Handbook of Research on

Advancements in Cancer Therapeutics

Sumit Kumar, Moshahid Alam Rizvi, and Saurabh Verma

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Handbook of Research on Advancements in Cancer Therapeutics

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Dedicated to: *Dr. G. P. Dutta, Ph.D., FNA, FNASc., Former Deputy Director, CDRI, Lucknow*
Who taught me and many others from Chemotherapy to Cell Biology.

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With the evolution of the tissue system and division of function among differentiated cells/tissues, the property of controlled cell growth also evolved in animals. It is when this very control is lost that cancers develop. The immune system's ability to distinguish between self and non-self is central to impeding cancer progression. However, cancer cells in time can develop multiple ways of escaping immune control. Even today, cancer remains a disease of baffling complexity on account of its diverse origin and pathogenesis. Classical methods like surgery, radiation, and chemotherapy have failed to make the cut as idyllic therapy, especially considering the encumbering side-effects and high failure rate. Alternative therapeutic strategies that exploit the immune system itself have proved promising. One of these is monoclonal antibody therapy. In this chapter, the relationship between the immune system and cancer and various forms of immunotherapy are discussed in detail.

In this chapter, computational approaches for the discovery of new drugs that are useful for diagnosis and treatment of disease will be described in three parts. MD technique uniquely supports protein design attempts by giving information about protein dynamics associated with atomic-level descriptions of the relationship between dynamics and function. The purpose of molecular docking is to provide an estimate of the ligand-receptor complex structure using computational methods. By this estimation, the mechanism of drug binding and action are described by determining the three-dimensional simulation of drug and drug-induced macrostructure. ADME characteristics are physicochemically significant descriptors and pharmacokinetically relevant properties used to design more effective drugs and new analogs. As a result, in-silico calculations can provide robust preliminary information as to drug activity and mechanism in the drug production process, as well as in vitro and in vivo studies.

Breast cancer is a carcinoma of mammary glands, which starts off as abnormal proliferation of ductal cells. This could, then, become either benign tumours or metastatic carcinomas. It is one of the most common causes of deaths because of cancer, and is one of the most common types of cancer in women in the whole world. India along with the US and China accounts for one-third of the breast cancer burden. The breast cancer carcinogenesis is attributed to epigenetics, which is the study of the reversible changes in the phenotype without any change in the DNA sequence. Genes, which are concerned with proliferation, anti-apoptosis, invasion, and metastasis, have been seen undergoing epigenetic changes in breast cancer. Cancer can be caused either by global hypomethylation (causing activation of oncogenes and leading to chromosomal instability) or by locus-specific hypermethylation (causing repression of gene expression and genetic instability due to inactivation of DNA repair genes). Other epigenetic mechanisms involved in carcinogenesis are histone modification and nucleosomal remodeling.

Chapter 7

The authors aim to describe valuable information and experimental reviews that may help to develop and design different formulation, which can boost up the overall efficiency of the final product. Further, they explained the overall efficiency, method of preparation, target delivery approaches, drawbacks, and other characteristics in relation to lipids, peptides, polymers, and vaccines. In addition, they also propose to uncover the physico-chemical properties, in-process manufacturing issues, and external factors that influence the fate of a medicine. That major includes the excipients, method of preparation, dose, delivery route, chemical and biological properties, drug-drug interaction, drug-body interaction, patient compliance, modifications in lipid based nano-vectors, polymer-mediated delivery systems, conjugate delivery systems, and others. In conclusion, by the end of this chapter, the authors are able to explain a robust mode of delivering active constituents more safely and economically to the target site by showing maximum bioavailability.

Chapter 8

Cancer has been the most deleterious disease in recent times, and unfortunately its spread is increasing. Systemic treatment with chemotherapeutics remains the conventional way of treating many cancers, despite the serious damage long-term chemotherapy can cause in healthy tissues. Many therapeutic

strategies have achieved popular practical applications, but drug delivery systems still face challenges associated with safety, and this has led to the development of safer drug delivery methods composed of biocompatible substances. In this respect, lipid-, polymer-, and peptide-based drug delivery systems have been proposed as safer candidates for cancer therapy. These delivery methods are expected to as biodegradable systems with low cytotoxicity for cancer therapy. Therefore, in this chapter, the authors discuss use of lipids, polymers, and peptides as delivery vehicles for chemotherapeutic agents and their structural characteristics.

Chapter 9

Cancer is a major killer disease caused by uncontrolled growth and invasion of cells. Apoptosis is the cell's natural mechanism of death, which maintains tissue homeostasis. Any mutation that disturbs the apoptotic pathway leads to deregulated proliferation, resistance, and evasion of apoptosis. This evasion is one of the hallmarks of malignant developments. Apoptosis takes place via two distinct pathways i.e. the intrinsic and the extrinsic pathways. These pathways use cleaved caspases to execute apoptosis which in turn cleave many downstream proteins to kill the cells. They can also be inhibited through various means that include up-regulation of anti-apoptotic and down-regulation of pro-apoptotic factors. The authors here aim to impart a comprehensive understanding of the biochemical characteristics of these pathways that render scientists target these pathways and assess apoptosis restoring abilities of the novel drugs and natural products for cancer treatment.

Chapter 10

Cancer has been a worldwide topic in the medical field for a very long time. As angiogenesis is essential for tumor growth and metastasis, controlling tumor-associated angiogenesis is a promising tactic in limiting cancer progression. In cancer patients, multidrug resistance (MDR) is most widely used phenomenon by which cancer acquired resistance to chemotherapy. This resistance to chemotherapy occurs due to the formation of insulated tumor microenvironment which remains a major hurdle in the

cure of various types of cancer. The mechanisms that cause malignant growth of cells include cell cycle control, signal transduction pathways, apoptosis, telomere stability, and interaction with the extracellular matrix. This chapter focuses on current strategies to suppress tumor angiogenesis for cancer therapy, various mechanisms involved in the development of MDR in cancer cells, which in turn will help us to identify possible strategies to overcome these MDR mechanisms and a variety of procedures that involves targeting apoptotic and telomerase pathways to suppress tumor progression.

Nanotechnology-based drug-delivery systems, as an anticancer therapy tool, have shown significant potentials for the diagnosis and treatment of cancer. Recent studies have demonstrated that cancer therapy could be efficiently achieved by combinatorial therapies, approaches using multiple drug regimens for targeting cancers. However, their usages have been limited due to shorter half-lives of chemotherapeutic agents, insignificant targetability to tumor sites and suboptimal levels of co-administered conventional drug moieties. Thus, nanotechnology-based drug-delivery systems with effective targetability have played a crucial role to overcome the limitations and challenges associated with conventional therapies and also have provided greater therapeutic efficacy. Herein, the authors have focused on various drug-incorporated combinatorial nanocarrier systems, the significance of various receptors-associated strategies, and various targeted delivery approaches for chemotherapeutic agents.

In a conventional oral or intravascular drug delivery approach, therapeutic factors are distributed throughout the body and only a limited part of the drug reaches to tumor site. Packaging of cytotoxic agents in drug delivery systems like nanoparticles could enhance its delivery to specific targets in the tumors and could be potential candidate for therapeutics advancement. Targeted drug delivery holds the potential to overcome the present therapeutics of cancer by selective delivery of an arbitrary amount of drug at the tumor site. Loading of cytotoxic agents in drug delivery systems could enhance its delivery to specific targets based on strategy to reach the tumor site. This chapter explores the detailed of innovative methods of drug delivery, challenges of targeted drug delivery, and their implications.

Targeted drug delivery in cancer treatment is a very convenient method for increasing the effectiveness of drugs and reducing their toxic side effects. Nano drug delivery systems have unique physical, chemical, mechanical, and optical properties. Nanoparticles, which have large surface areas and functional groups for the binding of therapeutic agents, benefit the drug distribution with nanoparticle formulations and can provide new features. They also enable personal oncology for diagnosis and treatment, which is appropriate for the personal molecular profile structures of cancer patients. The tumor-targeted active substances are attached to nanoparticles and the active substance loaded nanoparticles are targeted to the tumor area; these nanoparticles can be used with a high tendency to bind and specificity, to target tumor antigens or vessels. This chapter, besides traditional chemotherapy and radiotherapy methods in the field of cancer treatment, is aimed to give information about targeted drug delivery systems for cancer cell targeting without damaging normal tissues.

Chapter 14

Around 40% of new chemical entities and drugs are lipophilic or poor aqueous soluble in nature. Among them many anti-cancer drugs are also consist lipophilic properties. Available poorly water soluble anti-cancer drugs are paclitaxel, etoposide, and docetaxel. To get better stability of those anti-cancer drug via encapsulation and searching suitable carrier system for the controlled release, design and development requires of anhydrous nano carrier system. However, to deliver and entrapment of these kind of anti-cancer drugs are very essential with avoidance of water free preparation to get suitable controlled release application and achieve targeting site. The primary objective of proposed chapter is to develop and design novel stable anhydrous or non-aqueous nano emulsion carrier system and provide suitable carrier system for poorly aqueous soluble anti-cancer drugs. Another important aim is to design and develop better stabilizing agent by combining different type of surfactant, co-surfactant, and co-solvent.

Chapter 15

Cancer is the one of the deadliest diseases and takes the lives of millions of people every year across the world. Due to disease heterogeneity and multi-factorial reasons, traditional treatment such as radiation therapy, immunotherapy, or chemotherapy are effective only among a small population of the patients. Tumors can have different fundamental genetic causes and protein expressions that differ from one patient to another. This variability among individual lends itself to the field of precision and personalized medicine. Following the completion of human genome sequencing, significant progress has been observed in the characterization of human epigenome, proteome, and metabolome. Pharmacogenetics and pharmacogenomics use this sequence to study the genetic causes of individual variations in drug response and the simultaneous impact of change in genome that decide the patient's response to drug respectively. On summation, identify the subpopulation of patient and provide them tailored therapy thus increasing the effectiveness of treatment. All these evolved the field of precision or personalized medicine that plays a crucial role in cancer prevention, prognosis, diagnosis, and therapeutics. These tailored therapies are characterized by increased efficiency and reduced toxicity. Not all cancers have genetic variability; some are also influenced by polymorphism of gene encoding enzymes that play an important role in pharmacokinetics of drug. The discoveries of cancer predisposition genes allow diagnosis of a patient at risk of cancer development and let them make the decision on précised individual risk modification characteristic. The use of CYP2D6 genotyping for breast cancer, mutation in KRAS in colorectal cancer, genomic variation in EGFR in small lung cancer, melanoma are some of the examples of importance of cancer predisposition genes. In recent times, distinct molecular subtypes of cancers have been identified with requirement of different treatment for each subtype. Precision medicine shifts the trend from reaction to prevention and forestalls disease progression.

Chapter 16

Stem cells are pluripotent cells having capacity of self-renewal and produce various types of mature cells. Cancer stem cells are known to be responsible for drug resistance and tumor relapse, yet stem cells offer multiple avenues to treat same. Stem cells have been employed for treating of blood and immune systems damaged during chemotherapy and radiotherapy. Stem cell transplantation is emerged as critical therapy in cancer treatment, yet other potential applications of stem cells in cancer treatment are largely unexplored or underutilized. Recently, stem cells reengineered express different cytotoxic agents. It has shown to cause tumor regression and enhance the animal survival in preclinical studies. Stem cell therapy can be also employed for targeted drug delivery, gene delivery, and even used as virus to target cancer cell. In recent years, research is devoted on stem cells worldwide for new and newer application. Although the field of stem cells is nascent and raises many ethical concerns, scientific responsibilities, and future challenges, scientific community are still hopeful and filled with optimism. Currently, stem cell therapy represents the beginning of the new era in cancer treatment and giving a ray of hope to clinicians and also patients who are suffering from untreatable diseases and desperately looking for new therapies. In the present chapter, the authors mainly shed light on potential applications of stem cells to treat cancer. At the end, they also discussed the factor influencing stem cell therapies and current challenges in stem cell therapy.

Self-renewal is the most important property of stem cells. Parallel to this, cancer stem cells (CSCs) have an indefinite proliferative ability that drives tumorigenesis. The conventional treatment of cancer includes chemotherapy, radiotherapy, and surgery, which decreases the tumour size. Contrary, targeted therapy against CSCs initially does not shrink the tumour but ultimately causes tumour degeneration. Nanobiotechnology, RNA interference, microRNA are emerging fields with a vital role in targeted therapy against CSCs. The non-protein encoding microRNAs has a major role in cancer treatment since they regulate gene expression during post-transcription. RNAi technology can silence the gene of interest with potency and specificity inhibiting tumour growth. In nanoparticles-based RNA interference, nanocarriers protect RNAi molecules from immune recognition and enzymatic degradation. The cancer cell gene expression profiling using next-generation sequencing helps in understanding the underlying cancer cell mechanisms. The current chapter deals with novel concepts in the treatment of cancer.

Cancer is the most prevalent and dangerous disease, and it leads to millions of deaths worldwide. Generally, metastatic cancer cells are not eradication by conventional surgical operative or chemotherapy-based treatment. New pathways have been established in various arenas such as unique biology, modulators regulatory mechanism, directional migration, self-renewal, etc. The individual pathways can be employed as therapeutic carriers, specific drug targeting, generation of acquiring nature immune cells, and regenerative medicine. The present scenario, stem cell therapy, focused on a promising tool for targeted cancer treatment. Stem cells also utilized as viruses and nanoparticles carry to enhance the primary therapeutic application in various dimensions such as cancer target therapy, regenerative medicine, immune-modulating therapy, and anticancer drugs screening. Furthermore, the rapid development in next-generation sequencing techniques and cancer genomics and proteomics analysis approaches are making therapeutics targeting organ-specific cancer more precise and efficient.

In this chapter, the authors first review nano-devices that are mixtures of biologic molecules and synthetic polymers like nano-shells and nano-particles for the most encouraging applications for different cancer therapies. Nano-sized medications additionally spill especially into tumor tissue through penetrable tumor vessels and are then held in the tumor bed because of diminished lymphatic drainage. This procedure is known as the enhanced penetrability and retention (EPR) impact. Nonetheless, while the EPR impact is generally held to improve conveyance of nano-medications to tumors, it in certainty offers not exactly a 2-overlay increment in nano-drug conveyance contrasted with basic ordinary organs, bringing about medication concentration that is not adequate for restoring most malignant growths. In this chapter, the authors likewise review different obstructions for nano-sized medication conveyance and to make the conveyance of nano-sized medications to tumors progressively successful by expanding on the EPR impact..

Chapter 27

The importance of monocytes in modulating the lymphocyte dependent tumor necrosis is a target for cancer therapeutics. Monocytes produce a plethora of chemokine receptors. Lymphocyte to monocyte ratio is one of the negative factors in cancer patients. It is being targeted for treatment of abnormal lymphocytopenia and monocytosis in untreatable metastatic cancer patients. The aim of the chapter is to throw light on the circadian and psychological factors that modulate the progression of cancer and identify novel targets for controlling transformation of preneoplasms to neoplasms, invasiveness, and metastasis.

Preface

Cancer is the second leading cause of death worldwide. Despite the fact that scientific discoveries have hugely added to the existing knowledge, improvement in cancer therapeutics is remains same. Complexity of cancer demands an integrated approach from both cancer biology and pharmaceutics. The recent discoveries in targeted therapies and personalized medicines have generated greater hope and optimism. Therefore, the book has covered both the conventional and newer anticancer modalities, recent discoveries, and advancements in cancer treatment. Our primary objective is to update the medical and scientific fraternity about recent advances in cancer therapeutics. We have included the nascent personalized system of medicine and big data-driven cancer treatment in addition to the traditional chemotherapy. Our book will bridge the gaps among basic research, pharmaceutical drug development processes, regulatory issues, translational experimentation, and clinical application. The major goal of the book is the dissemination of the recent discoveries such as immunotherapies, synthetic lethality, carbon beam radiation, and other exciting targeted therapies based on rigorous evidence to benefit research trainees, oncologists, radiation oncologists, surgical oncologists, scientific community, and also to anyone who is merely interested in to know more about cancer biology and available therapies.

Apoptosis is the most fine-tuned process leading to the efficient removal of cells harboring damaged DNA. Deregulation of the apoptotic pathway results in many anomalies, including cancer. This stems from either deregulation of the apoptotic pathway or overactivation of the cell survival pathway. The identification of critical molecular events is panacea in anticancer therapeutics. Recent advancements in biological science had transformed our understanding and made an inbound opportunity in cancer therapeutics. In this context, molecular therapy and informatics are key to the future cancer treatment.

The book is written to cater the need biological science graduates, people working in cancer biology, therapists, and the normal public who may be interested in understanding the most fundamental aspects of cancer and the available therapeutic opportunities. This book will provide a valuable resource material for research trainees, oncologists, clinicians, and cancer biologists.

The first chapter titled “Cancer Immunotherapy: Beyond Checkpoint Inhibitors” has extensively discussed about the immunostimulants, immunotoxins, antibodies, fusion proteins, engineered cytotoxic T cells, engineered immunocytokines, vaccines, checkpoint inhibitors, CAR T-cell therapy, and nanomedicine. The chapter aims to evaluate the anti-tumor response of immunotherapies, reduce the immune suppression, and enhance the immunogenicity of tumors. The CAR T cell is the first living cell-based therapy approved by the FDA in 2017. The subsequent identification of different molecular functions of T-cell costimulatory molecules has greatly improved our understanding. Nanomedicine is a unique advantage for drug-delivery vehicles and can work in unison with immunotherapy. For immuno-oncology applications, cancer nanomedicine can be developed beyond drug-delivery platforms. A greater emphasis

is placed on actively modulating host anticancer immunity using nanomaterials that could provide new avenues for developing novel cancer therapeutics. The integration of nanotechnology with the CAR T cells offer many advantages over the standard chemotherapy. The sustained bioavailability of a low molecular weight drug can be increased by its nanoparticle formulation, whereas a nanosized drug carrier minimizes their elimination through the liver or kidney. The drug can be used to attract immune cells at tumor site, activate dendritic cells and significantly improve the clinical applications of immunotherapy. Identification of inhibitory molecular pathways during immunotherapy like B-cell maturation agent also allows us to target these pathways. These approaches can stimulate the immune response and reduce the unwanted side effect by inhibiting the immunosuppressive response.

The monoclonal antibodies have a long history in disease treatment starting from late 19th century for diphtheria and tetanus treatment in animals by Behring and Kitasato. The path-breaking 'hybridoma technology' by Kohler and Milstein gave further impetus to antibody-based therapy, as given us an ability to produce monoclonal antibodies in unlimited amounts. The first therapeutic monoclonal antibody approved by FDA was Muromonab, a murine monoclonal antibody against the CD3 receptor of T lymphocytes in 1986. The first monoclonal antibody in cancer treatment was approved in 1997 by the US FDA to treat non-Hodgkin's lymphoma. Monoclonal antibodies are the most employed and approved for cancer treatment due to hassle-free treatment. Monoclonal antibodies have carved their niche as a form of targeted therapy and immunotherapy in the ever-challenging realm of oncology. Technological advances as well as increased insights into immuno-oncology have allowed monoclonal antibody-based therapy to grow leaps and bounds. Certainly, it shall be interesting to see how it evolves further. Salonee Martins et al. comprehensively discusses the monoclonal antibodies in cancer therapeutics., in the chapter titled "Therapeutic Approaches to Employ Monoclonal Antibody for Cancer Treatment".

The development of informatics in the last two decades has given a massive push in drug discovery, including anticancer therapeutics. The chapter titled "Advancements in Cancer Therapeutics: Computational Drug Design Methods Used in Cancer Studies" by Serda Kecel, Gunduz, Bilge Bicak, and Aysen E. Ozel had explored the computer-based drug designing process in anticancer drug discovery. The author described the computational approaches for the discovery of new drugs in cancer diagnosis and treatment in three-part. MD technique uniquely supports protein design and giving information about protein dynamics at the atomic level. The purpose of molecular docking is to estimate the ligand-receptor complex and mechanism of drug binding. ADME characteristics are other physicochemically relevant descriptor and pharmacokinetically relevant properties that can be used for designing more effective drugs and analogs. The in-silico calculations provide us a piece of robust preliminary information on drug activity.

Advancement in human genome sequencing has given us ample information about the role of somatic aberrations in cancer initiation, development, and aggressiveness. This discovery has inspired interest among cancer researchers; many are now using genetic engineering therapeutic methods to improve cancer regression and seeking a possible cure for the disease. The large gene therapy sector offers various therapies that are likely to become effective in preventing cancer deaths. The latest clinical trials of third-generation vaccines for a wide variety of cancers have produced promising results. Cancer virotherapy, which uses viral particles replicating within the cancer cell, is an emerging treatment method that shows great promise. The latest developments in gene-editing techniques such as CRISPR, Cas9, TALENs, and ZFNs are being used to make cancer a manageable condition. Gene-based therapeutic approaches include gene transfer, oncolytic virotherapy, and immunotherapy. Gene Transfer or gene editing is the most recent treatment method that allows the insertion of new genes into the cancer cell

to mediate the cancerous cell's slow growth or death. Gene transfer is a very flexible technique, and a wide range of genes and vectors are being used in clinical trials and demonstrated positive results. Gene therapy is expected to play a significant role in potential cancer therapy as a part of a multi-modality procedure. The Gene Therapies in Cancer Treatment is beautifully covered by Shubhjeet Mandal et al., in the chapter titled "Gene Therapy and Gene Editing for Cancer Therapeutics".

The chapter titled "Gene Editing and Gene Therapies in Cancer Treatment" by Gyanendra Tripathi et al. has comprehensively discussed the cancer vaccines, gene-editing technology in anticancer treatment.

Dr. Umesh Kumar et al. discussed the role of epigenetics in breast cancer development in the chapter titled "Epigenetic Regulation of Breast Cancer". The topic is selected based on the sheer number of women who are affected by it. Breast cancer carcinogenesis is also attributed to epigenetics or reversible changes in the phenotype without any change in the DNA sequence. Genes that are concerned with proliferation, anti-apoptosis, invasion, and metastasis have been seen undergoing epigenetic changes in breast cancer. Cancer can be caused either by global hypomethylation (causing activation of oncogenes leading to chromosomal instability) or by locus-specific hypermethylation (causing repression of gene expression and genetic instability due to inactivation of DNA repair genes). Other epigenetic mechanisms involved in carcinogenesis are histone modification, and nucleosomal remodeling are also discussed.

Lipid-mediated drug delivery systems are frequently employed to deliver payloads consisting of either drugs, or drug conjugates, or antibodies, or even siRNA at the desired location through a lipid-based nanoparticle system. The chapter titled "Lipids, Peptides, and Polymers as Targeted Drug Delivery Vectors in Cancer Therapy" by Mani Sharma, et al. discussed the lipid-based drug delivery system. Authors have also discussed the advancement in research, different modifications, and improvements in the delivery of chemotherapeutic drugs. The authors' main emphasis is to uncover the most recent and smart technologies that offer a unique way to deliver chemotherapy drugs at the desired target without affecting healthy cells. Author discussed various approaches to modulate and upgrade the drug delivery system in cancer were discussed with a particular emphasis on cationic & anionic lipids, pH-responsive polymers, redox-mediated delivery, enzymes, temperature, and light-activated delivery.

The chapter titled "Use of Lipids, Polymers, and Peptides for Drug Delivery and Targeting to Cancer Cells or Specific Organs" by Sampan Attri et al., is another chapter which also focused on anticancer drug delivery. Many anticancer therapeutic strategies have achieved popular practical applications; however, drug delivery systems still face enormous challenges associated with poor safety, low solubility, and untargeted location delivery. This has led to the development of safer drug delivery methods composed of biocompatible substances and tailored molecules. In this respect, the addition of lipid, polymer, and peptide-based drug delivery systems in the book is paramount importance. These delivery methods, consisting of biodegradable systems with low cytotoxicity for cancer therapy, are also covered.

The chapter titled "Apoptotic Pathway: A Propitious Therapeutic Target for Cancer Treatment" by Durdana Yasin et al. discussed the molecular mechanism of apoptosis and how apoptosis deregulation results in cancer development. The authors have also discussed the deregulated apoptotic proteins, which can be targeted for anticancer therapeutics.

Multidrug resistance is the most common phenomenon observed in cancer treatment, where cancer acquired resistance to chemotherapy. This resistance to chemotherapy occurs due to the formation of an insulated tumor microenvironment, which remains a major hurdle in the cure of various cancer types. The chapter titled "Strategies to Suppress Tumor Angiogenesis and Metastasis, Overcome Multidrug Resistance in Cancer, Target Telomerase and Apoptosis Pathways" by Deepti Sharma et al., have beautifully covered the topic and comprehensively discussed the strategies to suppress tumour angiogenesis

in anticancer therapy. Various mechanisms that are known to involve in the development of MDR in cancer cells are also discussed. It will undoubtedly help in the identification of possible strategies and target to overcome MDR.

The chapter titled "Receptor-Based Combinatorial Nanomedicines: A New Hope for Cancer Management" by Harshita et al., have covered the Nanotechnology-based drug delivery systems in anticancer therapeutics. Nanotechnology has significant potentials in the diagnosis and treatment of cancer as it provides flexibility in the loading of multiple drugs destined to a certain location. Recent studies have demonstrated that cancer therapy could be efficiently achieved by combinatorial therapies and approaches using multiple drug regimens to target cancers. However, their usages have been limited due to shorter half-lives of chemotherapeutic agents, insignificant targetability to tumor sites and sub-optimal levels of co-administered conventional drug moieties. Thus, nanotechnology-based drug delivery systems with effective targetability will play a crucial role in overcoming the limitations and challenges associated with conventional therapies and will provide greater therapeutic efficacy.

The toxicity to normal cells is the biggest concern in anticancer therapeutics. Targeted drug delivery holds the potential to overcome this problem by selective delivery of a required amount of drug at the tumor site. Loading of cytotoxic agents in drug delivery systems could enhance its delivery to specific targets based on strategy to reach the tumor site. The chapter "Targeted Drug Delivery in Cancer Treatment" by Farhad Bano et al., explores the innovative methods of drug delivery, challenges in targeted drug delivery, implications, and how to overcome it.

The chapter "Advancements in Cancer Therapeutics: Targeted Drug Delivery in Cancer Treatment" by Bilge Bicak, Serda Kecel Gunduz and Aysen E. Ozel had discussed the targeted drug delivery to overcome the toxic side effects of conventional drugs. Nano drug delivery systems have unique physical, chemical, mechanical and optical properties. Nanoparticles with large surface areas and functional groups for the binding of therapeutic agents benefit the drug distribution with nanoparticle formulations and provide new features. Nanotechnology also enables personalized medicine systems, as it is appropriate for cancer patients' personal molecular profile structures. The tumor-targeted active substances are attached to nanoparticles and targeted to the tumor area. These nanoparticles can be used with a high tendency to bind and specificity to target tumor antigens or vessels. Besides traditional chemotherapy and radiotherapy methods in cancer treatment, the authors also aimed to give information about targeted drug delivery systems for cancer cell targeting without damaging normal tissues.

The chapter titled "Surfactant Based Anhydrous Nano Carrier System for Poorly Aqueous Soluble Anti-Cancer Drugs" by Shekhar Verma et al. discussed the carrier system for enhancing the solubility of the drug system. It is a good topic as around 40 percent of the cancer drugs (i.e., paclitaxel, etoposide, and docetaxel, etc.) are lipophilic and poorly soluble in an aqueous medium. The Primary objective of the chapter is to develop and design novel stable anhydrous or non-aqueous nanoemulsion carrier system and provide suitable carrier system for poorly aqueous soluble anticancer drugs. The authors discussed the topic with keeping an aim to design and develop better-stabilizing agents by combining different types of surfactant, co-surfactant, and co-solvent.

The chapter titled "A Voyage to the Sea of Precision Medicine in Cancer" by Nerethika Ravichandiran et al. had discussed the precision medicine in very detail. It is a nascent topic and requires significant attention. Tumor's gene and protein expressions differ from one patient to another. This variability among individuals leads to a huge variation in treatment outcomes. Considerable progress has been made in this field after the completion of human genome sequencing, characterization of the human epigenome, proteome, and metabolome. Pharmacogenetics and pharmacogenomics use this sequence to study the

genetic causes of individual variations in drug response and the simultaneous impact of change in genome that decide the patient's response to drugs, respectively. In summary, identifying the subpopulation of the patient and providing them tailored therapy would increase the effectiveness of treatment. The evolution of precision or personalized medicine plays a crucial role in cancer prevention, prognosis, diagnosis, and therapeutics in the future. These tailored therapies are characterized by increased efficiency and reduced toxicity. Not all cancers have genetic variability; some are also influenced by polymorphism of gene encoding enzymes that play an essential role in drug pharmacokinetics. The discoveries of cancer predisposition genes allow the diagnosis of a patient at risk of cancer development and let them decide on précised individual risk modification characteristics. The use of CYP2D6 genotyping for breast cancer, mutation in KRAS in colorectal cancer, genomic variation in EGFR in small lung cancer, and melanoma are examples showing the importance of cancer predisposition genes. In recent times, distinct molecular subtypes of cancers have been identified with different treatment requirements for each subtype. Precision medicine shifts the trend from reaction to prevention and forestalls disease progression.

The chapter "Role of Stem Cells in Cancer Therapeutics" by Madhu Rani, Sumit Kumar, and M. M. A Rizvi had discussed the different kinds of hematopoietic cells that can be used for anticancer drug delivery and also targeting the cancer cells.

The chapter "Cancer Stem Cells and Advanced Novel Technologies in Oncotherapy" by Shalini Sakthivel et al., had discussed the stem cells and advanced technologies such as siRNA, Next Genome Sequencing, gene expression profiling, nanobiotechnology, miRNA, etc. that can be used in the development of better anticancer therapeutics.

The chapter titled "Recent Research and Development in Stem Cell Therapy for Cancer Treatment: Future Promising and Challenges" by Nagendra Kumar Chandrawanshi et al. discussed the stem cell-based therapy for targeted cancer treatment. Stem cells are used as viruses and nanoparticles to enhance the primary therapeutic application in various dimensions such as cancer target therapy, regenerative medicine, immune-modulating therapy, anticancer drugs screening, etc. Furthermore, the development in next-generation sequencing techniques and cancer genomics and proteomics analysis approaches makes therapeutics targeting organ-specific cancer more precise and efficient.

