E}{\partial N}\text{, at constant entropy} \tag{2}$$

Further, Gyftopoulos and Hatsopoulos (1968) put forward a definition of χ of a system where the electronic chemical potential (μ) was considered as the negative of χ. Now, following Mulliken's (1934, 1935) prescription for χ, the Mulliken chemical potential can also be expressed as a finite difference approximation of the derivative of E with respect to N as:

$$\mu_{Mulliken} = -\chi_{Mulliken} = -\frac{IP + EA}{2} = \left[\frac{\partial E(N)}{\partial N}\right]_{N=N_0} . \tag{3}$$

Pearson (1963, 1966, 1968a, 1968b, 1987) introduced the concept of "hardness" or "softness" in chemistry during the study of Lewis acid-base reactions

$$A + :B \rightarrow A:B$$

where A, an acid, acts as an electron acceptor whereas B, a base is the electron-pair donor. Global hardness (η) implies the extent of compactness of the electron cloud encompassing the nucleus/nuclei of an atomic/molecular system whereas global softness (S) is the reciprocal of η and hence represents the extent to which the electronic environment surrounding the nucleus/nuclei of an atomic/molecular species tends to loosen itself. Parr and Pearson (1983) expressed the qualitative concept of η quantitatively in the form of a mathematical descriptor. It is defined as the first derivative of μ or the second derivative of E as a function of N at a fixed external potential $v(\mathbf{r})$ as follows:

$$\eta = \left(\frac{\partial \mu}{\partial N}\right)_{v(\mathbf{r})} = \left(\frac{\partial^2 E}{\partial N^2}\right)_{v(\mathbf{r})} . \tag{4}$$

The convexity in the E vs. N curve signifies that the value of η would be always positive and from the finite difference approach the corresponding curvature equals to difference between IP and EA, which actually represents hardness. Hence

$$\eta = \left(\frac{\partial \mu}{\partial N}\right)_{v(\mathbf{r})} = \left(\frac{\partial^2 E}{\partial N^2}\right)_{v(\mathbf{r})} = IP - EA\,. \tag{5}$$

Therefore, η of a given system describes the extent of its reluctance towards the electron shift to another system in a general charge-transfer phenomenon in between a donor-acceptor couple.

Global softness (S) (Pearson, 1987), a reciprocal of the global hardness (η) of a system, is expressed as:

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$$S = \frac{1}{\eta} = \left(\frac{\partial N}{\partial \mu}\right)_{v(\mathbf{r})}. \tag{6}$$

Electrophilicity (ω) (Parr et al., 1999; Chattaraj et al., 2006, 2007, 2011) and its recently reported mathematical refinement, net electrophilicity ($\Delta\omega^{\pm}$) (Chattaraj et al., 2009) are also important descriptors from the perspective of constructing useful SAR based models

In chemistry electrophiles represent those species, which have a special affinity towards accepting electrons. In general, electrophiles are electron-deficient species, which prefer to combine with electron-rich systems, nucleophiles. In an experiment of the human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein p7 (NCp7) with various electrophiles, Maynard et al. (1998) found that the decay rates of fluorescence varied almost linearly with the ratio of the square of χ and η, χ^2/η. This ratio could further be rationalized as the ability of an electrophile to attract electrons from a nucleophile in forming a covalent bond. This observation of Maynard et al. (1998) led Parr et al. (1999) to describe electron-attracting power of a system in a more precise way.

Consequently, a new reactivity descriptor namely electrophilicity index (ω) was introduced in chemistry. Different from the approach of Maynard et al., (1998) Parr's postulate of ω was based on a thermodynamic background and thus ω becomes a measure of the favorable change in energy upon saturation of a system with electrons. ω as prescribed by Parr et al. (1999) is:

$$\omega = \frac{\mu^2}{2\eta} = \frac{\chi^2}{2\eta}. \tag{7}$$

Eqution 7 resembles $W = V^2/R$, where W, V and R are electrical power, voltage and electrical resistance, respectively. Hence, ω describes the electrophilic power of a system. Few reviews summarizing the different application of ω in understanding the chemical reactivity of different systems are also available in literature (Chattaraj et al., 2006, 2007, 2011).

In order to correlate the associated energy changes between the interacting acceptors and donors in a charge-transfer process, based on the second-order Taylor series energy expansion formula as a function of the number of electrons (N) in the intervals between N - 1 and N, and N and N + 1, Gazquez et al. (2007) showed that the electrodonating (ω^-) and the electroaccepting (ω^+) powers might be defined as:

$$\omega^- = \frac{(\mu^-)^2}{2\eta^-} \tag{8a}$$

$$\omega^+ = \frac{(\mu^+)^2}{2\eta^+} \tag{8b}$$

where μ^- and μ^+ are the chemical potential for electron donation and electron acceptance, respectively, whereas η^- and η^+ are the hardness for electron donation and electron acceptance, respectively. It was also proved that μ^- and μ^+ could be equalized to μ and η^- and η^+ could be equalized to η. Therefore, ω^- and ω^+ become equivalent with ω and thereafter ω, ω^- or ω^+ may be represented in terms of μ and η as $\omega^- =$

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$\omega^+ = \omega = \mu^2/2\eta$. Further, Gazquez et al. (2007) proposed two different expressions for ω^- or ω^+ depending on two different approaches; one following the original formula as above and can be expressed as:

$$\omega^+ = \frac{EA^2}{2(IP - EA)} \tag{9}$$

$$\omega^- = \frac{IP^2}{2(IP - EA)} \tag{10}$$

and other by exploiting an alternative expression for energy as (Gazquez et al., 2007):

$$\omega^+ = \frac{(IP + 3EA)^2}{16(IP - EA)} \tag{11}$$

$$\omega^- = \frac{(3IP + EA)^2}{16(IP - EA)}. \tag{12}$$

It was also reported that the alternative expression of ω^- or ω^+ provided better correlations than the original one (Gazquez et al., 2007).

Chattaraj et al. (2009) recently put forward the concept of net electrophilicity ($\Delta\omega^{\pm}$) to describe the resultant electron-accepting power of a molecule arising from the combined attractive and repulsive effects due to the presence of both electrons and nuclei within a molecule. This dual descriptor ($\Delta\omega^{\pm}$) assesses the electrophilicity of a molecule relative to its own nucleophilicity, therefore is more meaningful descriptor in representing the "electrophilic power" of a system. Thus, net electrophilicity ($\Delta\omega^{\pm}$) in terms of ω^+ and ω^- can be expressed as:

$$\Delta\omega^{\pm} = \left\{\omega^+ - \left(-\omega^-\right)\right\} = \left(\omega^+ + \omega^-\right) \tag{13}$$

or,

$$\Delta\omega^{\pm} = \left\{\omega^+ - \left(\frac{1}{\omega^-}\right)\right\}.$$

The polarizability (α) of an atom or a molecule is the lowest order response of its electron cloud with respect to an external weak electric field (Dalgarno, 1962; van Vleck, 1932). The static dipole polarizability (α) can be defined as the second derivative of the total electronic energy (E) with respect to the external homogeneous electric field as:

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$$\alpha_{xy} = -\left(\frac{\partial^2 E}{\partial F_x \partial F_y}\right)_{F=0} \tag{14}$$

where F_x and F_y are the electric field components along x and y direction. Qualitatively, polarizability (α) varies inversely with η (Ghanty & Ghosh, 1993, 1996; Fuentealba & Reyes, 1993; Pal & Chandra, 1995: Pearson, 1986; Politzer, 1987) and is proportional to S. A linear correlation was found between α and the third power of S (Chattaraj & Poddar, 1998; Simon-Manso & Fuentealba, 1998; Vela & Gázquez, 1990).

The magnetizability (ξ) (Griffith, 1999) for a molecule is a measure of the linear response of its electron cloud with respect to an externally applied magnetic field and is expressed as:

$$\left|\xi = -\left(\frac{\partial^2 \varepsilon(B)}{\partial B^2}\right)\right|_{B=0} \tag{15}$$

where B is the external magnetic field.

A softer molecule is also found to be more polarizable and hence more magnetizable (Chattaraj et al., 2007).

In addition to the various CDFT based global reactivity descriptors, the local reactivity descriptors like Fukui function ($f(\mathbf{r})$), philicity ($\omega(\mathbf{r})$), local softness ($s(\mathbf{r})$), local hardness ($\eta(\mathbf{r})$) and electron density ($\rho(\mathbf{r})$) are also important in providing valuable information regarding the reactivity pattern at a local atomic site in a molecule.

Information about $\rho(\mathbf{r})$ is important to get insights into site-selectivity for a molecule and can be determined in terms of the first order variation in energy (E) as a function of the external potential ($v(\mathbf{r})$) (Parr & Yang, 1989, 1995).

$$\rho(\mathbf{r}) = \left(\frac{\delta E}{\delta v(\mathbf{r})}\right)_N . \tag{16}$$

The Fukui function ($f(\mathbf{r})$) (Ayers & Levy, 2004; Fukui et al., 1952, 1954; Fukui, 1975, 1982; Parr & Yang, 1984; Yang et al., 1984) is very useful to evaluate quanlitatively the extent of local response at a particular atomic site of a molecule. It is defined as the differential change in $\rho(\mathbf{r})$ due to an infinitesimal change in the number of electrons (N).

$$f(\mathbf{r}) = \left(\frac{\partial \rho(\mathbf{r})}{\partial N}\right)_{v(\mathbf{r})} = \left(\frac{\delta \mu}{\delta v(\mathbf{r})}\right)_N . \tag{17}$$

Now, due to the discontinuity in the derivative in eq. 17, for integral values of N, by applying the finite difference and frozen core approximations three different types of Fukui functions can be defined as follows: (Ayers & Levy, 2004; Parr & Yang, 1984)

For nucleophilic attack:

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$$f^{+}(\mathbf{r}) = \left(\frac{\partial\rho(\mathbf{r})}{\partial N}\right)^{+}_{v(\mathbf{r})} \approx \rho_{N+1}(\mathbf{r}) - \rho_{N}(\mathbf{r}) \approx \rho_{LUMO}(\mathbf{r})\,. \tag{18a}$$

For electrophilic attack:

$$f^{-}(\mathbf{r}) = \left(\frac{\partial\rho(\mathbf{r})}{\partial N}\right)^{-}_{v(\mathbf{r})} \approx \rho_{N}(\mathbf{r}) - \rho_{N-1}(\mathbf{r}) \approx \rho_{HOMO}(\mathbf{r})\,. \tag{18b}$$

For radical attack:

$$f^{0}(\mathbf{r}) = \frac{1}{2}\left[f^{+}(\mathbf{r}) + f^{-}(\mathbf{r})\right]. \tag{18c}$$

Yang and Mortier (1986) further proposed a coarse-grained atom by atom representation of the Fukui function, called condensed-to-atom Fukui function. Based on a finite-difference approach, they can be represented as:

$$f_k^{+} = q_k(N+1) - q_k(N) \text{ for nucleophilic attack} \tag{19a}$$

$$f_k^{-} = q_k(N) - q_k(N-1) \text{ for electrophilic attack} \tag{19b}$$

$$f_k^{0} = [q_k(N+1) - q_k(N-1)] \,/\, 2 \text{ for radical attack} \tag{19c}$$

where q_k is the electron population at a particular k^{th} atomic site in a molecule.

Therefore, condensed-to-atom Fukui function can be easily computed through the data obtained from the population analysis.

Mathematically, global softness (S) can be expressed as an integral of the individual local softness ($s(\mathbf{r})$). A normalized condition between S and $s(\mathbf{r})$ can be written as:

$$S = \int s(\mathbf{r})dr \tag{20}$$

$s(\mathbf{r})$ is connected to $f(\mathbf{r})$ through a chain rule as:

$$s(\mathbf{r}) = \left(\frac{\partial\rho(\mathbf{r})}{\partial\mu}\right)_{v(\mathbf{r})} = \left(\frac{\partial\rho(\mathbf{r})}{\partial N}\right)_{v(\mathbf{r})}\left(\frac{\partial N}{\partial\mu}\right)_{v(\mathbf{r})} = f(\mathbf{r}).S \tag{21}$$

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f(**r** *)* acts mainly as an intra-molecular reactivity descriptor by describing the reactivity of the atomic sites in a particular molecule whereas unlike *f(***r***)*, *s(***r***)* acts as an inter-molecular reactivity descriptor by comparing and correlating the tendency of interaction between a pair of neighboring molecules during chemical response.

Local hardness (*η(***r***)*) (Ghosh & Berkowitz, 1985; Yang & Parr, 1985) quantified the unwillingness of a particular atomic site in a molecule towards chemical attack. However, *η(***r***)* cannot be normalized to η as was done for *s(***r***)* to *S*. *η(***r***)* can be expressed as:

$$\eta(\mathbf{r}) = \frac{1}{N}\int \frac{\delta^2 F[\rho(\mathbf{r})]}{\delta\rho(\mathbf{r})\delta\rho(\mathbf{r}')}\rho(\mathbf{r}')d\mathbf{r}' \tag{22}$$

where F[ρ(**r**)] is the Hohenberg-Kohn universal functional. *η(***r***)* can also be expressed as:

$$\eta(\mathbf{r}) = \left(\frac{\delta\mu}{\delta\rho(\mathbf{r})}\right)_{v(\mathbf{r})}. \tag{23}$$

However, due to the dependence of $\rho(\mathbf{r})$ on $v(\mathbf{r})$, the above expression is ambiguous and hence cannot be considered as a basic definition for $\eta(\mathbf{r})$. Unlike η and S, $\eta(\mathbf{r})$ and $s(\mathbf{r})$ do not have a reciprocal relationship. They can be linked as:

$$\int \eta(\mathbf{r})s(\mathbf{r})dr = 1 \tag{24}$$

Further, the global hardness can be obtained from *η(***r***)* and *f(***r***)* as: (Chattaraj et al., 2007)

$$\eta = \int \eta(\mathbf{r})f(\mathbf{r})dr \tag{25}$$

Chattaraj et al. (2003) introduced the concept of philicity (*ω*$^{\alpha}$*(***r***)*), which represents the aptitude of a local atomic site in a molecule towards towards reactivity (electrophilicity or nucleophilicity).