The chapter "Utilization of Bio-Imaging in Cancer Study" by Muneesh Kumar Barman et al. discussed the biological imaging in cancer research, diagnosis, and treatment of various cancers. Cancer research employs various bio-imaging techniques such as fluorescence microscopy, FRET, FRAP, TPEF, SGH, etc. to study the complexity and characteristics of different cancer cells. The development of live-cell imaging has also helped in understanding the important biological processes which differentiate cancer cells from their environment. Advancement in cancer diagnosis has taken place with the development of sophisticated radiology techniques like MRI, CT scans, and FDG-PET. Also, the development of novel nanotechnology-based probes has improved the quality of both cancer research and diagnosis. The author also discussed some of the bio-imaging techniques in detail which are being used in the field of cancer studies.

The chapter titled "Chemopreventive and Therapeutic Potential of Phytopharmaceuticals Against Oral Cancer: Evidence-Based Reports From Preclinical Studies in Animal Model" by Dharmeswar Barhoi et al., have discussed the phytopharmaceuticals as alternative medicine in the prevention of oral carcinogenesis over conventional drugs. The utility of Phytopharmaceuticals (such as resveratrol, sulforaphane, quercetin, etc.) had been discussed in detail.

The chapter "Nutrition and Cancer" by Shazia Ali et al. discussed the role of diet in cancer treatment. The diet component includes folic acid, selenium, vitamin D, vitamin B-12, chlorophyll, and antioxidants

such as the carotenoids, kryptoxanthin, lycopene, and lutein have been discussed in relation to cancer risk, development, and therapy.

The chapter titled "Functional Mechanisms of Green Tea Polyphenols and Their Molecular Targets in Prevention of Multiple Cancer" by Zubair Bin Hafeez, et al., have discussed the regulation of NF-kB pathway by green tea polyphenols in different cancer types. Natural antioxidants have been shown to possess chemopreventive and chemotherapeutic potential via targeting NF-kB signaling, among which tea polyphenols are the most extensively studied chemopreventive molecules.

The chapter "Natural Product Compounds for Breast Cancer Treatment" by Bui Thanh Tung discussed the utility of natural product compounds in breast cancer treatment over conventional therapy. The chapter has included the molecular targets of breast cancer as well as bioactive compounds that can be used to target them.

The chapter titled "Cancer: Clinical Trials Design and Principles" by Rashi Rai et al., covered the most ignored yet important topic. The clinical trial design is key to the successful development of any drug. Clinical trials are essential to govern the impact of a new possible treatment. Clinical trial studies in cancer have provided successful treatment leading to longer survival span in the patients. This chapter provides detailed information on different aspects of clinical trials in cancer research. Protocols outlining the design and method to conduct a clinical trial in each phase are discussed. The process and the conditions applied in each phase (I, II, and III) is also precisely covered. The recent innovations in clinical design strategies and artificial intelligence to increase trial success rates are also discussed.

The chapter titled "Phytopharmaceuticals in Cancer Treatment" by Khalid Umar Fakhri et al. discussed the synergistic activity of the phytochemicals, emphasizing anticancer effects. This chapter aims to present the fundamental mechanism of phytochemicals in combination therapy.

The chapter titled "Obstructions in Nanoparticles Conveyance, Nano-Drug Retention, and EPR Effect in Cancer Therapies" by Khalid Umar Fakhri et al. review the nano-devices that are mixtures of biologic molecules and synthetic polymers like nano-shells and nanoparticles for anticancer therapies. The authors have also discussed the enhanced penetrability and retention effect in nanomedicine.

The book's main focus is nanomedicine, targeted system of medicine, precision-based therapeutics, and natural product-based medicinal system. The book is suitable for graduate students, clinicians, and researchers working in the cancer biology field. Our book will serve as an important source of information in cancer therapeutics and is expected to guide future treatments.

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It is a great coincidence to release this book in the auspicious centenary year and month of Jamia Millia Islamia, the top most Central University of India. The Editor (Moshahid Alam Rizvi) is deeply indebted to the Vice-Chancellor Prof. Najma Akhtar and the administration team of Hall of Boys Residence for their unflinching support and encouragement during the course of this book writing. We further wish to congratulate the JMI fraternity for this extraordinary feat.

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Chapter 1
Cancer Immunotherapy:
Beyond Checkpoint Inhibitors

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ABSTRACT

Cancer immunotherapy has become a powerful clinical strategy as well as an established pillar for the treatment of cancers to improving the prognosis of many cancer patients with a broad variety of solid tumors as well as blood cancers. The primary goals of immunotherapy are (a) to increase anti-tumor response, (b) decrease the immune suppression, and (c) to enhance the immunogenicity of tumors. This chapter aims to discuss the mechanism and different types of immunotherapies used for different cancers. It will also focus on recombinant products including immunostimulants, immunotoxins, antibodies, fusion proteins, engineered cytotoxic T cells, engineered immunocytokines, vaccines, checkpoint inhibitors, CAR T-cell therapy, and nanomedicine. Although immunotherapy has a rare side effect, it is not fully understood. The development of new strategies has been on the clinical trial to enhance the benefit of cancer patients to meet with challenges of limited efficacy and/or toxicity.

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INTRODUCTION

Immunotherapy used to enhance the power of the host immune system for the treatment of malignancies. It has become a powerful clinical strategy for treating and improving the prognosis of many solid and hematological cancer patients. The primary goal of immunotherapy is (a) to increase anti-tumor response, (b) decrease the immune suppression (c), and to enhance the immunogenicity of tumors. Cancer immunotherapy uses recombinant products, including immunostimulants, immunotoxins, monoclonal antibodies, fusion proteins, engineered cytotoxic T cells, engineered immunocytokines, vaccines, checkpoint inhibitors, CAR-T cell therapy, and the nanomedicine. Although immunotherapy has a rare side effect, however, it is not fully understood. The expansion of new strategies has been on the clinical trial to enhance cancer patients' benefit to meet with challenges of limited efficacy and toxicity.

Brief History of Cancer Immunotherapy

Cancer immunotherapy approval started in 1986 with interferon-α2a (IFN-α2a) and IFN-α2b for hairy cell leukemia, Kaposi sarcoma, and other hematological malignancies. In 2012, food and drug administration (FDA) approved Aflibercept due to its use in combination with a chemotherapy regimen (consists of 5-fluorouracil, leucovorin as well as irinotecan) for the metastatic colorectal cancer treatment. New immunotherapy called chimeric antigen receptor-T (CAR-T) cells licensed since 2017, outside clinical trials. The CAR-T cells found with very potent antitumor activity listed in Table 1.

Table 1. CAR-T cells clinical trials

Clinical trial	Patient group	Response rate	Complete remission rate	Overall survival	Reference
ELIANA (Novartis)	Children & young adults with relapsed and refractory B-ALL	81%	81%	Median survival 19.1 months	Maude SL et al., 2018
MSKCC	Adults with relapsed B-ALL	--	83%	Median survival 12.9 months	Park JH et al., 2018
ZUMA-1 (Kite Pharma)	Adults with refractory large B-cell Lymphoma	82%	54%	52% at 18 months	Neelapu SS et al., 2017
JULIET (Novartis)	Adults with relapsed DLBCL or Follicular Lymphoma	64%	43% DLBCL 71% follicular lymphoma	DLBCL median survival 22.2 moths, follicular lymphoma not reached	Schuster SJ et al., 2017
CRB-401 (Celgene/ Bluebird)	Relapsed and refractory multiple myeloma	89%	22%	Not available	Berdeja JG et al., 2017

The Nobel Prize in Chemistry, 2018, awarded jointly to George P. Smith and Gregory P. Winter for the discovery of "phage display of peptides and antibodies," and the other half of Nobel Prize to Frances H. Arnold for the "directed evolution of enzymes." Their pioneer work together utilizes the processes of evolution for the creation of novel biological compounds. These tools transformed the production of pharmaceuticals, such as monoclonal antibodies (mAbs) and renewable fuels. (The Nobel Prize in Chemistry 2018, Frances H. Arnold, George P. Smith, Sir Gregory P. Winter)

Monoclonal Antibodies (mAbs) as Cancer Immunotherapy

Antibodies that are produced by identical, single, unique clones of immune cells (B-cells) fusing them with an immortal myeloma cell, to form hybridoma cell lines (Hybridoma Technology). In 1975 Köhler and Milstein used hybridomas to generate mAbs. The most common methods for production of mAb *in vivo* considered as Hybridoma technology (Zaroff & Tan, 2019). The use of hybridoma technology makes an *In vivo* diagnostic that is an invasive diagnostics tool into noninvasive for diagnosing disease progression through the analysis of biomarkers without using biologic samples.

The antibody-based diagnostics *in vivo* used for very high specified imaging technology such as magnetic resonance imaging (MRI), positron emission tomography (PET), ultrasound, and (FMT) fluorescent molecular tomography (Bannas et al., 2015; Sohn et al., 2015). A tagged antibody targets a precise location for diagnostic imaging. This idea of conjugating a full-length antibody or an antibody fragment to a nanoparticle, be it a radioisotope, fluorophore, or positron emitter, would not have been possible without hybridoma-based antibody discovery. It was only through hybridoma technology that fully natural mAb variable domains that did not adversely impact a patient's immune system during an examination discovered.

The mAbs used not only for the diagnostics but also as therapeutics because of extremely high affinity can specifically bind to the target cells. Due to the property of high specificity and affinity, scientists began investigating the therapeutic potential of mAbs as immunomodulators, inhibitors, and metabolic activators. The therapeutic antibodies established as 'standard of care' agents for several human cancers (Table 2). The mAbs possess unique and multiple clinically relevant antitumor mechanisms, including antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, and the induction of T cell immunity through cross-presentation. Antibodies mediated targeting the tumour microenvironment components, and tumour antigens can provide synergistic and enhanced therapeutic benefits. The IgG Fc receptors (FcγRs) contribute an essential link between the cellular immune system and the therapeutic antibodies to warrant mAb to illicit adaptive immunity. Thus, antibodies alone or in combination with chemotherapy contribute the prolonged antitumor responses. An antitumor effect of mAb can utilize the synergistic effect when used in combination with other immunomodulatory approaches such as radiotherapy, chemotherapy targeted therapy agents, vaccines, or other immunomodulators.

Table 2. Recombinant Products for Cancer Therapy such as immunostimulant interferons, mAbs, immunotoxins, Funsion proteins and vaccines Approved by FDA (Pranchevicius and Vieira, 2013)

Approval year	Drug	Drug class	Therapeutic indications	Organism class	Strains
1986	IFN-α2a IFN-α2b	Immunostimulant-interferon Immunostimulant-interferon	Hairy cell leukemia, Kaposi sarcoma Hairy cell leukemia, Kaposi sarcoma, malignant melanoma, follicular lymphoma	Human Human	*Escherichia coli* *Escherichia coli*
1992	Aldesleukin	Immunostimulant-interleukin	Metastatic renal cell carcinoma, metastatic melanoma	Human	*Escherichia coli*
1997	Rituximab	mAb	Non-Hodgkin lymphoma and CLL	Chimeric murine/human	Mammalian cell (Chinese hamster ovary)
1998	Trastuzumab	mAb	Metastatic breast cancer, gastric cancer	Humanized	Mammalian cell (Chinese hamster ovary)

continues on following page

Table 2. Continued

Approval year	Drug	Drug class	Therapeutic indications	Organism class	Strains
1999	Denileukin diftitox	Immunotoxin	CTCL	Human	*Escherichia coli*
2000	Gemtuzumab ozogamicin	Antibody-conjugated	CD33-positive acute myeloid leukemia	Humanized	Mammalian cell
2001	Alemtuzumab	mAb	B-cell CLL	Humanized	Mammalian cell (Chinese hamster ovary)
2002	Yttrium-90 Ibritumomab Tiuxetan	Antibody-conjugated	Non-Hodgkin lymphoma	Murine	Mammalian cell (Chinese hamster ovary)
2003	Iodine-131 Tositumomab	mAb	CD20-positive, follicular, non-Hodgkin lymphoma	Murine	Mammalian cell
2004	Cetuximab Bevacizumab	mAb mAb	Metastatic colorectal cancer Metastatic colorectal cancer and HER2-negative metastatic breast cancer	Chimeric murine/human Humanized	Mammalian (murine myeloma) cell Mammalian cell (Chinese hamster ovary)
2006	Quadrivalent HPV Panitumumab	Vaccine mAb	Cervical, vulvar, vaginal and anal cancer caused by HPV 16 and 18, genital warts caused by HPV 6 and 11 Metastatic colorectal carcinoma	Viral Human	VLPs of the major capsid (L1) protein of HPV 6, 11,16 and 18 Mammalian cell (Chinese hamster ovary)
2009	Cervarix MEDI 501	Vaccine	Cervical cancer with HPV types 16 and 18	Viral	L1 protein of oncogenic HPV types 16 and 18, *Trichoplusia ni* insect cells
	Ofatumumab	mAb	CLL	Human	Recombinant murine cell line (NS0) using standard mammalian cell
2010	Sipuleucel-T	Vaccine	Castrate-resistant (hormone-refractory) prostate cancer	Human	Patient's peripheral blood mononuclear cells
2011	Ipilimumab	mAb	Unresectable or metastatic melanoma	Human	Mammalian cell (Chinese hamster ovary)
	Brentuximab vedotin	Antibody-conjugated	Hodgkin lymphoma, systemic anaplastic large-cell lymphoma	Chimeric murine/human	Mammalian cell (Chinese hamster ovary)
2012	Pertuzumab	mAb	HER2-positive metastatic breast cancer	Humanized	Mammalian cell (Chinese hamster ovary)
	Ziv-aflibercept	Fusion protein	Metastatic colorectal cancer	Human	Mammalian cell (Chinese hamster ovary)

Conjugated mAbs (Immunotoxins) as Cancer Immunotherapy

The immunotoxins are antibodies conjugated with a toxin that is poison. Immunotoxins assemble from a fusion of a highly selective cell ligand known as a targeting moiety (it can be antibody or antibody-fragments, a carbohydrate antigen, a growth factor, or a tumor-related antigen) (Choudhary et al., 2011), and a toxin obtained from plants or human cells or microbial pathogens (bacteria, fungi) (Pennell, 2002). It works as a protein synthesis inhibitor because it binds to the targets, thereby internalizing the immunotoxins and destroying it. Immunotoxins considered potent agents (Kreitman, 2001). The Diphtheria toxins and Pseudomonas toxins are important bacterial toxins used to produce these immunotoxins (Madhumathi et al., 2012).

The therapeutic application is to effectively deliver the toxin to the undesirable cells, for instance, those infected by HIV-1, or participate in immunopathologic reactions, immunosuppression and explored for their

anticipation in cancer immunotherapy including metastatic ovarian carcinoma, melanoma; colorectal, and breast cancers; Hodgkin non-Hodgkin lymphoma; T cell lymphoma and B cell lymphoma. Interestingly the immunotoxins work as "magic bullets" and effectively spot the target destruction. However, it has become clear that most of these "bullets" are not as precise as desired and have serious side effects, such as vascular leak syndrome, aphasia, paresthesia, myalgia, neuropathy, encephalopathy, thrombocytopenia, renal insufficiency, liver destruction, proteinuria, hypoalbuminemia, hematuria, dyspnea, and tremors.

In the majority of cases, toxins themselves have proved quite immunogenic. The first immunotoxins, Gemtuzumab ozogamicin, were approved by the FDA for clinical use in 2000. Gemtuzumab is a monoclonal antibody formed when fusing the anti-CD33 antibody-gemtuzumab with ozogamicin, an anticancer agent. CD33 expressed in healthy hematopoietic stem cells and most leukemic blast cells, but the intensity reduces with stem cell maturation. When it bound the antigen receptor and internalized, and the active agent is released to kill the cell—the drug approved for the treatment of AML (acute myeloid leukemia) in above 60 years old patients. Currently, human IL-2 and Diphtheria toxin (truncated) have been approved by the FDA for the treatment of cutaneous T-cell lymphoma. Another toxin, having an anti-CD22 Fv and truncated Pseudomonas serotoxin, induced treatment of hairy-cell leukemia (Kreitman et al., 2001). Immunotoxins may be used against hematological malignancies and solid tumors but have a better response against the first because they are large enough to go across the tumor tissue. Furthermore, there are other problems in reaching major effectiveness: immunogenicity, toxicity (namely, cardiac toxicity and side effects of the digestive system), and the other molecular imbalance. Madhumathi, in 2012 and other researchers, has suggested using an immunosuppressant, the humanization of the components size reduction, and the removal of some toxin epitopes that are close to B cell epitopes to overcome these side effects. Currently, many clinical trials in phases I and II conducted to verify new immunotoxins' efficacy and security. Only one FDA-approved immunotoxin, called denileukin diftitox (Table 2), is a combination of an interleukin-2 and Diphtheria toxin fusion protein. The denileukin diftitox used for recurrent cutaneous T-cell lymphoma (CTCL) therapy; however, many trials testing are undergoing the treatment of other cancers (Choudhary, 2011). Denileukin diftitox performance limited because of its weak affinity for the IL-2 receptor, related to the absence of CD122 (Madhumathi, 2012) and denileukin, which can induce the development of a human antitoxin antibody response by the second treatment (Prince et al., 2010). Table 2.

Immunostimulants in Cancer Immunotherapy

Immunostimulant is the materials that can modulate the immune system by augmenting the function of one or more of the system's components. It is of two types (i) Specific immunostimulants called vaccines and (ii) Non-specific immunostimulants (general stimulants). Immunostimulants can stimulate the innate or/ and adaptive immune system for a potential immunotherapeutic response when the tumor causes its clinical manifestations. The first recombinant drugs developed were interferon-alpha2a (IFN-α2a) and interferon-α2b (IFN-α2b), and approved for use in 1986. The interferons (IFN-α, IFN-α2a, IFN-α2b) are the class of cytokines with multifunctional properties, which can induce pro-apoptotic gene expression, causing direct effects on cancer cells and inhibiting angiogenesis. IFN-induced antitumor immunity results by activating T cells and dendritic cells. The only type I interferons, and specifically IFN-α, have applications in anticancer therapy for the treatment of different blood cancers (Table 2). IFN-α is capable of promoting the differentiation of human monocytes into dendritic cells (DCs) that present cancer cell antigens to T cells, which triggers an immune response, thereby DC discovered as vaccines. The FDA

was approved the second recombinant drug IL-2 (aldesleukin), produced from Escherichia coli. IL-2 was used to treat metastatic renal cell carcinoma (RCC) and metastatic melanoma because it alleviates and stimulates T-cells Production and expansion (Table 2).

Fusion Proteins in Cancer Immunotherapy

The fusion proteins are small proteins often engineered from an extracellular receptor domain, and the Fc-portion of receptor immunoglobulin G (IgG) can quickly move to the tumor tissues. These proteins can work as inhibitors for one or more receptors, thereby inhibiting the EGFR signaling and stimulating the immune effector cell recruitment (Weidle, e al., 2012). A fusion protein, Aflibercept (brand name Eylea and Zaltrap), was approved in 2012 by the FDA; namely, Ziv-aflibercept used in combination therapy regimen, and chemotherapy consists of leucovorin, 5-fluorouracil, and irinotecan for advanced colorectal cancer (CRC) treatment. Aflibercept produced by Regeneron Pharmaceuticals and approved in Europe and the United States can bind with VEGF-A, VEGF-B, and P1GF (placenta growth factor), thereby inhibits the angiogenesis (Weidle 2012, Gaya, 2012), (Table 2).

Immunocytokines in Cancer Immunotherapy

The immune cytokines is a fusion protein of cytokine-antibody. It is one of the classical methods for targeted cytokine activity. It used for better efficacy and low toxicity of localized over systemic administration of therapeutic cytokines (Jackaman, 2003; Gutbrodt, 2012; van Horssen, 2006). Immunocytokines furnishes a different route for achieving localized cytokine action. It can utilize to target disease antigens through their antibody moieties to potentiate the effector functions by their cytokine constituent. The most commonly used cytokines, including IL-2, (interleukin) IL-7, IL-12, IL-15, GM-CSF, IFN-α, IFN-γ, and TNF-superfamily, have been used development of immune cytokines with potential for tumor immunotherapy (Chang 2009, Kontermann 2012). Interleukin-2 is part of the body's natural response to microbial infection, stimulates the proliferation of cytotoxic T-cells (CD8+) and NK cells, which both acts on tumor cells to stop tumor progression (Boyma et al., 2006). Because of its anti-tumorigenic activity, high dose IL-2 (HD) therapy approved for the treatment of and renal cancer and metastatic melanoma (Boyman, 2006; Atkins, 1999; Rosenberg, 1998). However, HD IL-2 therapy can cause an adverse effect called vascular leak syndrome (VLS). Symptoms arising from VLS can be life-threatening, making intensive patient management a requirement for the use of IL-2 immunotherapy (Rosenberg, 1998).

According (Kiefer and Neri review 2016), a bio-distribution study explains five significant types of the pharmacokinetic behavior of immunocytokines, these are as follows:

(a) immunocytokines which can be efficiently delivered at the tumor site by fusion to antibodies (e.g., IL2, IL4, IL6, IL10, IFNa, TNF) (23–28) (Gutbrodt et al., 2013; Hemmerle et al., 2014; Hess et al., 2014; Doll et al., 2013; Frey et al., 2011; Hemmerle et al., 2013).
(b) immunocytokines which can be efficiently delivered to the tumor in some immunocytokine format but not in others (e.g., IL12) (Gafner et al., 2006)
(c) immunocytokines which are trapped by receptors at low doses, but which regain tumor-targeting performance at higher doses (e.g., when a cognate receptor saturated in vivo (e.g., IFNg, GM-CSF) (Hemmerle et al., 2014; Kaspar et al., 2007).

(d) immunocytokines which are too negatively or positively charged, or only too significant, thus preventing efficient extravasation (e.g., VEGF164 vs. VEGF120 in the mouse) (Halin et al., 2002)
(e) immunocytokines which are extensively glycosylated and rapidly captured by the asialoglycoprotein receptor in the liver and, as a consequence, removed from circulation (e.g., IL9 produced in certain experimental conditions, B7 proteins) (Halin et al., 2002, Venetz et al., 2005).

The pro-inflammatory cytokines at the site of the disease can arbitrate various biological activities. For instance, IL2, IL12, and TNF payloads mediate a massive infiltration of leukocytes (mainly T cells and Natural Killer cells) into the tumor mass, which may be responsible for the therapeutic activity of the products (Carnemolla et al. 2002 Halin C, et al.,2002; Borsi et al. 2003; Halin et al. 2003). Some cytokines, such as IL2 and TNF trigger the endothelial cells at disease area, support and augment uptake of therapeutic agents within the tumor mass (Halin, et al. 2003; Hornick et al., 1999). The mechanism of antitumor activity of immunocytokine products can be challenging to prove, even depletion experiments in immunocompetent mice facilitate the task to assess the contribution of CD4+ T cells quantitatively, CD8+ T cells and NK cells (Zhu et al. 2015 et al., Hemmerle, 2014). Alternative views on the contribution of tumor-targeting to therapeutic activity have recently proposed for IgG-based immunocytokines (Tzeng et al., 2015). Various types of anti-cancer therapeutic agents to synergize with immunocytokine products, including external beam radiation (Rekers et al. 2015; Zegers et al. 2015; Rekers NH, 2015; van den Heuvel, et al., 2015) certain cytotoxic drugs (Hemmerle 2013, Borsi L, et al. 2002; Moschetta et al. 2012), immunological checkpoint inhibitors (Schwager et al., 2013), anti-cancer immunoglobulins are acting via antibody-dependent cell-mediated cytotoxicity (ADCC) mechanisms (Schliemann et al. 2009) and other immunocytokine products (Halin C, et al. 2003; Hemmerle T, Neri D 2014; Balza E, et al. 2010).

L19-TNF is a fusion protein, consisting of the L19 antibody in scFv format, fused to human TNF (a homotrimeric). The product well tolerated (up to 13 μg/Kg) in a monotherapy dose-escalation trial in which a Maximal Tolerated Dose was not established (Spitaleri et al. 2013). Currently, the product investigates in combination with doxorubicin for the treatment of patients with metastatic soft tissue sarcoma, based on robust preclinical and clinical findings (Hemmerle et al., 2013).

Checkpoint Inhibitors in Cancer Immunotherapy

Tumor immunotherapy using an immune checkpoint inhibitor is one of the immunotherapy types, which may elicit an immune response that causes inflammation to organs in the body. It blocks the checkpoint proteins from binding to their partner proteins, prevents the signal, and allowing the T cells to kill cancer cells (Figure 1). It is FDA approved now for the treatment of a broad range of cancers. (National Cancer Institute, 2019)

How Do The Checkpoint Inhibitors Function?

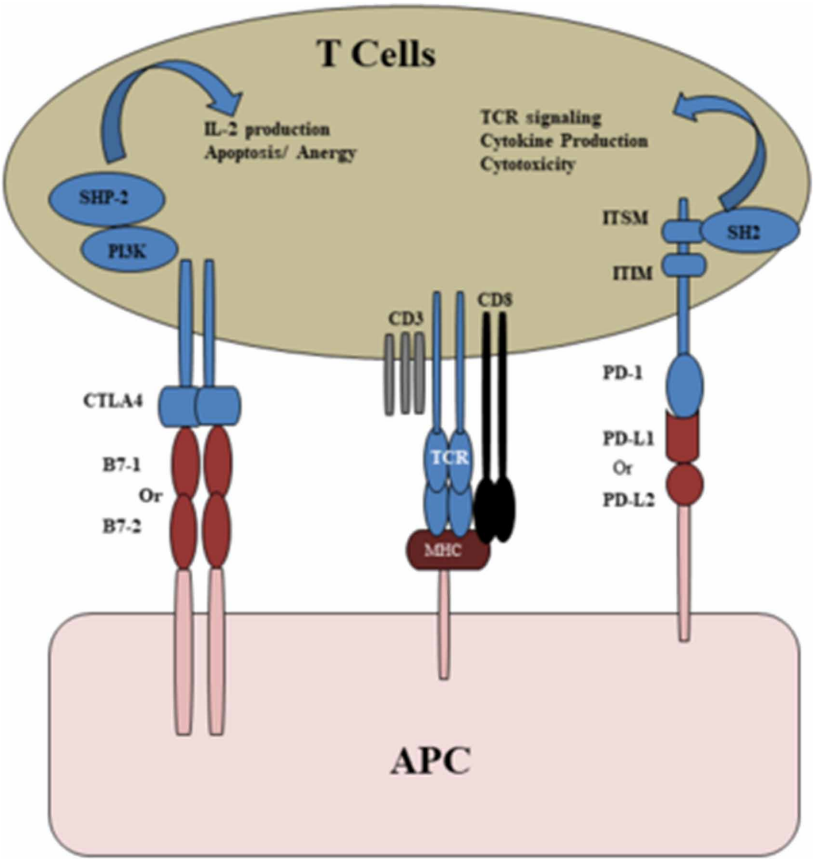
Immune checkpoints' functional role is to prevent an immune response from being so strong that it destroys healthy cells. The proteins present on the T cells recognize and bind to their ally proteins on the cancer cells. When the checkpoint and partner proteins interact together, send switch "off" signal to the T cells, prevent the immune system from smashing cancer. Some checkpoint inhibitor acts on checkpoint

protein, CTLA-4, and PD-1 and its ligand PD-L1. Few tumor cells decline the T lymphocyte reaction by producing a greater number of PD-L1 (Figure 2).

Which Cancers Treated With Immune Checkpoint Inhibitors?

Immune checkpoint inhibitors approved for treating some patients with a variety of cancer types, including Bladder, Breast, Cervical, Colon, Head and neck cancers, Hodgkin lymphoma, Liver cancer, Lung, RCC (Renal cell cancer), Skin, Stomach, and colorectal cancer. A summary of costimulatory and coinhibitory receptors, and the ligands, immunologic expression pattern, biological function, and molecular mechanisms presented. Molecular functions (i.e., downstream signaling) reflect predominant currently known mechanisms, but additional mechanisms are likely to contribute significantly (Figure 2).

Figure 1. Immune checkpoint blockade in hematologic malignancies; Interaction of T cells with antigen Presenting cells (APC)
(Adopted with modification from Armand et al., 2015).



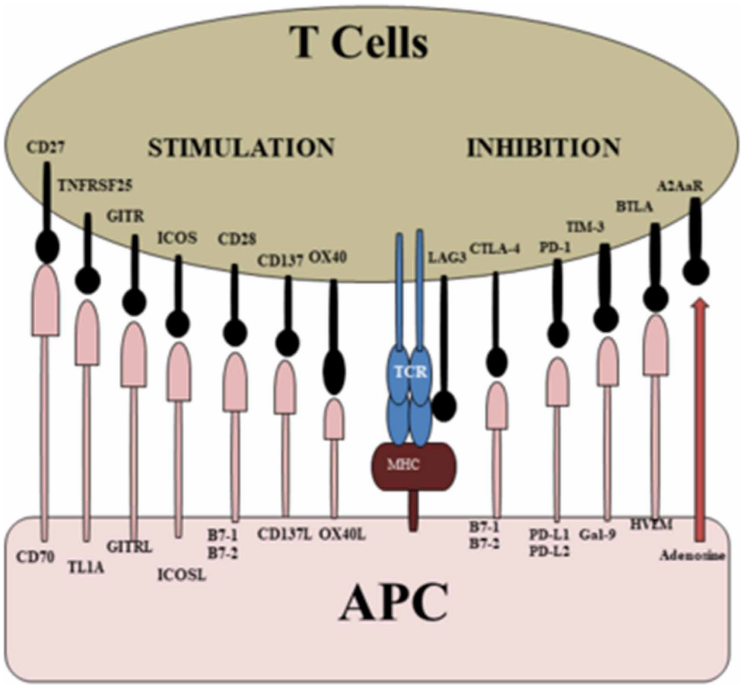
Common Side Effects of Immune Checkpoint Inhibitors

The adverse effect of the drug depends on health status before treatment and types of cancer and the dose receiving. According to the National Institute of Health (United States), the most common adverse effects of checkpoint inhibitors include Diarrhea, Rashes, and Fatigue; however, the rarer side effects are general inflammation that can be widespread in the whole body. Inflammation in the skins can lead to changes in skin color, rash, and feeling itchy. Similarly, inflammation in the lungs results in chest pains and cough. Abdominal pain and diarrhea can be caused by inflammation in the colon. The inflammatory diseases such as Diabetes, Hepatitis, Hypophysitis, Myocarditis, Nephritis, are caused by the inflammation in the pancreas, liver, pituitary gland, heart muscle, and kidney, respectively.

Details of Checkpoint Inhibitors

The FDA approved anti-CTLA4 (ipilimumab) in 2011, for the treatment of metastatic melanoma. Till now, an additional five checkpoint inhibitor therapies developed, attacking the PD-1/PD-L1, have approved for a broad range of tumor therapy listed in Table 2. Furthermore, anti–PD-1 (nivolumab) and ipilimumab combination therapy approved for the treatment of advanced melanoma with favorable outcomes compared with either monotherapy. The CTLA4 and PD-1 (negative costimulatory molecules) attenuate T-cell activation also learn the conceptual advancement and is related to the mechanisms of action of anti–PD-1 and anti-CTLA4 therapies in the context of antitumor immunity. (Table 3, Figure 2.)

Figure 2. Stimulatory and inhibitory co-receptor. A partial list of currently known stimulatory and inhibitory T-cell co-receptors are shown together with their cognate ligands.
HVEM: Herpes Virus Entry mediator



Mechanisms of CTLA4-Mediated Negative Costimulation

T-cell activation is linked with CTLA4 expression and immediately overexpressed after T-cell receptor (TCR) engagement (signal 1), with its expression reaching a peak 24 to 72 hours after (Walunas et al., 1994, Brunner et al., 1999). CTLA4 decreases TCR signaling by competing with the costimulatory molecule (CD28 for the B7 ligands B7-1 (CD80) and B7-2 (CD86) (Linsley 1994, Linsley 1991, van der Merwe 1999), both B7-1 and B7-2 impart positive costimulatory signals through CD28 (Lanier, 1995). signal 2), competitive inhibition of both B7-1 and B7-2 molecules by CTLA4 is essential to impair the T-cell activation effectively.

Table 3. The FDA-approved Checkpoint Inhibitor therapies used in different cancers (Wei et al., 2018)

Therapeutic agent	Cancer type	FDA approval year
Ipilimumab	Melanoma	2011
Nivolumab	Melanoma	2014
Pembrolizumab	Melanoma	2014
Nivolumab	Non–small cell lung cancer	2015
Pembrolizumab	Non–small cell lung cancer	2015
Ipilimumab + nivolumab	Melanoma (BRAF wild-type)	2015
Ipilimumab	Melanoma (adjuvant)	2015
Nivolumab	Renal cell carcinoma	2015
Nivolumab	Hodgkin lymphoma	2016
Atezolizumab	Urothelial carcinoma	2016
Nivolumab	Head and neck squamous cell carcinoma	2016
Pembrolizumab	Head and neck squamous cell carcinoma	2016
Ipilimumab + nivolumab	Melanoma (any BRAF status)	2016
Atezolizumab	Non–small cell lung cancer	2016
Pembrolizumab	Hodgkin lymphoma	2017
Avelumab	Merkel cell carcinoma	2017
Avelumab	Urothelial carcinoma	2017
Durvalumab	Urothelial carcinoma	2017
Nivolumab	Urothelial carcinoma	2017
Pembrolizumab	Urothelial carcinoma	2017
Pembrolizumab	MSI-high or MMR-deficient solid tumors of any histology	2017
Nivolumab	MSI-high, MMR-deficient metastatic colorectal cancer	2017
Ipilimumab	Pediatric melanoma	2017
Nivolumab	Hepatocellular carcinoma	2017
Pembrolizumab	Gastric and gastroesophageal carcinoma	2017
Durvalumab	Non–small cell lung cancer	2018
Ipilimumab + nivolumab	Renal cell carcinoma	2018

At the immunologic synapse, CTLA4 is stabilized by B7 ligand binding, allowing it to accumulate and effectively outcompete CD28 (Pentcheva-Hoang, 2004). The CTLA4 attenuates positive costimulation by CD28 and thus limits CD28 downstream signaling, which is primarily mediated by PI3K and AKT (Kane, 2001, Pages 1994), results in strong regulation of TCR signal amplitude and, thus, T-cell activity. The scientist has reported biallelic genetic deletion of Ctla4 leads to massive lymphoproliferation that mice succumb to death at 3 to 4 weeks of age (Chambers et al., 1997, Waterhouse et al., 1995, Tivol et al., 1995), as its central role in regulating T-cell activation, negative costimulation by CTLA4 is critical for tolerance.

The cell-extrinsic suppressive function of CTLA4 mainly mediated through Tregs (Friedline 2009, Read, 2006). Therefore, specific loss of CTLA4 in Tregs is sufficient to induce aberrant T-cell activation and give rise to autoimmunity (Wing et al., 2008; Jain et al., 2010), indicates that Treg-derived CTLA4 is very important to maintain immunologic tolerance, although it is unlikely that Treg-derived CTLA4 is enough to maintain T cell-mediated tolerance. Refer to Table 4 and Figure 1 and 2. CTLA4 expression mechanism by Treg cells may attenuate T-cell activation in a cell-extrinsic manner by limiting the availability of the B7 ligands B7-1 and B7-2 for CD28-mediated positive costimulation of nearby effector T cells. CTLA4 also has cell-extrinsic contributions within the effector compartment. CTLA4 expressed by effector T cells can compete for B7 ligands in trans (Corse et al., 2012).

Mechanisms of PD-1–Mediated Attenuation of T-Cell Activity

The primary functions of PD-1 are to maintain peripheral tolerance and maintain T-cell responses within a desired physiologic range. PD-1 regulates the activation of T-cells by interacting with PD-L1 and PD-L2 (Latchman et al., 2001; Freeman et al., 2000; Dong et al., 1995). Importantly, PD-1 expressed upon activation of B and T lymphocytes (Agata 1995). Further, PD-1 ligands expression is widely present in the nonlymphoid tissues; PD-1 acts primarily to diminish the T-cell activation. In response to inflammatory cytokines, IFNγ, the PD-L1 gets overexpressed and to a lesser degree of PD-L2. Hence, PD-1 regulation of T-cell activity occurs in response to cytolytic and effector T-cell function in an inducible manner. This molecular mechanism reflects a contrast in the regulation approach utilized by the involvement of CTLA4 and PD-1 (Table 4). This report suggests the PD-1 directly regulates TCR signaling to attenuate T-cell activity. However, recent evidence indicates that CD28 is a primary target for PD-1–induced attenuation of T-cell signaling (Hui et al., 2017). Functionally, PD-1 is crucial for homeostasis of peripheral tolerance, as suggested by the autoimmune disease that develops on the genetic deletion of Pdcd1 genes, which encodes for PD-1 proteins. The loss of Pdcd1 gene leads to the induction of autoimmune disease (lupus-like) dilated cardiomyopathy in BALB/c mice and aged C57BL/6 mice autoimmune (Nishimura et al., 1999, 2001). Recent studies evident new functional roles for the PD-1/PD-L1 signaling. For instance, macrophage expression of PD-L1 may lead to ongoing eviction of T cells from the tumor microenvironment (Kortlever et al., 2017), suggests that PD-1 signaling may regulate T-cell trafficking and migration and tumor cell-intrinsic function (Kleffe et al., 2015). Future studies are needed to study the degree to which such mechanisms contribute to therapeutic efficacy noncanonically.

Table 4. Summary of the biological and molecular functions of T-cell Costimulatory molecules (Wei, et al., Review 2018)

Molecule	Ligand(s)	Receptor expression pattern	Biological function	Molecular function	References
Coinhibitor					
CTLA4	B7-1 (CD80), B7-2 (CD86)	Activated T cells, Treg	Negative T-cell costimulation (primarily at priming); prevent tonic signaling and/or attenuate high-affinity clones	Competitive inhibition of CD28 costimulation (binding of B7-1 and B7-2)	(33-34), 36, 54, 145; (147- 149)
PD-1	PD-L1, PD-L2	Activated T cells, NK cells, NKT cells, B cells, macrophages, subsets of DC; as a result of inflammation	Negative T-cell costimulation (primarily in periphery); attenuate peripheral activity, preserve T-cell function in the context of chronic antigen	Attenuate proximal TCR signaling, attenuate CD28 signaling	(150; 50- 55) ; (154 -159)
PD-L1	PD-1, B7-1 (CD80)	Inducible in DC, monocytes, macrophages, mast cells, T cells, B cells, NK cells	Attenuate T-cell activity in inflamed peripheral tissues	PD-1 ligation; cell-intrinsic mechanism unclear	(50- 51; 160)
LAG3	MHC-II, LSECtin	Activated CD4 and CD8 T cells, NK cells, Treg	Negative regulator of T-cell expansion; control T-cell homeostasis; DC activation	Competitive binding to MHC-II; proximal LSECtin mechanism unknown	161 - 167)
TIM3	Galectin-9, PtdSer, HMGB1, CEACAM-1	Th1 CD4 and Tc1 CD8, Treg, DC, NK cells, monocytes	Negative regulation of Type 1 immunity; maintain peripheral tolerance	Negative regulation of proximal TCR components; differences between ligands unclear	(87; 168-171)
TIGIT	PVR (CD155), PVRL2 (CD112)	CD4 and CD8, Treg, TFH, NK cells	Negative regulation of T-cell activity; DC tolerization	Competitive inhibition of DNAM1 (CD226) costimulation (binding of PVR), binding of DNAM1 in *cis*; cell-intrinsic ITIM-negative signaling	144, 145, 172–176)
VISTA	Counter-receptor unknown	T cells and activated Treg, myeloid cells, mature APC	Negative regulation of T-cell activity; suppression of CD4 T cells	Increase threshold for TCR signaling, induce FOXP3 synthesis; proximal signaling unknown	(140, 141, 146, 147, 177, 178)
Costimulatory					
ICOS	ICOSL	Activated T cells, B cells, ILC2	Positive costimulation; Type I and II immune responses; Treg maintenance; TFH differentiation	p50 PI3K recruitment (AKT signaling); enhance calcium signaling (PLCγ)	(179–186)
OX40	OX40L	Activated T cells, Treg, NK cells, NKT cells, neutrophils	Sustain and enhance CD4 T-cell responses; role in CD8 T cells and Tregs	Regulation of BCL2/XL (survival); enhance PI3K/AKT signaling	(187–193)
GITR	GITRL	Activated T cells, Treg, B cells, NK cells, macrophages	Inhibition of Tregs; costimulation of activated T cells, NK cell activation	Signal through TRAF5	(194–200)
4-1BB (CD137)	4-1BBL	Activated T cells, Treg, NK cells, monocytes, DC, B cells	Positive T-cell costimulation; DC activation	Signal through TRAF1, TRAF2	(201–205)
CD40	CD40L	APCs, B cells, monocytes, nonhematopoietic cells (e.g., fibroblasts, endothelial cells)	APC licensing	Signal through TRAF2, 3, 5, 6; TRAF-independent mechanisms?	(206–209)
CD27	CD70	CD4 and CD8 T cells, B cells, NK cells	Lymphocyte and NK cell costimulation; generation of T-cell memory	Signal through TRAF2, TRAF5	(210–214)

Chimeric Antigen Receptor T Cells (CAR-T) in Cancer Immunotherapy

The next generation of T cell therapy took the form of autologous T cells derived from the patient and harvested from tumor sites. These tumor-infiltrating lymphocytes (TILs) are stimulated in vitro and re-delivered to the patient to induce an endogenous anti-tumor immune response (Figure 4) This therapy's efficacy has limited, owing logistically to difficulty in T cell isolation from tumors, as well as difficulty generating sufficient active cytotoxic T cells from TIL harvests, perhaps due to T cell exhaustion induced by the tumor and tumor microenvironment (Wherry & Kurachi, 2015). The CAR-T cells concept first discovered by Eshhar (Gross et al., 1989). The CAR has both extracellular domain (from Mab) and intracellular domain CD3ζ (T-cell receptor component for downstream signaling pathways) and a costimulatory domain (usually 4-1BB or CD28) that allows the T-cells to have sustained antitumor activity. It is now globally revolutionizing for haemato-oncology patients treatment, with the first two CAR-T cell products licensed by the FDA in 2017 (Listed in Table 1)

CAR-T cell therapy is one of the most promising immunotherapy techniques of cancer treatment involving Chimeric Antigen Receptor T cell. It is a cell-based technique used in personalized tumor therapy using the patient's white blood cells such as T cells (autologous T-cells) specific for a tumor antigen after ex vivo modification and expansion infuse to the same patients (Kochenderfer et al., 2010). CAR-T therapy mainly use in clinical trials now (Table 1). The CAR-T-cells are supposed to keep working for years, so cancer shouldn't come back. Nevertheless, some experts say it's too early to know if that will happen.

How to make CAR-T cells? (Illustrated in figure 3)

a) First, collect the T cells from the cancer patients
b) Treat the T cells in the laboratory and modify it genetically
c) Make special receptors called CARs that are chimeric antigen receptors
d) Now CARs allow the T cells to recognize an antigen (or marker) at the surface of cancer cells and activate T cells.
e) Now, this chimeric T cells to kill these cancer cells.
f) Then infused back into the patient's blood.
g) These CAR-T cells can recognize and destroy certain cancers.

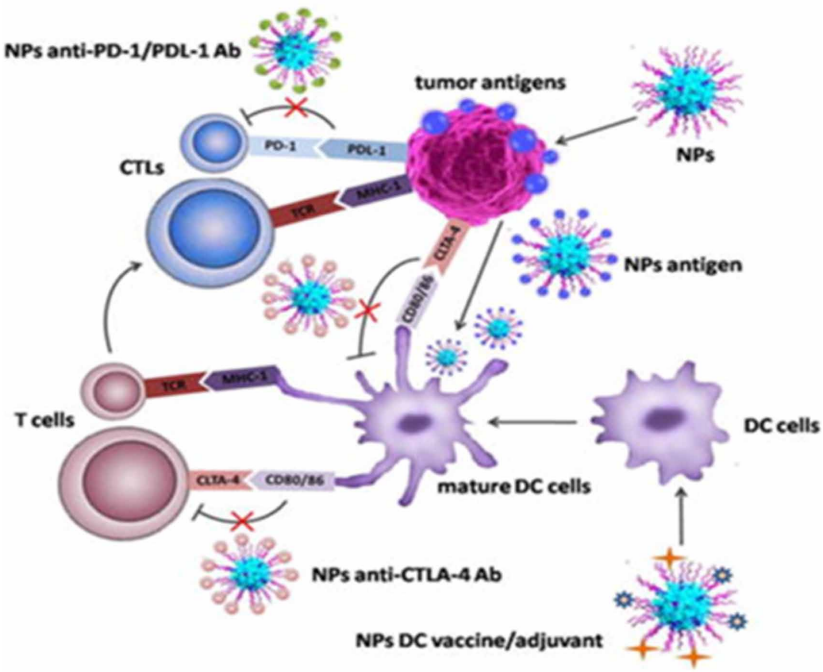
The progression of cancer recognition and immunity typically cycles through the release of TAAs, presentation of TAAs by APCs, priming of T cells by activated APCs, migration of T cells back to the tumor, killing of tumor cells by T cells, and release of more TAAs. NP approaches to cance immunotherapy should focus on improving the progression of these steps via delivery of antigen and immune modulators that increase response and reduce immunosuppressive mechanism.

Steps for CAR-T Cells Preparation and Treatment

This technique is simple, but its completion required a few hours steps listed in (Figure 3)

Collection of T-cell: A particular machine is required to collect T cells from the patient's blood. The separation of WBC from the whole blood is called leukapheresis (a particular type of apheresis). In this case, two iv.(intravenous) lines used in the arms veins. One iv. carries blood to the machine, and the other returns blood to the body.

Figure 3. Cancer immunity cycle



Step 1: Patients can lie on the bed or sit in a reclining chair. Patients can relax or can do some work like listen to music, read, work on computers.

Step 2: T-cell modification. Now the separated WBC containing T-cells can be transferred to a laboratory where a new gene added, which makes the cells sprout, unique surface proteins. The chimeric antigen receptors, or CARs, allow the T cells to spot and attach to antigens on tumor cells. The lab grows of millions or billions of these new cells. The new cells now called CAR T cells. The procedure usually takes a few weeks, though the time can be different for each person.

Step 3: Treatment with Low-dose chemotherapy. Patients may get a low dose of chemotherapy for a few days to cut back on other immune cells while waiting for the CAR-T cells to grow. This chemotherapy can reduce the spread of tumor cells.

Step 4: Infusion. The frozen CAR T cells can ship to the hospital or cancer center where patients treated. It is like a blood transfusion technique; CAR-T cells put back in the patient's vein in the arm through an iv.

The CAR-T cells will do a better job finding cancer. Furthermore, once they start attacking tumors, CAR-T can multiply so that more cancer cells targeted.

Step 5: Recovery. Two to three months needed to recover from CAR T therapy.

After discharge from the hospital, patients advised staying near the hospital for at least the first month so the treating doctors could watch for side effects and complications. Patients gradually recover, may feel very tired, and less desire for food. It needs to ease back into a healthy life slowly.

Why CAR-T Cell Therapy?

CARs are fusion proteins of one or more T-cell receptor intracellular signaling domains and selected a single-chain fragment variable domain from a specific monoclonal antibody. Now, genetic modification of this T-cell may be carried out either via viral-based gene transfer methods or nonviral methods, for instance, a direct transfer of transcribed-mRNA in vitro by electroporation or CRISPR/Cas9 methods. The FDA approved CAR-T therapy in the year 2017. It approved for the treatment of acute lymphocytic leukemia (ALL) in kids and young adults and certain types of adult non-Hodgkin's lymphoma. The clinical trials are going on for the testing of other types of blood cancer. This therapy is different from stem cell therapy or other cancer therapies. Cancer patients who have a history of relapsed or refractory may be eligible for CAR-T therapy (Table. 3)

CAR-T therapy used for the treatment option of:

1. Relapsed, refractory B-cell acute lymphoblastic leukemia
2. Relapsed, refractory B-cell non-Hodgkin's lymphoma
3. Other types of cancers and medical conditions

Conditions for CAR-T Therapy

These are FDA-approved conditions for CAR-T cell therapy:

i) B-cell precursor acute lymphoblastic leukemia (ALL), in people up to 25 years of age
ii) Diffuse large B-cell lymphoma (DLBCL)
iii) Primary mediastinal large B-cell lymphoma
iv) Large B-cell lymphoma transformed from follicular lymphoma
v) High-grade B-cell lymphoma
vi) Aggressive B-cell lymphoma not otherwise specified (NOS)

Surgery, chemotherapy, radiotherapy (chemo-radio), and stem cell therapy for hematological cancer are always the first choices of cancer treatments. However, if they fail to respond after at least two tries, or cancer relapses after treatment, CAR T may be an option for few patients, it could be the last chance for survival and cure. During the body's immune surveillance system, occasionally, T cells fail to recognize and act on the tumor cells because it's too much like healthy cells or fails to launch a full-on attack, which allows cancer to grow unlimitedly. That's where CAR T comes in. It powers up the immune system by adding a specific receptor, so it's easier for T cells to find and latch onto cancer cells. CAR-T is known as autologous immunotherapy because it uses the body's immune system, and therefore, a donor is not required.

Possible Side Effects CAR-T Therapy

Side effects are generally reversible. Most of these side effects go away, but they can be life-threatening for some people. CAR-T cell infusion requires the patients to stay in the hospital for days to weeks to monitor and manage side effects. Typically, the reaction happens within hours to days after the infusion. CAR-T is affecting the immune system as well as other changes in the body.

Side effects may include:

i) Cytokine release syndrome (CRS): In many clinical trials of the licensed products, 57–97% of patients found with CRS (Maude et al., 2018; Neelapu et al., 2017; Schuster et al., 2017), which leads to 47% patient admission in intensive therapy unit (ITU) (Maude et al., 2018).

The CRS causes fever, fast heart rate, low blood pressure, and low blood oxygen and breathlessness. When CAR T-cells start attacking tumor cells and elicit an immune response in the patients. Hence, for some people, CRS may feel like a bad case of flu. CRS can be treated by prescribing an arthritis medicine named tocilizumab (Actemra).

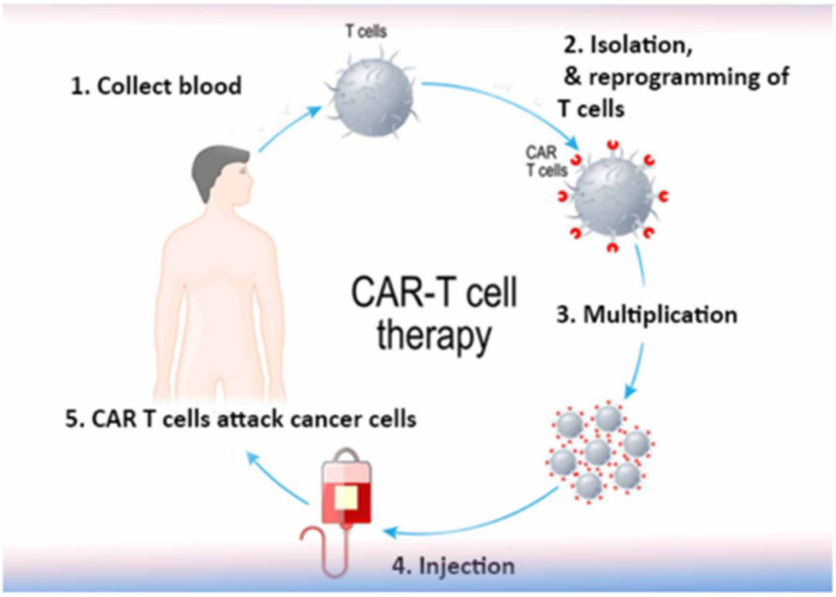
ii) Low blood counts from the conditioning chemotherapy
iii) Neurologic effects known as neurotoxicity, which can cause confusion, tremors, or difficulty with communication. It usually happens in the first two months after CAR-T infusion
iv). Serious infection. CAR T cells can also kill the B cells, resulting in a decrease in fighting with germs and foreign invaders, therefore, more likely to get serious infections.
v) Development of new cancer. After CAR T therapy, the patient may get a new type of cancer, or old cancer might reoccur. So, Doctors should watch for signs of disease for the rest of their life. The long-term toxicity of CAR-T cell therapy still needs to study. Discuss with the clinician about the potential risks of treatment.

Enhancing Cancer Immunotherapy With Nanomedicine

Nanomedicine is the therapeutics composed of or formulated in carrier materials typically smaller than 100 nm. Nanomedicine originally developed was to increase the uptake of chemotherapy agents by tumors and to reduce their off-target toxicity. Many studies found that a nanoparticle (NP) linked with chemotherapeutic drugs some encouraging result, many NP approved for the treatment of different cancer types (Laprise-Pelletier, 2018, Figure 4). The conceptualization of chemotherapeutics into a nanoparticle has several advantages over the standard chemotherapy. The sustained bioavailability of a low molecular weight drug is increased by its formulation in the nanoparticle, whereas a nanosized drug carrier minimizes their elimination through the liver or kidney (Matsumura, 2008). The permeability and accumulation of chemotherapeutical nanoparticle are passively targeting the tumor tissue resulting in low systemic toxicity and increased drug concentration inside more than the treatment with standard chemotherapy (Maeda et al., 2000).

To date, a variety of NP structures have been used as vehicles to deliver a broad spectrum of molecular cargos, stabilize their cargoes' biological activity, increase cargoes' solubility in biological fluids, and reduce systemic side effects. Indeed, several NP-based formulations delivering cytotoxic drugs have proven successful in the clinic (Shao, 2015). Thus, NPs provide ideal immunotherapy delivering candidates to overcome the associated challenges. Nanoparticles improve the pharmacokinetics and biodistribution of their cargo, which can reduce side effects. General approaches for improving NP pharmacokinetics and biodistribution include maintaining the size around 100 nm, keeping the Zeta-potential within ten mV, and grafting PEG onto the particles' surface (Wilhelm et al., 2016). Besides, NPs can target and stimulate the immune system, thereby producing cytokines that mediate humoral and cellular immunity.

Figure 4. Steps in CAR-T cell therapy; 1. Collection of Blood, 2. Isolation and reprogramming of T cells, 3. Multiplication, 4. Injection, 5. CAR T function



Today, a variety of NPs, including VLPs, cationic liposomes, dendrimers, micelles, gold NPs, are used for cancer vaccination and immunomodulator delivery. (Figure 4)

Activation of Dendritic Cells (DCs) Through NPs

DCs are the immune system's professional APCs capable of promulgating a host of antigen-specific immune responses against pathogens. Thus, immunotherapeutic strategies utilize DCs to present antigens as a means of cell-mediated therapeutic vaccination in individuals with advanced malignancies (Anguille et al., 20014). In this strategy, DCs are trained ex vivo with antigens then adoptively transferred back into patients for vaccination. Despite demonstrating an increase in antigen-specific CTL responses after immunization at metastatic tumor sites, this method still lacks examples of clinical therapeutic effectiveness in many advanced tumors (Anguille, et al., 2014). Furthermore, this strategy can prove technically challenging and expensive (Liu, & Irvine, 2015; Palucka &Banchereau, 2012; Sehgal et al., 2014; Cruz et al., 2012). Therefore, in situ DC targeting with antigen and adjuvant laden NPs loaded with antigens and adjuvants may significantly improve the clinical applications of DC-mediated immunotherapies (Sehgal, et al., 2014).

DCs in Vivo

Maji et al. (2016) investigated the role of cationic liposomes on the maturation and antigen presentation capacity of DCs. They found that cationic liposomes were taken up more efficiently by DCs and transported to different cellular sites for MHC processing than anionic liposomes and neutral liposomes. Rietscher et al. evaluated the use of hydrophilic polyethyleneglycol (PEG)-b-PAGE-b-poly (lactic-co-

glycolic acid) (PPP) as a platform for prophylactic vaccination (Rietscher et al., 2016). When PPPs loaded with model ovalbumin (OVA) antigen, they found that T cell activation by APCs significantly increased in vitro compared to delivering the free and soluble OVA antigen.

DCs Targeting

The TME has several unique physiological characteristics that complicate immunotherapy, including irregular vascularization, hypoxic conditions, low extracellular pH, and increases in proteolytic activity (Estrella et al., 2012). Furthermore, the TME can produce an immunosuppressive environment by releasing soluble cytokine mediators and attracting immune suppressive cell types, such as tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and myeloid-derived suppressive cells (MDSCs) (Dunn et al., 2004). These features of TME are associated with treatment resistance and poor clinical prognosis. Therefore, new cancer immunotherapeutic approaches demand control over the TME to reverse the immunosuppressive conditions. Using NPs to target immunosuppressive cells in the TME offers a promising strategy to eliminate this tumor-induced immunosuppression.

Virus-Like Particles (VLPs)

Virus-like particles (VLPs) are highly versatile NPs (20–100 nm) derived from viruses that cannot replicate (Buonaguro et al., 2011). VLPs can be easily engineered via site-directed mutagenesis or bioconjugation to load immunogenic ligands, target immune cells, or augment vaccine efficacy (Smith, 2013). Today, the majority of VLP-based vaccines are in clinical development against viral pathogens, such as HIV and HPV (Zhang et al., 2015). However, Lizotte et al. recently demonstrated that inhalation of VLPs generated from cowpea mosaic virus (CPMV) reduced lung metastases of established B16F10 melanoma and generated potent systemic anti-tumor immunity against relatively non-immunogenic B16F10 in the skin (Lizotte et al., 2016). These VLPs also promoted anti-tumor immune effects in ovarian, colon, and breast cancer models at various locations (e.g., subcutaneous, lung, mammary pad). Li et al., reported the recombinant bacteriophage MS2 VLPs, whose coat protein engineered to bind prostate acid phosphatase antigen mRNA via a 19-nucleotide RNA aptamer, induced robust immune responses and protected mice against prostate cancer challenge (Li et al. 2014).

Cationic Liposomes

Cationic liposomes have been used extensively as immunotherapy and vaccine delivery systems, especially for nucleic acids, to enable prolonged therapeutic and antigen delivery. (Bal et al., 2011). In Zhou et al.'s study, cationic liposomes complexed with CpG (CpG lipoplex) prevented the proliferation of tumor cells, prolonged the survival time of tumor-bearing mice induced higher IFN-γ production compared to naked CpG (Zhou et al., 2010). In another study, Mansourian et al. demonstrated that 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)-cholesterol-dioleoylphosphatidylethanolamine (DOPE) liposomes loaded with p5 antigenic peptide and CpG greatly enhanced CTL responses and inhibited tumor progression compared to soluble p5 and CpG30. Zaks et al. assessed the adjuvant vaccine effects of cationic liposomes complexed to TLR agonists in mice (Zaks et al., 2006). They found that cationic liposomes complexed to nucleic acids were particularly useful adjuvants for eliciting CD4+ and CD8+ T cell responses against peptide and protein antigens compared to control treatment.

Gold NPs

Gold NPs (Au NPs) readily phagocytosed by mononuclear cells (Ahn et al., 2014). As a result, Au NPs have several favorable characteristics as nanocarriers for antigen delivery. Jon Sangyong's laboratory reported that intramuscular administration of Au NP-based cancer vaccines could enable effective cancer prevention and treatment in vivo (Lee et al., 2012). In their study, a large proportion of injected Au NPs carrying antigens drained into the local LNs. Besides, Au NPs induced active humoral and cellular immunity against an endogenous TAA. Thus, Au NPs immune simulating effects could serve as vaccine platforms for cancer therapy without additional adjuvants. Almeida et al. demonstrated that Au NP delivery of OVA (Au NP-OVA) and of CpG (Au NP-CpG) enhanced the efficacy of both agents and induced strong antigen-specific responses (Almeida et al., 2015). Besides, Au NP-OVA delivery without CpG was sufficient to promote significant antigen-specific responses, subsequent anti-tumor activity, and prolonged survival in vivo tumor models (Almeida et al., 2015. The results again point to Au NPs as a possible self-adjuvant platform. Ma et al. synthesized SM5-1-conjugated Au NPs (Au-SM5-1 NPs) and investigated their anti-cancer efficacy in hepatocellular carcinoma. Compared with SM5-1 alone, Au-SM5-1 NPs significantly inhibited the tumor growth of both subcutaneous and orthotopic hepatocellular carcinoma tumor models (Ma et al., 2016).