The relation between the local electrophilicity (*ω(***r***)*) and the global electrophilicity (ω) can be expressed as:

$$\omega = \omega\int f(\mathbf{r})dr$$

$$\text{or, } \omega = \int \omega f(\mathbf{r})dr = \int \omega(\mathbf{r})dr \text{ thus, } \omega(\mathbf{r}) = \omega f(\mathbf{r}). \tag{26}$$

Therefore, from the expression it is obvious that *ω(***r***)* includes information about both ω and *f(***r***)* but without the knowledge of ω, *f(***r***)* alone cannot give any clue about *ω(***r***)*. One can apply this concept of *ω(r)* in several types of chemical reaction. Therefore, it will be better to represent philicity as *ω*$^{\alpha}$*(***r***)* in stead of *ω(***r***)*. α has signs of +, – and 0 describing nucleophilic, electrophilic and radical attacks, respectively. Therefore:

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$$\omega^{\alpha}\left(\mathbf{r}\right)=\omega.f^{\alpha}\left(\mathbf{r}\right)\ \text{and},\quad \omega_{k}^{\alpha}=\omega.f_{k}^{\alpha} \tag{27}$$

where ω_k^{α} is the condensed-to-atom local philicity variant for the k^{th} atomic site in a molecule.

The concept of group philicity (ω_g^{α}) (Parthasarathi et al., 2004a) was also put forward in order to describe the reactivity of a group or an atomic assembly. It is generally expressed as a summation of the individual ω_k^{α} over all the related atoms. Therefore:

$$\omega_{g}^{\alpha}=\sum_{k=1}^{n}\omega_{k}^{\alpha} \tag{28}$$

where n describes the number of atoms present in the reacting group and $\alpha = +, -$ and 0 represents nucleophilic, electrophilic and radical attacks, respectively.

The efficacy of various CDFT based global and local reactivity descriptors in predicting chemical reactivity are guided by the various electronic structure principles like the principle of maximum hardness (PMH) (Ayers & Parr, 2000; Chattaraj et al., 1995, 2000; Chattaraj, 1996; Pan et al., 2013; Pan & Chattaraj, 2013; Parr & Chattaraj, 1991; Parr & Zhou, 1993; Pearson, 1987, 1993), minimum electrophilicity principle (MEP) (Chamorro et al., 2000; Pan et al., 2013; Parthasarathi, 2005), minimum polarizability principle (MPP) (Chattaraj & Sengupta, 1996, 1997; Chattaraj et al., 1999), minimum magnetizability principle (MMP) (Tanwar et al., 2006).

With the structural changes and/or substituent effects in a system, the variation of related conceptual DFT based reactivity descriptors led light into the quantitative correlation between chemical reactivity and toxicity. They indeed become important tools in building effective regression models for QSAR/QSPR/QSTR analyses.

4. ELECTROPHILICITY INDEX, NET ELECTROPHILICITY INDEX, AND THEIR LOCAL VARIANTS IN PREDICTING TOXICITY

Electrophilicity index (ω), net electrophilicity index ($\Delta\omega^{\pm}$) and the local variants of ω were found to be very effective tools in predicting toxicity. Some examples are given below.

4.1 Prediction of Toxicity of Various Alkali, Alkaline-Earth, Transition-Metal Ions and Arsenic Ions

Roy et al. (2009) attempted to predict the toxicity of arsenic (As^{3+} and As^{5+}) ions by choosing a training set composed of various alkali and alkaline-earth metal ions (Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}) and transition metal ions (Cr^{3+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) along with their experimental toxicity (50% Lethal concentration, LC_{50}) in order to get single-parameter regression models. Two training sets composed of experimental toxicity (50% Lethal dose, LD_{50}) (Leermakers et al., 2006; Hughes, 2002) of two different sets of arsenic derivatives were also considered in order to predict their toxicity by employing several selected parameters. The corresponding atomic number (Z), energy (E), electrophilicity (ω) and the experimental as well as the calculated toxicity values expressed in terms of LC_{50} at the B3LYP/6-31+G(d) level are provided in Table 1. The calculated LC_{50} values in terms of Z and ω were computed as follows:

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$$\text{Calc. } LC_{50} = -0.0181 \times Z + 0.5272; \quad (29)$$

$R = 0.923$, $SD = 0.054$, $N = 10$

$$\text{Calc. } LC_{50} = -7.1 \times 10{-}04 \times \omega + 0.2987; \quad (30)$$

$R = 0.793$, $SD = 4.337$, $N = 10$.

In both Z and ω models, two ions were found to be outliers, being Li^+ and K^+ in case of Z- model and Cu^{2+} and Zn^{2+} in case of ω- model. However, the Z- model was found to be better in prediction of toxicity of ions than that of ω- model but being the same Z value, Z- model could not differentiate the toxicity of As^{3+} and As^{5+}. On the other hand, ω- model can predict the toxicity of As^{3+} and As^{5+} correctly, As^{3+} ($LC_{50} = 0.217$) being more toxic than As^{5+} ($LC_{50} = 0.169$).

Further, efficacy of different global descriptors like number of nonhydrogenic atoms (N_{NH}) and ω and different local descriptors like philicity (ω^+_{As}) and atomic charges (Q_{As}) at As site were tested in building QSTR taking two sets of As-derivatives, whose experimental toxicity were known in terms of LD_{50} obtained from two different biological assays (Leermakers et al., 2006; Hughes, 2002).

Table 1. Atomic number (Z), energy (E, au), electrophilicity index (ω, eV), and experimental and calculated LC_{50} values for various selected alkali, alkaline-earth, and transition-metal ions along with arsenic ions at the B3LYP/6-31+G(d) level

Metal Ions	Z	E	ω	Expt. LC_{50}	Calc. LC_{50}	
					Z	ω
Training set						
Li^+ [a]	3	−7.28459	22.033	0.215	–	0.283
Na^+	11	−162.08125	16.607	0.398	0.328	0.287
Mg^{2+}	12	−199.22743	37.645	0.250	0.310	0.272
K^+ [a]	19	−599.72502	12.909	0.479	–	0.290
Ca^{2+}	20	−676.86695	28.515	0.173	0.165	0.278
Cr^{3+}	24	−1042.22188	495.511	0.019	0.093	−0.053
Mn^{2+}	25	−1149.83984	168.557	0.132	0.075	0.179
Fe^{3+}	26	−1261.27545	413.554	0.0003	0.056	0.005
Co^{2+}	27	−1381.56785	204.105	0.022	0.038	0.154
Ni^{2+}	28	−1507.03577	331.966	0.069	0.020	0.063
Cu^{2+} [b]	29	−1639.25180	100.906	0.002	0.002	–
Zn^{2+} [b]	30	−1778.15610	65.992	0.007	−0.016	–
Toxicity prediction of arsenic ions						
As^{3+}	33	−2231.61591	115.148	–	–	0.217
As^{5+}	33	−2227.48829	182.990	–	–	0.169

[a]Outliers in the Z model;[b]Outliers in the ω model.

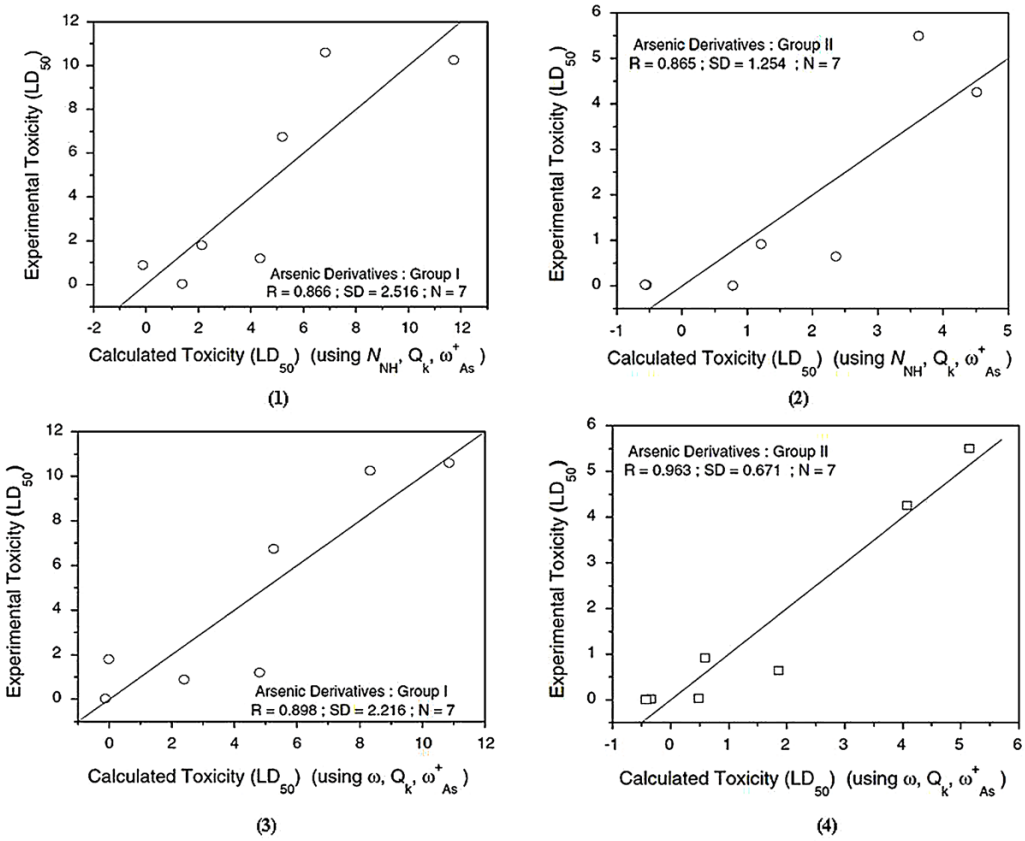
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In both sets, seven As-derivatives were included (Set-I members were Arsenite (As(III)), Arsenobetaine (AsB), Arsenocholine (AsC), Monomethyl arsonic acid (MMA), Dimethyl arsinic acid (DMA), Trimethyl arsine oxide (TMAO), Tetramethylarsonium ion (TeMA) and Set-II members were Arsenate [As(V)], Arsenite [As(III)], Arsenobetaine (AsB), Arsenic trioxide (As_2O_3), Monomethyl arsonic acid (MMA), Dimethyl arsinic acid (DMA), Trimethyl arsine oxide (TMAO)). For each individual descriptor, one-parameter regression analyses were performed. It was found that N_{NH} has reasonably good predictive power towards the toxicity of As-derivatives in both the sets except the case of TMAO molecule.

ω showed better prediction in set-II than in set-I. On the other hand, ω^+_{As} showed better prediction in set-I than in set-II and Q_{As} could not predict the toxicity of As(III) in both the sets as well as As_2O_3 in set-II. Nevertheless, none of these descriptors could predict the toxicity of all seven molecules in both the sets. Then possible combinations of these four descriptors were tested towards the prediction of toxicity. Two-parameter regressions were not found that much suitable in predicting toxicity of all the As-derivatives; however, three-parameter regressions composed of one global (N_{NH} or ω) and two local parameters (ω^+_{As} and Q_{As}) were capable to represent the toxicity of all the derivatives reasonably well (see Figure 1).

*Figure 1. Experimental versus calculated toxicity (LD50) values using three-parameter (N_{NH}, ω^+_{As}, and Q_{As}) regression model for the complete set of set-I (**1**) and II (**2**) As-derivatives and three-parameter (ω, ω^+_{As}, and Q_{As}) regression model for the complete set of set-I (**3**) and II (**4**) As-derivatives*
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The corresponding regression models could be represented as follows:

Set-I:

$$\text{Calc.LD}_{50} = 1.983 \times N_{\text{NH}} - 5.360 \times \omega^{+}_{\text{As}} - 35.926 \times Q_{\text{As}} + 15.402 \quad (31)$$

$R = 0.866$, $SD = 2.516$, $N = 7$

$$\text{Calc.LD}_{50} = 1.805 \times \omega - 16.312 \times \omega^{+}_{\text{As}} - 85.353 \times Q_{\text{As}} + 51.415 \quad (32)$$

$R = 0.898$, $SD = 2.216$, $N = 7$

Set-II:

$$\text{Calc.LD}_{50} = 0.484 \times N_{\text{NH}} - 3.232 \times \omega^{+}_{\text{As}} - 16.968 \times Q_{\text{As}} + 10.065 \quad (33)$$

$R = 0.865$, $SD = 1.254$, $N = 7$

$$\text{Calc.LD}_{50} = 4.724 \times \omega - 10.881 \times \omega^{+}_{\text{As}} - 54.889 \times Q_{\text{As}} + 23.380 \quad (34)$$

$R = 0.963$, $SD = 0.671$, $N = 7$

It was also found that the toxicity prediction power of the combination, ω, ω^{+}_{As} and Q_{As} is better than that of the combination of N_{NH}, ω^{+}_{As} and Q_{As}.