The future clinical translation of NP-based approaches will undoubtedly require further optimization of nanostructure parameters, such as the stability, biodistribution, pharmacokinetics, toxicity of component compounds (e.g., cationic dendrimers), and size. However, the ever-increasing understanding of immunology and nanotechnology will undoubtedly engineer remarkable mechanisms to modulate immune responses.

Vaccines in Cancer Immunotherapy

Vaccines constitute active immunotherapy against cancer. Most vaccines aim to enhance an immune response against a tumor employing tumor antigen-specific cytotoxic T lymphocytes (CTLs) because these cells can directly kill malignant cells. (Eriksson, 2008). The vaccines researchers used several tumor antigens, including cell-surface molecules (proteins, peptides or lysates) and cells or autologous tumor cell lines or lysates of allogeneic (Dillman, 2011).

The ideal target for cancer vaccines is Tumor-specific antigens (TSAs) because these antigens are critical molecules for cancer progression and tumorigenesis. The tumor-associated antigens (TAAs) are not specific and can be found in tumors with the same histology and tumors of different origins and even in specific healthy cells. (Bele, 2012). TAAs trigger only a weak immunological response compared to TSA, due to self-antigen tolerance (Vergati, 2010).

Currently many cancer vaccines available including recombinant life (viral or bacterial) vector vaccines, nucleic acid vaccines (RNA or DNA or replicon), peptide vaccines, whole-cell vaccines (DC- or tumor cell-based), viral-like particle (VLP) vaccines, edible vaccines and combined approaches (e.g., prime-boost vaccination). (Bolhassani et al.,2009; Palena, 2006).

Certain vaccines autologous or allogeneic tumor cells removed by surgery and treated in the laboratory, generally using radiation (to avoid neoplasia formation). In some instances, the cells can modify by adding chemicals or new genes so that the immune system recognizes them as foreign, after which the cells injected into patients. However, tumor cell vaccines can potentially cause autoimmunity and

anergic status of T cells increased because of devoid of costimulatory molecules on the tumor cells. (Kumar, 2010).

Cancer vaccines can also develop using single proteins or combinations of proteins, including heat shock proteins, peptides, anti-idiotype antibodies, and fusion proteins. Other advantages of these vaccines are that their production, storage, and distribution easy and that their cost-effectiveness is higher than that of tumor cell-based vaccines (Vergati, 2010). Additionally, TSAs are preferable because these antigens can produce a more personalized immune response to the tumor cells. Nevertheless, the problem is, such vaccines can start an autoimmune reaction, therefore, the specific human leukocyte antigen limit the use of vaccines', Furthermore, the immunogenicity is weak in a single protein and the low capacity for evenly activating CD4 and CD8 receptors (Bele, 2012).

Vector-based vaccines develop to introduce recombinant genes (TAAs, cytokines, or costimulatory molecules) on different vectors such as viruses, bacteria, or yeast into antigen-presenting cells (APC). 11 The professional APCs can now elicit an immune response against the tumor cells. Also, the vectors have a lower cost of production than proteins or whole-tumor cell vaccines. Nevertheless, individual vectors can provoke an immunological reaction against themselves. The significant vectors in use are vaccinia, adenovirus, Saccharomyces cerevisiae, Salmonella, and Listeria monocytogenes. (Gulley, 2010).

DNA vaccines based on the capacity of vectors to transport DNA that encodes protein antigens and inserts them into immune cells, which instructs the cells on how to initiate the desired response against a tumor. DNA vaccine has several edges, for example, the possibility of mobilizing both the cell-mediated (CMI) and the antibody-mediated (humoral arms) immune response in the animals; more accessible and less expensive production than protein-based vaccines; and transgene expression that to happen over a long period, which avoids the repetitive booster vaccinations dose. However, in early clinical trials, DNA vaccines have not adequately induced a robust immune response, demonstrating low immunogenicity (Aldrich et al., 2010; Schlom, 2012).

Cancer vaccines may be two types (a) prophylactic cancer vaccines: in which cancer vaccines can prevent infection by oncolytic viruses. (b) therapeutic cancer vaccines: the development of cancer in high-risk individuals to treat existing cancer (Bolhassani et al., 2011). Many challenges in making a prophylactic vaccine are that there are several strains of the virus. Nevertheless, certain prophylactic vaccines, such as the human papillomavirus (HPV) vaccine, have already shown excellent results. These vaccines largely invented to perceive the etiologic carcinogenic agents, viz. HBV, which is responsible for HCC (hepatocellular carcinoma). The HPV vaccine, made of a recombinant L1 protein that forms a VLP, is strain-specific and intended to prevent approximately 70% of cervical cancer cases by preventing infection with just two oncogenic strains, HPV 16 and 18 (Armstrong, 2010). Examples of HPV-associated cancer vaccines are Cervarix MEDI 51 and the quadrivalent HPV vaccine. Moreover, the second is used to prevent genital warts caused by HPV 6 and 11 and is expressed in yeast, whereas Cervarix expressed in baculovirus (Bharadwaj, 2009). Therapeutic vaccines mainly aim to prime antigen-specific T cells and reprogram memory T cells, effectively transforming one type of immunity into another (e.g., regulatory to cytotoxic). (Palucka et al., 2011) Nevertheless, therapeutic vaccine engineering may encounter several barriers, including the incomplete knowledge of tumor physiopathology and the variable immune response to antigens. A therapeutic vaccine currently on the market is sipuleucel-T, designed to treat castrate-resistant (hormone-refractory) prostate cancer. This vaccine is composed of many types of leukocytes, such as monocytes, T and B lymphocytes, and macrophages; because of this complex composition, the vaccine's precise mechanism of action is unknown. (Dillman, 2011) Sipuleucel-T received FDA approval after 225 patients experiencing advanced metastatic androgen-independent prostate cancer survived ap-

proximately four months longer than the control group in a clinical trial. (Higano, 2009) The success of sipuleucel-T, despite the side effect of flulike symptoms and the expensive cost, can only be the start of a rise of success in the area of cancer vaccines (Nemunaitis, 2011).

Future Perspectives of Cancer Immunotherapy

The advancement in cancer therapy has been seen remarkably from 1980 to date, have given new faith for cancer patients with poor prognosis. These advancements were possible with the support of new technology like biotechnology, bioengineering as well as nanotechnology. In the beginning, hybridoma technology was developed then with progressive development in science and technology to produce many recombinant drugs with more specificity, effectiveness, and lower side effects. The first recombinant immunostimulants used was IFN-α, which augment the immune nonspecifically to kill the cancer cells. Further, IL-2 was being used as an adjuvant in cancer vaccines to enhance the effective response on cancer immunity.

Surgery, chemotherapy, radiotherapy (chemo-radio), and stem cell therapy for hematological cancer are always the first choices of cancer treatments. However, if they fail to respond after at least two tries, or cancer relapses after treatment, CAR T may be an option for few patients, it could be the last chance for survival and cure. These days, different antibodies and their fragments are available in the market due to high specificity and good clinical response, whether used singly or in combination with drugs, toxins or radionuclides to enhance the benefit of cancer patients like nanomedicine to meet with challenges of limited efficacy and significant toxicity.NP approaches to cancer immunotherapy should focus on improving the progression of these steps via the delivery of antigen and immune modulators that increase response and reduce immunosuppressive mechanisms. Nanomedicine is a unique advantage for drug-delivery vehicles for cancer therapeutics. For immuno-oncology applications, cancer nanomedicine can be developed beyond drug-delivery platforms. A greater emphasis on actively modulating host anticancer immunity using nanomaterials provides new avenues for developing novel cancer therapeutics.

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Chapter 2
Therapeutic Approaches to Employ Monoclonal Antibody for Cancer Treatment

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ABSTRACT

With the evolution of the tissue system and division of function among differentiated cells/tissues, the property of controlled cell growth also evolved in animals. It is when this very control is lost that cancers develop. The immune system's ability to distinguish between self and non-self is central to impeding cancer progression. However, cancer cells in time can develop multiple ways of escaping immune control. Even today, cancer remains a disease of baffling complexity on account of its diverse origin

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and pathogenesis. Classical methods like surgery, radiation, and chemotherapy have failed to make the cut as idyllic therapy, especially considering the encumbering side-effects and high failure rate. Alternative therapeutic strategies that exploit the immune system itself have proved promising. One of these is monoclonal antibody therapy. In this chapter, the relationship between the immune system and cancer and various forms of immunotherapy are discussed in detail.

CANCER: AN OVERVIEW

'Cancer' is a generic term for a large group of diseases typified by the unrestrained growth and spread of abnormal cells. This is the result of a multistage process involving the transformation of normal cells. A failure to contain the progression from a pre-cancerous lesion to a malignant tumour and its subsequent spread throughout the body can prove fatal. As of 2018, an estimated 9.6 million deaths were attributed to it, making it the second leading cause of death at the global level. Around 70% of these deaths occur in low- and middle-income countries; the reason being a lack of timely and quality diagnosis and treatment. [WHO, 2018]

Onset of cancer may be initiated by inherited or acquired genetic mutations, such as translocation, chromosomal gain/loss, or changes in glycosylation; or epigenetic alterations like DNA methylation. They occur in oncogenes and tumour suppressor genes, recognized as promoters and inhibitors of cell growth, respectively [Pinho & Reis, 2015; Sharma et al.; 2010; Akhavan-Niaki & Samadani, 2013]. Besides, many risk factors have also been identified, which may contribute to the occurrence and growth of cancer. These may be changeable or avoidable e.g. dietary and behavioural factors, infectious diseases; or are unchangeable-like immune deficiencies. [American Cancer Society]

Certain terminologies must be considered while dealing with the field of oncology. A mass of anomalous cell growth is called a 'tumour'. It is can be either 'benign' if localized at the site of origin (primary site), or 'malignant'. cancerous, with ability to migrate and invade other locations of the body (secondary site) [Lodish et al., 2000]. The latter is the result of 'metastases', and is the main cause of deaths linked to cancer [Chambers & Werb, 2015].

Metastases is a multi-step cascade whereby the cancer cells from lone solid tumours acquire distinct characteristics over time; thus, enabling their escape from the primary site, followed by dissemination through the circulation and finally, colonization of distant organs. [Chambers & Werb; 2015, Lambert et al., 2017; Gonzalez et al., 2018]. Metastases remains a significant hindrance in the treatment and complete cure of cancer [Weigelt et al., 2005]. The process is summarized as follows:

First, is the invasion of the local tissue at the primary tumour site by the metastatic cancer cells. These cells secrete enzymes like matrix metalloproteinases that degrade extracellular matrix proteins, allowing them to detach from the primary site. This is followed by intravasation into blood or lymph vessels. Here, only the cancer cells that survive migrate through the blood circulation or lymphatic flow using signalling mechanisms and reach distant secondary sites. The adaptation and proliferation of these cells requires sufficient supply of nutrients and oxygen, along with waste removal. This is afforded by the induction of angiogenesis, which is regulated by a wide variety of cytokines, interleukins and growth factors [Hanahan & Weinberg, 2011; van Zijl et al., 2011; Pantel & Brakenhoff, 2004; Reymond et al., 2013; Rundhaug J. E., 2003; Nishida et al., 2006; Blanpain C., 2013].

Cancers are classified based on site of origin. 'Carcinomas' and 'sarcomas' originate from the epithelium and mesenchyme respectively, while glands give rise to 'adenocarcinomas' [Blanpain C., 2013]. Those that arise in the bone marrow are called 'myelomas' or 'leukaemia's'; whereas those that are within the lymphatic system and found to have an effect on lymphoid organs are known as 'lymphomas' [Sehn L. H., 2015; Fermand et al., 1958].

In view of the variation in cancer pathophysiology and pathogenesis, consequently there exist differences in treatments.

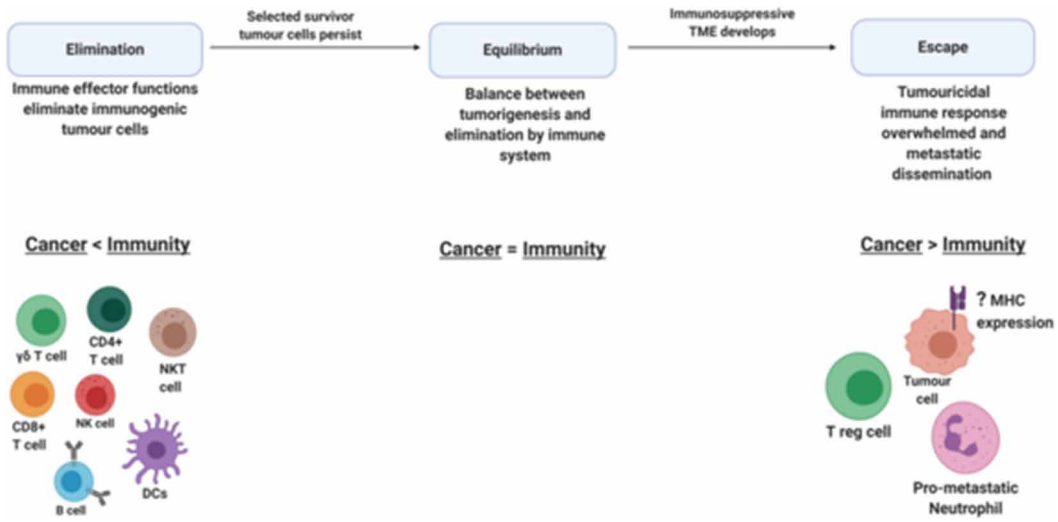
CANCER AND THE IMMUNE SYSTEM

The immune system is indispensable in the protection of an organism against infection and trauma; from aggressors of exogenous and endogenous origin [Candeias & Gaipl., 2016].It was Rudolph Virchow who first discovered the relation between the immune system and cancer in the 1860's [Adams et al., 2015]. In recent years it has become evident that both, the innate and adaptive immune system play a role in not only the curbing, but also as the promotion of cancer development.

The complex dynamic mechanism of 'immunoediting' maintains the balance between immune surveillance and cancer progression. It is principally composed of 3 phases that aid the cancer elimination, dormancy and escape respectively; namely, elimination, equilibrium, and escape [Schreiber et al., 2011].

Given that the purpose of this chapter is to enlighten one on the utilization of the immune system for cancer therapy, it is critical to first and foremost comprehend the immune system and its role in oncogenesis; which will be highlighted upon in this section (Fig. 1).

Figure 1. The process of immunoediting in cancer (Created with BioRender.com)



The Immune Surveillance and Control of Cancer

The immune system prevents tumour formation in various ways. It works toward prompt elimination of invading pathogens and resolution of the inflammatory response; this is because inflammation contributes to tumorigenesis. It also attempts to stem viral infections so as to prevent the formation of virus-induced tumours. Lastly, is 'immune surveillance', which is the ability of the immune system to specifically recognize and eliminate tumour/cancerous cells via the TAAs they express [Swann & Smyth, 2011].

The theory of cancer immunosurveillance by Burnet and Thomas hypothesized that the immune system can identify and terminate nascent transformed cells, thereby preventing neoplasia. However, this idea gained strong support only with the use of genetically modified organisms as experimental models; and immunosurveillance was recognized as a more generalized process in immunoediting. On the other hand, the protective function of the immune system against tumour development in humans particularly, was suggested by epidemiological studies involving transplant patients. They revealed that, those receiving immunosuppressive treatments, were more likely to develop cancer [Dunn et al., 2002, Dunn et al., 2004].

Direct immune-mediated tumour killing comprises two vital participants: CD8+ cytotoxic T cells of the adaptive immune system, and NK cells of the innate immune system. This mechanism has been found to act in the primary tumour [Corthay A., 2014] in addition to disseminated cancer cells.

Generally, host proteins are treated as 'self-antigens' by the immune system due to the normal state of 'immune tolerance'. But as tumour cells acquire a high number of mutations, they express mutated proteins i.e. neoantigens or TAAs, which are identified as 'non-self'. In addition to the direct alteration of the antigen itself, changes may occur in protein quantity, processivity and subsequent presentation. All in all, this favours recognition by the immune system, resulting in its activation and eventual killing of tumour cells. [Mellman et al., 2011; Chen & Mellman, 2013].

On the tumour cell surface, TAAs are presented with MHC. Once the TAA-MHCI-complex is recognized by the CD8+ T cells via their antigen-specific TCR, they get activated. These CD8+ T cells proliferate, and generate a pool of CTLs. The CTLs can identify the tumour cells expressing the TAAs and bring about their apoptosis through granzymes and perforin, or even by Fas receptor-ligand interaction. [Chen & Mellman, 2013; Janeway et al., 2001].

Nevertheless, the tumour cell recognition/killing ability of the CD8+ cytotoxic T cells warrants prior priming; via their recognition of TAAs presented by APCs like DCs i.e. appropriate co-stimulation must occur first. Thus, DCs present TAAs with MHC I which prime and activate CD8+ cytotoxic T cells; and also, present TAA-MHC II complexes to activate CD4+ helper T cells. Cytokines like IL-2 and IFNs are released by the activated CD4+ Th1 and Th2 helper T cells. Of these, particularly those secreted by the Th2 cells play a role in CTL activation and response [Chen & Mellman, 2013; Janeway et al., 2001].

In contrast to the above, NK cells do not recognize TAAs, and priming is of no necessity. Rather, via NCRs, NKG2D, CD16, DNAM1 etc. which are NK cell receptors, they directly identify molecules characteristically found on the tumour cells and other such stressed cells. For e.g. NKG2D binds to MICA/B that may be expressed on the tumour cell. Generally, the normal expression of MHC I on host cells makes them impervious to NK-mediated lysis; since they activate receptors on NK cells that inhibit NK cell mediated apoptosis. However, the down-regulated expression of MHC by tumour cells to evade immune recognition is identified as a 'missing-self' state by the NK cells. Then, NK-induced apoptosis takes place by any of these mechanisms: TNF-α induced release of perforin and granzymes; by

Fas receptor-ligand interaction; by ADCC due to Fc receptorCD16; or by cytokines released e.g. IFN-γ, which then facilitates APC activation and maturation. [Wu & Lanier, 2003; Waldhauer & Steinle, 2008].

IFN-γ signalling magnifies both specific immunity and general non-specific inflammatory response against cancer. IFN-γ signalling causes an upsurge in the expression of MHC I and stimulates MHC II expression on APCs. This increases their TAA presenting ability to T cells and consequently, an increased CTL cell mediated tumour killing. Besides, IFN-γ also broadly activates myeloid cell differentiation; as well as ROS production by macrophages, neutrophils and NK cells and their secretion of inflammatory cytokines. The latter enables recruitment of more innate and adaptive effector cells [Trinchieri & Perussia, 1985; Farrar & Schreiber, 1993; Ding et al., 1988].

Thus, we see it is imperative to note that the TAAs that arise during oncogenesis are central to the immune response against cancer.

Aside from the aforementioned, several other components of the innate and adaptive immunity also influence the various facets of tumour initiation, growth/ rejection and metastasis. These diverse interactions between the immune system and cancer cells are briefly described below.

Innate Immunity and Cancer

The innate immune system has the crucial task of attempting to mitigate cancer mediated inflammation, which furthers genomic instability, epigenetic modifications, proliferation and enhancement of anti-apoptotic pathways in cancer cells, angiogenesis, and ultimately metastases. It also plays a part in initiating the adaptive immune response. [Hanahan & Weinberg, 2011; Chen & Mellman, 2013; Dunn et al., 2006]

Complement proteins are able to recognize TAAs on the surface of cancer cells, subsequently allowing complement-mediated death [Pio et al., 2014].

Neutrophils have been found to both encourage and allay the progression of cancer. Their granules contain proteases that facilitate metastases, as they aid cleavage of extracellular matrix proteins. Also, enzymes such as NADPH oxidase are part of their phagolysosomes. They oxidize superoxide radicals as well as other ROS. These ROS cause genetic modifications due to DNA damage, and therefore by extension, promote cancer development; as well as disrupt tumour cell membrane by instigating cytotoxicity. [Gregory & Houghton, 2011].

Adaptive Immunity and Cancer

The adaptive immune system employs more specific mechanisms whilst targeting cancer, by exploiting the effector functions of lymphocytes and APCs [Warrington et al., 2011].

CD4+ T cells recognise TAA-derived exogenous peptides presented by MHC class II molecules on APCs; while CD8+ T cells recognize endogenous peptides derived from the TAA, presented by MHC class I molecules on cancer cells [Chen & Mellman, 2013, Warrington et al., 2011].

When CD4+ T cells are activated by MHC II-TAA complexes on APCs, it primes them for successive exposures to that precise TAA; accordingly, memory T cells are formed [Chen & Mellman, 2013; Harris & Drake, 2013]. IL-2 produced during this activation process promotes T cell proliferation [Minami et al., 1993].

Thymus-dependent (involving 2 types of signals between the T helper cells and B cells, namely; (i) TCR with TAA-MHC II complex and (ii) a costimulatory signal due to CD40 ligand-CD40 interaction)

or thymus-independent (in absence of the costimulatory signal) B cell activation will lead to secretion of TAA specific antibodies. This results in initiation of cancer cell lysis via ADCC or CDC [Janeway et al., 2001].

*DCs, $\gamma\delta$ T cells, macrophages, and NKT cells act as bridge between the innate and adaptive immune system. DCs and macrophages, act as phagocytes as part of the innate immunity, and as APCs in adaptive immunity. NKT cells and $\gamma\delta$ T cells partake in immune response against cancer cells via IFN-γ secretion. This activates the NK/CD8+ T cell effector functions, which then leads to cancer cell lysis via granzymes or perforin. This cytotoxic effect can also be brought about by NKT cells' interaction with DCs via CD40 ligand-CD40 interaction; enabling the secretion of IL-12, which then activates NK/CD8+ T cells.$\gamma\delta$ T cells on the other hand, have receptor NKG2D, which binds MICA/B on tumour cells. This will promote perforin secretion, and consequently tumour cell lysis. Besides this, $\gamma\delta$T cells recognize TAAs through CD16Fc receptor; in order to mediate ADCC. These cells can also bind to heat shock proteins and other self-antigens, found to be upregulated in the TME [Waldhauer & Steinle, 2008; Terabe & Berzofsky, 2008; Palucka & Banchereau, 2012; Gogoi & Chiplunkar, 2013].

CANCER THERAPIES: TRADITIONAL AND NOVEL APPROACHES

Complete surgical removal of the primary tumour would ideally lead to full recovery; if not for the presence of metastasis, which greatly complicates the treatment process. Chemotherapy and/or radiation therapy are the standard treatments in use for cancer patients. [Davidson et al., 2014; NCI-Surgery to treat cancer, 2015].

Chemotherapy makes use of cytotoxic drugs administered in oral or intravenous manner. Since this is a systemic form of therapy, the drugs travel throughout the body and reach even the metastatic cancer cells that have spread far from the original tumour site. Chemotherapeutic drugs include alkylating agents, nitrosoureas, antimetabolites, anti-tumour antibiotics, corticosteroids and inhibitors of topoisomerase and mitosis. Depending on their mechanism of action, these drugs target different phases of the cell cycle. Due to their rapidly proliferating nature, cancer cells are better targets than normal cells for chemotherapy. However, side effects are the norm, as normal cells like hematopoietic cells, cells of the alimentary tract, reproductive cells and hair follicles are also likely to be damaged in the process. [Olsen & Naseman, 2018; Andersin & Matey, 2018; Copur et al.,2018].

Radiation therapy exploits ionizing radiation in the form of high-energy electromagnetic wavelike x-rays and gamma rays; or particulate forms like electron beams and protons. These create breaks in the cellular DNA, and hence affect subsequent growth and proliferation of cancer cells in particular. Still, this also damages the normal healthy cells nearby, leading to significant side effects. Like surgery which is aimed only at the site where cancer is found, radiation is a local form of treatment; at least when referring to external and internal (also named brachytherapy) radiation. This excludes systemic radiation wherein radioactive drugs are given orally or intravenously [Morgan et al., 2018; NCI-Radiation Therapy to treat cancer, 2019].

Aside from the crucial side effects caused by conventional therapies mentioned above, the most critical drawback lies in their failure to treat cancer as a disease of great complexity and heterogeneity [Dagogo-Jack & Shaw, 2018]. Hence, research in the field of oncotherapy is constantly geared toward discovering novel efficient therapeutic approaches; particularly in the realm of targeted therapy.

Nanomedicine has been shown to improve bioavailability, concentration and release profile of chemotherapeutic drugs at tumour site by providing biocompatible and biodegradable delivery systems [Martinelli et al., 2019]. Similarly, extracellular vesicles which have been implicated in tumour microenvironment modification and metastases, are now bioengineered as drug delivery vehicles [Kumar et al., 2016]. The pro-apoptotic and anti-proliferative nature of natural antioxidants and phytochemicals resulted in their use as adjuvants in anti-cancer therapies [Chikara et al., 2018; Singh et al., 2016]. Targeted therapy and immunotherapy are widely being explored, due to its specific mode of action. The idea is to precisely identify and attack certain types of cancer cells, leaving the surroundings unaffected, thereby reducing side-effects [Bazak et al., 2015]. Gene therapy for the expression of pro-apoptotic and tumour suppressor genes, and RNA mediated gene silencing of anti-apoptotic and oncogenes are being evaluated at clinical trial level[Lebedeva et al., 2003;Shanker et al., 2011;Vaishnaw et al., 2010]. Invasive surgeries may be substituted with precision methods like thermal ablation and magnetic hyperthermia [Brace C, 2011; Hervault & Thanh, 2014]. Radiomics and pathomics are promising methods that manage data collection, therapy design and prediction of response, clinical outcome and cancer recurrence; by merging information obtained during diagnostic and therapeutic procedures [Yu et al., 2016, Aerts H. J., 2016].

CANCER IMMUNOTHERAPY

Hanahan and Weinberg endeavoured to organize the complexities of cancer biology into a small number of commonly shared traits or hallmarks, namely: self-sufficient growth signalling, insensitivity to anti-growth signalling, evasion of apoptosis, unlimited proliferative ability, sustained angiogenesis, and invasion of neighbouring tissue and dissemination i.e. metastasis. The list was further extended by the addition of another two hallmarks: escaping the immune response and reprogramming energy metabolism [Hanahan & Weinberg, 2011; Hanahan & Weinberg, 2000]. Of these, it is the circumvention of the immune response, that provides the basis for cancer immunotherapy.

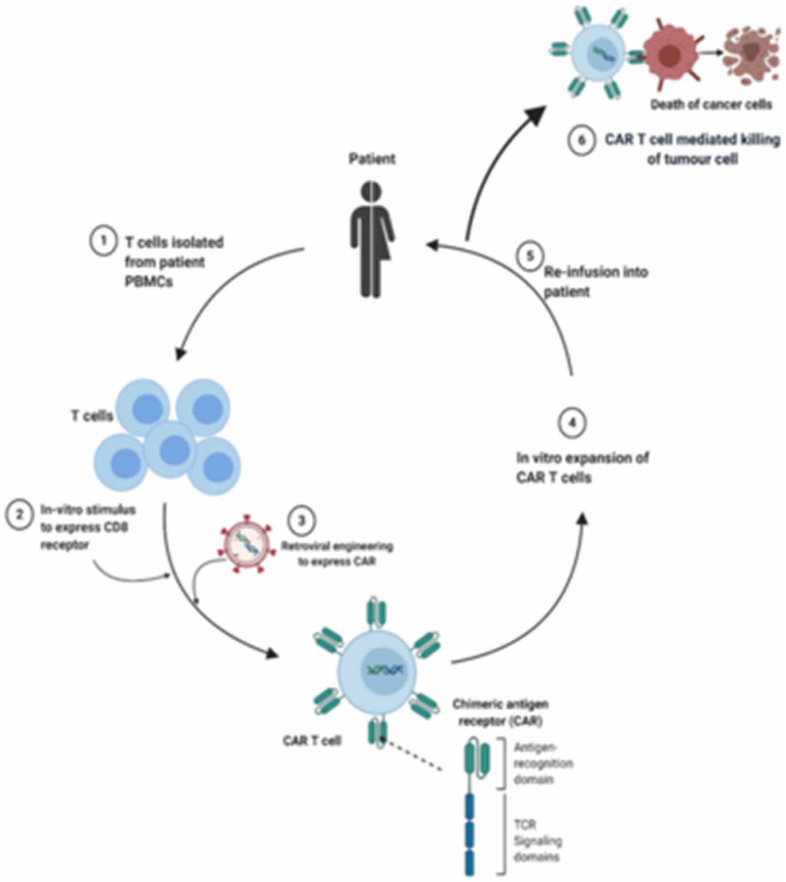
Immunotherapy utilization as cancer treatment was first attempted by Coley in 1891. He injected streptococcal extracts into cancer patients; with the intent of stimulating the immune system. The assumption was that the infection so produced would consequently result in shrinkage of the malignant tumour. He was successful in this trial, although his results were not accepted at the time, due to an absence of adequate controls. Immunotherapy employs components of the innate and adaptive immune system to target cancer cells. The chief objective is to re-activate and /or direct the patient's immune response which has been silenced or evaded by the cancer [Adams et al., 2015; Mellman et al., 2011; Hoos & Britten, 2012; Visage & Joubert, 2010].

Immunotherapy has the Following Advantages

First and most importantly, side effects of conventional therapy are avoided due to its specificity of action. Metastatic cancer and cancer stem cells can also be targeted in the event of appropriate stimulation of the immune system. Furthermore, activated and tumour-specific immune cells can access areas otherwise unreachable by surgery. Since it directly targets the tumour and/or the TME, immunotherapy therefore makes it plausible to have therapy that is personalized, customized, less toxic as well as having lesser side effects [Harris & Drake, 2013; Seledtsov et al., 2015; Dimberu & Leonhardt, 2011].

Cancer immunotherapies can be classified into different types: vaccines, recombinant cytokines, small molecules, autologous T cells and monoclonal antibodies (Fig. 2). They are briefly introduced below.

Figure 2. CAR-T cell therapy (Created with BioRender.com)



1) Vaccines

Cancer vaccines aim to immunize patients against TAAs, thereby inducing tumour-specific host immune response; such as anti-tumour T cells. They are usually administered with adjuvants, for e.g. DCs, that initiate and boost the immune response. The vaccines can be DNA- based encoding TAAs. Or they could be peptide-based, comprised of TAA derived immunogenic epitopes [Seledtsov et al., 2015; Özlük et al., 2017; Farkona et al., 2016; Papaioannou et al., 2016].

Example:

- Provenge™ - DC-based cancer vaccine, US FDA approved in 2010 for the treatment of advanced prostate cancer. It consists of autologous APCs like DCs, and a recombinant protein PAP-GM-CSF. Prior to administration, the autologous APCs are activated against PAP, a tumour specific

antigen expressed in prostate cancer tissue. While the PAP directs the immune response toward the cancer, the GM-CSF stimulates APC growth. [Cheever & Higano, 2011; Shi et al., 2006].

Note: Vaccines against oncoviruses like hepatitis B virus and human papillomavirus that potentially lead to hepatocellular carcinoma and cervical cell carcinoma respectively, are not to be considered in this category. [Schiller & Lowy, 2010]

2) Recombinant Cytokines

Cytokines are a broad group of small proteins that facilitate and modulate haematopoiesis, as well as the immune and inflammatory response. They are produced by, and act upon immune and non-immune cells; mediating cell signalling and cell-cell communication [Zhang & An, 2007; Rich et al., 2013]. In the field of cancer immunotherapy, the commonly used cytokines include interleukins, interferons, and GM-CSF [Barbaros & Dikmen, 2015; Visage & Joubert, 2010, Mellman et al., 2011].

Examples (FDA approved):

- Proleukin- recombinant IL-2 for renal cancer and melanoma treatment. Via IL-2 receptors, it promotes activation of immune cells like T cells [Adams et al., 2015; Mellman et al., 2011; Minami et al., 1993; Nelson B.H., 2004].
- Sylatron™- Pegylated IFN-α2b for treatment in resected melanoma patients. While IFN-α2b acts as an anti-inflammatory repressing proliferation of cells, the polyethylene glycol masks the immunogenicity of IFN-α2b until it reaches its target. [Adams et al., 2015, Dunn et al., 2006, Patel & Walko, 2012].

3) Small Molecules

Examples (FDA approved):

- Plerixafor-antagonistic to the binding interaction of SDF-1 to the chemokine receptor CXCR4; hence, impedes cancer metastasis and improves deployment of hematopoietic stem cells; particularly of use in pancreatic ductal adenocarcinoma patients[Adams et al., 2015; Uy et al., 2008;Tögel et al., 2005].

Imiquimod-agonistic for TLR7 on DCs and macrophages. Subsequent TLR7 activation induces proinflammatory cytokine release, suppression of Tregs, and promotes NK cell activation by Th1 cells. The NK cells then eliminate cancer cells [Adams et al., 2015].

4) Autologous T-cell Therapy

Immunologically active T cells are administered to the patient; which are autologous in origin i.e. the patient's own T cells are used. Its purpose is T-cell redirection so as to specifically target and destroy tumour cells. This method comprises firstly, the harvesting of immune cells from the peripheral blood or tumour of the patient. This is followed by isolation of T cells, and *ex vivo* expansion of tumour-specific

T cells. Finally, these are re-infused into the patient [Mellman et al., 2011; Mayor et al., 2016; Neves & Kwok, 2015].

A variation of this is the CAR-T cell therapy. Here, the autologous T cells are genetically altered via retroviral gene transfer, to express CAR. CAR is a fusion protein that has TAA-specific Ab variable region (extracellular) such as scFv, linked to downstream TCR-based activation motif (intracellular). Thus, the CAR-T cells combine the specificity of an Ab with the signalling mechanism of the TCR. Upon interacting with the target TAA on the cancer cell, these T-cells activate, proliferate and exhibit cytotoxicity in order to kill the cancer cell [Rini B., 2014; Magee & Snook, 2014].

Examples (FDA approved):

- Tisagenlecleucel-First US FDA approved CAR-T cell therapy. Used to treat acute lymphoblastic leukaemia. It is engineered to target CD19 found on B cells [Kymriah, 2017].
- Axicabtagene ciloleucel – It also targets CD19 on B cells; for treatment of relapsed/refractory large B-cell lymphoma in adults [Yescarta, 2017].

5) Monoclonal Antibodies

Since this is the focus of our chapter, it will be dealt with in a detailed manner in the upcoming sections.

ANTIBODIES- AN OVERVIEW OF STRUCTURE AND FUNCTION

A single antibody molecule comprises 4 polypeptide chains in total: 2identical heavy chains (50 kDa) and 2 identical light chains (25kDa); which are linked by inter-chain disulphide bonds and non-covalent interactions. Thus, the Ab molecule is a heavy chain-light chain heterodimer. While each heavy chain consists of a variable domain (VH) at the N-terminus and three constant domains (CH1, CH2, CH3), the light chains consist of an N-terminal variable domain (VL) and a C-terminal constant domain (CL). There exists a 'hinge region' between the domains CH1 and CH2 [Schroeder & Cavacini, 2010].

The Ab molecule is subdivided into two distinct functional units:

- Fab-formed by association between light chain and the heavy chain VH and CH1 domains. It has 3CDRs that together form the antigen-specific binding site.
- Fc-formed by the distal part of the hinge region and the heavy chain CH2, CH3 domains [Schroeder & Cavacini, 2010].

Abs are divided into 5 classes-IgM, IgD, IgG, IgE and IgA, on the basis of their heavy chain constant region sequences. IgG and IgA are further subclassified. The light chains are either of lambda or kappa type. [Schroeder & Cavacini, 2010].

Of these, IgG which is one of the most abundant proteins in human serum; is the Ab class used for therapy purposes. Most mAb therapeutics belong to the IgG1 subclass and they mostly have light chain of kappa type [Schroeder & Cavacini, 2010; Grilo & Mantalaris, 2019].

Abs link the adaptive immune system (through Fab) with the effector functions of the innate immune system (through Fc). The Fc region initiates various immune effector mechanisms like-the initiation of

CDC and ADCC; thus, capable of killing target cells. The latter is brought about by cross-linking FcγR on NK cells, neutrophils, macrophages or DCs [Schroeder & Cavacini, 2010].

THERAPEUTIC MONOCLONAL ANTIBODIES- A HISTORY

In the late 19th century, Behring and Kitasato successfully treated animals for diphtheria and tetanus, by transferring serum from animals immunized with diphtheria and tetanus toxins, respectively. This marked the advent of passive antibody-based therapy, which then began to be widely used for the treatment of various infectious diseases. [Kaufmann S., 2017] However, serum therapy generally faced issues because of its polyclonal nature, disparity between lots, high production costs, and toxicity associated with heterologous sources; while human serum therapy particularly encounters problems of large-scale availability and if unscreened, a risk of transmitting diseases. [Casadevall et al., 2004].

Important information concerning Ab structure, diversity and generation began to be revealed around the mid-20th century onward. Significantly, Brunet's 'clonal selection theory' explained that a single B cell produces an Ab of single specificity i.e. a monoclonal Ab against a single antigenic epitope [Cooper M. D., 2015].

The path-breaking 'hybridoma technology' gave impetus to antibody- \based therapy once again, due to its ability to produce mAbs in unlimited amounts. Kohler and Milstein won the Nobel Prize in 1984 for this technique. It involved the fusion of splenocytes from a rat that was immunized with a specific Ag and mouse myeloma cells, thus generating hybrid cells; each secreting mAbs of murine origin [Köhler & Milstein, 1975].

Thus finally, Ehrlich's idea of a 'magic bullet' came to pass, given the mono-specificity of mAbs; and the hybridoma technique enabling the possibility of a wide range of applications in biochemistry, molecular biology, cell biology, and importantly in clinical research-in which therapeutics would benefit most.[Strebhardt & Ullrich, 2008]

The first therapeutic mAb was Muromonab, a murine mAb against CD3 receptor of T lymphocytes. It was approved in 1986 by US FDA and EMA. It functioned as an immunosuppressant in the control of transplant rejection [Norman et al., 1987]. Unfortunately, this success was not replicated, as a result of safety and/or efficacy inadequacies. This was primarily due to murine nature of the mAbs. It was observed in clinical trials, that when the murine mAbs are repeatedly administered, the mAbs half-life and efficacy is reduced with each subsequent administration. This is because of the HAMA response, resulting in adverse reactions *in vivo* [Hwang & Foote, 2005; Liu J. K., 2014; Oldham & Dillman, 2008; Teillaud J. L., 2012].

So as to avoid the problems generated by the HAMA response, whilst preserving the binding specificity of the murine mAbs, r-DNA technology began to be used to create chimeric and humanized antibodies [Levene et al., 2005; Simpson & Caballero, 2014]. The first chimeric Ab, was Abciximab; an anti-GPIIb/IIIa Fab. In 1994, it was US FDA approved. The purpose was to inhibit platelet aggregation in treatment of cardiovascular diseases [Morrison et al., 1984; Foster & Wiseman, 1998].

THERAPEUTIC MAbs AGAINST CANCER

mAbs as immunotherapy target specific TAAs or immunomodulators; and in doing so direct the patient's own immune response toward cancer elimination. It exploits both, the Fab region's specificity towards the target Ag, as well as the Fc region's ability to partake in the immune effector mechanisms [Harris & Drake, 2013].

The first of its type was Rituximab, a chimeric anti-CD20 IgG1 approved in 1997 by US FDA for treatment of non-Hodgkin's lymphoma [Maloney et al., 1997; Maloney et al., 1997].

Of all the available immunotherapeutic strategies, mAbs are usually the most employed and approved. The chief types of cancer targeted are breast, colon and lymphomas [Sathyanarayanan & Neelapu, 2015]. But then again, many side effects have been documented: flu-like signs and symptoms, nausea/vomiting, diarrhoea, skin rashes, breathing difficulties, bleeding, etc. [Oldham & Dillman, 2008].

Categories

Therapeutic mAbs in cancer immunotherapy are broadly divided into two categories, depending on whether they are naked Abs or have been modified to improve therapeutic value.

1) Naked mAbs

These are self-acting, non-conjugated mAbs that are directly used for therapeutic purpose. The mechanisms of cancer cell killing include mediation of CDC/ADCC or direct induction of apoptosis. They may also target the tumour microenvironment or immune checkpoints so as to hamper tumorigenesis. (Explained further in sections 8.3 and 8.4)

Examples grouped based on their targets (FDA approved):

- CD20
  Rituximab-chimeric mAb for B-cell non-Hodgkin's lymphoma therapy [Neves & Kwok, 2015; Hallek M., 2006].
  Obinutuzumab-humanized mAb used to treat chronic lymphocytic leukaemia and non-Hodgkin's lymphoma [Al-Sawaf et al., 2017].
  Ofatumumab-human mAb in chronic lymphocytic leukaemia treatment [Simpson & Caballero, 2014; Al-Sawaf et al., 2017].

- CD52
  Alemtuzumab-humanized mAb for treatment of chronic lymphocytic leukaemia [Neves & Kwok, 2015, Hallek M., 2006].

- CD38
  Daratumumab-human mAb for multiple myeloma treatment [Sanchez et al., 2016].

- HER2
  Trastuzumab-humanized IgG1 mAb used for invasive breast cancer therapy [Peddi & Hurvitz, 2014; Weiner et al., 2010].
  Pertuzumab-humanized mAb in the treatment of breast cancer [Simpson & Caballero, 2014].

- GD2 glycolipid
  Dinutuximab-chimeric mAb in therapy of neuroblastoma of high-risk nature, in children [Dhillon S., 2015].

- SLAMF7
  Elotuzumab-humanized mAb in the treatment of multiple myeloma. [Magen & Muchtar., 2016].

- RANKL
  Denosumab-human for solid tumour bony metastases treatment [Simpson & Caballero, 2014; Redman et al., 2015].

- VEGF
  Bevacizumab-humanized mAb used to treat various cancers-NSCLC, metastatic colorectal, ovarian and breast cancer [Mayor et al., 2016; Weiner et al., 2010].
  Ramucirumab-humanized mAb in the treatment of gastric cancer, non-small cell lung cancer and breast cancer [Mayor et al., 2016].

- EGFR
  Cetuximab-chimeric IgG1 mAb in therapy of many cancers- metastatic colorectal cancer, NSCLC and squamous cell cancer [Mayor et al., 2016, Levene et al., 2005].
  Panitumumab-humanized IgG2 mAb for treatment of metastatic colorectal cancer [Neves & Kwok, 2015, Scott et al., 2012].
  Necitumumab-human mAb in the treatment of metastatic NSCLC [Mayor et al., 2016].

- CTLA-4
  Ipilimumab- human IgG1 mAb used in metastatic melanoma treatment [Mellman et al., 2011; Redman et al., 2015].

- PD-1
  Pembrolizumab-is a humanized mAb in the treatment of melanoma and metastatic NSCLC [Mayor et al., 2016; Lee et al., 2016].
  Nivolumab-human mAb utilized for treatment of various cancers- melanoma, NSCLC, renal cell carcinoma and Hodgkin's lymphoma [Redman et al., 2015, Lee et al., 2016].

- PD-L1
  Atezolizumab-humanized mAb for metastatic NSCLC therapy [Krishnamurthy& Jimeno,2017].
  Avelumab-human mAb in the treatment of many cancers- NSCLC, renal cell carcinoma, ovarian and stomach cancers [Kaplon & Reichert, 2018].

Durvalumab-human mAb in metastatic urothelial carcinoma treatment [Kaplon & Reichert, 2018].
Note: Suffixes used in mAb nomenclature based on species of origin [Lu et al., 2020]

- Murine mAb-*momab*
  Entire Ab is of murine origin.

- Chimeric mAb-*ximab*
  The variable regions are of murine origin, and the remainder is human in origin.

- Humanized mAb-*zumab*
  Only the CDRs are murine in origin.

- Human mAb-*umab*
  Entire Ab is of human origin.

2) Modified mAbs

In this category, the mAbs have been altered in various ways for the purpose of enhancing their usefulness as immunotherapeutic agents.

a. Antibody-Drug Conjugates

Consist of TAA-specific mAb conjugated to a chemotherapeutic drug. This combines the specificity of a mAb with the cytotoxicity of a drug; but with decrease in damage to non-target cells and hence lesser side-effects. This especially allows the use of drugs with considerably high potency and toxicity [Beck et al., 2017; Peters & Brown, 2015]

Examples (FDA approved):

- Gemtuzumab ozogamicin-humanized anti-CD33 mAb that is conjugated with calicheamicin (binds minor groove of DNA with certain sequence specificity and causes double-strand breaks), used in the treatment of acute myelogenous leukaemia [Oldham & Dillman, 2008; Levene et al., 2005; Sathyanarayanan & Neelapu, 2015; Huryn & Wipf, 2008].
- Brentuximab vedotin-chimeric anti-CD30 mAb which is conjugated with monomethyl auristatin E (inhibits tubulin polymerization); used in therapy of Hodgkin's or systemic anaplastic large cell lymphoma [Simpson & Caballero, 2014; Redman et al., 2015, van de Donk& Dhimolea, 2012].

b. Antibody-Radioisotope Conjugates

With advantages similar to the Ab-drug conjugates, these too enable direction of radiotherapy selectively to cancer cells [Larson et al., 2015].

Examples (FDA approved):

- Ibritumomab tiuxetan-murine anti-CD20 mAb conjugated with yttrium-90. It is utilized to treat B cell non-Hodgkin's lymphoma [Simpson & Caballero, 2014, Scott et al., 2012].

- Tositumomab-murine anti-CD20 mAb labelled with iodine-131; used for treatingnon-Hodgkin's lymphoma patients; especially those not responding to conventional chemotherapy. [Shadman et al., 2016]

c. Bispecific Antibodies

Here, a single Ab concurrently binds two different targets. Protein engineering is used to link two antigen binding domains (Fab/scFv), thus generating an Ab of dual specificity. One arm binds tumour cells i.e. is TAA- specific, the other binds activating receptors on cytotoxic cells, like T cells (e.g. CD3 ε-chain) or NK cells. This results in recruitment of the cytotoxic effector cells to the target cancer cells [Labrijn et al., 2019; Weiner G. J., 2015; Strohl W. R., 2018].

They may also be altered to exhibit functions unique to even a mixture of the parental Abs from which they are derived. [Labrijn et al., 2019; Weiner G. J., 2015; Strohl W. R., 2018].

Examples (FDA approved):

- Catumaxomab-first approved bispecific antibody. It targets CD3 and EpCAM; and is used in treatment of solid tumours in malignant ascites [Heiss et al., 2010]
- Blinatumomab-targetsCD3 and CD19. Hence, it acts as a bispecific T-cell engager. It is used to treat B-cell precursor acute lymphoblastic leukaemia [Gökbuget et al., 2018].

*Other such modified mAbs include Immunocytokines (cytokines fused to an Ab to enhance delivery specificity) [Neri D., 2019], Immunoliposomes (Fab/scFvis conjugated to liposomal delivery systems) [Ohradanova-Repic et al., 2018]and CAR-T cells described previously in section 4 (4).

Mechanisms of Action of Naked mAbs

Unconjugated, TAA-specific mAbs function either by disruption of signalling that partakes in tumorigenesis, and/or initiation of tumour-specific immune responses like ADCC/CDC [Harris & Drake, 2013; Papaioannou et al., 2016] These mechanisms include:

i. Inhibition of signalling pathways involved in tumorigenesis

Here, the mAbs target either the receptor or its corresponding ligand (such as growth factors, proangiogenic factors), thereby blocking their interaction and downstream signal transduction (Fig. 3).

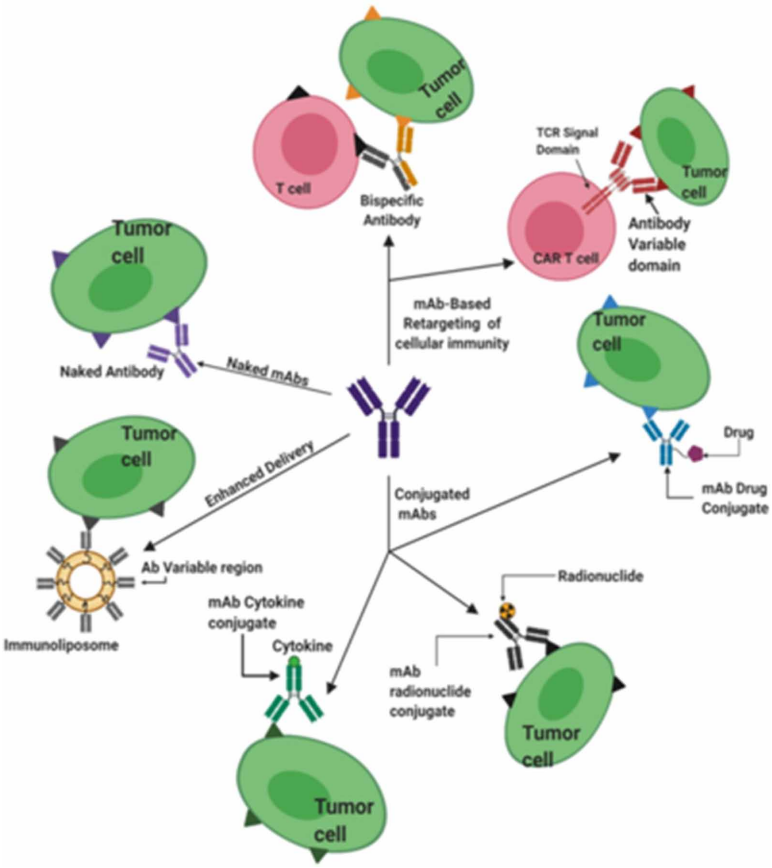
The purpose is to impede cancer progression by inhibiting processes like cancer cell proliferation, angiogenesis, etc.

Examples: Cetuximab and Bevacizumab [Sunada et al., 1986; Li et al., 2005; Ellis & Hicklin, 2008]

ii. Antibody-dependent cellular cytotoxicity

First, the mAb binds to the TAA on tumour cell surface. Next, Fc domain of this mAb is recognized by the FcγR of macrophages and NK cells. The resultant cross-linking of these receptors causes release of cytotoxic agents like perforin and granzyme; this leads to tumour cell apoptosis [Chung et al., 2014; Wang et al., 2015].

Figure 3. Types of mAb based strategies for cancer therapy (Created with BioRender.com)



Examples: Rituximab, Trastuzumab, Cetuximab [Boyerinas et al., 2015; Glassman & Balthasar, 2014].

Certain studies demonstrated the importance of Fc-FcγR interactions in immune mediated anti-tumour activity of mAbs. For e.g. (i)Polymorphisms in the FcγR were corelated with clinical response rates to Trastuzumabin breast cancer [Musolino et al., 2008], and (ii) FcγR-deficient mice showed reduced anti-tumour activity by Rituximab and Trastuzumab as compared with wild-type mice [Clynes et al., 2000].

iii. Complement-dependent cytotoxicity

In this process, the mAb binds to TAA on surface of the target tumour cell. Successive binding of complement proteins triggers a reaction cascade. Ultimately membrane attack complexes are formed in the cell membrane and cell lysis occurs [Zhou et al., 2008].

Examples: Rituximab and Alemtuzumab [Glassman & Balthasar, 2014].

Studies with Rituximab highlighted the relationship between the complement system and therapeutic effects of mAbs in cancer. For e.g. (i) anti-tumour activity of Rituximab was eliminated in C1q knockout mice [Di Gaetano et al., 2003], and(ii) C1qA gene polymorphisms are associated with clinical response to Rituximab in follicular lymphoma patients [Racila et al., 2008].

*Note that most clinically approved mAbs that mediate ADCC are also capable of activating the complement system. One exception is, Alemtuzumab which mediates CDC, but not ADCC [Lundin et al., 2002].

Additional mechanism: Induction of adaptive immunity via cross-presentation

Certain studies involving Rituximab suggested that the adaptive immune system imparts long-term benefits in mAb therapy, months after the beginning of treatment [Cartron et al., 2004]

This may be the consequence of cross-presentation by DC's, triggered by mAb mediated ADCC. The resultant apoptotic tumour cells may be engulfed by DCs, which then process and present TAAs with MHC I and II. As explained previously in section 2.1 and 2.3, this presentation ultimately leads to CTL mediated killing of tumour cells as well as the activation of CD4+ T cells. The latter can then activate B cells for the production of TAA specific Abs. [Dhodapkar et al., 2002]

Targets

1) Targeting tumour cells

Most commonly, therapeutic mAbs in cancer are designed to impede ligand binding to and/or signalling via growth factor receptors that are increasingly expressed in tumorigenesis (Fig. 4). The purpose of these mAbs is to induce apoptosis in tumour cells, regulate their growth, and/or increase tumour sensitivity to chemotherapy [Adams & Weiner, 2005]. The growth factor receptors often overexpressed in solid tumours are members of the epidermal growth factor receptor family: EGFR, HER2, HER3, and HER4.

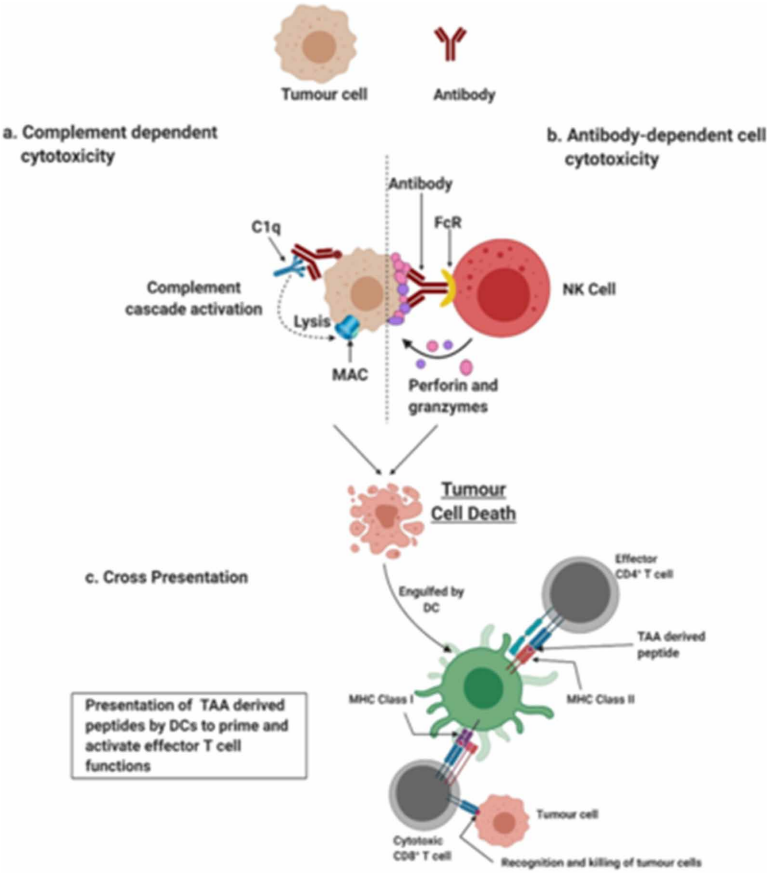
- EGFR
  Cetuximab and Panitumumab block the activating ligand binding and prevent receptor dimerization, hence inhibiting EGFR-mediated signal transduction. Additionally, the former mAb promotes ADCC/CDC [Sunada et al., 1986; Li et al., 2005, Kim R., 2009]

- HER2
  ~ 30% invasive breast cancers display a gene-amplification and overexpression of this growth factor receptor. In some lung, ovary, prostate and gastrointestinal tract adenocarcinomas, it is found to be overexpressed, but rarely gene amplified. Unlike EGFR, it has no known ligand. Hence, targeting mAbs like Trastuzumab and Pertuzumab must perturbHER2-mediated signal transduction by inhibiting its dimerization and internalization. The former mAb also activates immune responses against tumour cells. [Chen et al., 2003; Hudis C. A., 2007; Franklin et al., 2004]

- HER3
  MM-121is a human mAb that preventsHER3 phosphorylation. It has been observed to retard growth of xenograft tumours in mice. [Schoeberl et al., 2009].

- HER4
  Unlike the other members of the EGFR family, HER4 has been found to be both increasingly and decreasingly expressed on tumour cells. This may be attributable to multiple isoforms of HER4. A mAb against select isoforms was found to downregulate their expression and thus, reduce tumour cell proliferation. This was due to the inhibition of phosphorylation and cleavage of HER4 by the mAb. [Hollmén et al., 2009]

Figure 4. Immune mediated tumour cell killing by mAbs, a) Complement Dependent Cytotoxicity, b) Antibody-Dependent Cell Cytotoxicity, c) Cross Presentation (Created with BioRender.com)



2) Targeting the tumour microenvironment

Of the possible approaches that focus on key events in the TME, one such involves targeting the process of angiogenesis.

- VEGF
  This is a growth factor expressed by solid tumours, that stimulates angiogenesis upon binding its corresponding receptor VEGFR on vascular endothelium.

Bevacizumab, binds VEGF, and prevents this receptor-ligand interaction [Ellis & Hicklin, 2008].

- VEGFRs
  Ramucirumab blocks the aforementioned interaction by binding VEGFR [Krupitskaya & Wakelee, 2009].

- PDGFR
  PDGF is a proangiogenic mediator like VEGF. PDGF- PDGFR signalling maintains the endothelial support system, which in turn stabilizes and promotes angiogenesis. [Hirschi et al., 1998]This interaction has garnered importance in light of a surge in bevacizumab resistant tumours due its increased therapeutic use; this resistance lead to upregulation of proangiogenic mediators such as PDGF. In pre-clinical studies, the blocking of PDGFR-mediated signalling via a PDGFRβ-specific human Abdemonstrateda synergistic effect with anti-VEGFR2 therapy. [Shen et al., 2009].
  The TME is also seen to have immunosuppressive effects by means of various cytokines and growth factors produced by both the tumour cells and surrounding stroma.

- TGFβ
  It plays a role in inhibition of T cell activation, differentiation, and proliferation. This enables it to promote immune evasion in cancer. Links have been observed between high levels of TGFβ in the plasma and poor clinical outcomes in cancer. GC-1008, is a human anti-TGFβ Ab studied as therapy for metastatic kidney cancer or malignant melanoma patients [Rabinovich et al., 2007; Grütter et al., 2008; Petrausch et al., 2009].

- CD25
  CD25 is the α-chain of the high affinity IL-2 receptor [Minami et al., 1993]. mAbs against this, like daclizumab have been shown to deplete Treg cells and repress tumour formation in metastatic breast cancer patients. [Rech & Vonderheide, 2009].

3) Targeting immune cells

In this approach, mAbs are used that target co-receptors (inhibitors/repressors or activators/stimulators) present on immune cells, that participate in immune regulation. These mAbs act as blocks of immune checkpoints. The objective is the reversal of tumour mediated immunosuppression and augmentation of patient's own anti-tumour immune response. Therefore, this method can be applied for several cancer types [Weiner G. J. 2015; Emens et al., 2017; Littman D. R., 2015].

As mentioned previously, the first evidence that the immune system can be exploited in cancer treatment was given by Coley, when he observed the regression and elimination of solid tumours in patients that were either suffering from streptococcal skin infections or administered with streptococcal extracts [Coley W. B.,1891].

When Sharma and Allison demonstrated that the targeting of a negative regulator of T cells, namely CTLA-4 resulted in enhanced tumour cell killing, it provided the necessary stimulus for the discovery and targeting of several checkpoints found to be deactivated or activated in malignant tumours [Sharma & Allison, 2015; Krummel & Allison, 1995;Ishida et al., 1992].

Below are mAbs acting as antagonists of immune checkpoint molecules like CD40, CTLA- 4, PD-1 and PD-L1.

- CD40
  It belongs to the family of TNFR. It is expressed by various APCs. Upon its engagement, an up-regulation of costimulatory molecules and production of proinflammatory cytokines occurs, which then facilitates cross-presentation of antigens [Quezada et al., 2004; Krummel & Allison, 1995] Carcinomas of many organs like the ovary, cervix, breast, prostrate, etc. have displayed CD40 expression[Elgueta et al., 2009]
  Dacetuzumab, a humanized anti-CD40 IgG1 has displayed Fc-mediated ADCC as well as phagocytosis of tumour cells by macrophages. [Oflazoglu et al., 2009]. It has been studied as treatment for non-Hodgkin's lymphoma patients. [Advani et al., 2009].