4.2 Prediction of the Toxicity ($pIGC_{50}$) of 252 Aliphatic Compounds on *Tetrahymena Pyriformis*

Chattaraj et al. (2007) used global descriptors like the number of carbon atoms (N_C), number of nonhydrogenic atoms (N_{NH}) in a molecule, ω and local descriptors like ω^{+}_{max}, ω^{-}_{max}, q_k^{max} computed at the HF/6-311G(d) level in modeling effective QSTR of a large number of aliphatic compounds on *T. pyriformis*. Here ω^{+}_{max} and ω^{-}_{max} referred to the ω_k^{α} at an atomic site in which it was maximum. q_k^{max} implied atomic charge at the kth site having maximum charge. By studying 252 aliphatic compounds, it was found that apart from the set of aliphatic amines, the atom number (N_C and N_{NH}) was an important descriptor whereas ω was also turned out as an effective descriptor in most of the cases. The predictability towards toxicity of two-parameter models based on N_C and ω was also found almost same with respect to the corresponding three-parameter models built by N_C, ω and any one descriptor from N_{NH}, ω^{+}_{max}, ω^{-}_{max}, q_k^{max}. Different regression equations adopted in one (N_C)- and two (N_C, ω)-parameter models are given Table 2. N_C-based single parameter model could predict toxicity fairly in most of the cases. In few cases like carboxylic acids, halogenated acids, amino alcohols and amines, it did not work. However, except amines the predictability towards toxicity of the model was found to improve significantly in coupling N_C with ω. In cases of amines, q_k^{max} and ω^{-}_{max} were also found to be important in describing toxicity. With the observation that in many cases, similar results were obtained, N_C was termed as the crude alternative

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Table 2. Regression models for different groups of aliphatic compounds for estimating their toxicity towards Tetrahymena pyriformis

Molecules	Regression Equations	R	SD
Aliphatic electron acceptors			
Diols (N = 10)	$pIGC_{50} = 0{\cdot}4497 \times N_C - 4{\cdot}0855$	0·9683	0·2781
	$pIGC_{50} = 0{\cdot}8059 \times \log P - 1{\cdot}4688$	0·9892	0·1617
	$pIGC_{50} = -12{\cdot}4224 \times \omega + 0{\cdot}3554 \times N_C + 7{\cdot}6094$	0·9826	0·2070
	$pIGC_{50} = 10{\cdot}0678 \times \omega + 0{\cdot}9625 \times \log P - 10{\cdot}5043$	0·9934	0·1270
Halogenated alcohols (N = 11)	$pIGC_{50} = 0{\cdot}3271 \times N_C - 2{\cdot}0248$	0·8923	0·3852
	$pIGC_{50} = 0{\cdot}7783 \times \log P - 1{\cdot}3735$	0·9486	0·2561
	$pIGC_{50} = -6{\cdot}2863 \times \omega + 0{\cdot}1982 \times N_C + 4{\cdot}5793$	0·9424	0·2855
	$pIGC_{50} = -4{\cdot}0784 \times \omega + 0{\cdot}5772 \times \log P + 2{\cdot}7468$	0·9646	0·2169
Saturated alcohols (N = 32)	$pIGC_{50} = 0{\cdot}4144 \times N_C - 3{\cdot}0801$	0·9634	0·3456
	$pIGC_{50} = 0{\cdot}7745 \times \log P - 2{\cdot}0034$	0·9903	0·1777
	$pIGC_{50} = 0{\cdot}8927 \times \omega + 0{\cdot}4261 \times N_C - 3{\cdot}9484$	0·9636	0·3451
	$pIGC_{50} = 1{\cdot}6835 \times \omega + 0{\cdot}8138 \times \log P - 3{\cdot}5796$	0·9907	0·1739
Carboxylic acids (N = 28)	$pIGC_{50} = 0{\cdot}1116 \times N_C - 0{\cdot}9678$	0·6676	0·2917
	$pIGC_{50} = 0{\cdot}2857 \times \log P - 0{\cdot}7006$	0·9586	0·1070
	$pIGC_{50} = -5{\cdot}4426 \times \omega + 0{\cdot}0338 \times N_C + 4{\cdot}8562$	0·8801	0·1860
	$pIGC_{50} = -0{\cdot}3944 \times \omega + 0{\cdot}2715 \times \log P - 0{\cdot}2924$	0·9589	0·1066
Halogenated acids (N = 11)	$pIGC_{50} = 0{\cdot}2257 \times N_C - 1{\cdot}3481$	0·6564	0·3632
	$pIGC_{50} = 0{\cdot}4620 \times \log P - 0{\cdot}8744$	0·8107	0·2285
	$pIGC_{50} = 1{\cdot}8930 \times \omega + 0{\cdot}0976 \times N_C - 2{\cdot}2827$	0·9186	0·1903
	$pIGC_{50} = 1{\cdot}6012 \times \omega + 0{\cdot}2001 \times \log P - 1{\cdot}8388$	0·9169	0·1762
Mono esters (N = 31)	$pIGC_{50} = 0{\cdot}3645 \times N_C - 2{\cdot}7969$	0·9189	0·3710
	$pIGC_{50} = 0{\cdot}7599 \times \log P - 2{\cdot}0274$	0·9645	0·2396
	$pIGC_{50} = -10{\cdot}9131 \times \omega + 0{\cdot}2554 \times N_C + 8{\cdot}1384$	0·9352	0·3330
	$pIGC_{50} = -3{\cdot}0902 \times \omega + 0{\cdot}6960 \times \log P + 1{\cdot}0027$	0·9655	0·2365
Diesters (N = 20)	$pIGC_{50} = 0{\cdot}2861 \times N_C - 2{\cdot}884$	0·9299	0·3382
	$pIGC_{50} = 0{\cdot}6338 \times \log P - 1{\cdot}3322$	0·9539	0·2632
	$pIGC_{50} = -4{\cdot}8166 \times \omega + 0{\cdot}1999 \times N_C + 1{\cdot}1227$	0·9636	0·2460
	$pIGC_{50} = -4{\cdot}2407 \times \omega + 0{\cdot}4687 \times \log P + 1{\cdot}7763$	0·9790	0·1834
Aldehydes (N = 13)	$pIGC_{50} = 0{\cdot}2230 \times N_C - 1{\cdot}4027$	0·8980	0·2459
	$pIGC_{50} = 0{\cdot}4628 \times \log P - 0{\cdot}8864$	0·9227	0·1988
	$pIGC_{50} = -2{\cdot}5248 \times \omega + 0{\cdot}1228 \times N_C + 1{\cdot}3002$	0·9332	0·2008
	$pIGC_{50} = -2{\cdot}1731 \times \omega + 0{\cdot}2904 \times \log P + 1{\cdot}2280$	0·9496	0·1664
Ketones (N = 15)	$pIGC_{50} = 0{\cdot}4147 \times N_C - 3{\cdot}4470$	0·9850	0·2249
	$pIGC_{50} = 0{\cdot}7720 \times \log P - 2{\cdot}0314$	0·9872	0·2048
	$pIGC5_0 = -3{\cdot}2176 \times \omega + 0{\cdot}38989 \times N_C - 0{\cdot}6459$	0·9855	0·2211
	$pIGC_{50} = -1{\cdot}4487 \times \omega + 0{\cdot}7511 \times \log P - 0{\cdot}8080$	0·9873	0·2041

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Table 2. Continued

Molecules	Regression Equations	R	SD
Aliphatic electron donors			
Amino alcohols (N = 18)	$pIGC_{50} = 0{\cdot}2255 \times N_C - 2{\cdot}3296$	0·4711	0·4596
	$pIGC_{50} = 0{\cdot}3533 \times \log P - 1{\cdot}0529$	0·5829	0·2468
	$pIGC_{50} = 8{\cdot}9875 \times \omega + 0{\cdot}1464 \times N_C - 7{\cdot}4431$	0·9152	0·2100
	$pIGC_{50} = 8{\cdot}5520 \times \omega + 0{\cdot}2282 \times \log P - 6{\cdot}3481$	0·9377	0·1697
Acetylenic alcohols (N = 13)	$pIGC_{50} = 0{\cdot}3332 \times N_C - 2{\cdot}2125$	0·8942	0·4218
	$pIGC_{50} = 0{\cdot}5506 \times \log P - 0{\cdot}8071$	0·8842	0·3891
	$pIGC_{50} = 3{\cdot}7452 \times \omega + 0{\cdot}3707 \times N_C - 5{\cdot}0494$	0·9080	0·3947
	$pIGC_{50} = 0{\cdot}2523 \times \omega + 0{\cdot}5538 \times \log P - 0{\cdot}9858$	0·8843	0·3890
Unsaturated alcohols (N = 25)	$pIGC_{50} = 0{\cdot}4093 \times N_C - 3{\cdot}3473$	0·8580	0·3311
	$pIGC_{50} = 0{\cdot}7587 \times \log P - 1{\cdot}6861$	0·9315	0·2185
	$pIGC_{50} = -2{\cdot}2250 \times \omega + 0{\cdot}3641 \times N_C - 1{\cdot}7327$	0·9136	0·2622
	$pIGC_{50} = -0{\cdot}0271 \times \omega + 0{\cdot}7568 \times \log P - 1{\cdot}6679$	0·9315	0·2185
Amines (N = 25)	$pIGC_{50} = 0{\cdot}1136 \times N_C - 1{\cdot}3456$	0·2711	0·3965
	$pIGC_{50} = 0{\cdot}4609 \times \log P - 1{\cdot}1380$	0·8534	0·1833
	$pIGC_{50} = -0{\cdot}1700 \times \omega + 0{\cdot}1116 \times N_C - 1{\cdot}2295$	0·2723	0·3964
	$pIGC_{50} = 0{\cdot}9307 \times \omega + 0{\cdot}4792 \times \log P - 1{\cdot}7367$	0·8641	0·1792
	$pIGC_{50} = 0{\cdot}1162 \times q_k^{max} + 2{\cdot}1524 \times \omega^-_{max} + 0{\cdot}0669 \times N_C - 1{\cdot}8782$	0·8692	0·2037
	$pIGC_{50} = 0{\cdot}0681 \times q_k^{max} + 1{\cdot}3490 \times \omega^-_{max} + 0{\cdot}2802 \times \log P - 1{\cdot}4885$	0·9429	0·1293

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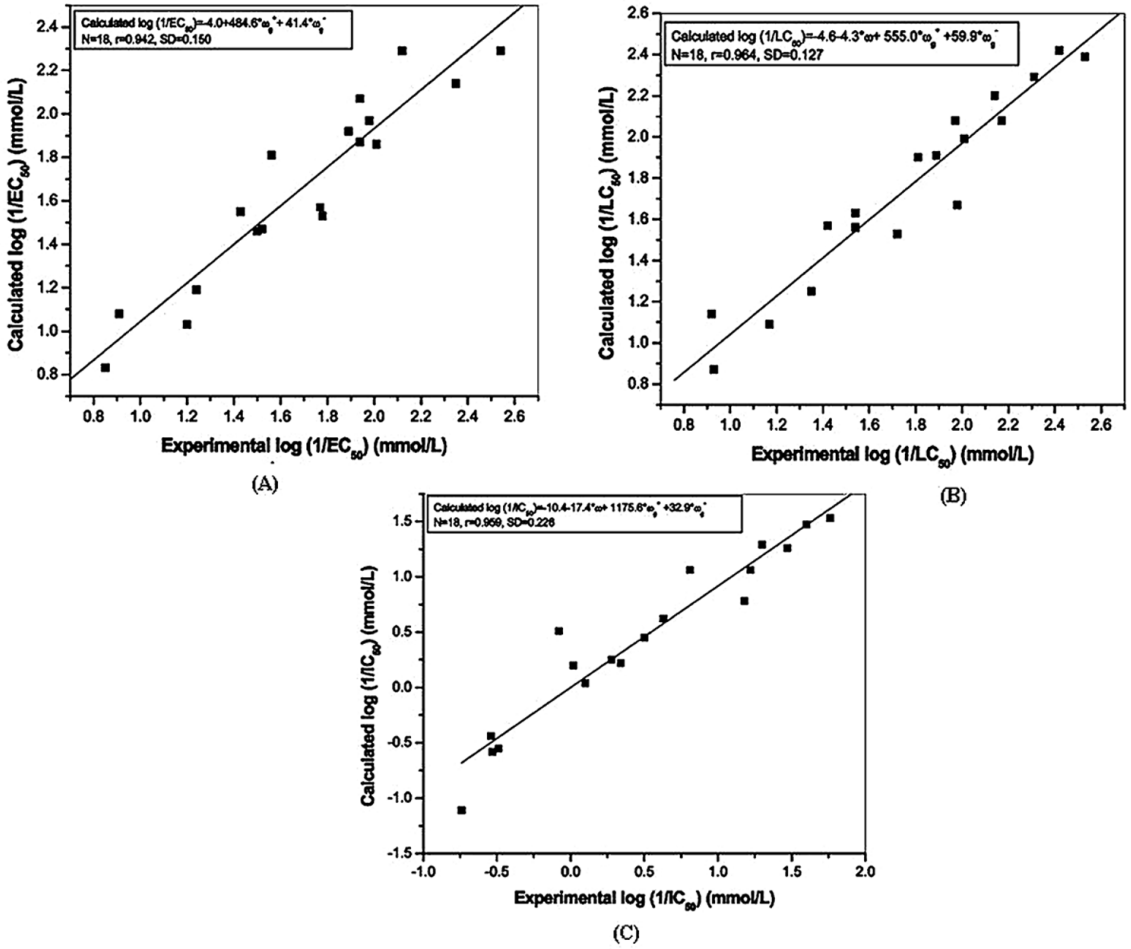
of log P. The plots of experimental $pIGC_{50}$ versus calculated $pIGC_{50}$ values are displayed in Figure 2. Quite large R^2, R^2_{cv} and variance adjusted to degrees of freedom, R^2_{adj} values implied the effectiveness of these models in building QSTR.

4.3 Prediction of Toxicity of Several Polyaromatic Hydrocarbons (PAH)

The global and local electrophilicities computed at the B3LYP/6-31G(d) level were used to predict the toxicity of several polyaromatic hydrocarbons (PAH) (Roy et al., 2006c). The toxicity was represented in terms of pIC_{50}, which could be defined as the molar concentration of these chemicals required to displace 50% of radio-labeled tetrachlorodibenzo-p-dioxin (TCDD) from the arylhydrocarbon (Ah) receptor. As a training set, dependent and independent variables were considered as the experimental pIC_{50} values of the electron acceptor toxin like polychlorinated dibenzofurans (PCDF) and ω, respectively. Further, this model was also employed on another set of polychlorinated biphenyls (PCB). General structures of PCDFs, and PCBs are provided in Tables 3 and 4, respectively, along with the atom numbering, identitity number and substitution pattern. The fractional number of electrons (ΔN) shifted from the PCDFs, PCBs and amines (A) to the nucleic acid bases/DNA base pairs (B) was also used as a descriptor to describe toxicity. The positive values of ΔN for PCDFs and PCBs implied their electron accepting nature whereas the negative values for

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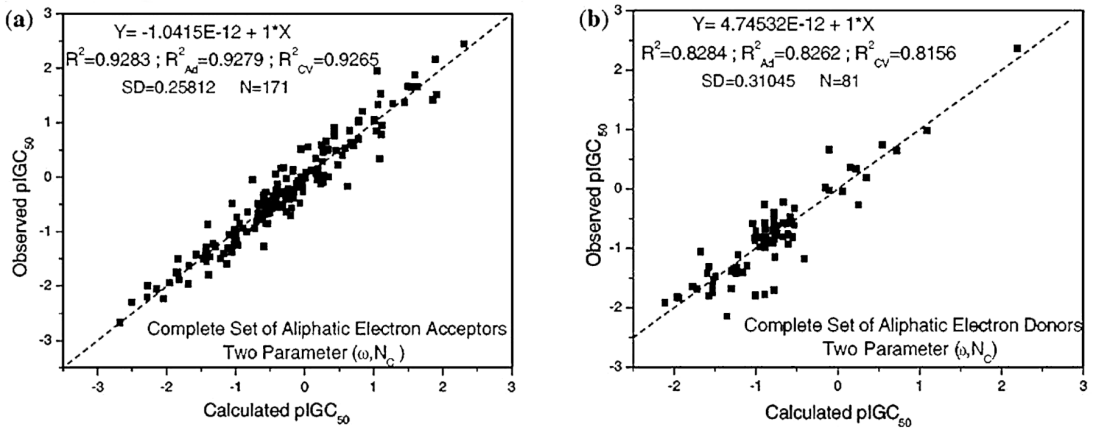
Figure 2. Observed versus calculated $pIGC_{50}$ values using two-parameter (ω, N_C) regression models for the (a) Complete set of aliphatic electron acceptors and (b) complete set of aliphatic electron donors
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amines recognized them as electron donors. Reasonably good correlation was found to exist between ΔN (in between toxin and guanine) and ω having R = 0.991. The correlation between ΔN and exp. pIC_{50} was 0.881 whereas that obtained from the two-parameter regression based on ΔN and ω and exp. pIC_{50} was 0.892 (see Figure 3). Similarly, in cases of interactions in between PCDFs and GCWC (Guanine-Cystosine Watson-Crick) base-pairs, good correlations were observed. The calculated parameters (ω and ω^{+}_{max}) along with the experimental and predicted pIC_{50} values of the training set of PCDFs and PCBs are given in Table 5. In case of PCDFs, ω values gave a correlation R value of 0.891 whereas in case of PCBs, it was 0.834 (see Figure 4). The toxicity of aliphatic amines expressed by the 50% inhibitory growth concentration (IGC_{50}) towards ciliate fresh-water protozoa *Tetrahymena pyriformis* was also assessed. Due to the lack of global nucleophilicity, local nucleophilicity (ω^{+}_{max}) was applied as a descriptor for this set. This model was then further employed for the test set of amino alcohols. It was found that despite the occurrence of a very good correlation, there was some cross validation problem. Additionally, the use of the amines and amino alcohols together as both training and test sets provided fair correlation.