- CTLA4
  CD28 usually binds to the B7 (CD80/86) costimulatory molecules expressed on surface of APCs, as part of the T cell activation process. CTLA-4 by contrast, negatively regulates T cell activation, by inhibiting the co-stimulatory signal of CD28.This is because it is a CD28 homologue, and binds CD80 and CD86 with an affinity higher than that of CD28 itself. [Leach et al., 1996]
  Studies have revealed that CTLA4 blockade leads to the development of immunological memory, as well as prevention and reversal of antigen-specific CD8+ T cell tolerance in a manner reliant on CD4+ T cells. Further work gave a plausible reason for its anti-tumour nature: the enhancement of effector T cell activity, together with Treg cell inhibition. [Leach et al., 1996; Shrikant et al., 1999; Peggs et al., 2009]
  Ipilimumab was the first immunomodulator mAb approved in 2011 by FDA and EMA. By its anti-CTLA-4 nature, it reinstates CD28 activity. This leads to arise activated T cells levels, which bring about anti-tumour response [Ascierto & Marincola, 2014].

- PD-1 or PD-L1
  PD-L1 plays a major role in suppressing the adaptive immune functions. When it binds the inhibitory checkpoint molecule PD-1 found on activated T cells, it mediates an inhibitory signal transduction; thereby reducing proliferation of antigen specific T cells [Ishida et al., 1992]
  Anti-PD-1 mAbs like Pembrolizumab and Nivolumab and anti-PD-L1 mAbs like Atezolizumab, Avelumab and Durvalumab block these inhibitory interactions that may suppress anti-cancer immune response [Alsaab et al., 2017].
  mAbs targeting other such negative (LAG-3, TIM-3, VISTA) and positive (ICOS, OX40, 4-1BB) regulators of immune response are also being developed and studied. [Mahoney et al., 2015; Triebel et al., 1990; Sakuishi et al., 2010; Wang et al., 2011; Fan et al., 2014; Curti et al., 2013; Melero et al., 1997].

Production Methodologies

Hybridoma Technology

In 1975, Kohler and Milstein showed that Ab secreting cell lines could be established routinely and maintained *in vitro*. This was accomplished by fusing two cells, each having properties necessary for the successful production of a hybrid cell line that is both immortal and produces Abs indefinitely. The two cells commonly used as fusion partners are Ab-secreting B cells isolated from immunized animals and myeloma cells. While the latter provide the appropriate genes for unlimited cell division, the former provide the desired functional Ig genes [Köhler & Milstein,1975]

As per the classical method, first mice are immunized with the target Ag. Then, their harvested splenocytes are fused with an Ig non-secreting, drug-sensitive myeloma cell line. The resultant hybrid cells are individually cloned in microwell plates. The supernatants from the microwells are then screened for specific Abs. Those cells from the positive wells are grown further as source of mAbs. [Köhler & Milstein,1975]

However, this method faced a major issue: When used for therapeutic purposes, their allogenic nature leads them to be recognized by the patients' immune systems as foreign. This resulted in HAMA response, which rapidly inactivates and eliminates them. [Hwang & Foote, 2005; Steinitz M., 2009]

So, why did the hybridoma method fail when implemented for the production of human Mabs? A suitable drug-resistant myeloma cell line is not available. Also, antigen-sensitized splenocytes of human origin are not as accessible in comparison to those that are murine. [Steinitz M., 2009]

Given the drawbacks of mAbs of non-human origin, in the next sections, we will concentrate on the techniques used in the production of humanized and human therapeutic mAbs for cancer.

1) Humanization of murine mAbs

Initially, chimeric mAbs were developed by chemical exchange of heavy and light chain constant regions of the murine mAb, with those of human origin [Boulianne et al., 1984]. Further replacement of the murine components of mAbs; and by extension further reduction in their immunogenicity, was achieved with the polymerase chain reaction technology. It involves grafting donor murine mAb CDR residues and nominal yet crucial framework residues onto acceptor human Ab frameworks. Thus, it enables preservation of the affinity and specificity of the parental murine mAb to the desired target, but with reduced immunogenicity risk [Jones et al., 1986].

The process of CDR grafting was first developed by G.P. Winter in 1986. This classic and simple method remains a popular technique in humanized mAb production [Jones et al., 1986]. The technique opened up a range of possibilities in clinical application of mAbs, especially against diseases that demand long-term therapeutic approaches, such as cancer. In 1997, Daclizumab, an anti-IL-2 receptor mAb became the first US FDA approved humanized mAb developed by CDR grafting. It is used to prevent transplant rejection [Tsurushita et al., 2005; Gorman & Clark, 1990; Mountain & Adair, 1992]

Humanization of murine mAbs is preferred particularly when the murine mAbs have already been characterized in detail [Kashmiri et al., 2005]. Also, murine mAbs offer easier availability, lower cost and production time; thus, making large-scale humanization a possibility. Besides, the humanized mAbs more effectively exert effector functions as dictated by the human Fc domain [Lu et al., 2020].

Since the entire constant region and nearly all of the Fab region comprise human sequences in humanized Abs, it greatly improved clinical tolerance of mAb therapeutics. A study showed that when [Rebello et al., 1999] a humanized anti-CD52 mAb was compared with its parent mAb of murine origin, the former displayed a significant immunogenicity reduction. Nevertheless, the murine CDRs in humanized Abs could evoke immune response in humans. For example, Abs were detected against Trastuzumab in a fraction of metastatic breast cancer patients treated with the same [Cobleigh et al., 1999].

Certain framework residues of the parent murine mAb may play a vital role in contributing to Ab binding to the target antigen. These amino acids can be identified by analysis of Ab-Ag complex using X-ray crystallography, cryo-electron microscopy or *in silico* homology modelling [Choi et al., 2015]. Once the desired amino acids and their positions have been ascertained, they can be incorporated to increase affinity of the humanized Ab.

Humanness score tools like H-score, G-score, and T20 score analyser are available [Abhinandan & Martin, 2007; Thullier et al., 2010; Gao et al., 2013] for assessment of the degree of humanness of the mAbs variable regions.

2) Phage Display Library

Display technology utilize various micro-organisms like phage, yeast, bacteria and viruses or even mammalian cells and ribosomes to display repertoires of human Ig variable gene segments [as scFvs/Fabs] on their surface; for the purpose of creation and isolation of mAbs of desired specificity *in vitro*[Steinitz M., 2009;Carter P. J., 2006;Hoogenboom H. R., 2005;Ho et al., 2006].

In 1985, George P. Smith [Smith G. P., 1985] fused foreign protein genes with those of filamentous bacteriophage M13 coat protein using rDNA technology. The resultant phage displayed the foreign proteins as fusions with phage coat protein on its surface. This work laid the foundation for the phage display technique of today. Phage display was the first method for *in vitro* Ab selection. It was also instrumental in the generation of fully human mAbs by G.P. Winter in 1990 [Jones et al., 1986, McCafferty et al., 1990].

The first step in this method is Ab-library construction. Collections of Ig genes can be obtained from donors either naïve or immunized. While libraries of the former afford a lower risk of immunogenicity due to the similarity to the human Ig germ line (if human donors are used); the latter allows selection of high affinity Abs to the target of choice, as a consequence of *in vivo* affinity maturation. Recombinant libraries may thus be constructed from variable regions of Ig genes of these donor B cells. Synthetically constructed libraries may also be obtained using fixed frameworks, within which are randomized CDR sequences [Lu et al., 2020].

The next step is the selection of the desired mAbs by iterative cycles of affinity screening against the target antigen. This process is called 'bio-panning'. This enables the enrichment of even the rarest antigen-binding phage clones, ultimately selecting for Abs of the highest specificity [Lu et al., 2020, Carter P. J., 2006, Hoogenboom H. R., 2005].

The phage display method is not constrained by immunological tolerance; being an *in vitro* method. The presence of an appropriate library can permit quick identification of high affinity Abs for the generation of therapeutics. Therapeutic Abs can be designed to contain desirable properties of affinity, specificity, cross-reactivity and stability [Lu et al., 2020].

This method offers various advantages in the realm of cancer therapy. As whole cells can be used in Ab selection, prior unidentified antigens on the tumour cell surface may be revealed [Wrublewski DT., 2019]. This is of significance in CSC research. These cells have stem cell like properties, and are re-

sponsible for initiation and maintenance of tumours [Batlle & Clevers, 2017].In this manner, Abs against CSC markers such as CD133 and CD44 have been produced[Swaminathan et al., 2013, Nilvebrant et al., 2012].Investigation of the tumour microenvironment is made possible since, tumour biopsies may be used in the panning process[Larsen et al., 2015;Sun et al., 2009;Larsen et al., 2015].

Examples of cancer therapeutic mAbs (FDA approved):

- Necitumumab-anti-EGFR human mAb used to treat squamous NSCLC. It was developed by screening a non-immunized phage-displayed Fab library against high EGFR-expressing epidermal carcinoma cells [de Haard et al., 1999; Diaz-Serrano et al., 2019].
- Ramucirumab-anti-VEGFR2 human mAb identified by panning a phage-displayed human naïve Fab library against VEGFR2 extracellular domain; used in therapy for gastric cancer, metastatic colorectal cancer and, metastatic NSCLC [Arrieta et al., 2017; Aprile et al., 2016].
- Avelumab-anti-PD-L1 human mAb generated from a phage-displayed naïve Fab library [Frenzel et al., 2016]. Used in the urothelial and Merkel-cell carcinoma therapy [Rodriguez-Vida& Bellmunt, 2018].

3) Human antibody-producing transgenic mice

In this technique, heavy and light chain genes of human Ig are knocked-in to replace those of the mouse. Therefore, the transgenic mouse so created, will produce fully human Ab expressing B cells after immunization. These B cells can be immortalized via hybridoma technology and screened for desired mAb [Steinitz M., 2009]

In 1985, Alt and colleagues first suggested introducing human Ig genes into the mouse genome, with the intent of human Ab production in the resultant transgenic mice [Alt et al., 2003]. Longberg et al developed the first such transgenic mouse strain 'HuMabMouse' [Lonberg et al., 1994] in 1994; wherein human IgH and IgK were introduced into mice deficient in murine IgH and IgK.

Unlike the aforementioned strain that displayed the production of mouse endogenous Ig, the 'XenoMouse' generated in 1997 by Mendez et al produced only human Abs; which were of higher diversity. This was because the size of human IgH (~ 1 Mb) and IgK (~ 700 Kb) introduced were larger that of HuMabMouse (<80 kb) [Mendez et al., 1997, Kashyap et al., 2008, Jakobovits et al., 2007]

In human mAb production, these transgenic animals are advantageous given the following: humanization is not needed, more diversity of Abs generated along with Ab optimization due to *in vivo* affinity maturation and clonal selection [Brüggemann et al., 2015].

There exist certain drawbacks however. Stability of the human Ig loci is worrisome. During development of these strains, the large size of the human Ig loci present difficulties. The entirety of the human Ig genome is not transferred; hence human Ig gene repertoire is incomplete. Also, the absence of mouse constant regions lowers the efficiency of human Ab generation, class-switching and somatic hypermutation. Besides this, availability and cost of the knock-out/knock-in mice required are an issue. [Steinitz M., 2009; Brüggemann et al., 2015; Xu et al., 2007].

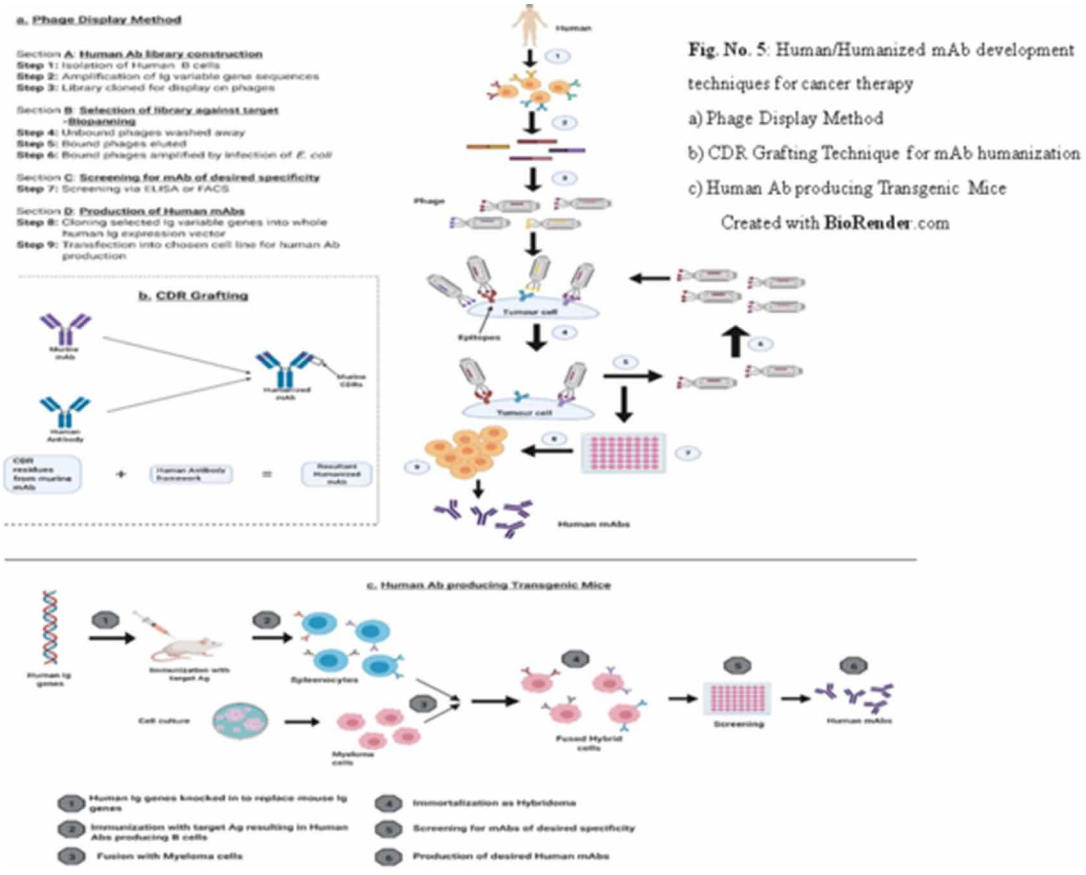
Examples of cancer therapeutic mAbs (FDA approved):

- XenoMouse was used to produce Panitumumab, an anti-EGFR. [Berardi et al., 2010].
- HuMabMouse was used to produce Ipilimumab [Gibney et al., 2019].

CURRENT CHALLENGES AND FUTURE PROSPECTS

In the last several years, mAbs have gained increasing importance as therapeutics and have become a major treatment approach studied and applied for numerous diseases; particularly in oncological diseases. [Grilo & Mantalaris, 2019]. Technological advances in mAb generation and engineering have only served to provide impetus to the field.

Figure 5. Human/Humanized mAb development techniques for cancer therapy, (a) Phage Display Method, (b) CDR Grafting Technique for mAb humanization, (c) Human Ab producing Transgenic Mice, (Created with BioRender.com)



However, despite their highly specific nature, therapeutic mAbs have been shown to cause adverse effects like anaphylactic response and early drug clearance; thereby affecting its pharmacokinetic properties. [Chirmule et al., 2012] This may be due to patient's genetic background/previous immunity or the drug's molecular characteristics/formulation/manufacturing process/schedule. [Jefferis R., 2016].

Human and humanized mAbs are predominant in the field of therapeutic Abs. [Grilo & Mantalaris, 2019]. Still, as previously mentioned in section 8.5 (1), even humanized mAbs have been seen to have a certain degree of immunogenicity. Fully human mAbs should in theory, be even less immunogenic than

the chimeric or humanized forms. Still, several such Abs are reported to have induced marked immune responses, perhaps because the Fv region of even the fully human mAb is not identical to that of human germline. In a study with Adalimumab, anti-drug Abs were induced in up to 30% of the patients Bartelds et al., 2007; Goupille P., 2016]. This where i*n silico* identification of immunogenic sequences may aid [Hamze et al., 2017] engineering of less immunogenic mAbs.

Since the panning process for Ab selection through phage display is subject to bias, the binding epitope may be optimized via *in vitro* affinity maturation. This would entail mutagenesis by random or targeted approach and *in silico* modelling of Ab-Ag interaction for the purpose of improvement of Ab affinity toward the target Ag. [Batista & Neuberger, 1998; Chowdhury P. S., 2003; Balint & Larrick, 1993; Lamdan et al., 2013]

The stability of therapeutic mAbs is still unsatisfactory. This may be achieved by the use of stabilizing agents like surfactants [Agarkhed et al., 2018]; or by improving the molecular structure itself. The latter makes use of protein engineering to incorporate amino acid residues that increase stability [Cutois et al., 2016; Lawrence et al., 2007].

A variation in response rates between patients to cancer mAb therapy occurs due to a wide range of reasons. The tumour microenvironment affects the ease of access of therapy to the tumour. Development of cancer resistance to treatment due to genetic and epigenetic changes is unavoidable due to the highly proliferative nature of cancer cells, and tendency of cancer cells to activate alternative pathways as per changes in the environment. The type and stage of cancer may render the therapy ineffective. Greater diversity of individual immune system e.g. HLA locus diversity corelates with better response rates and survival. The administration of prior chemotherapy would reduce efficacy of the therapy as it reduces the competency of the immune system. Since gut microflora assists in the maintenance of a healthy immune system, therefore it should contribute to the success of the treatment[Sambi et al., 2019; Nesse et al., 2015; Zugazagoitia et al., 2016; Weiner L. M., 2015; Zaretsky et al., 2016; Chowell et al., 2018; Cerf-Bensussan, & Gaboriau-Routhiau, 2010;Gopalakrishnan et al., 2018]

Response rates to cancer therapeutic mAbs can be improved in the future in many possible ways. Discovery of additional conserved biomarkers on tumour surface is necessary, so that the therapy may be applied to a diverse patient population. Resistance to therapy may be overcome by use of combination therapy. Increased efficiency of mAb therapy would be achieved if used as first-line treatment, enabling robust anti-cancer host immune response. Tracking the mutational status of the cancer could help predict the likelihood of a therapy being effective. Probiotics and prebiotics can help to alter or diversify the gut microflora, which may improve immune response. Nanotechnology is being exploited for designing carrier systems to enhance delivery. [Sambi et al., 2019; Ventola C. L., 2017; Zaretsky et al., 2016; Par doll D., 2015; Kwang et al., 2013]

There has been a failure to bring the field of immunotherapeutic up to the mark with respect to paediatric cancer. The reasons are most likely to be variability in cancer type, location, and difference in immune cell composition in adults as compared with children. Hence, ascertaining immune cell differences must be the focus, in order that proper cancer immunotherapies are selected [Boklan J., 2006].

Immune checkpoints play a crucial role in the modulation of immune responses and maintenance of self-tolerance. Immune checkpoint therapy gained wide recognition when the Nobel Prize for Physiology/ Medicine in 2018 was awarded to Allison and Honjo for their work on immune checkpoints. This form of therapy revolutionized the approach to cancer treatment that attempts to engage the immune system. Hence, more and more are the checkpoint molecules being discovered and targeted for mAb therapeutics [Donini et al., 2018; Pardoll D., 2012; Nobel Prize in Physiology or Medicine, 2018].

Improved success by combination of mAbs with other forms of cancer therapy such as chemotherapy, radiation therapy, vaccines and other immunomodulatory approaches have been reported; this would be worthwhile exploring further in the future. [Taylor et al., 2007; Bonner et al., 2010; Khan et al., 2006; van Elsas et al., 1999]

9. CONCLUSION

Monoclonal antibodies have carved their niche as a form of targeted therapy and immunotherapy in the ever-challenging realm of oncology. Technological advances as well as increased insights into immuno-oncology have allowed mAb therapy to grow leaps and bounds; and certainly, it shall be interesting to see how it evolves even further in the future.

ABBREVIATIONS

Ab/Abs: Antibody/Antibodies
Ag: Antigen
APC(s): APC antigen presenting cell(s)
ADCC: Antibody-dependent cellular cytotoxicity
CAR: Chimeric antigen receptor
CAR-T: Chimeric antigen receptor T cell
CD: Cluster of differentiation
CDC: Complement dependent cytotoxicity
CDR: Complementary-determining region
CSC: Cancer stem cell
CTL: Cytotoxic T lymphocytes
CTLA-4: Cytotoxic T-lymphocyte associated antigen -4
CXCR4: C-X-C chemokine receptor-4
DNAM1: DNAX accessory molecule-1
EGFR: Epidermal growth factor receptor
EMA: European Medicines Agency
EpCAM: Epithelial cell adhesion molecule
Fab: Antigen-binding fragment
Fc: Fragment crystallizable region
FcγR: Fcγ receptors
FOXP3: Forkhead box protein-3
GM-CSF: Granulocyte–macrophage colony-stimulating factor
HAMA: Human anti-mouse/murine antibody
HER: Human epidermal growth factor receptor
ICOS: Inducible T cell co-stimulator
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin

LAG-3: Lymphocyte activation gene- 3
mAb: Monoclonal antibody
MHC: Major histocompatibility complex
MICA/B: MHC class I chain-related protein A/B
NCR: Natural cytotoxicity receptor
NKG2D: NK cell group-2D
NK cell: Natural killer cells
NSCLC: Non-small cell lung cancer
PAP: Prostatic acid phosphatase
PBMC: Peripheral blood mononuclear cell
PCR: Polymerase chain reaction
PD-1: Programmed cell death protein 1
PDGF: Platelet-derived growth factor
PDGFR: Platelet-derived growth factor receptor
PD-L: Programmed death-ligand
RANKL*:* Receptor activator of nuclear factor-κB ligand
ROS: Reactive oxygen species
r-DNA: Recombinant-DNA
scFv: Single chain fragment variable
SDF-1: Stromal cell-derived factor-1
SLAMF7: Signalling lymphocytic activation molecule family-7
TAA(s): Tumour-associated antigen(s)
TCR: T cell receptor
TGF-β: Transforming growth factor
TIM-3: T-cell immunoglobulin mucin-3
TME: Tumour microenvironment
TNF-*α*: Tumour necrosis factor-alpha
TNFR: Tumour necrosis factor receptor
Treg cells: Regulatory T cells
US FDA: United States Food and Drug Administration
VEGF: Vascular endothelial growth factor
VEGFR: Vascular endothelial growth factor receptor
VISTA: V-domain Ig suppressor of T cell activation

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Chapter 3
Advancements in Cancer Therapeutics:
Computational Drug Design Methods Used in Cancer Studies

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ABSTRACT

In this chapter, computational approaches for the discovery of new drugs that are useful for diagnosis and treatment of disease will be described in three parts. MD technique uniquely supports protein design attempts by giving information about protein dynamics associated with atomic-level descriptions of the relationship between dynamics and function. The purpose of molecular docking is to provide an estimate of the ligand-receptor complex structure using computational methods. By this estimation, the mechanism of drug binding and action are described by determining the three-dimensional simulation of drug and drug-induced macrostructure. ADME characteristics are physicochemically significant descriptors and pharmacokinetically relevant properties used to design more effective drugs and new analogs. As a result, in-silico calculations can provide robust preliminary information as to drug activity and mechanism in the drug production process, as well as in vitro and in vivo studies.

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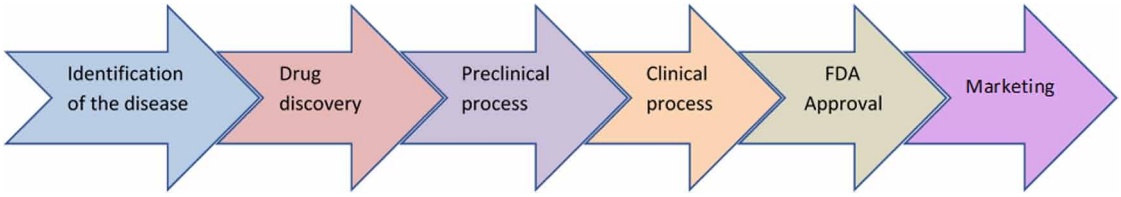
INTRODUCTION

Drugs are a very important factor that affect human life and health. Unfortunately, the discovery of molecules that result in safe and effective drugs involves a process that has high cost at production, development and testing stages; they must go through certain stages as a result of the experiments performed in the laboratory, and then include clinical research. Drug research contains several phases. Phase 1 covers a period of 1 to 1.5 years, in which the pharmacokinetic properties of the drug, its toxicity, and its effect on body functions are determined. Phase 2 is a clinical trial period that lasts 1 to 3 years to determine therapeutic dose limits in order to investigate the clinical efficacy and safety of the product. Phase 3 lasts 3 to 4 years. At this stage, the phase 1 and phase 2 periods are tested. Phase 4 is a process for further clinical investigation of the approval of approved products, indications for administration. These basic studies, which include finding a new molecular structure that can be used as a drug, finding new uses of existing molecular structures, and re-evaluating the adverse effects of a drug, are conducted through clinical tests, thus covering a long and costly process. The drug design, application and development mechanism is shown in Figure 1.

Figure 1. The drug design, application and development mechanism



Designing and developing the most effective drug in a short time and with lower costs attracts the attention of many scientists working in different fields. In the process of designing the most effective drug, in silico (applied in computer environment) methods are preferred because it minimizes time and cost. The appropriate drug structures obtained in accordance with the calculations made with in silico methods allow for more rational drug designs by reducing the processes of organic synthesis with high budget. The aim of molecular modeling methods that define molecular systems at the atomistic level is to show how atoms and molecules can interact with a three-dimensional image and simulation, and to determine the structure of these interaction mechanisms. These models can also be used to interpret existing observations or to predict new chemical behaviors. In the drug design process, in silico methods have become a valuable and necessary tool for the modeling of molecular structures that have been nominated for drugs, for increasing the effectiveness of drugs, and for the design of new drug molecules with unknown molecular structure. With these methods, it is possible to examine the relationship between chemical structure and function from small systems to large biologic molecules and material groups. Molecular biology, protein science, drug design, electronic and photonic materials, and polymer science are among these areas. With the help of in silico methods, information can be obtained from the microscopic details of the system up to the macroscopic properties. In other words, it is possible to understand the biochemical and physicochemical properties of molecules by performing a perfect calculation with these methods under high conditions such as high pressure and temperature. The contribution of modern

computer-aided drug design to the discovery of drugs is an indisputable fact, and is understood to have been used by large pharmaceutical companies in many commercially available drugs.

The most preferred methods for drug design will be described in two parts.

Molecular Dynamic Methods
Molecular Docking Methods

MOLECULAR DYNAMIC METHODS

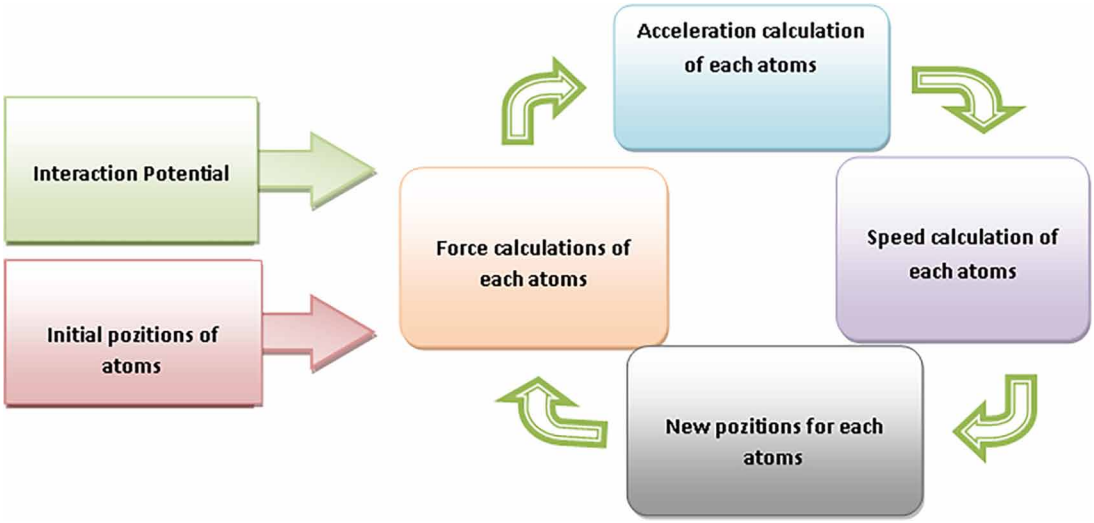
Various experimental techniques may provide information about the dynamics of proteins and other biomolecules, but they generally report their spatial and temporal averaging properties, not as the individual molecules in the protein (Dror, Dirks, Grossman, Xu, & Shaw, 2012). The quantum mechanical behavior of molecules at the subatomic level is defined by the time-dependent Schrödinger equation, but a direct solution of this equation is practically impossible for the calculation of biologic macromolecules. The standard method for simulating the movements of such molecules is known as molecular dynamics (MD) simulation, in which the positions and velocities of the particles representing each atom in the system are determined according to the classic physics laws (Dror et al., 2012). Experimental techniques and rational design strategies, as well as MD methods, which model atomic-level motions computationally based on first-principles physics in the design and engineering of proteins, have been preferred to simulate the movement of proteins according to classic dynamics. One of the strongest aspects of MD is that, thanks to the simulations, dynamic molecular interactions that contribute to protein stability and function provide protein design by revealing atomistic details. MD simulations can also be used as a virtual scanning tool to sort, select, define, and evaluate potential designs (Childers & Daggett, 2017). MD is a physical method used to study the interaction and movement of atoms and molecules according to Newton's physics. In this method, inter- and intra-molecular interactions are taken into account. The MD method allows the determination of the new state of a known system after a period of time, by mathematical equations, so that the molecular structure-function-motion relationship is determined and the behavior of the system according to time is revealed. MD is based on the solution of Newton's equation of motion for small time intervals for an N-particle system. The force acting on any particle in the system (i. particle) is calculated as the sum of the forces applied to that particle by other particles in the system. From this force expression, the acceleration and velocity of the particle are obtained. The new position of the particle is calculated from the acceleration and velocity expression and the force and energy are calculated for the particle in the new position. These calculations are repeated continuously to determine the behavior of the N-particle system over time (Frenkel & Smit, 2002; Van Gunsteren & Berendsen, 1990).

The calculation diagram of the MD method is given in Figure 2.

$$\boldsymbol{F_i} = m_i \frac{d^2 \boldsymbol{r_i}(t)}{dt^2} \; (i=1, 2, 3, \ldots, \mathrm{N}) \tag{1}$$

The position and mass of the i. atom are expressed by their sequence r_i and m_i, respectively.