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*Figure 3. Variation of charge transfer (ΔN) with (***A***) electrophilicity index (ω), (***B***) the experimental biological activity (pIC_{50}) during interaction of polychlorinated dibenzo furan with guanine and (***C***) Observed versus calculated pIC_{50} values of polychlorinated dibenzo furan using electrophilicity index (ω) and N with guanine (For* **C***, the regression model is: pIC_{50} = 4.033*ω − 13.596*ΔN − 5.954)*
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4.4 Structure-Toxicity Analysis on Chloroanilines

Padmanabhan et al. (2006a) further developed QSTR for aquatic toxicity ($\log(IGC_{50}^{-1})$) of a set of nine chloroanilines (CA) against *T. pyriformis* at the B3LYP/6-311++G(d,p) level as given in Table 6. The experimental and calculated $\log(IGC_{50}^{-1})$ are also given in Table 6. The regression equation considering experimental $\log(IGC_{50}^{-1})$ value (Schultz, 1999) as a dependent variable and ω as an independent variable was as follows:

$$\log(IGC_{50}^{-1}) = -4.414 + 56.035\ \omega \tag{35}$$

$N = 9$, $r^2 = 0.968$, SD = 0.144.

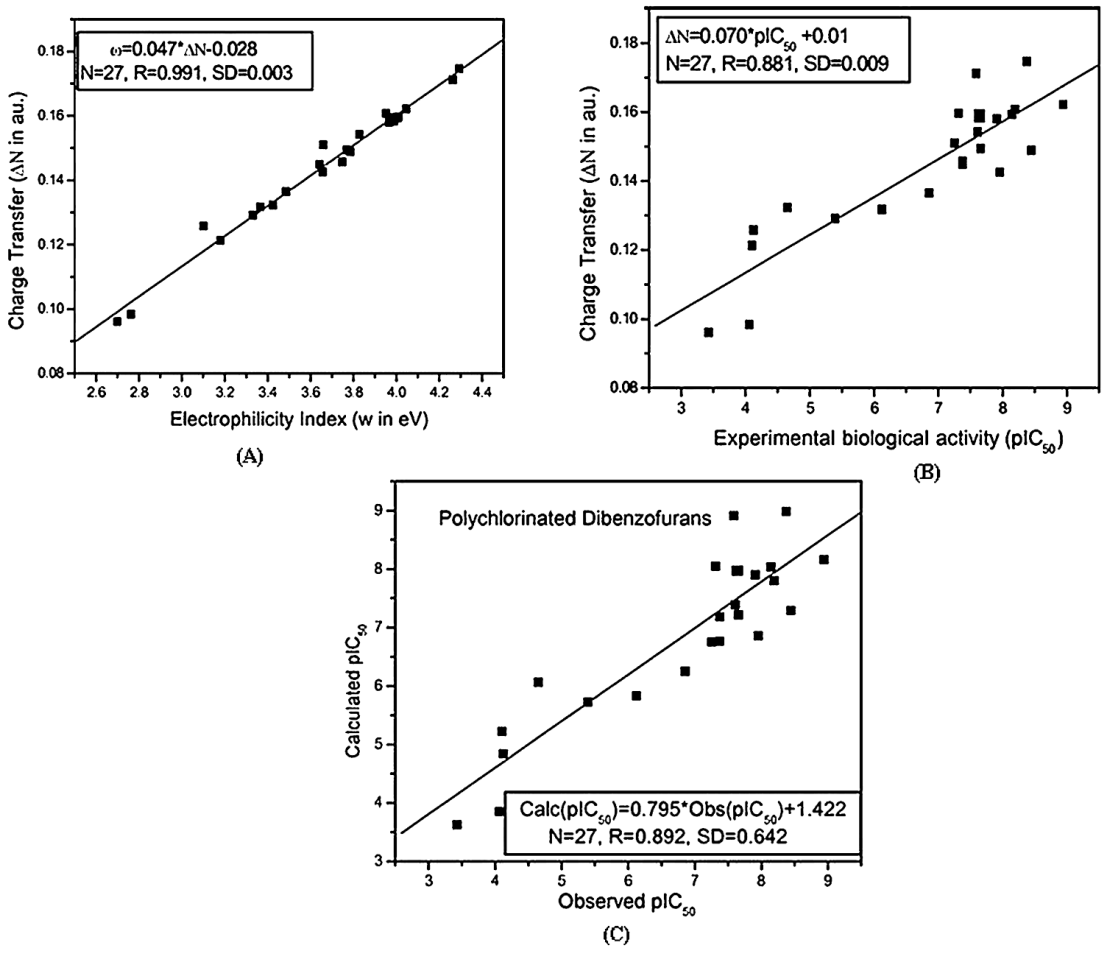
The plot between exp. and calc. $\log(IGC_{50}^{-1})$ (see Figure 5) showed that ω could describe the aquatic toxicity of these CA systems superbly having a correlation coefficient (r) of 0.984.

4.5 Prediction of Toxicity of Phenol, Nitrobenzene, and Benzonitrile-Derivatives

ω and its local variants, local electrophilicity index (ω^{α}_{k}) computed at the HF/6-31G(d) level were also employed in order to describe the toxicity of a large number (174) of aromatic compounds towards *T. pyriformis*, mainly belonging to the family of phenols, nitrobenzenes and benzonitriles (Roy et al., 2006b). At first, depending on their electron donating/accepting nature measured by comparing their χ values with those of the nuclei acid bases (adenine, thymine, guanine, cytosine and uracil) and DNA base pairs (GCWC and ATH), they were divided into two classes viz., 97 phenol derivatives having electron donating group and 77 nitrobenzenes and benzonitriles having electron accepting group. The regression models were used for the complete set of aromatic donors as

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Figure 4. (A) Calculated versus observed values of biological activity (pIC_{50}) for training set of PCDFs, (B) predicted versus observed values of biological activity (pIC_{50}) for test set of PCBs in gas phase
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$$\log(IGC_{50}^{-1}) = -0.0703\ \omega + 0.7706\ \omega^{-}_{O\max} - 6.10^{-05}E_{HF}\ 0.6179 \quad (36)$$

N=97, R=0.898, $R^2_{adj} = 0.804$, $R^2_{CV} = 0.795$, SD=0.290

and that for the complete set of aromatic acceptors as

$$\log(IGC_{50}^{-1}) = 0.6612\ \omega - 1.2673\ \omega^{+}_{C\max} - 4.10^{-05}E_{HF}\ 1.1752 \quad (37)$$

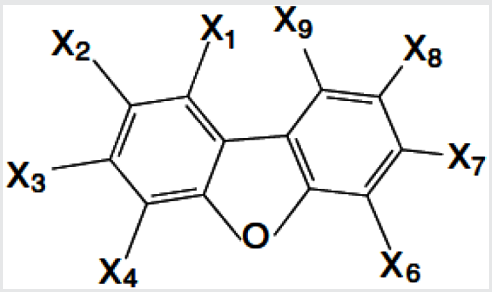
N=77, R=0.902, $R^2_{adj} = 0.811$, $R^2_{CV} = 0.800$, SD=0.359

The corresponding plots of exp. $\log(IGC_{50}^{-1})$ vs. calc. $\log(IGC_{50}^{-1})$ are displayed in Figure 6.

Three-parameter regression models based on global electrophilicity (ω), local philicity descriptor at the O site ($\omega^{-}_{O\max}$) in case of phenol derivatives (aromatic donors) and at the C site ($\omega^{+}_{C\max}$) in cases

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Table 3. Polychlorinated dibenzofurans with identity number (ID) representing the substitution pattern

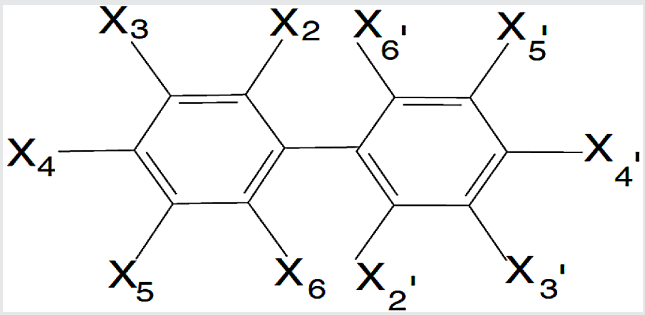


ID	X_1	X_2	X_3	X_4	X_6	X_7	X_8	X_9
1	H	Cl	H	H	H	H	H	H
2	H	H	H	Cl	H	H	H	H
3	H	Cl	H	H	Cl	H	H	H
4	H	Cl	H	H	H	H	Cl	H
5	Cl	H	Cl	H	Cl	H	H	H
6	Cl	H	Cl	H	H	H	Cl	H
7	H	Cl	Cl	Cl	H	H	H	H
8	H	Cl	Cl	H	H	H	Cl	H
9	H	Cl	H	H	Cl	Cl	H	H
10	H	Cl	Cl	Cl	Cl	H	H	H
11	H	Cl	Cl	Cl	H	H	Cl	H
12	H	Cl	Cl	H	H	Cl	Cl	H
13	Cl	Cl	H	Cl	Cl	Cl	H	H
14	Cl	Cl	Cl	Cl	H	H	Cl	H
15	Cl	Cl	Cl	H	H	Cl	Cl	H
16	H	Cl	Cl	Cl	H	Cl	Cl	H
17	Cl	Cl	Cl	Cl	H	Cl	Cl	H
18	H	Cl	Cl	Cl	Cl	Cl	Cl	H
19	H	Cl	Cl	H	Cl	H	Cl	H
20	Cl	Cl	Cl	H	Cl	H	H	H
21	Cl	Cl	Cl	H	H	Cl	H	H
22	Cl	H	Cl	Cl	H	Cl	Cl	H
23	H	Cl	Cl	Cl	H	Cl	H	Cl
24	Cl	Cl	Cl	H	H	Cl	H	Cl
25	Cl	Cl	Cl	H	H	Cl	H	H
26	Cl	H	Cl	Cl	H	Cl	Cl	H
27	H	Cl	Cl	Cl	H	Cl	H	Cl

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Table 4. Polychlorinated biphenyls with identity number (ID) representing the substitution pattern



ID	X_2	X_3	X_4	X_5	X_6	X_2'	X_3'	X_4'	X_5'	X_6'
28	Cl	H	H	H	H	H	Cl	Cl	Cl	H
29	Cl	Cl	Cl	H	H	H	Cl	Cl	H	H
30	Cl	H	Cl	Cl	H	H	Cl	Cl	H	H
31	Cl	Cl	Cl	Cl	H	H	Cl	Cl	H	H
32	Cl	H	Cl	H	H	Cl	H	Cl	H	H
33	Cl	H	Cl	H	Cl	H	Cl	Cl	Cl	H

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of nitrobenzene and benzonitrile derivatives (aromatic acceptors) and total Hartree-Fock energy (E_{HF}) could predict toxicity towards the *T. pyriformis* larger than 80% of cross-validation variance of data of these systems.

4.6 QSPR Model for the Prediction of Lipophilic Behavior of Polychlorinated Biphenyl Derivatives

Padmanabhan et al. (2006b) successfully developed effective QSPR model for the prediction of the lipophilic behaviour ($\log K_{ow}$) of a set having large number (133) of polychlorinated biphenyl (PCB) derivatives by using semi-empirical AM1 method. The effectiveness of a QSPR model depends on the accuracy in the selection of appropriate descriptors. CDFT based global descriptors like ω was employed in addition to the other descriptors viz., energy of the lowest unoccupied molecular orbital (E_{LUMO}) and number of chlorine substituents (N_{Cl}). Assuming experimental $\log K_{ow}$ values (Patil, 1991) of the training set of 100 PCB derivatives as dependent variable and these three descriptors along with their several combinations as independent variables, the regression models were developed (see Table 7). It was found that each descriptor individually could describe lipophilic behavior quite well ($r^2 = 0.81$-0.88); however the three-parameter model based on the combination of the ω, E_{LUMO} and N_{Cl} provided the best prediction.

This model was able to predict largest variation in data ($r^2 = 0.914$) with leave-one-out cross-validated squared correlation coefficient ($r^2_{cv} = 0.909$) having lowest SD value of 0.225. Figure 7 depicts the plots between exp. $\log K_{ow}$ vs. calc. $\log K_{ow}$ for the training set of 100 PCBs and the data set of 133 PCBs. In both cases, the three-parameter regression models gave excellent correlation coefficient, r value of 0.956 for these cases. Therefore, it clearly showed the efficacy of these descriptors in explaining lipophilic behavior of PCBs.