Figure 2. Calculation diagram for MD method



The force expression is also equal to the negative gradient of potential energy:

$$\boldsymbol{F_i} = -\vec{\nabla}_i V\left(r_1, r_2, \ldots, r_N\right) \tag{2}$$

These two equations are equalized to each other;

$$-\frac{\partial V}{\partial r_i} = m_i \frac{d^2 r_i(t)}{dt^2} \tag{3}$$

The following equations are reached from the solution of the equation of motion.

$$\boldsymbol{a} = \frac{\boldsymbol{F}}{m} \tag{4}$$

$$\boldsymbol{v} = \boldsymbol{v}_0 + \boldsymbol{at} \tag{5}$$

$$\boldsymbol{r} = \boldsymbol{ro} + \boldsymbol{v}_0 t + \frac{1}{2}\boldsymbol{a}t^2 \tag{6}$$

In order to perform orbit calculations, it is necessary to have knowledge of the atoms, their initial position, initial velocity distributions, and the accelerations that can be obtained by the gradient of potential energy. These cycle calculations continue until the total potential energy for the system becomes constant. With this method, if the speed and location of each atom are known at any time, it is possible to determine the state of the system at any time in the past or future. MD also allows examining the

experimental behavior of a system that is difficult or impossible to observe. Parameters such as the number of particles (N), temperature (T), and pressure (P) of the system give the thermodynamic state of that system. Several of the ensembles used in MD simulations are given in Table 1 (Menteş, 2009).

Table 1. The ensembles used in Molecular Dynamic (MD) simulation

Ensembles		
Microcanonic ensembles NVE	• Number of Particles (N) • Constant Volume (V) • Constant Energy (E)	Corresponds to isolated systems.
Canonical ensembles NVT	• Number of Particles (N) • Constant Volume (V) • Constant Temperature(T)	Corresponds to all systems
Isobaric-isothermal ensembles NPT	• Number of Particles (N) • Constant Pressure (P) • Constant Temperature (T)	Corresponds to all systems

The force fields used for molecular systems can be classified as having two basic components, such as internal and external forces. There are some limitations in the calculation of energy related to the deviations in their angular values between the equilibrium and the reference values. In the force field function, these angular expressions refer to the energy change of the system when the bonds are stretched or shortened or bent. In more complex force field functions, bond stretching, angle bending, dihedral rotations, and the electrostatic and van der Waals interactions between the non-bonded atoms in the system are also included. A force field is used to define these energies, which is a potential energy function used to estimate the forces between interacting atoms and to calculate the total energy of the system (De Vivo, Masetti, Bottegoni, & Cavalli, 2016). Thus, together with these force fields, all interactions occurring between atoms are modeled according to reality and results compatible with experimental data are obtained. The potential energy function, which defines the force field, includes the potentials for intra- and inter-molecular interactions. The intra-molecular interactions include bond stretching, angle bending, dihedral rotations potential terms, while the inter-molecular interactions are based on electrostatic interactions and van der Waals interactions (Field, 1999; Hedman, 2006).

$$U_{intra\text{-}molecular} = U_{bond\text{-}stretching} + U_{angle\text{-}bending} + U_{dihedral\text{-}rotations} \tag{7}$$

$$U_{inter\text{-}molecular} = U_{electrostatic} + U_{Van\ der\ Waals} \tag{8}$$

Bond Stretching Potential Energy

The interaction between at least two atoms in the molecule is generally determined by the bond length potential, which is usually defined by a quadratic equation. It is assumed that there is a spring between the two atoms and the coefficient of the function, depending on the bond type and the atoms it contains, is considered to be equal to the spring constant. In biomolecules, the bonds have a stiff degree of freedom, so energy is only correct for values close to the equilibrium length.

$$U_{bond-stretching} = \sum_{i,j} k_{i,j}{}^{b} \left(r_{i,j} - r_{i,j}{}^{0} \right)^2 \quad (9)$$

$k_{i,j}^{b}$; corresponds to the spring constant,
$r_{i,j}^{0}$; distance between atoms i and j at equilibrium
$r_{i,j}$; distance between atoms i and j at momentary displacement

Angle Bending Potential Energy

The interaction between at least 3 atoms bound by two chemical bonds is defined by the angle bending potential. As in the bond stretching potential energy, it is assumed that there is a spring between the two bonds and this constant is expressed as $k_{i,j,k}^{a}$ the potential function coefficient. Angles are also have a stiff degree of freedom in biomolecules, so the energy function is only correct for values close to the equilibrium angle.

$$\mathrm{U}_{angle\text{-}bending} = \sum_{i,j,k} k_{i,j,k}^{a} \left(\theta_{i,j,k} - \theta_{i,j,k}^{0} \right)^2 \quad (10)$$

$\theta \mathrm{i}_{,j,k}$; instantaneous angle value,

$\theta_{i,j,k}^{0}$; angle value at the moment of equilibrium

Dihedral Rotation Potential Energy

A dihedral angle is defined as the angle between two intersecting planes which composed of four atoms. In chemistry, a torsion angle is defined as a special name of a dihedral angle, defining the geometric relationship of two parts of the molecule linked by a chemical bond. Torsions can occur on single chemical bonds within the molecule and these torsions affect the total energy of the molecule. Molecule rotates around single chemical bonds with a little energy from the environment. These turns are not completely free, but are braked.

$$U_{dihedral-rotation} = \sum_{i,j,k,l} k_{i,j,k,l}{}^{c} \left[1 \pm \mathrm{Cos}\, n \left(\phi_{ijkl} - \phi_{ijkl}{}^{o} \right) \right] \quad (11)$$

where $k_{ijkl}{}^{c}$: force constant between atoms, $\phi_{i,j,k}$: instantaneous angle value, $\phi_{i,j,k}{}^{o}$: angle value at the moment of equilibrium

Electrostatic Interaction Potential Energy

It is used to calculate the potential energy between charged atoms in the molecule. This potential is directly proportional to the charge of atoms and is inversely proportional to the distance between atoms.

$$U_{electrostatic} = \sum_{i,j}^{N} \frac{q_i q_j}{\varepsilon r_{ij}} \tag{12}$$

q_i and q_j; the charges on the atoms,
r_{ij}; the distance between atoms;
ε; the dielectric constant of the environment

Van der Waals Interaction Potential Energy

Although the orders of electrons around the atoms in the molecule are on an average symmetrical, a small fluctuation in the electron distribution means that the symmetrical arrangement can be asymmetrical for a moment. When the instant electronic settlement changes, instant dipoles occur, this induces interactions with all neighboring atoms and causes atoms to attract each other. When the two atoms arrive at a very close distance, a repulsion force occurs between the surrounding electron clouds and this repulsion is greater than the induced attraction between atoms. At the optimum distance, which is 2.5-2.7 Å between two unbound atoms, called the van der Waals contact distance, repulsion and attraction forces known as London dispersing forces are formed. These bonds are weak due to the instant dipole interaction and are called van der Waals bonds. Although van der Waals interactions are 100 times smaller than chemical bond energy, their contribution to the energy of the molecule is important because it takes place between many atoms. The Lennard-Jones potential (Eq.13) is used to identify this energy. The interaction potential of van der Waals is a short-range interaction.

$$U_{vdw} = \sum_{i,j} 4\varepsilon_{i,j} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{6} \right] \tag{13}$$

$\sigma i_{j;}$ distance between particles where the potential is zero,
$\in_{ij}$; is the depth of the potential well
r_{ij}; represents the distance between atoms.

Along with all these interaction potentials, the system's total potential energy equation is obtained. Some of the most commonly known force fields used in MD are; AMBER, CHARMM, GROMOS, and OPLS force fields. Nowadays, with the emergence of innovative hardware and software codes, it is possible to run MD simulations from nanoseconds to microseconds within a few milliseconds. This enables a detailed examination of the conformational area, including large biomolecules. The exact description of the path followed by the ligand bound to a target protein can be determined by the MD trajectories, and protein-ligand binding, which is critical to drug discovery is also performed by this method using thermodynamic and kinetic data. Some of the programs used to perform MD simulation are given below;

AMBER: The Amber software package performs biomolecule simulations using the AMBER force field. This program is written in the Fortran 90 and C programming languages (Case et al., 2018).

CHARMM: The name for the commonly used force fields for molecular dynamics and their associated molecular dynamic simulation and analysis is the name of the computer software package. This program enables the production and analysis of a wide variety of molecular simulations (Brooks et al., 1983).

GROMACS: This program, GROningenMAchine for Chemical Simulations, was written at the University of Groningen in the early 1990s using the source code system (SCCS). GROMACS is a versatile program that solves Newton's equations with specific approaches for systems containing hundreds of particles while performing molecular dynamic simulations. First, this program, designed for biochemical molecules such as proteins, lipids, and nucleic acids containing complex bound interactions, is extremely fast in calculating unbound interactions. It also uses non-biologic systems such as polymers. It runs 3-10 times faster than many simulation programs. The command line is executed through the interface and the files are used as input and output. The calculation, progress, and estimated end time (ETA) provide a comprehensive view of the feedback, trajectory viewer, and trajectory analysis. In addition, the availability of different force fields makes GROMACS a suitable program (Berendsen, van der Spoel, & van Drunen, 1995; Hess, Kutzner, Van Der Spoel, & Lindahl, 2008; Lindahl, Hess, & Van Der Spoel, 2001). The GROMACS program is compatible with GROMOS, OPLS, AMBER, and ENCAD force field (Van Der Spoel et al., 2005).In addition to these programs, NAMD, LAMMPS, and GROMOS programs can also be provided for MD. MD studies published (Budama-Kilinc, Cakir-Koc, Kecel-Gunduz, Zorlu, et al., 2018; Kumar, 2017; Pavlin et al., 2018; Vettoretti et al., 2016).

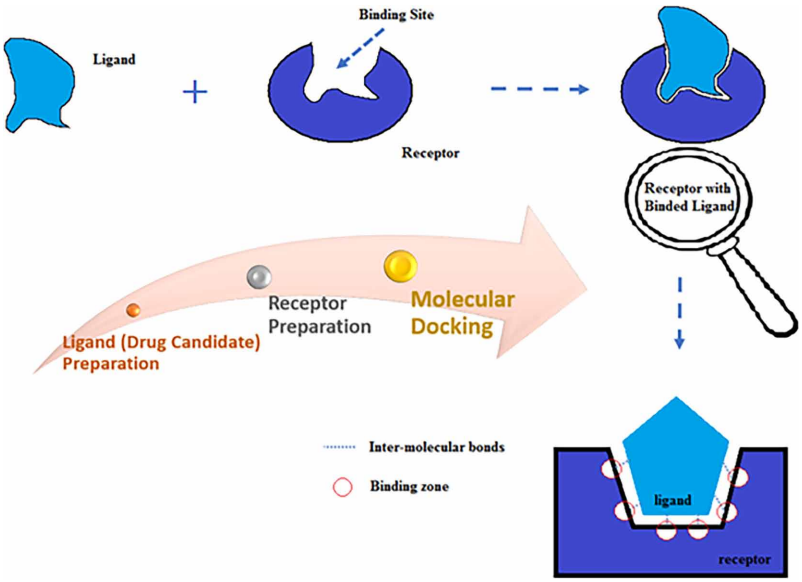
The are some limitations of MD simulations such us timescales, force field accuracy, covalent bonds during MD simulations. For numerical stability, the short time steps simulations are required. Despite the simulation times increased from nanoseconds to microseconds, simulation timescales remain a challenge for MD calculations. Although molecular mechanical force fields have undergone significant improvements, they are naturally approximations and contain some limitations. In addition, the fact that the formed covalent bonds cannot be broken or regenerated imposes limitations on calculations during MD simulations.

MOLECULAR DOCKING METHOD

Molecular docking methods are of great importance in the planning process of potential new drugs. The aim of molecular docking is to provide a prediction of the ligand-receptor complex structure and to determine the best conformation of the ligand to obtain a ligand-receptor complex with the lowest energy using calculation methods(Khan T, 2018). The molecular docking approach can be used to model the interaction at the atomic level between small molecules and a macromolecule like proteins or enzymes; this allows us to elucidate the basic biochemical processes by characterizing the behavior of small molecules at the binding site of the target macromolecules (McConkey, Sobolev, & Edelman, 2002; X.-Y. Meng, Zhang, Mezei, & Cui, 2011). The diagram of molecular docking is shown in Figure 3.

The interactions of target macromolecule-drug candidate molecules are achieved with the help of intermolecular bonds. In addition to ionic and covalent bonds, the most common bonding of hydrogen bonds and van der Waals interactions are of great importance in receptor-ligand interactions. As a result of these interactions, effects on efficacy and selectivity are investigated by linking the drug candidate molecule to the target macromolecule. The structure-activity relationships of the molecules that may be drug candidates before the synthesis steps are investigated in molecular docking studies (Ece & Sevin, 2013).

Figure 3. The diagram of molecular docking



Many programs such as AutoDock Vina, GOLD, FlexX, and Glide are currently used for molecular docking studies. Molecular docking programs can differ from each other due to the difference of the search algorithms and scoring functions used in determining a docking pose.

The process of ligand docking contains two steps and the diagram of ligand docking process is seen in Figure 4.

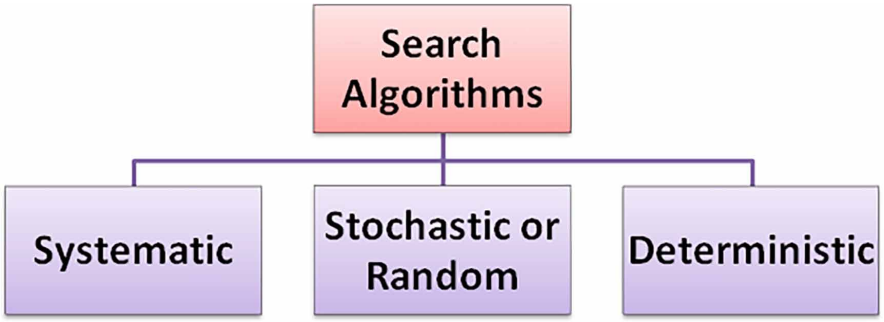
1- Estimation of ligand conformation and orientation called the pose by search algorithms.
2- Evaluation of binding affinity by scoring functions.

Figure 4. The diagram of ligand docking process

Search Algorithms

In molecular docking, search algorithms are used to discover the free energy field to find the best ligand poses. According to the method used to investigate ligand flexibility, search algorithms are divided into three main groups: systematic, stochastic (or random) and deterministic searches Figure 5. Table 2 presents different search engine available for search algorithms.

Figure 5. The diagram of search algorithms



Systematic search algorithms investigate all degrees of freedom of the ligand. The ligand conformation number is associated with the number of rotatable bonds. The greater the number of rotatable bonds, the great the number of combinations that can be affected. Therefore, for the placement to be more practical, geometric / chemical constraints are applied to the initial screening of ligand poses, and the filtered ligand conformations are subjected to more precise correction / optimization processes (Huang & Zou, 2010).

Stochastic or random methods can form randomly changeable degrees of freedom, including translational, rotational, and conformational, of the ligand at each step. These ligand poses are evaluated according to the probability criteria to decide whether or not to reject each one. There are several different ways in which random method algorithms operate such as the Monte Carlo method, the genetic algorithm method, evolutionary algorithms, and particle swarm optimization (Guedes, de Magalhães, & Dardenne, 2014; Huang & Zou, 2010).

Deterministic methods, which is the third category, include methods such as molecular simulations by considering a ligand as a flexible body (Guedes et al., 2014). Simulations provide details of the movement of individual particles as a function of time (Dastmalchi, 2016). These methods are based on calculations performed with Newton's equations of motion.

Table 2. Some examples of search algorithms

Systematic Search	Stochastic Search
GLIDE (Friesner et al., 2004)	Auto Dock (Morris, Goodsell, Huey, & Olson, 1996)
DOCK (Ewing, Makino, Skillman, & Kuntz, 2001)	GOLD (Jones, Willett, Glen, Leach, & Taylor, 1997)
FlexX (Rarey, Kramer, Lengauer, & Klebe, 1996)	Molegro Virtual Docker (Sochacka, 2014)
SLIDE (Schnecke & Kuhn, 1999)	CDocker(Wu, Robertson, Brooks III, & Vieth, 2003)
FRED (McGann, 2012)	MOE_Dock(Corbeil, Williams, & Labute, 2012)

Scoring Functions

During the docking process, the search algorithm searches for many ligand conformations. After the conformations are determined with the search algorithms in the first step, scoring functions separate the correct exposures from false exposures or separate the binders from inactive compounds within a

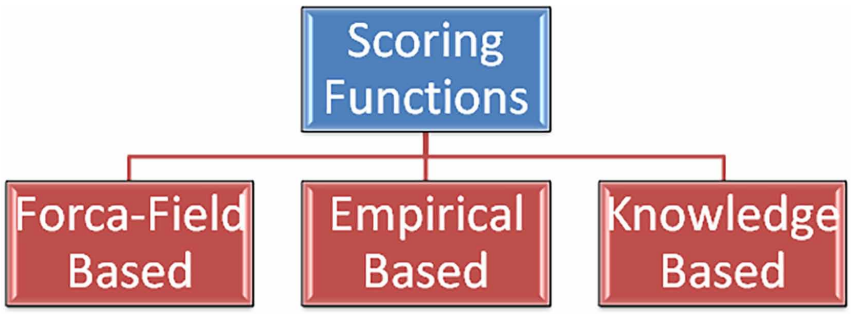
reasonable calculation period. The basic aim of scoring functions is to approximately predict the binding affinity between two molecules using some mathematical functions after the docking process (Jain, 2006). Scoring functions can be divided into force field-based, empirical-based, and knowledge-based scoring functions. The diagram of scoring functions were seen in Figure 6 and Table 3.

The scoring functions based on force field, fundamentally consider the non-bonded terms of the molecular mechanic's force field; these functions predict the interaction energy between the receptor and the ligand (E. C. Meng, Shoichet, & Kuntz, 1992). Scoring functions based on the classic force field determine the binding energy by calculating the sum of bond (stretching/bending/torsional) terms, the electrostatic and van der Waals interactions. Electrostatic and van der Waals interactions are estimated using the Coulomb formulation and Lennard-Jones potential function, respectively (Ferreira, dos Santos, Oliva, & Andricopulo, 2015). The challenges in force field-based scoring functions are how to account for solvation and entropy contributions. The simplest method is to use a distance-dependent dielectric constant ε(rij) such as the force field scoring function:

$$E= \sum_{i,j}\left(\frac{A_{ij}}{r_{ij}^{12}}-\frac{B_{ij}}{r_{ij}^{6}}+\frac{q_i q_j}{\varepsilon\left(r_{ij}\right)r_{ij}}\right) \tag{14}$$

Force-field-based scoring functions can be extended by adding hydrogen bonds, solvation, and entropy contributions. The force-field-based functions that adjust with other techniques such as free-energy perturbation methods (FEP) can be used to improve the accuracy in predicting binding energies (Ece & Sevin, 2013; Huang & Zou, 2010; McConkey et al., 2002; X.-Y. Meng et al., 2011).

Figure 6. The diagram of scoring functions



In empirical scoring functions, the binding energy includes various energy components such as van der Waals energy, electrostatic energy, hydrogen bonding, desolvation terms, hydrophobic effect, and binding entropy. With increased numbers of crystal structures of various macromolecules (such as protein or enzyme)-ligand complexes with known binding affinities, an empirical scoring function could be developed by training on the binding constants of many receptor-ligand complexes (Ece & Sevin, 2013; Huang & Zou, 2010; McConkey et al., 2002; X.-Y. Meng et al., 2011). However, because of the simplicity of the employed energy terms, empirical functions are faster than force-field-based methods(Ferreira et al., 2015).

$$\Delta G = \sum_i W_i . \Delta G_i \tag{15}$$

where ΔG_i symbolize empirical energy terms (Van der Waals energy, electrostatics energy, hydrogen bonding, desolvation terms, binding entropy, hydrophobicity effect etc.) and the corresponding coefficients W_i are identified by fitting the binding affinity data of a training set of receptors–ligand complexes known three-dimensional structures(Böhm, 1994; Eldridge, Murray, Auton, Paolini, & Mee, 1997; Head et al., 1996; Jain, 1996; Wang, Liu, Lai, & Tang, 1998).

A knowledge-based scoring function, the third approach of scoring function, uses statistical analysis of crystal structure of ligand-receptor complexes to obtain inter-atomic distances between ligand and macromolecule (protein or enzyme). The principle of knowledge-based scoring functions is based on the potential of the mean force obtained by the inverse Boltzmann relation. For receptor–ligand studies, the potentials are calculated by;

$$w(r) = -k_B T In\left(\frac{\rho(r)}{\rho^*(r)}\right) \tag{16}$$

where k_B is the Boltzmann constant, Tis the temperature of the system, ρ(r) express the number density of the receptor-ligand atom pair at distance r in the training set, and ρ*(r) express the pair density in a reference state where the interatomic interactions are zero.

Knowledge based scoring functions present better balance between accuracy and velocity compared with force field and empirical based scoring functions(Böhm, 1994, 1998; Dastmalchi, 2016; Eldridge et al., 1997; Ferreira et al., 2015; D. Gehlhaar, Bouzida, & Rejto, 1999; D. K. Gehlhaar et al., 1995; Head et al., 1996; Huang & Zou, 2010; Jain, 1996; Krammer, Kirchhoff, Jiang, Venkatachalam, & Waldman, 2005; X.-Y. Meng et al., 2011; Wang, Lai, & Wang, 2002; Wang et al., 1998).

Table 3. Examples of scoring functions applied in used molecular docking programs

Force Field-Based	Empirical	Knowledge-Based
Dock	Glide Score	SMoG (DeWitte & Shakhnovich, 1996)
AutoDock	LUDI (Böhm, 1994)	DrugScore(Gohlke, Hendlich, & Klebe, 2000)
GoldScore(Jones et al., 1997)	ChemScore(Eldridge et al., 1997)	RF Score (Ballester & Mitchell, 2010)
Molegro Virtual Docker	PLP (D. K. Gehlhaar et al., 1995)	PoseScore(Fan et al., 2011)

The Ligand and the Receptor Docking Types

There are three different docking types including ligand/target flexible or rigid based upon the objectives of docking studies.

Rigid Ligand-Rigid Receptor

The search space is limited due to only three translational and rotational degrees of freedom in the rigid ligand and rigid receptor. In this method, the calculations are performed using the predetermined conformations of the ligand or allowing an amount of the overlaps between atoms of the receptor-ligand.

Flexible Ligand-Rigid Receptor

The flexibilities of ligand and receptor are very important for correctly predicting drug binding because the ligand and receptor can change their conformations to form the best pose having minimum energy. However, the flexibility of the receptor is a problem because it increases cost. Therefore, when the accuracy and calculation time are considered, the ligand is kept flexible and the receptor is kept rigid. Flexible ligand and rigid receptor are the most commonly used method.

Flexible Ligand-Flexible Receptor

Flexible ligand-receptor complex studies can be realized by MD simulations. This method includes all the degrees of freedom of the ligand and the receptor, but it cannot create sufficient sample for a docking study. Also, it has high computational cost (X.-Y. Meng et al., 2011).

The common challenges of docking method such as ligand- and receptor- conformation, flexibility and cavity detection. The flexible receptor docking, especially the backbone flexibility at the receptors, is still a major challenge for existing docking methods.

Some molecular docking programs used are discussed below:

Autodock Vina

AutoDock Vina is fast and effective for most systems, predicting optimum docked conformations using coordinate files for receptor and ligand. AutoDock Vina shows good performance for typical biologic size and composition. It uses rigid receptor and this simplification decreases the size of the conformational space and the computational effort of scoring each trial conformation (Trott & Olson, 2010). In docking calculations with AutoDock Vina, a configuration file must be prepared. The configuration file contains the ligand and receptor information, the central Cartesian coordinates of the area to be connected, and the width of the docking region.

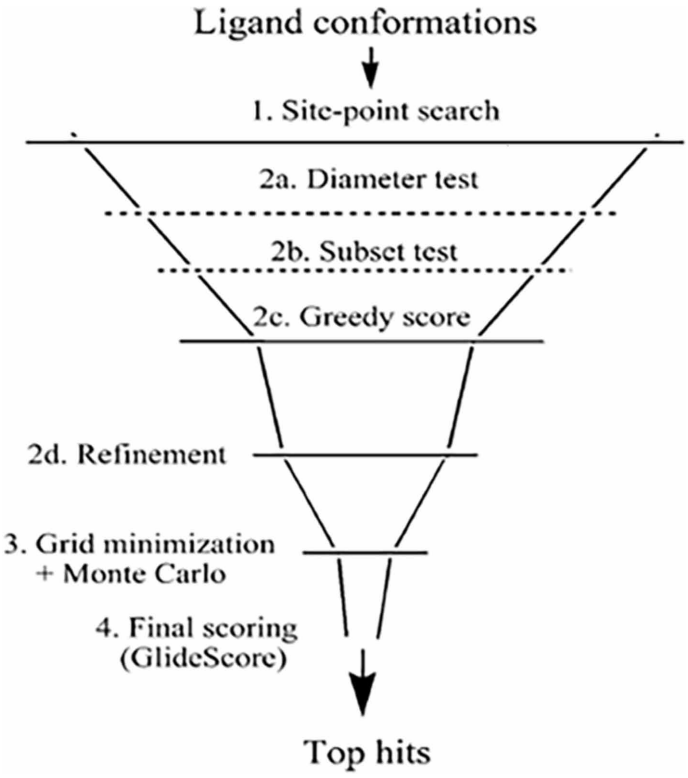
The AutoDock binding affinities of the NAC molecule, which was extensively used as a pharmaceutical prodrug for the treatment of cataract, and a vaccine against to Toxoplasma gondii were investigated in our previous studies(Budama-Kilinc, Cakir-Koc, Kecel-Gunduz, Kokcu, et al., 2018; Cakir-Koc, Budama-Kilinc, Kokcu, & Kecel-Gunduz, 2018). In addition, Phe-Tyr dipeptide (Kecel-Gündüz et al., 2018) with greatest ACE-inhibitory activity was also investigated as a pharmaceutical drug for the treatment of hypertension by using AutoDock Vina.

The RMSD values obtained as a result are very important for the evaluation of docking success. The crystal structures of ligands are taken as a reference to obtain RMSD values. The comparison of structure-ligand poses and the docked poses is performed using the results of successful docking studies. The upper RMSD limit accepted is 2 Å (Dastmalchi, 2016).

Glide (Grid-Based Ligand Docking with Energetics)

Glide is planned to search the positional, orientational, and conformational space available to the ligand, retaining sufficient computational speed to screen large libraries. The aim of Glide is to search possible positions of the ligand in the active site of the receptor using a series of hierarchical filters. Glide presents the full range of fast and accurate options, from the HTVS (High-throughput virtual screening) to the SP (standard precision). HTVS and SP docking use the same scoring function. HTVS decreases the number of intermediate conformations along the docking funnel and the thoroughness of the final torsional refinement and sampling. Additionally, XP (extra precision) performs more extensive sampling compared with SP. The aim of the XP Glide methodology is to semiquantitatively rank the capability of candidate ligands to bind to a specified conformation of the receptor (Friesner et al., 2004; Friesner et al., 2006; Halgren et al., 2004). Glide docking "funnel", showing the Glide docking hierarchy was seen in Figure 7.

Figure 7. Glide docking "funnel", showing the Glide docking hierarchy
(Friesner et al., 2004)



In our last published study, we analyzed the structural behavior of a skin protective tripeptide Gly-His-Lys (GHK) with anti-oxidant and anti-cancer properties. To reveal the mechanism of interaction between GHK tripeptide and Fibroblast Growth Factor, the hydrogen bonding interactions were investigated by Molecular docking calculations using Glide SP module of the Schrodinger Software program. In this

study, the ADME profile of the GHK tripeptide was also carried out using the Qik-Prop tool, and the distribution and absorption values in different tissues were determined and its potential for being able to be a drug was revealed (Kokcu et al., 2019).

ADMEPHARMACOKINETICS

The determination of the ADME profile of molecules is very important because the pharmacokinetic information of drug candidate molecules, such as ADME properties, are taken into consideration during drug development studies at different points in biologic systems. It is determined by the ADME profile that a drug candidate can be easily absorbed by the mouth, easily transported to the target area in the body, and can be easily eliminated from the body. The determination of many properties such as molecular weight, brain/blood partition coefficient, skin permeability, and percent human oral absorption helps in the production and development of effective and beneficial drugs. Lipinski's 5 rules provide information on whether a drug candidate has the ability to become an orally active drug. Lipinski determined four simple physicochemical parameters depending on 90% of the active drugs taken orally that reached the phase II clinical stage. These four simple physicochemical parameters are as follows(Lipinski, 2004; Lipinski, Lombardo, Dominy, & Feeney, 1997):

- Not more than 5 hydrogen bond donors (expressed as the sum of OHs and NHs)
- Not more than 10 hydrogen bond acceptors (expressed as the sum of oxygen and nitrogen atoms)
- A molecular mass less than 500 Daltons
- An octanol-water partition coefficient log P not greater than 5

Some important ADME pharmacokinetic properties are given as follows; and the diagram of formation of ADME profiles are also given in Figure 8.

Molecular Weight (MW)

The molecular weight can be calculated as the sum of the masses of atoms in a molecule. Many fundamental physicochemical properties such as molecular weight, hydrophobicity and polarity reveal very important properties for drug discovery(Bickerton, Paolini, Besnard, Muresan, & Hopkins, 2012). For example, the molecular weight of a peptide-based active drug that is targeted to the kidney because of targeting ability and a high degree of safety plays an important role for increasing efficacy and reducing toxicity (Xu, Zhang, Dang, & Jiang, 2018). MW should be below 500 Daltons according to Lipinski's 5 rules, although broad synthetic trials may cause the size of chemicals to increase. Most successful drugs have molecular weights below 500 Daltons (Hefti, 2008). The masses of elements commonly found in the structure of drug molecules are listed in Table 4.

Hydrogen Bond Acceptors and Donors (HBA and HBD)

According to the Lipinski's 5 rules, an orally active drug in general should have no more than 10 hydrogen bond acceptors (all nitrogen or oxygen atoms) and no more than 5 hydrogen bond donors (the total number of nitrogen–hydrogen and oxygen–hydrogen bonds).The number of nitrogen and oxygen

atoms is very important in determining the number of hydrogen bond donors and acceptors. The number of hydrogen bond donors is defined as the sum of the nitrogen-hydrogen and oxygen-hydrogen bonds and the number of the hydrogen bond acceptors is also defined as the sum of the nitrogen and oxygen atoms in the structure.