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Table 5. Experimental and calculated biological activity (pIC_{50}) in gas phase for the training set of polychlorinated dibenzofurans and polychlorinated biphenyls

ID	ω (eV)	ω^{+}_{max} (eV)	Observed pIC_{50}	Calculated pIC_{50}
1	2.763	0.888	4.061	3.800
2	2.699	0.720	3.429	3.583
3	3.101	0.883	4.125	4.947
4	3.180	0.893	4.103	5.216
5	3.366	1.306	6.123	5.847
6	3.425	1.007	4.653	6.047
7	3.331	1.156	5.396	5.728
8	3.486	1.488	6.858	6.254
9	3.659	1.551	7.255	6.841
10	3.748	1.551	7.379	7.144
11	3.769	1.633	7.657	7.215
12	3.785	1.824	8.444	7.269
13	3.952	1.717	8.194	7.836
14	3.967	1.683	7.911	7.887
15	4.005	1.756	8.147	8.016
16	4.046	1.902	8.943	8.155
17	4.263	1.612	7.587	8.892
18	4.293	1.756	8.376	8.994
19	3.828	1.621	7.610	7.415
20	3.642	1.57	7.379	6.784
21	3.657	1.718	7.954	6.835
22	3.989	1.629	7.657	7.962
23	3.988	1.629	7.657	7.958
24	4.010	1.575	7.313	8.033
25	3.657	1.718	7.954	6.835
26	3.989	1.621	7.623	7.962
27	3.988	1.622	7.623	7.958
28	3.109	0.476	5.584	4.975
29	3.329	0.432	6.134	5.721
30	3.424	0.440	5.762	6.044
31	3.597	0.461	6.057	6.631
32	2.866	0.318	4.442	4.150
33	3.112	0.406	4.577	4.985

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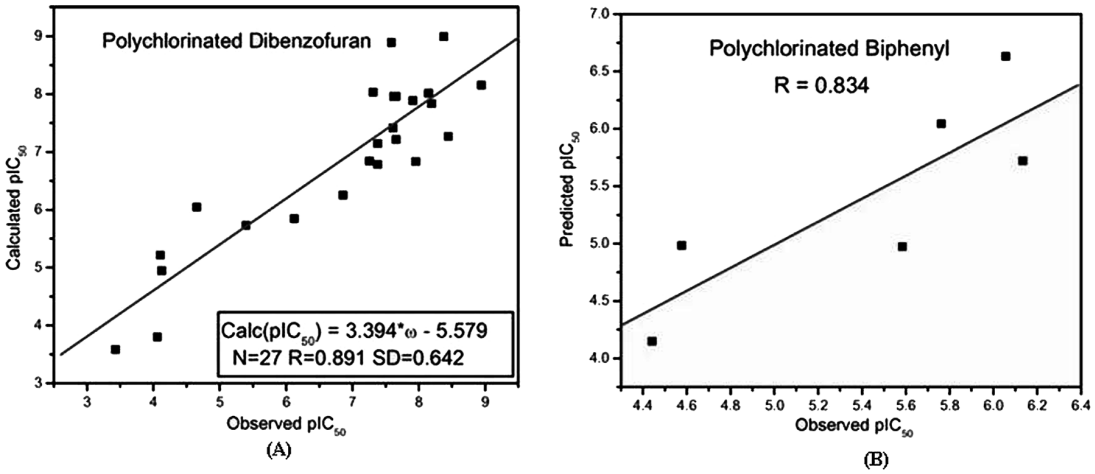
Table 6. Experimental and calculated toxicity (log(IGC_{50}^{-1})) values against T. pyriformis of the selected chloroanilines at the B3LYP/6-311++G(d,p) method

System	Log(IGC_{50}^{-1})		Residual[b]
	Observed[a]	Calculated	
2-CA	-0.17	-0.09	-0.08
3-CA	0.22	-0.02	0.24
4-CA	0.05	0.01	0.04
2,5-C2A	0.58	0.72	-0.14
3,4-C2A	0.56	0.67	-0.11
3,5-C2A	0.71	0.75	-0.04
2,4,5-C3A	1.30	1.44	-0.14
2,3,4,5-C4A	1.96	1.83	0.13
2,3,5,6-C4A	1.76	1.66	0.10

[a] The values from reference 179. [b]Difference between the experimental and calculated toxicity (log(IGC_{50}^{-1})) values. The number before CA indicates the position of chloro group and CxA indicates the presence of x number of chloro groups.

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Figure 5. A plot between experimental and calculated toxicity (log(IGC_{50}^{-1}) values of the selected chloroanilines against T. pyriformis
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4.7 QSPR Models to Predict the Enthalpy of Vaporization of 209 Polychlorinated Biphenyl Congeners

The ω, E_{LUMO} and N_{Cl} were further employed to model QSPR in order to predict the enthalpy of vaporization ($\Delta_{vap}H_m$) of a set of 209 PCBs by using semi-empirical AM1 level of computations (Padmanabhan et al., 2007). A small data set containing only 17 PCBs, whose experimental $\Delta_{vap}H_m$ values were known, (Puri et al., 2001) was considered in building regression models. In general, data set is used to divide into training set and test set but here due to small number of data set, it was only considered as the train-

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Figure 6. Experimental versus calculated log (IGC_{50}^{-1}) values for aromatic donors (a) and for aromatic acceptors (b)
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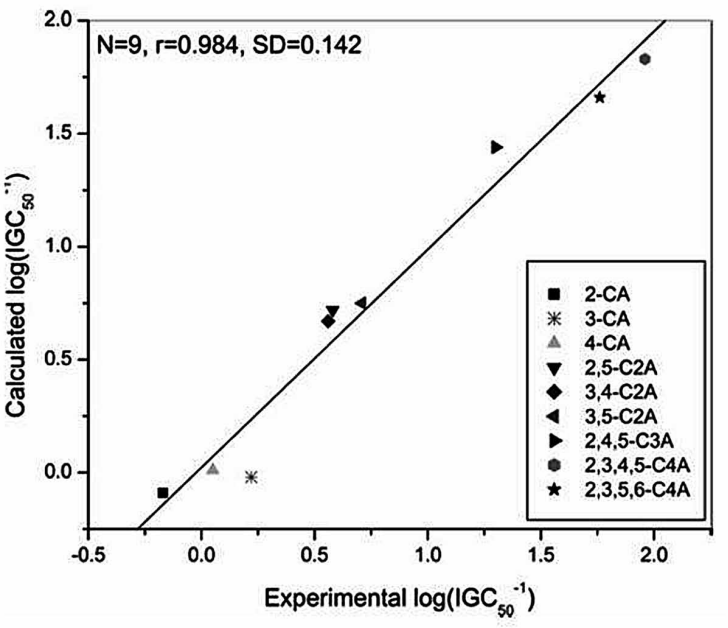


Table 7. Regression models for logK_{ow} using various descriptors for the training set of 100 PCB congeners

S.No.	Regression Equation	r^2	r^2_{cv}	SD
1	$LogK_{ow} = 4.617–75.219*E_{LUMO}$	0.811	0.806	0.330
2	$LogK_{ow} = 4.234 + 0.406*N_{Cl}$	0.842	0.838	0.302
3	$LogK_{ow} = -2.446 + 81.693*\omega$	0.877	0.874	0.266
4	$LogK_{ow} = 4.262 + 0.243*N_{Cl} - 36.119*E_{LUMO}$	0.892	0.888	0.251
5	$LogK_{ow} = -10.523 + 91.620*E_{LUMO} + 176.280*\omega$	0.905	0.901	0.235
6	$LogK_{ow} = -0.038 + 0.173*N_{Cl} + 51.015*\omega$	0.906	0.902	0.234
7	$LogK_{ow} = -5.967 + 0.115*N_{Cl} + 58.070*E_{LUMO} + 121.280*\omega$	0.914	0.909	0.225

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ing set. The different regression models based on the exp. $\Delta_{vap}H_m$ values as dependent parameters and individual ω, E_{LUMO} and N_{Cl} desciptors or their different combinations as the independent parameters along with the obtained r^2, r^2_{cv} and SD values are provided in Table 8.

The regression equation developed by each individual descriptor could predict $\Delta_{vap}H_m$ with high r^2 value (0.82-0.92); however the best correlation was provided by the three-parameter regression equation developed by ω, E_{LUMO} and N_{Cl} desciptors, which described the maximum variation in the data (r^2 = 0.976) having leave-one-out (LOO) cross-validated squared correlation coefficient, r^2_{cv} value of 0.948. This model could explain $\Delta_{vap}H_m$ value with higher efficiency (in terms of higher r^2_{cv}) than that model prescribed by Puri et al. (2001), where they obtained r^2_{cv} value of 0.852. Another advantage of this model was that it could provide excellent result even based on least expensive AM1 level of computations.

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Figure 7. Experimental versus calculated values of logK_{ow} with electrophilicity, E_{LUMO} and N_{Cl} as descriptors for the model constructed using (a) the training set of 100 PCBs; (b) the data set of 133 PCBs (Reprinted with permission from Padmanabhan et al., 2006b. Copyright © 2006, Elsevier).

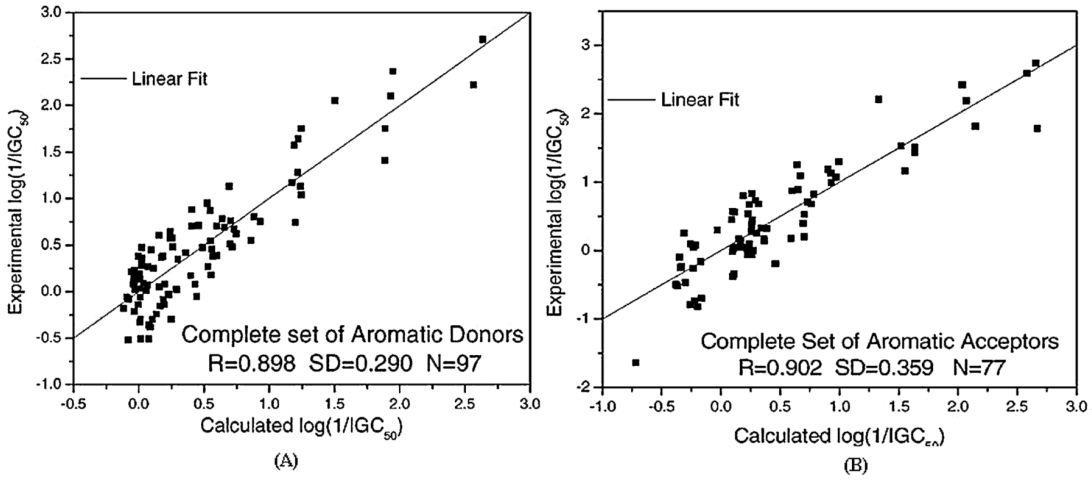


Table 8. Regression models for vaporization enthalpy ($\Delta_{vap}H_m$ (298.15 K) in kJ/mol) using various descriptors for the dataset of 17 PCBs[a]

S.No.	Regression Equation	r^2	r^2_{cv}	SD
1	$\Delta_{vap}H_m = 73.8 - 936.5*E_{LUMO}$	0.819	0.764	5.9
2	$\Delta_{vap}H_m = 65.2 + 6.1*N_{Cl}$	0.908	0.879	4.2
3	$\Delta_{vap}H_m = -27.5 + 1159.4*\omega$	0.921	0.904	3.9
4	$\Delta_{vap}H_m = 67.0 + 4.4*N_{Cl} - 309.0*E_{LUMO}$	0.928	0.884	3.9
5	$\Delta_{vap}H_m = 12 + 2.9*N_{Cl} + 654.9*\omega$	0.951	0.918	3.2
6	$\Delta_{vap}H_m = -151.0 + 2583.6*\omega + 1240.4*E_{LUMO}$	0.968	0.943	2.6
7	$\Delta_{vap}H_m = -103.6 + 1.7*N_{Cl} + 994.7*E_{LUMO} + 2009.0*\omega$	0.976	0.948	2.3

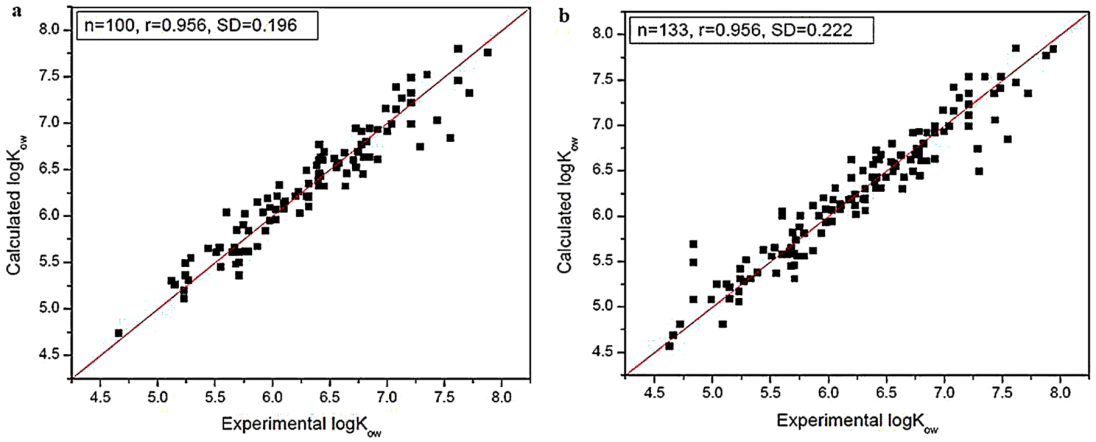
[a]Experimental data as obtained from ref 184.

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Now, another set of 27 PCDFs with their experimentally reported $\Delta_{vap}H_m$ values (Nakajoh et al., 2006) were considered to further test this model. Here 22 PCBs were taken as training set and remaining five PCBs as test set. It was found that the best regression equation in the previous set could describe maximum variation in the data ($r^2 = 0.915$) having leave-one-out cross-validated squared correlation coefficient, r^2_{cv} value of 0.856. It was also capable of predicting the $\Delta_{vap}H_m$ values of the test set within 4.2 kJ/mol error bars. Now including these five test set candidates in the data set, for all 27 PCBs, this model was able to represent the $\Delta_{vap}H_m$ values with $r^2 = 0.858$ and $r^2_{cv} = 0.758$. The plots between the exp. $\Delta_{vap}H_m$ and calc. $\Delta_{vap}H_m$ values for both the first set and the second set are given in Figure 12. In case of the first set, correlation coefficient (r) was 0.988 and in the second set it was 0.926. Based on this model, the $\Delta_{vap}H_m$ values for the remaining 183 PCB derivatives were predicted (see Figure 8).

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Figure 8. Exp. vs. calc. $\Delta_{vap}H_m$ values for the dataset of (a) 17 PCBs, (b) 27 PCBs, in the general set for (c) 193 PCBs and (d) 183 PCBs
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4.8 QSAR Model in Predicting Biological Activity of Testosterone and Estrogen Derivatives

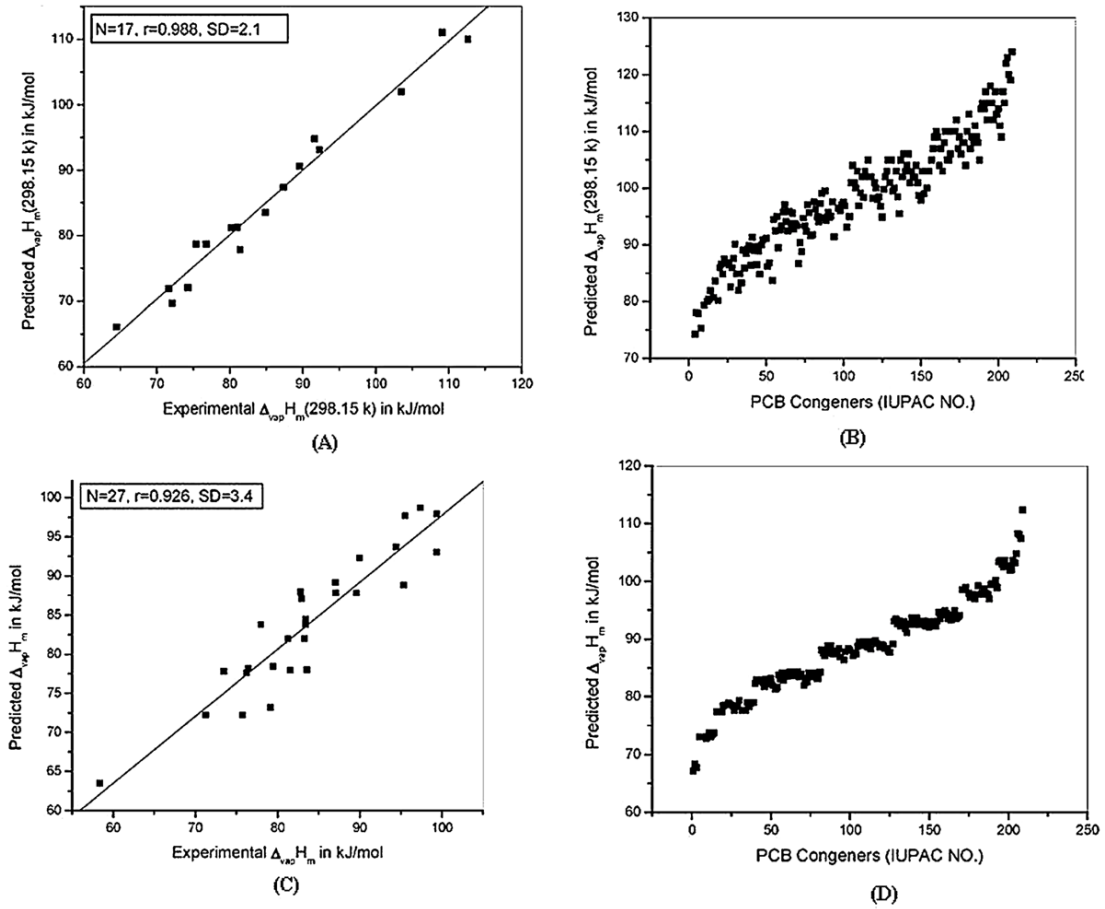
Parthasarathi et al. (2004b) further employed electrophilicity to assess its efficacy as a probable biological activity descriptor in modeling QSAR for testosterone and estrogen derivatives. The geometry optimizations of all the systems were carried out at the semi-empirical AM1 level whereas the single point energy calculations at the B3LYP/6-31G(d) level were performed with those geometries obtained at the AM1 level. The biological activity of various testosterone derivatives was described by means of relative binding affinity (RBA) (Saartok et al., 1984), androgenic potency (Counsell et al., 1962), relative androgenic activity (Liao et al., 1973), therapeutic index (Sala & Baldratti, 1957), testosterone-binding globulins (TeBG) binding affinity (Cramer et al., 1988), relative competition indices (Liao et al., 1973), binding affinity for rat ventral prostate receptor protein (Wolff, 1955) and myotrophic to androgenic potency in temporal activity (Kochakian, 1976) (MAPT) whereas the same for estrogen derivatives was measured in terms of RBA values (Gao et al., 1999). In Figure 13, some representative plots between calculated ω and different biological activities of testosterone derivatives are presentated. In every case, a linear correlation between them was found; however, the correlations of RBA, relative competition indices and myotrophic to androgenic potency in temporal activity with ω were found to be better than the others. The corresponding regression models used to generate the plots were inserted within the Figures (see Figure 9). In all cases R^2 values were found to range from 0.61 to 0.90. It was also noted that B3LYP result was better than that of AM1 in predicting biological activity.

Similarly, in case of estrogen derivatives ω could predict their biological activity towards RBA having R^2 value in the range of 0.73 and 0.87. Therefore, it clearly showed that ω could predict biological activity of these systems quite efficiently.

Roy et al. (2007) also showed that the number of atoms in a molecule (N_A) along with ω values could explain the biological activities of testosterone and estrogen derivatives reasonably well ($R \approx 0.8$).

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Figure 9. Relationship between various biological activities of testosterone derivatives with electrophilicity index
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Pasha et al. (2005) and Singh et al. (2004) developed QSAR models based on frontier orbitals information (E_{HOMO}, E_{LUMO}) and different CDFT based descriptors like η, S, χ, μ as well as different energy descriptors like total energy, heat of formation, electronic energy, and the highest negatively charge on an atom to predict different biological activities of testosterone and estrogen derivatives. They found that the regression models made by η and different energy descriptors possess better predicitivity than the other combinations. They argued that η represents the situation in these cases in a better way due to the associated maximum hardness principle. Srivastava et al. (2005) further showed that some other descriptors like IP and EA of an atom in a molecule, atomic softness could predict the biological activity of testosterone derivatives quite well, The differences in softness values of estrogen derivatives and lysine, histidine, tyrosine and cysteine receptors were also found to be an effective parameter in describing the biological activities of estrogen derivatives (Pasha et al., 2005). Rokhina & Suri (2012) further built QSPR to represent the estrogenicity of the natural estrogen hormones. They found that the information of frontier molecular orbital energies is very important to interpret this activity.

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5. GROUP PHILICITY TOWARDS MODELING QSAR

In addition to the conventional global electrophilicity Padmanabhan et al. (2006c) used group philicity (ω_g^+, ω_g^-) computed at the B3LYP/6-31G(d) level to model QSAR for ecotoxicity of chlorophenols (CPs). The data set was made by 18 CPs, which was divided into training set having 16 CPs and test set containing two CPs. Then various regression models were developed by these descriptors in order to describe the experimental toxicity ($\log(1/EC_{50})$) (Devillers & Chambon, 1986) of the training set against *Daphnia magna* taking $\log(1/EC_{50})$ as a dependent variable whereas ω, ω_g^+ and ω_g^- as independent variables (see Table 9). It was found that two-parameter regression equation made by ω_g^+ and ω_g^- was capable of describing maximum variation data (88.7%) having large r^2_{cv} value of 0.826. The SD was also found to be the least for this model. This model was also found to predict the experimental $\log(1/EC_{50})$ value for the test set within ± 0.05 error bar. Now, regression analyses were carried out for all 18 CPs (data set) (see Table 9). In this set also, the combination of ω_g^+ and ω_g^- could explain maximum variation data (88.9%) along with r^2_{cv} value of 0.838. The second best model was three-parameter regression composed by ω, ω_g^+ and ω_g^-, which could also predict 88.9% variation data, however, the r^2_{cv} value for this model was 0.812. The plots of calc. $\log(1/EC_{50})$ vs exp. $\mathrm{Log}(1/EC_{50})$ are given in Figure 10(A), which shows a correlation coefficient of 0.942 for the best model. Further, these descriptors were used to explain the experimental toxicity of CPs against *B. rerio*, and *Bacillus*. It was found that in case against *B. rerio*, three-parameter regression model (ω, ω_g^+ and ω_g^-), being a very good model, could explain 92.8% variation data ($r^2_{cv} = 0.878$) having correlation coefficient value (r) of 0.964 (see Figure 10(B))

Table 9. Regression models for the toxicity [log(1/EC$_{50}$)] against D. magna using various descriptors for a training Set of 16 CPs and data set of 18 CPs

S.No.	Regression Equation	r^2	r^2_{cv}	SD
	For training set			
1	$\log(1/EC_{50}) = -2.2 + 198.0*\omega_g^-$	0.515	0.374	0.348
2	$\log(1/EC_{50}) = -0.9 + 28.7*\omega$	0.731	0.671	0.259
3	$\log(1/EC_{50}) = -1.4 + 24.8*\omega + 42.0*\omega_g^-$	0.741	0.630	0.264
4	$\log(1/EC_{50}) = -3.8 + 545.6*\omega_g^+$	0.874	0.835	0.177
5	$\log(1/EC_{50}) = -3.5 + 3.5*\omega + 491.3*\omega_g^+$	0.877	0.802	0.178
6	$\log(1/EC_{50}) = -4.0 - 0.6*\omega + 492.3*\omega_g^+ + 43.26*\omega_g^-$	0.887	0.792	0.182
7	$\log(1/EC_{50}) = -4.0 + 485.3*\omega_g^+ + 41.9*\omega_g^-$	0.887	0.826	0.175
	For data set			
1	$\log(1/EC_{50}) = -2.2 + 196.2*\omega_g^-$	0.529	0.409	0.326
2	$\log(1/EC_{50}) = -0.8 + 28.4*\omega$	0.704	0.644	0.260
3	$\log(1/EC_{50}) = -1.6 + 22.4*\omega + 65.5*\omega_g^-$	0.731	0.634	0.254
4	$\log(1/EC_{50}) = -3.8 + 545.5*\omega_g^+$	0.876	0.842	0.167
5	$\log(1/EC_{50}) = -3.6 + 3.3*\omega + 496.4*\omega_g^+$	0.879	0.817	0.171
6	$\log(1/EC_{50}) = -3.9 - 0.6*\omega + 477.3*\omega_g^+ + 40.2*\omega_g^-$	0.889	0.812	0.170
7	$\log(1/EC_{50}) = -4.0 + 484.6*\omega_g^+ + 41.4*\omega_g^-$	0.889	0.838	0.164

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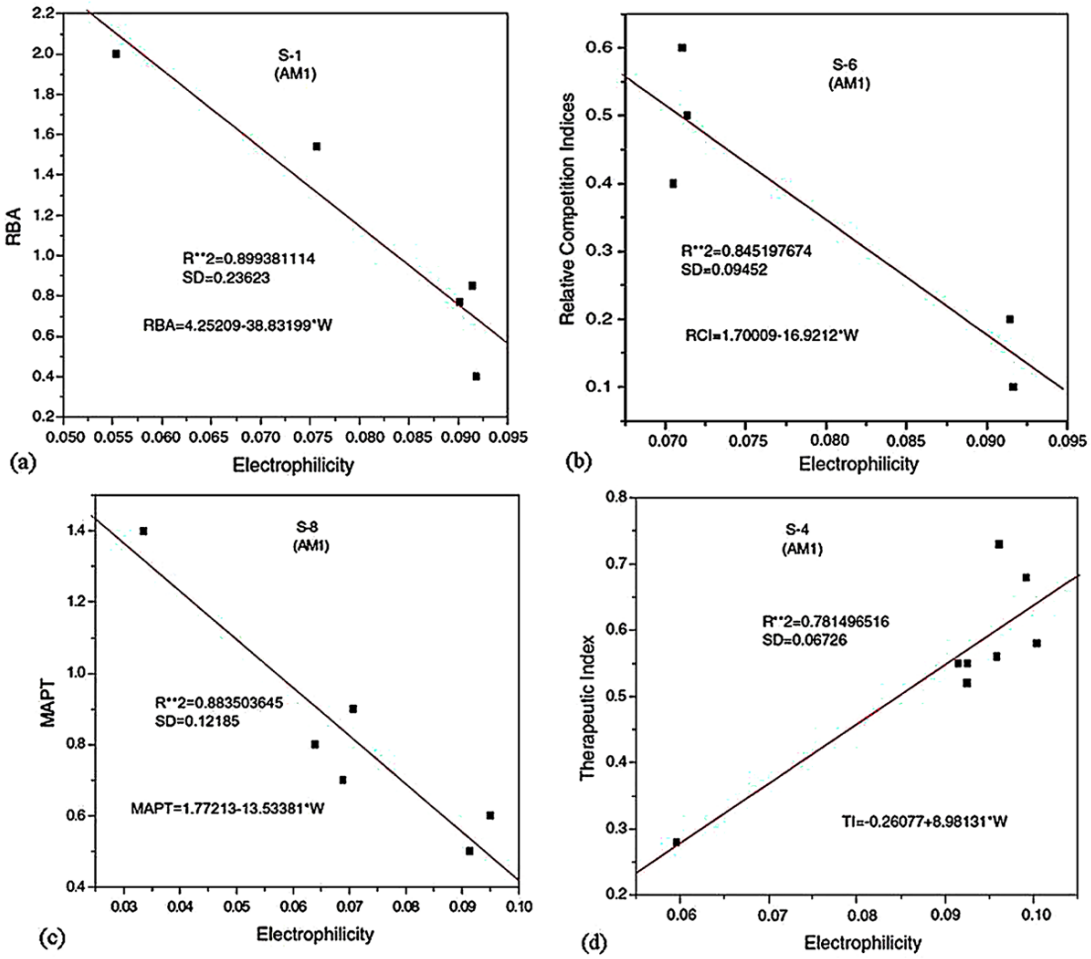
whereas in case against *Bacillus* this model was able to describe maximum variation in data (91.9%) along with r^2_{cv} value of 0.863 and r value of 0.959 (see Figure 10(C).

Therefore, this result implied that group philicity along with electrophilicity are very much effective in representing the toxicity of CPs against *D. magna*, *B. rerio*, and *Bacillus*. It in turn showed the importance of these reactivity indices as effective descriptors in ecotoxicological studies.

6. EFFECT OF CHOSEN FUNCTIONALS IN BUILDING GLOBAL REACTIVITY DESCRIPTORS BASED QSAR/QSPR/QSTR MODELS

Vijayaraj et al. (2009) assessed the effect of studied functionals on the ionization potential (*IP*) and the electron affinity (*EA*) of the systems, which were generally used to compute the CDFT based reactivity descriptors. Therefore, the dependence of *IP* and *EA* on the chosen functionals would raise questions

Figure 10. Plots between experimental and calculated toxicity of CPs against (A) D. magna, (B) B. rerio, and (C) Bacillus
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about the level of theory chosen to construct QSAR, QSPR and QSTR based on the reactivity descriptors. In other words, the predictive power of a regression model might depend on the chosen level of theory. In order to explore the efficacy of various density functionals along with different basis sets in calculating *IP* and *EA*, hence, to benchmark, systematic computations were performed considering a series of chlorinated benzenes (CBs). Some common functionals of pure, GGA, hybrid, and meta and hybrid meta GGA types were employed. It was found that a good agreement between computed and experimental *IP* and E*A* values could be attained with the increase in the rigor of the level of computation. The prediction of *IP* and *EA* of CBs, computed using both Koopmans' approximation and ΔSCF method, was most accurately made by the use of M05-2X functional. Further, in between Koopmans' approximation and ΔSCF method, *IP* and *EA* computed by ΔSCF method matched more closely with the corresponding experimental values than that by the Koopmans' approximation.

Now, using those descriptors computed at different levels of theory, different regression models were designed to predict the toxicity of nine CBs against *Poecilia reticulata*. Different descriptors along with different approximations were included. It was found that although the computation of global reactivity descriptors at different levels and/or different basis sets yielded considerably different values, the mean deviation of a certain property among all the studied systems with respect to the chosen method/basis set was comparable. It meant that with the change in studied method and/or basis set, the values of computed global reactivity descriptors would be changed, however, the magnitude of the difference in between the systems were more or less same in all the methods. This finding was important in this sense that the efficacy of a regression model and hence the correlation coefficient would not alter significantly with the change in level of theory. For example, in spite of good agreement with the experimental *IP* and *EA* using ΔSCF method, it did not improve the correlation coefficient with respect to that obtained using Koopmans' theorem. In general, to construct a QSAR/QSPR/QSTR model, one has to consider a data set containing a large number of systems. Therefore, it can be concluded that if one considers computationally a very cheap level of theory to model QSAR/QSPR/QSTR, it can be possible to get reliable results similar to the one constructed from the results of a high level of theory, which will in deed save a lot of computational time.

7. CONCLUSION

In order to better understand the variation of property, activity and toxicity with the structural change of a molecular motif, the Quantitative-Structure-(Property/Activity/Toxicity) Relationship models are widely applied. Various types of methods and models were employed to construct effective regression equations. Both, global and local reactivity descriptors based on Conceptual Density Functional Theory are found to be very effective indices in order to predict biological activity or property or toxicity of a set of systems. We have specially drawn attention on the efficacy of the regression models built based on the different combinations of the parameters viz., electrophilicity index, net electrophilicity index, group philicity along with their local variants, atomic charge, number of carbon or Cl or non-hydrogenic atoms, atomic number, chemical potential, LUMO energy and Hartree-Fock energy in predicting the toxicity of various aliphatic compounds, polyaromatic hydrocarbons, chloroanilines, phenol, nitrobenzene, benzonitrile-derivatives, halogen, sulfur and chlorinated aromatic compounds, testosterone, estrogen derivatives, and different alkali, alkaline-earth, transition-metal, arsenic ions as well. Although individual

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descriptors were found to vary with the change in level of theory and /or basis set, the efficacy of the overall QSAR/QSPR/QSTR models depend hardly on the level of theory. Therefore, very low level of theory can also yield reliable results.

Therefore, the reactivity descriptors within the purview of conceptual DFT will be a good choice in predicting the desired properties of a set of systems.

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KEY TERMS AND DEFINITIONS

Aliphatic Compounds: These are non-aromatic compounds made by carbon and hydrogen.

Electrophilicity: It is a measure of 'electrophilic power' of a molecule. It can be estimated as the electronegativity squared divided by twice the hardness.

Energies of Frontier Molecular Orbitals: They are the energies of the highest occupied molecular orbital and lowest unoccupied molecular orbital, respectively.

Group Philicity: It is a condensed philicity summed over a group of relevant atoms.

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Net Atomic Charge: It is a non-integer charge at each atom in a molecule in elementary charge units.

Net Electrophilicity: It is the electron-accepting power of a molecule relative to its own electron-donating power.

Polyaromatic Hydrocarbons: These are compounds made by only carbon and hydrogen and contain multiple aromatic rings.

Polychlorinated Biphenyls: These are compounds containing two phenyl rings and several chlorine atoms are attached with phenyl rings.

***Tetrahymena Pyriformis*:** These are free-living ciliate protozoa, generally found in freashwater ponds.

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APPENDIX: LIST OF SYMBOLS AND ABBREVIATIONS

SAR: Structure-Activity Relationship.
QSAR: Quantitative Structure-Activity Relationship.
QSTR: Quantitative Structure-Toxicity Relationship.
DFT: Density Functional Theory.
CDFT: Conceptual Density Functional Theory.
LSFER: Linear Solvation Free Energy Relationship.
CoMFA: Comparative Molecular Field Analysis.
QSPR: Quantitative Structure-Property Relationship.
PCA: Principal Component Analysis.
η**:** Hardness.
χ**:** Electronegativity.
ω**:** Electrophilicity.
$f(\mathbf{r})$**:** Fukui function.
f_k**:** Condensed Fukui function.
$s(\mathbf{r})$**:** Local softness.
$\eta(\mathbf{r})$**:** Local hardness.
$\omega(\mathbf{r})$**:** Philicity.
IP: Ionization Potential.
EA: Electron Affinity.
μ**:** Chemical Potential.
E: Energy.
N: Number of Electrons.
$v(\mathbf{r})$**:** External Potential.
$\Delta\omega^{\pm}$**:** Net Electrophilicity.
HIV-1: Human Immunodficiency Virus type 1.
NCp7: Nucleocapsid Protein p7.
W: Electrical Power.
V: Voltage.
R: Electrical Resistance.
α**:** Polarizability.
ξ**:** Magnetizability.
$\rho(\mathbf{r})$**:** Electron Density.
q_k**:** Atomic Charge at k^{th} site.
ω_g^{α}**:** Group Philicity.
PMH: Principle of Maximum Hardness.
MEP: Minimum Electrophilicity Principle.
MPP: Minimum Polarizability Principle.
MMP: Minimum Magnetizability Principle.
$\mathbf{LD_{50}}$**:** 50% Lethal Dose.
$\mathbf{LC_{50}}$**:** 50% Lethal Concentration.
Z: Atomic Number.

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N_{NH}**:** Number of Nonhydrogenic Atoms.
R or r: Coefficient of correlation.
$\mathbf{R^2_{CV}}$ **or** $\mathbf{r^2_{CV}}$**:** Variance of Leave-one-out Cross-Validation.
PAH: Polyaromatic Hydrocarbons.
TCDD: Tetrachlorodibenzo-p-Dioxin.
Ah: Arylhydrocarbon.
PCDF: Polychlorinated Dibenzofurans.
CA: Chloroanilines.
PCB: Polychlorinated Biphenyl.
RBA: Relative Binding Affinity.
TeBG: Testosterone-Binding Globulins.
MAPT Myotrophic to Androgenic Potency in Temporal Activity.

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Chapter 59
Quasi-SMILES for Nano-QSAR Prediction of Toxic Effect of Al_2O_3 Nanoparticles

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ABSTRACT

The level of malondialdehyde (MDA) in wet tissue of different organs is utilized as a measure of toxic effect. The numerical data on the concentration of MDA in wet tissue of liver, kidneys, brain, and heart of rat is examined as the endpoint which are impacted by different dose (mg/kg), exposure time (3 and 14 days) and single oral treatment of aluminium nano-oxide (Al_2O_3) with 30 nm or 40 nm. An attempt to develop predictive model for this endpoint has been carried out in this work. SMILES is a traditional tool to represent molecular structure for QSPRs/QSARs. In contrast to traditional SMILES, so-called quasi-SMILES can be a tool to build up quantitative features – property / activity relationships (QFPRs/QFARs) for endpoints which are not defined by solely molecular structure, but by a group of physico-chemical and/or biochemical conditions. The quasi-SMILES is the representation of the above eclectic conditions whereas the QFPR/QFAR are models of endpoints which are modified under impacts of these eclectic conditions.

1. INTRODUCTION

The influence of various nanomaterials on the everyday life gradually increase owing to their high functional potential be very useful materials for different applications (Vanić and Škalko-Basnet, 2013; Ma et al., 2013; Singh and Gupta, 2014; Melagraki and Afantitis, 2014; Panneerselvam and Choi, 2014; Potrč et al., 2015; Sauer et al., 2015; Speck-Planche et al., 2015). However, a tool to risk assessment for

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nanomaterials similar to quantitative structure – property /activity relationships (QSPRs/QSARs) (Toropova et al., 2012; Yilmaz et al., 2015) as research field is in an initial phase of the development (Muthu, 2012; Oksel et al., 2015). The solution of this task for regulatory purposes in the case of nanomaterials involved in the agriculture, food, cosmetics, drug discovery, etc. needs to be reached in the near future (Arts et al., 2014; Arts et al., 2015; Filon et al., 2015; Amenta et al., 2015).

In general, different measures of danger acting of nanomaterials upon cells are known (Long et al., 2009; Prabhakar et al., 2012; Diez-Ortiz et al., 2015; Toropova et al., 2015a; Hadrup et al., 2015). In particular, the level of malondialdehyde (MDA) in wet tissue of different organs is considered as a measure of toxic effect of nanomaterials (Long et al., 2009; Prabhakar et al., 2012).

Attempts which are aimed to build up models for such acting of nanomaterials using the traditional QSPR/QSAR approaches (De Abrew et al., 2015), as rule, are impossible excepting the cases of acting of small molecules together with a nanomaterial (Fourches et al., 2010; Toropov et al., 2013).

In the case of traditional materials where the molecular structure represented by SMILES is available to mathematical and computational analyses, the QSPR/QSAR is a tool to more or less satisfactory prediction of different endpoints (Veselinović et al., 2013; Achary, 2014a, 2014b; Comelli et al., 2014; Nesmerak et al., 2014; Worachartcheewan et al., 2014; Toropova and Toropov, 2014; Veselinović et al., 2015).

A possible way to build up such models can be expressed by paradigm: "Endpoint = f(SMILES)". In the case of nanomaterials where weak variation of molecular structure accompanied by intensive variation of conditions more appropriate paradigm is "Endpoint = f(Eclectic data)".

The quasi-SMILES can be the representation of eclectic data (Toropov and Toropova, 2015). The quasi-SMILES are analogies of traditional SMILES, but symbols involved in the quasi-SMILES are representations of features and/or conditions that are not representation of features of the molecular architecture only. (Toropova and Toropov, 2013; Toropov and Toropova, 2014; Toropova et al., 2015b).

The aim of this work is to build up models for level of MDA in wet tissue of different organs of rat under different action of Al_2O_3 nanoparticles.

2. METHOD

2.1. Data

The experimental data on the level MDA in wet tissue (nanomoles of MDA per gram wet tissue) of liver, kidneys, brain, and heart of rat under different conditions taken in the literature (Prabhakar et al., 2012). Table 1 contains the representation of different conditions of acting Al_2O_3 nanoparticles by quasi-SMILES. These data (quasi-SMILES together with endpoint values) were three times randomly split into the training, calibration, and validation set. The length of quasi-SMILES (the number of symbols) is important indicator for possibility of a model to be successful.

It is to be noted that length 1 for quasi-SMILES is nonsense, because in this case the prevalence of each attribute is zero in the training or in calibration set (the presence in both mentioned set is impossible), i.e. for each attribute, A, one can obtain:

$$N_{training}(A) \times N_{calibration}(A) = 0$$

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Table 1. The definition of quasi-SMILES

quasi-SMILES				**Endpoint, *E***
Organ	**Dose mg/kg**	**Days**	**Single oral treatment**	**Nanomoles of MDA per gram wet tissue**
L=Liver K=Kidneys B=Brain H=Heart	1=2000 2=1000 3=500	X=3 days Y=14 days	T=30 nm F=40 nm	
L	1	X	T	33.6
L	2	X	T	23.4
L	3	X	T	14.5
K	1	X	T	13.4
K	2	X	T	9.95
K	3	X	T	8.73
B	1	X	T	16.7
B	2	X	T	13.1
B	3	X	T	13.2
H	1	X	T	2.8
H	2	X	T	2.2
H	3	X	T	2.8
L	1	X	F	29.8
L	2	X	F	24.1
L	3	X	F	13.7
K	1	X	F	12.2
K	2	X	F	8.93
K	3	X	F	8.51
B	1	X	F	14.4
B	2	X	F	12.7
B	3	X	F	11.0
H	1	X	F	2.43
H	2	X	F	2.14
H	3	X	F	2.38
L	1	Y	T	14.8
L	2	Y	T	10.8
L	3	Y	T	10.6
K	1	Y	T	7.2
K	2	Y	T	9.0
K	3	Y	T	8.0
B	1	Y	T	12.3
B	2	Y	T	12.0
B	3	Y	T	11.6
H	1	Y	T	2.8
H	2	Y	T	2.8
H	3	Y	T	2.6
L	1	Y	F	13.6
L	2	Y	F	10.7
L	3	Y	F	9.33
K	1	Y	F	7.7
K	2	Y	F	8.6
K	3	Y	F	8.4
B	1	Y	F	11.6
B	2	Y	F	11.6
B	3	Y	F	12.0
H	1	Y	F	2.4
H	2	Y	F	2.6
H	3	Y	F	2.7

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$$P_{training}(A) \times P_{calibration}(A) = 0$$

where $N_{training}(A)$ and $N_{calibration}(A)$ are the numbers of attribute into the training and calibration sets, respectively; $P_{training}(A)$ and $P_{calibration}(A)$ are probabilities of presence of the attribute A in the training and calibration sets, respectively.

The length 2 gives possibility to obtain at least for part of attributes:

$$N_{training}(A) \times N_{calibration}(A) \neq 0$$

$$P_{training}(A) \times P_{calibration}(A) \neq 0$$

The total number of quasi-SMILES involved in built model has significant meaning:

Length 2 …$N_{max} = N_1 \times N_2$

Length 3 …$N_{max} = N_1 \times N_2 \times N_3$

Length 4 …$N_{max} = N_1 \times N_2 \times N_3 \times N_4$

For instance, two situations for quasi-SMILES with length 2 which are represented by a pairs of < Impact, Condition > are represented in Table 2. From practical point of view, the second situation is preferable, because 10 variables are more information about a phenomenon than 6 variables.

Table 2. Different eclectic sources for construction of the quasi-SMILES

Situation 1

	Impact 1	**Impact 2**	**Impact 3**
Condition 1	Endpoint 11	Endpoint 12	Endpoint 13
Condition 2	Endpoint 21	Endpoint 22	Endpoint 23

$Nmax = N_{condition} \times N_{impact} = 2 \times 3 = 6$

Situation 2

	Impact 1	**Impact 2**	**Impact 3**	**Impact 4**	**Impact 5**
Condition 1	Endpoint 11	Endpoint 12	Endpoint 13	Endpoint 14	Endpoint 15
Condition 2	Endpoint 21	Endpoint 22	Endpoint 23	Endpoint 24	Endpoint 25

$Nmax = N_{condition} \times N_{impact} = 2 \times 5 = 10$

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Using the formalism of the quasi-SMILES which are utilized to integrate eclectic data for the nanomaterials one can build up model for endpoints related to nanomaterials. Apparently, increase of length of quasi-SMILES is promoter of improving for the statistical quality of a model. In this work, the length of used quasi-SMILES is four.

2.2. Optimal Descriptors

The optimal descriptors are calculated as the following:

$$DCW(Threshold, N_{epoch}) = \sum CW(C_k) \quad (1)$$

where C_k is code of k-th feature (condition); $CW(C_k)$ is the correlation weight for C_k; The *Threshold* and N_{epoch} are parameters of the Monte Carlo optimization. The *Threshold* is a tool to define two classes of features (conditions): rare (noise) and not rare, i.e. active. The optimal descriptors are calculated with the correlation weights of active features. Correlation weights for rare features (conditions) are fixed equal to zero, i.e. these are not involved in building up model; The N_{epoch} is the number of epochs of the Monte Carlo optimization.

The *Threshold* = T* and N_{epoch} =N* which give preferable statistical characteristics for the calibration set of quasi-SMILES should be defined by means of several runs of the Monte Carlo optimization. These parameters should be used to build up the final model for endpoint, ***E*** (Toropova et al., 2015a):

$$E = C_0 + C_1 \times DCW(T^*, N^*) \quad (2)$$

Figure 1 represents the scheme of the selecting T* and N*.

The predictive potential of the model calculated with Equation 2 should be checked up with the external validation set.

All operations with quasi-SMILES are carrying out by the same algorithms which were developed for the traditional SMILES (Toropov et al., 2012a, b; Roy, 2015).

3. RESULTS AND DISCUSSION

The optimal descriptors give for three random splits the following models:

E =-1357.4 (±57.96) + 344.21 (±14.61) * *DCW(1,3)* (3)

n=26, r^2=0.7382, q^2=0.6524, s=3.55, F=68 (training set)

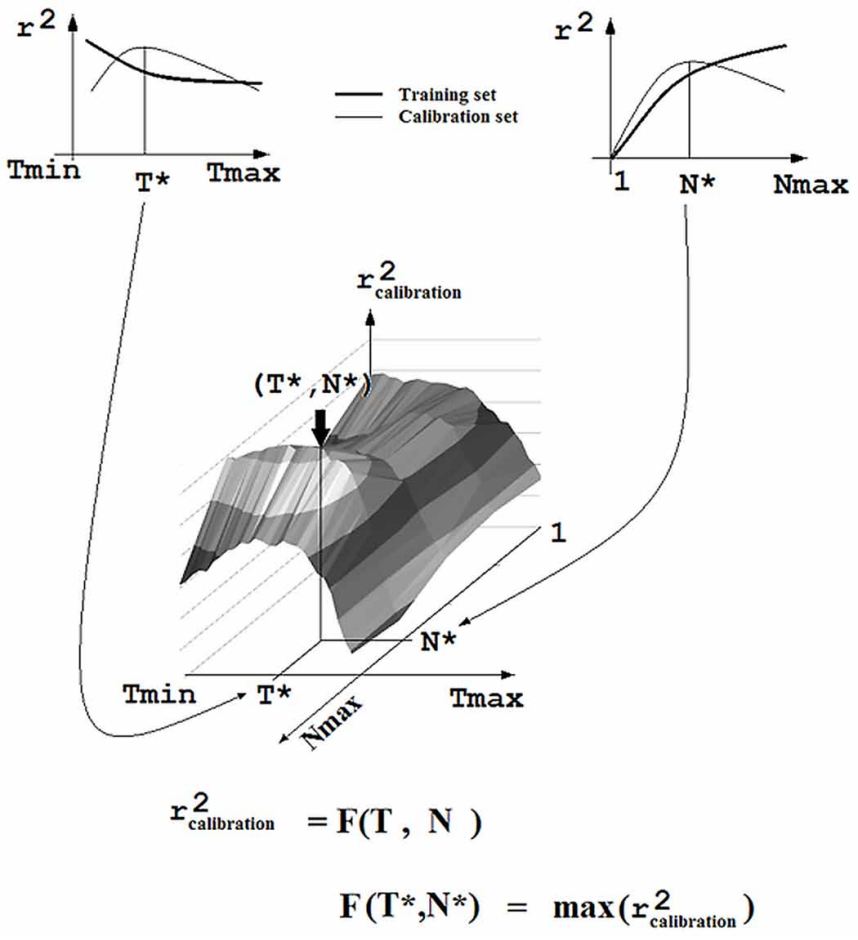
n=9, r^2=0.7456, s=4.30 (calibration set)

n=13, r^2=0.7735, s=3.42 (validation set)

E =-617.60 (±17.64) + 155.21 (± 4.379) * *DCW(1,3)* (4)

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Figure 1. The scheme of selecting of the T and N**



$$r^2_{calibration} = F(T, N)$$

$$F(T^*,N^*) = \max(r^2_{calibration})$$

n=29, r^2=0.7976, q^2=0.7479, s=3.52, F=106 (training set)

n=9, r^2=0.7711, s=3.76 (calibration set)

n=10, r^2=0.7234, s=3.51 (validation set)

E =-218.59 (± 7.953) + 54.172 (± 1.900) * *DCW(1,3)* (5)

n=28, r^2=0.7674, q^2=0.7007, s=3.76, F=86 (training set)

n=9, r^2=0.7041, s=2.71 (calibration set)

n=11, r^2=0.8254, s=4.08 (validation set)

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In Equations 3-5, the n is the number of quasi-SMILES in a set (i.e. training set, calibration set, or validation set); the r^2 is the correlation coefficient; the q^2 is leave-one-out cross-validated r^2; the s is root-mean squared error; and the F is Fischer F-ratio.

Table 3 contains the correlation weights $\boldsymbol{CW(C_k)}$ for split 1 (Equation 3), split 2 (Equation 4) and split 3 (Equation 5) together with frequencies of $\boldsymbol{C_k}$ in the training and calibration sets.

Table 4 contains quasi-SMILES together with the numerical data on $\boldsymbol{E}$ for the models.

The third distribution into the training set, the calibration set, and the validation set should be estimated as the worst since attribute of quasi-SMILES "L" is absent in the calibration set. However, even for this distribution the prediction for the endpoint characterized by quite satisfactory statistical quality.

The mechanistic interpretation of a QSPR/QSAR model is important principle of building up predictive model (OECD, 2007; REACH 2011). The mechanistic interpretation for models similar to ones suggested in this work can be defined as the following. Having the numerical data on the correlation weights of features obtained in several runs of the Monte Carlo optimization, one can classify the features as promoters of endpoint increase (if the correlation weight of a feature every time is large) and promoters of endpoint decrease (if the correlation weight of a feature every time is small). In the examined dataset (Table 3), one can select doses ('1' and '2') together with the liver ('L') as promoters of increase the endpoint. Only heart ('H') is the promoter of the endpoint decrease. It can be interpreted as the following: the high doses (2000 and 1000 mg/kg) give increase of nanomoles of MDA per gram in wet tissue, liver accumulates the MDA more intensively than other organs, and heart accumulates the MDA less intensively than other organs.

Thus, the thesis that optimal descriptor can be a translator of eclectic data into endpoint prediction is confirmed.

Table 3. Correlation weights of quasi-SMILES attributes (conditions) for calculation of the DCW(1,3) for split 1, 2, and 3; T and C are frequencies of $\mathbf{C_k}$ *in the training set and calibration set, respectively*

	Split 1, Eq. 3			**Split 2, Eq. 4**			**Split 3, Eq. 5**		
$\boldsymbol{C_k}$	$\boldsymbol{CW(C_k)}$	T	C	$\boldsymbol{CW(C_k)}$	T	C	$\boldsymbol{CW(C_k)}$	T	C
1	1.00499	6	5	1.10609	12	3	1.09869	10	2
2	1.00071	10	3	1.09139	9	3	1.03604	11	2
3	0.99649	10	1	1.07653	8	3	1.02655	7	5
B	0.99828	8	2	0.98928	7	1	1.10572	6	2
F	0.98857	11	6	0.99074	15	6	1.07396	14	4
H	0.96721	7	1	0.92433	8	3	0.90153	8	2
K	0.98800	3	5	0.97011	7	2	1.04558	7	5
L	1.00881	8	1	1.03275	7	3	1.20953	7	0
T	0.99670	15	3	0.99545	14	3	1.09827	14	5
X	0.99518	12	7	0.99811	15	4	1.08228	10	6
Y	0.98255	14	2	0.97300	14	5	0.99005	18	3

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Table 4. Distributions into the training (T), calibration (C), and validation (V) sets for splits 1, 2, and 3; together with experimental and calculated level ADM in wet tissue of rats

	Distribution				Nanomoles of MDA per gram wet tissue			
ID	1	2	3	quasi-SMILES	Experiment	Eq.3	Eq.4	Eq.5
1	T	T	T	L1XT	33.60	21.4061	23.8192	24.5742
2	T	C	V	L2XT	23.40	19.9312	21.5384	21.1799
3	V	V	V	L3XT	14.50	18.4786	19.2317	20.6659
4	T	T	T	K1XT	13.40	14.2407	14.0967	15.6922
5	C	V	C	K2XT	9.95	12.7658	11.8160	12.2980
6	T	T	C	K3XT	8.73	11.3132	9.5093	11.7840
7	C	T	V	B1XT	16.70	17.7815	17.0711	18.9502
8	T	T	C	B2XT	13.10	16.3066	14.7903	15.5559
9	V	C	V	B3XT	13.20	14.8539	12.4837	15.0419
10	T	C	T	H1XT	2.80	7.0876	6.9908	7.8889
11	T	T	T	H2XT	2.20	5.6127	4.7101	4.4946
12	T	T	C	H3XT	2.80	4.1601	2.4034	3.9806
13	C	T	T	L1XF	29.80	18.6066	23.0881	23.2568
14	V	T	T	L2XF	24.10	17.1317	20.8074	19.8625
15	T	T	V	L3XF	13.70	15.6791	18.5007	19.3486
16	C	T	C	K1XF	12.20	11.4412	13.3657	14.3749
17	V	T	T	K2XF	8.93	9.9663	11.0849	10.9806
18	C	V	T	K3XF	8.51	8.5137	8.7782	10.4667
19	C	T	V	B1XF	14.40	14.9819	16.3401	17.6328
20	T	V	T	B2XF	12.70	13.5070	14.0593	14.2386
21	T	V	V	B3XF	11.00	12.0544	11.7526	13.7246
22	C	T	T	H1XF	2.43	4.2881	6.2598	6.5715
23	V	C	V	H2XF	2.14	2.8132	3.9790	3.1772
24	T	T	C	H3XF	2.38	1.3606	1.6724	2.6633
25	V	T	V	L1YT	14.80	17.0578	19.9210	19.5775
26	T	V	T	L2YT	10.80	15.5829	17.6403	16.1833
27	T	T	T	L3YT	10.60	14.1302	15.3336	15.6693
28	V	V	C	K1YT	7.20	9.8923	10.1986	10.6956
29	C	T	T	K2YT	9.00	8.4174	7.9178	7.3013
30	T	T	T	K3YT	8.00	6.9648	5.6111	6.7874
31	T	T	T	B1YT	12.30	13.4331	13.1730	13.9536
32	T	V	T	B2YT	12.00	11.9582	10.8922	10.5593
33	T	V	T	B3YT	11.60	10.5056	8.5855	10.0453
34	V	T	T	H1YT	2.80	2.7392	3.0927	2.8922
35	T	T	T	H2YT	2.80	1.2643	0.8119	-0.5020
36	V	V	T	H3YT	2.60	-0.1883	-1.4947	-1.0160
37	T	C	V	L1YF	13.60	14.2582	19.1900	18.2602
38	T	T	T	L2YF	10.70	12.7833	16.9092	14.8659
39	T	C	T	L3YF	9.33	11.3307	14.6026	14.3519
40	V	C	T	K1YF	7.70	7.0928	9.4675	9.3783
41	C	C	T	K2YF	8.60	5.6179	7.1868	5.9840
42	V	T	C	K3YF	8.40	4.1653	4.8801	5.4700
43	V	T	T	B1YF	11.60	10.6336	12.4419	12.6362
44	T	T	T	B2YF	11.60	9.1587	10.1612	9.2419
45	T	T	C	B3YF	12.00	7.7061	7.8545	8.7280
46	T	T	T	H1YF	2.40	-0.0603	2.3617	1.5749
47	T	T	V	H2YF	2.60	-1.5352	0.0809	-1.8194
48	V	C	T	H3YF	2.70	-2.9878	-2.2258	-2.3333

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4. CONCLUSION

The suggested scheme of the prediction for level of malondialdehyde in wet tissue of rat under action of the Al_2O_3 nanoparticles (oral exposure) based on described quasi-SMILES (Table 1) gives quantitative prediction for this endpoint. Since the suggested model is validated with a group of distribution into visible the training and calibration sets and invisible validation set, the approach can be used for regulatory purposes according to OECD principles (OECD, 2007) and REACH (REACH, 2011).

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