Figure 8. The diagram of formation of ADME profiles

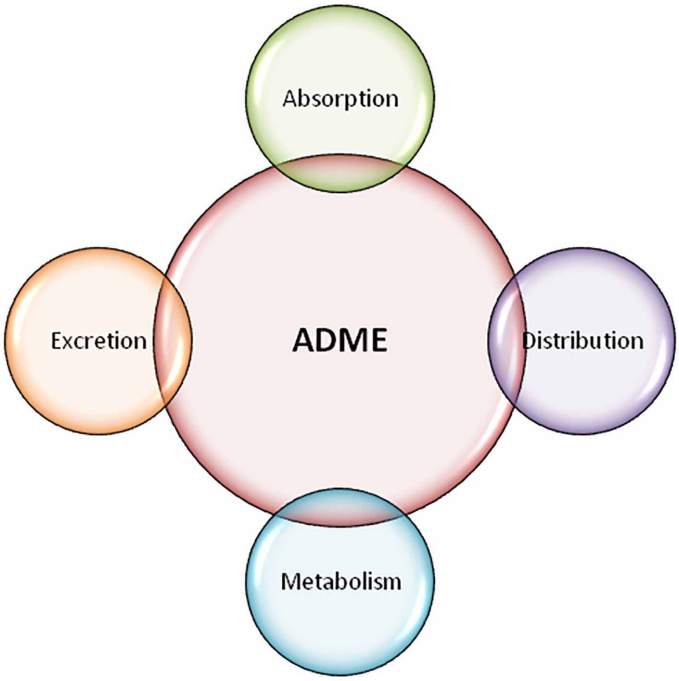


Table 4. The masses of organic chemistry elements commonly found in the structure of drug molecules for use in the calculation of molecular weights

Element	*Atomic Mass*
Carbon (C)	12.011
Nitrogen (N)	14.007
Oxygen (O)	15.999
Sulphur (S)	32.065
Chlorine (CI)	35.453
Fluorine (F)	18.998
Bromine (Br)	79.904
Phosphorus (P)	30.974

Octanol/Water Partition Coefficient (ClogP)

The octanol/water partition coefficient (Log P), as one of estimative factors for the Blood Brain Barrier (BBB), is a descriptor for aqueous media (Tihanyi & Vastag, 2011). Log P, which is the partition coefficient that gives a ratio of the two concentrations, offers a prediction about whether a molecule is hydrophilic or hydrophobic (lipophilic). The determination of Log P of a molecule can also give information about absorption, distribution, metabolism, and excretion.

Polar Surface Area (PSA)

Polar surface area (PSA) is a crucial parameter in physical chemistry studies for drug discovery because PSA is used to characterize the transportation process of drug molecules, such as the octanol-water partition coefficient, and PSA can be attributed to several reasons for drug absorption (Kubinyi & Folkers, 2008). The determination of the PSA is realized with the sum of the surface area of the oxygen and nitrogen atoms, called polar atoms, including their attached hydrogens in the molecule structure. PSA less than 140 $Å^2$ has a surrogate property for cell permeability. PSA must be less than 90 $Å^2$ to be used as a surrogate for BBB penetration (Hitchcock & Pennington, 2006; Pajouhesh & Lenz, 2005). PSA influences BBB permeability, as well as positive effects on intestinal absorption and peripheral circulation restriction problems (Clark, 2011). PSA also best defines the concepts of molecular polarity, H-binding properties, and solubility, which plays important roles in cell membrane penetration (Kubinyi & Folkers, 2008). To predict BBB penetration, van de Waterbeemd and Kansy (van de Waterbeemd & Kansy, 1992) performed a PSA study. In addition, Caco-2 permeability was estimated by using PSA (van De Waterbeemd, Camenisch, Folkers, & Raevsky, 1996).

The PSA can be obtained from the 3D structure of a molecule. However, 3D conformation of the molecule is needed to obtain the PSA. This situation may cause the process to be long and intensive. For this reason, Ertl et al. developed a predictive model that only needed a topology file for ease of PSA calculation. This predictive model was called the Topological Polar Surface Area (TPSA) (Ertl, Rohde, & Selzer, 2000).

Caco-2 Cell Permeability and MDCK Cell Permeability

Caco-2 and MDCK (Madin-Darby Canine Kidney) are preferred in cell-based models for drug studies. Caco-2 cells, which are obtained from human colonic adenocarcinoma, have many morphologic and functional properties of the intestinal epithelial cell barrier (Tihanyi & Vastag, 2011). It is an extensively used cell line in different in vitro cell culture studies to predict the intestinal permeability of drug candidates (Castillo-Garit, Marrero-Ponce, Torrens, & García-Domenech, 2008). In the literature, the range of Caco-2 permeability is accepted as $<1 \times 10^{-6}$ (0-20% poor), $1\text{-}10 \times 10^{-6}$ (20-70% moderate), $>10 \times 10^{-6}$ (70-100% good), respectively (Chaturvedi, Decker, & Odinecs, 2001; Yee, 1997). MDCK cells, which are also important in cell studies such as cell polarity, cell-cell adhesions, have an important role for drug development studies (O'Brien, Zegers, & Mostov, 2002). MDCK cell lines show similar properties to caco-2 cell lines regarding the observed permeability versus human intestinal absorption (Irvine et al., 1999; Tihanyi & Vastag, 2011), but it is an alternative and can be a useful tool for rapid membrane permeability screening.

Skin Permeability

The skin permeability parameter is important for development studies of drugs administered through the skin. The Kp permeability coefficient, which is given with units of cm/h, is beneficial for comparing the diversity of drugs and offers a prediction of their relative skin permeability. The prediction of skin permeability of any drug is linked to the skin of the species used. For example, the range of Kp is generally 10^{-5} - 10^{-3} cm/h for human skin (Ranade & Cannon, 2009).

Kp is given as following formula (Gupta et al., 2010),

$$K_p = \frac{K_m \times D}{h} \tag{17}$$

where K_m is distribution coefficient between stratum corneum and vehicle, and D is average diffusion coefficient (cm2/h), and h is thickness of skin (cm).

There are various models for the prediction of the skin permeability. The most frequently used of these models is the Potts-Guy relationship. The skin permeability prediction is obtained with an equation that depends on a molecule's molecular weight (MW) and octanol-water partition coefficient.

$$\text{Log Kp} = -2.74 + (0.71 \text{ x Log } P_{o/w}) - (0.0061 \text{ x MW}) \tag{18}$$

This equation helps for a prediction of skin permeability to determine whether a drug candidate is sufficiently permeable to be considered for transdermal delivery in the absence of experimental skin permeation data (Ranade & Cannon, 2009).

BBB

Blood vessels are an essential tool to transport oxygen and nutrients to different points of the body (Daneman & Prat, 2015). The micro-vascular system of the central nervous system, called the BBB, is important to identify unique characteristics such as the movement of ions, molecules, and cells between the blood and the brain (Daneman, 2012; Zlokovic, 2008). The control of CNS homeostasis provides to protect the neural tissue from harmful components such as pathogens and toxins. Any problem in the BBB can cause serious diseases such as stroke, edema, and brain trauma (Alvarez, Cayrol, & Prat, 2011; Daneman, 2012; Sandoval & Witt, 2008; Zlokovic, 2008). The BBB permits diffusion of small molecules that are polar and hydrophobic. It prevents the dissolution of large and hydrophilic molecules in the cerebrospinal fluid (CSF) (Johansen et al., 2018). Central nervous system-related drugs must first pass through the BBB (Carpenter et al., 2014).

HSA (Human Serum Albumin) Serum Protein Binding

One of the other important parameters in ADME is HSA binding. HSA, a carrier protein, is found in blood plasma and has the largest amount of the total protein in blood plasma (Lexa, Dolghih, & Jacobson, 2014; Simard, Zunszain, Hamilton, & Curry, 2006). HSA is responsible for the transport of hormones, fatty acids, and other compounds through the bloodstream (He & Carter, 1992). Plasma protein binding

(PPB) is one of the few factors that affects the penetration of drugs from plasma to target tissue (Bohnert & Gan, 2013). Interactions with serum proteins are factors that affect the distribution volumes. HSA also connects with passive permeability and penetration across the BBB(Howard, Hill, Galluppi, & McLean, 2010). Thus, the interactions of HSA and small molecules affect the ADME profiles of small molecules (Benet, Kroetz, Sheiner, Hardman, & Limbird, 1996; Kratz & Elsadek, 2012).

In reference (Kokcu et al., 2019), the pharmacokinetic parameters which are required for predicting the drug-like properties of GHK are listed in Table 5. GHK tripeptide has 340g/mol molecular weight, 6 hydrogen bond donors and 9 hydrogen bond acceptors, and the calculated value of octanol / water partition coefficient is -3.868. The rate of skin permeability (SP) is a very important pharmacokinetic property for the transdermal effect of drugs and cosmetics, especially in the fields of medicine and cosmetics. The calculated QP log Kp for skin permeability (Kp in cm/hr) value of GHK tripeptide is-9.697. It is important to know the ability to cross the blood brain barrier due to GHK's anti-anxiety activity. The calculated brain/blood partition coefficient (QPlogBB) is-2.441 and is within the recommended range of value (-3.0 – 1.2). Additionally, Human serum albumin (HSA) is important like the blood-brain barrier for the probability of being drug. The calculated QP log K hsa Serum Protein Binding value was determined as-1.556 (standard limits from -1.5 to 1.5).

Table 5. Docking score and calculated ADME properties of GHK Tripeptide

Property	Value	Recommended
Docking score (kcal/mol)	-7.513	
Polar surfacearea PSA (A˚2)	197.821	7.0 / 200.0
MolecularWeight, MW (g/mol)	**340.381**	**130.0 / 725.0**
QP Polarizability (Angstroms^3)	30.663M	(13.0 / 70.0)
QP logP forhexadecane/gas	12.735M	(4.0 / 18.0)
QP logP foroctanol/gas	**25.070M**	**(8.0 / 35.0)**
QP logP forwater/gas	21.918M	(4.0 / 45.0)
QP logP foroctanol/water	-3.868	(-2.0 / 6.5)
QP logS foraqueoussolubility	0.515	(-6.5 / 0.5)
QP logS -conformationindependent	0.636	(-6.5 / 0.5)
QP log K hsa Serum Protein Binding	-1.556	(-1.5 / 1.5)
QP log BB for brain/blood	**-2.441**	**(-3.0 / 1.2)**
No. of PrimaryMetabolites	8	(1.0 / 8.0)
Predicted CNS Activity (-- to ++)	--	
HERG K+ Channel Blockage: log IC50	-1.760	(concern below -5)
Apparent Caco-2 Permeability (nm/sec)	0	(<25 poor. >500 great)
Apparent MDCK Permeability (nm/sec)	0	(<25 poor. >500 great)
QP logKpfor skin permeability	**-9.697**	**(Kp in cm/hr)**
Jm, maxtransdermal transport rate	0	(micrograms/cm^2-hr)
LipinskiRule of 5 Violations	1	(maximum is 4)
% Human Oral Absorption in GI (+-20%)	0	(<25% is poor)

THE LIMITATIONS OF IN SILICO APPROACHES

Some limitations of MD simulations are timescales, force field accuracy, covalent bonds during MD simulations. For numerical stability, the short time steps simulations are required. Despite the simulation times increased from nanoseconds to microseconds, simulation timescales remain a challenge for MD calculations. Although molecular mechanical force fields have undergone significant improvements, they are naturally approximations and contain some limitations. In addition, the fact that the formed covalent bonds cannot be broken or regenerated imposes limitations on calculations during MD simulations.

The common challenges of docking method such as ligand and receptor conformation, flexibility and cavity detection. The flexible receptor docking, especially the backbone flexibility at the receptors, is still a major challenge for existing docking methods. There are different in silico ADME models because of different levels of complexities and throughputs. The throughput these models are limited by the extent of computation (Yu & Adedoyin, 2003).

CONCLUSION

Developing a new drug; It is a very demanding process that lasts about 10-16 years and costs about 1.2 billion dollars for synthesis, preclinical research, clinical trials (phases I, II and III), FDA approval and Phase IV studies. In addition, many animals should be sacrificed in pre-clinical studies. On the other hand, with computerized calculation methods, cost and time savings are achieved, while the number of experimental tests is minimized and fewer animals are sacrificed for toxicity tests. Drug design using computer programs plays a crucial role in interdisciplinary studies and as part of a drug discovery approach in the pharmaceutical industry, since it plays a vital role in the design and analysis of biologically new and more active molecules by reducing cost and time.

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Chapter 4
Gene Therapy and Gene Editing for Cancer Therapeutics

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ABSTRACT

Over the past two decades, developments in human genomics have shown that cancer in the host genome is caused by somatic aberration. This discovery has inspired interest among cancer researchers; many are now using genetic engineering therapeutic methods to improve the cancer regression and seeking a possible cure for the disease. The large gene therapy sector offers a variety of therapies which are likely to become effective in preventing cancer deaths. The latest clinical trials of third generation vaccines for a wide variety of cancers have produced promising results. Cancer virotherapy, which uses viral particles replicating within the cancer cell, is an emerging method of treatment which shows great promise. The latest developments in gene editing techniques, such as CRISPR, Cas9, TALENs, and ZFNs, are being used to help to make cancer a manageable condition. Gene therapy is expected to play a significant role in potential cancer therapy as a part of a multi-modality procedure.

INTRODUCTION OF GENE THERAPY

Gene therapy means an approach aimed at altering, removing or replacing anomalous gene(s) at a target cell (High KA & Roncarolo MG, 2019). These target cells may be primary malignant or metastatic nodules, circulating tumour cells or inactive stem cells, and unique cells like T-cell lymphocytes or dendritic cells. With the presence of more than 20,000 active genes in human cells exposed to multiple causes, whether inherited, environmental, infectious or random, infinite possibilities for gene mutation, aberration, deficiency or deletion have been expected, leading to clinical presentation of various medical disorders, including cancer (Dulbecco, 1986; Lander ES et al., 2001).

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A clear and succinct concept of gene therapy (there are many) is the use of any of a set of human disease treatment strategies that rely on the transfer of genetic material based on DNA into an organism (fda.gov). Gene delivery can be achieved in vivo by injecting the packaged gene directly into the blood, tissue, or cell. Additionally, the packaged DNA can be indirectly administered via ex vivo laboratory techniques. Somatic gene therapy which targets nongermline cells (nonegg and non-sperm cells) is currently consistent with the extension of biomedical science and medical therapy in which treatment does not go beyond the patient. Gene therapy can correct the basic pathophysiology of the disease in altering the genetic material of somatic cells. In addition to posing particular ethical problems, therapy of human germline cells, thus changing the genetic makeup of an offspring will represent a deviation from current medical practices (Strachan and Read, 1999).

In a subset of cancer patients and in paediatric cases, the main neoplastic events are germline mutations of the tumour suppressor or DNA repair genes. Germline mutations cause all of an individual's cells to become at risk for the development of cancer and are thus not appropriate for somatic cell gene therapy. Yet clonal selection of variant cells results in a population of cells with increasingly violent growth properties, in both somatic and germline mutations (Miller,1992).

In individuals with only somatic gene mutations, the insertion of a gene (such as a tumour suppressor gene) would alter the phenotype of a malignant cell only if the mutation is not dominant. In addition, both the degree of corrective cell therapy (possibly as high as 100 percent correction of all tumour cells) and the question of gene therapy in distal metastasis will need to be decided. Therefore, significant biological obstacles in the application of gene therapy to other types of cancer remain to be resolved. Indirect approaches were suggested, based on these formidable problems. These include: gene transfer of cytokines or other immune mediators to improve host immune responses, genetic alteration of neoplastic cells to promote immunogenicity, treatment of localized cancers with viral or bacterial enzyme encoding genes that transform prodrugs into toxic metabolites, or transfer of genes to provide enhanced resistance to traditional chemotherapy (Weichselbaum and Kufe, 1997).

HISTORY OF GENE THERAPY

The tradition of cancer therapy goes back to the 18th century, when surgery was the main treatment for early cancer stages, and patients experienced regular recurrences (DeVita *et al.,* 2012). The patients were treated with herbal remedies, castor oil, or arsenic until the disease spread. Radiation therapy was invented in 1895, which brought few cures (Curie and Curie, 1898). Many cases of spontaneous cancer regression after bacterial infection have been documented at that time (Lage, 2013). Following an erysipelas infection, a patient with soft tissue sarcoma went into remission in 1868 but this relapse lasted only a short time Nitrogen mustard was used in the treatment of lymphoma patients in 1943, and folic acid antagonists in childhood leukaemia contributed to temporary remission in 1948 (Goodman *et al*., 1946; Faber and Diamond, 1948; Hemminiki and Hemminki,2013). Chemotherapy care for cancer has also been making dramatic strides (DeVita, 2012).

In animal models, too, and subsequently in humans in 1956, viruses were found to be effective in regulating malignancies. Especially adenoviruses were studied more intensively in humans, with the subsequent development of gene therapy (Kelly and Russell, 2007; Atasheva et al., 2019; Atasheva S & Shayakhmetov DM et al., 2016). In 1987, immunotherapy was implemented in the treatment of lymphoma patients with subsequent FDA approval of the rituximab antibodies (1997) (Maloney *et al*., 1997).

The first gene therapy trial approved by FDA in the United States occurred for a patient with significant mixed immunodeficiency disorder in 1990 (Sheridan, 2011). Since then, many clinical trials have been conducted for cancer patients using various gene therapy approaches, with positive results documented in patients with chronic lymphocytic leukaemia, acute lymphocytic leukaemia, brain tumours, and others Several commercially authorized gene therapy drugs have been released including ONYX-15 (Onyx Pharmaceuticals) for refractory head and neck cancer (2005) (Chiocca *et al*., 2004); human papilloma virus vaccine (Gardasil) (Merck Sharp & Dohme) for cancer cervix prevention (2006) (Block *et al.,* 2006); and modified dendritic cells, silence-T (Provenge) (Dendreon Corporation, Seattle, WA), for minimally symptomatic, castration resistant metastatic prostate cancer (2010) (Kantoff *et al.,* 2010).

PRESENT APPROACHES IN CANCER GENE THERAPY

Despite the preclinical success of gene therapy, until recently, the clinical application of this therapeutic method has met with limited progress. Nonetheless, this base of clinical trials has demonstrated and enabled approaches to be developed to resolve the challenges to gene therapy clinical development (Baranyi L et al., 2013; Yang W et al., 2010). This includes targeting vectors, possible toxicity and the cost of generating vectors. Another obstacle to gene therapy is the lack of agreement as to its definition. The US Food and Drug Administration (FDA) describes gene therapy products as "products that mediate their effects through transcription and/or translation of transferred genetic material and/or incorporation into the host genome and that are administered as nucleic acids, viruses or genetically modified microorganisms. The products may be used to alter in vivo cells or transferred to ex vivo cells prior to delivery to the recipient". In practical terms, the aims of gene therapy are to deliver a transgene to an acceptable number of cells and at an acceptable rate of expression that is necessary to achieve therapeutic effects. Both requirements allow a vector to be used, and possibly a formulation that can accomplish these objectives. While this approach is both straightforward and appealing, because of the technological challenges, gene therapy has offered plenty so far and delivered nothing. There have been a number of preclinical research and clinical trials to develop gene transfer systems. As of August 2016, a total of 2409 gene therapy clinical trials worldwide were published in the Journal of Gene Medicine clinical trials database, the majority of which targeted cancer (64.5%) (Hu and Zhang, 2006). Because of the tropism of viral vectors and their superior gene transfer and expression efficiencies, transgene delivery through viral vectors was the predominant process, with usefulness of 75% in these clinical trials. In addition, naked DNA / RNA was 23%, and bacterial and yeast vectors were 2%. The main viral vector used was adenovirus (Adv) dependent (21 percent), with a near second (18.6 percent) of the retrovirus vectors (Allen RJ & Byrnes AP, 2019).

The first therapeutic gene was approved for use in China in 2003. It was an Adv serotype 5 vector developed to express p53 (Gendicine) for the treatment of patients with squamous cell carcinoma (HNSCC) in the head and neck (Peng, 2005). A second gene therapy drug, H101 (ONYX-015), an Adv vector modified to replicate and eliminate cancer cells with TP53 mutations, was approved in December 2005 (Hu and Zhang, 2006). Neovasculogen (a vascular endothelial growth factor [VEGF] transgene plasmid vector) was approved in Russia for the treatment of peripheral arterial disease in December 2011. Strimvelis (a retroviral vector used as part of ex vivo stem cell gene therapy) obtained authorisation from the European Medicines Agency (EMA) for the treatment of adenosine deaminase (ADA) deficiency and extreme combined immunodeficiency (SCID) in May 2016 (Hoggatt, 2016). As described later, a vec-

tor with a granulocyte-macrophage colony-stimulating factor (GM-CSF) transgene with herpes simplex virus (HSV-1) has been approved for melanoma treatment in the United States, the European Union and Australia (Harrington *et al.,* 2016). Regardless of these approvals, advances in targeting existing vectors and increasing the efficiency of gene transduction remain the major challenges in gene therapy (Manickan, E et al., 2013). Overcoming these challenges will promote the production of targetable vectors and will aid in the production of vectors that can be delivered intravenously, despite the systemic complexity of most malignancies. This chapter focuses on efficacy approaches and on the current therapeutic gene approaches. This also explores and reviews recent developments and suggests areas that need further progress to become a commonly accepted form of treatment for clinical gene therapy.

CASE STUDY 1: A PROMISING START OF ASHI DESILVA

The first gene therapy trial was performed by Dr Michael Blaese and Dr. French Anderson of the National Institutes of Health (NIH). There were only two patients in that trial in 1990: the first was Ashanthi DeSilva, who was only four years old at the time; the second was a nine-year-old girl who is simply referred to as patient 2 because of a wish to remain anonymous. Patient 2 only showed small progress after the procedure but it was a dramatic success for DeSilva.

All patients had a genetic disorder known as extreme combined immunodeficiency (SCID). SCID is an immune system disorder that makes combating even mild diseases, such as common cold or influenza, very difficult for those afflicted. The term "combined immunodeficiency" refers to both the T and B lymphocytes involved. In the initial descriptions of this disease the term extreme was used as most children had a serious clinical condition and died before their second birthday. By the time DeSilva was treated, however, diagnosis and improved therapies (other than gene therapy) ensured that most children lived a lot longer.

Today the term severe refers more to the lifestyle that patients with SCID must endure than to early death. Specific form of SCID suffered by DeSilva and patient 2 was due to a deficiency in an enzyme called adenosine deaminase (ADA). The gene for that enzyme is on chromosome 20's long arm. Human beings, being diploid organisms, obtain from both parents a copy of each chromosome; this is the way nature protects us from genetic abnormalities. An infant who receives one parent's faulty ADA gene and the other parent's healthy ADA gene does not develop SCID, because the defective gene is recessive to the normal gene. That is, a functioning copy of the protein is produced by the regular gene, thereby compensating for the faulty copy of the mutated gene. This is why ADA deficiency is considered an autosomal recessive genetic disorder, and why genetic disorders are rare in general. Symptoms only occur when both parents provide the infant with a faulty ADA gene.

The standard treatment for ADA deficiency is a bone marrow transplant or a drug called PEG-ADA which provides the patient with normal copies of the enzyme. Bone marrow transplants for DeSilva or patient 2 were not feasible due to a lack of suitable donors. In the months leading up to the trial both patients were being treated with PEG-ADA. Indeed, this procedure was a test entry prerequisite because it enhanced their safety, which would be helpful in case of any side effects arising from the study. Though PEG-ADA relieves many of SCID's symptoms, it isn't a cure. The drug provides an extracellular source of normal ADA but there is still a deficiency in the internal environment of each B and T lymphocyte. Consequently, the immune system does not function normally even with ADA supplements.

Both patients were accepted into the study in the expectation that their illness would be cured by gene therapy. DeSilva reacted surprisingly well to the drug, but as we shall see, the trial itself was but the tip of a scientific iceberg, based on retrospective studies dating back to the early 1970sThis work included identifying ADA as the source of clinical symptoms, isolating the ADA gene, and years of work that clarified the role of this gene, as well as how the genetic defect led to a crippled immune system. Preliminary research also focused on the details of the gene therapy procedure: the type of virus used as the gene vehicle, the joining of the isolated ADA gene to the virus, and the method used to supply the patient with the ADA-virus construct. All these things needed to be sorted out in depth, using animal models, before a human patient could be treated with gene therapy.

Clinical Procedure for ADA Gene Therapy

The treatment starts with the removal of all T lymphocytes from PEG-ADA-treated patients. The T cells are grown in tissue culture, and they are inserted into a normal ADA gene using a process called retroviral-mediated gene transfer, after which the gene-corrected cells are returned to the patient. The vector is a modified murine leukaemia virus (retrovirus) called LASN, where the ADA gene was inserted into it.

The protocol was conceived to have two sections. In Part 1, the patient was given consistently low numbers of gene-corrected T lymphocytes in order to build up the immune system and also to gain knowledge as to how long gene-corrected T cells live. A selection technique was used in Part 2A to increase the number of gene-corrected T cells that produce significant amounts of the ADA enzyme. Then, these enriched cells were given monthly to the patient for about six months. Part 2B increased the number of gene-corrected T cells to the therapeutic level expected (about 1 billion gene-corrected T cells per kilogram of the patient's body weight); then, 1 billion to 3 billion gene-corrected T cells were injected multiple times per kilogram, during which the patient was examined to assess if the immune system was functioning normally.

The DeSilva Clinical Trial

Ashi DeSilva became one of two patients enrolled in the first-ever trial of gene therapy in 1990. The jury was conducted by Blaese and Anderson, who included a total of 30 principal investigators and nurses. DeSilva's lymphocytes, as already mentioned, were isolated, grown in culture, and transduced with the LASN vector containing the ADA gene. Its immune response improved almost from the first day of the trial. Transfusions lasted for two years, during which they closely monitored her reaction to the court. In 1995 Blaese and other colleagues in the journal Science released a detailed account of the case. Both Patient 2 and DeSilva showed an improvement but the response from DeSilva surpassed the expectations of all. Within five to six months of starting the trial, DeSilva's T cell count increased rapidly and stabilized within normal range. ADA enzyme activity, which initially was almost undetectable in her lymphocytes, increased in concentration over the first two years of treatment, reaching a level roughly half that of a normal value, allowing her to live a normal life. DeSilva's immune response has weakened slightly after the trial but it remained within the usual range as of 2003, when she was 17 years old. The decline in her immune response may be due to an autoimmune response that kills some of her own T lymphocytes. This may occur when monocytes encounter vector antigens and present them to T lymphocytes, activating an adaptive response to the vector and any cells that contain it. The autoimmune

response would be mild in this particular case, since the retrovirus generally remains in the nucleus, thus minimizing the exposure of its antigens.

The DeSilva trial showed that gene therapy can be used to treat such genetic disorders. Procedural research grew significantly from only a few trials in the early 1990's to over 600 trials in 2003.

Most of these studies are designed to treat different forms of cancer, but a few try to cure SCID by combining gene therapy with stem cell therapy. Between 2000 and 2002, Italian, British, and French medical teams reported complete success in curing patients suffering from SCID-ADA and SCID-X1, an interleukin-deficient type of SCID, using transgenic stem cells. Such teams have used modified variants of the LASN vector developed by Anderson and his colleagues; this, combined with the use of transgenic stem cells, has significantly increased the percentage of positive tests. In this method, isolated stem cells are transfected with the therapeutic gene and then injected into the patient, where 43 mature T lymphocytes with enhanced functionality are transformed into Ashi DeSilva. For example, eight out of nine patients in British trial showed significant changes in their immune response function. Both of these trials, however, are only at the stage of Phase II or III, and have yet to be accepted as a standard treatment.

METHODS AND TOOLS OF GENE THERAPY

Cancer genomics evolve between primary cancer and metastases (Tran, B et al., 2012). For example, breast cancer mutations of the estrogen receptor gene (ESR J) were found in proportion of metastases but not in primary tumours (Toy *et al.,* 2013). Whole-exome sequencing of metastatic samples among the top 17 mutated genes was identified and only five mutated in primary tumors (Koboldt *et al.,* 2012). The evolution from minority clones to lethal metastases is followed by branched development. Thus, tumours with high levels of heterogeneity in intratumor and genomic instability may be more likely to escape targeted therapies such as gene therapy, unless such branched evolution is taken into account Therefore gene therapy with modest effectiveness is very difficult to achieve. Most approaches are currently for monogenic gene therapy, addressing one or more critical defects in the genes (Wang, F et al., 2019). Selecting the correct gene therapy mode is focused on evaluating the immune status and determining the molecular nature of the disease of a patient (Shirley and Heller, 2013). Ultimately, with the recent increase in knowledge of molecular biology of different medical conditions, a more sophisticated and systematic approach to gene therapy will become possible, with expected improvements.

Gene Transfer Delivery System

Several methods were developed, using various vectors, to facilitate the entry of genetic materials (transgenes) into target cells (Cucchiarini, M., 2016). They are generally classified into two main categories: viral and non-viral vectors (or bacterial). Viruses normally bind to target cells and as part of their replication cycle, introduce their genetic materials into the host cell. They will hold a load of other genetic material called "transgenes" when they reach target cells various methods have been used for non-viral vectors, using physical, chemical, and other genetic transfer modes (Baranyi and Dropulic, 2013). The movement of genetic material directly into cells is referred to as "transfection," whereas the transmission into cells carried by a viral or bacterial vector is referred to as "transduction." Non-viral methods have

the advantage of protection and ease of adjustment but have a lower efficiency of transfection compared to viral vectors (Baranyi *et al.,* 2013).

Physical Mediated Gene Therapy

DNA genetic material that is coated with gold or other mineral nanoparticles and reinforced by compressed air or fluid (gene gun) or ultrasound with their kinetic energy may force the genetic material into the target cell, followed by the release of DNA into its nucleus. They are ideally suited for the introduction of genes into the tissue or for vaccination of genes. The approach to electroporation gene therapy aims at achieving disruption of the cell membrane with high-voltage electrical pulses, resulting in the formation of nanopores through which naked DNA, foreign genetic materials and even chemotherapeutic agents can enter cells (Ahmad S et al., 2010). This method is ideally suited for gene transfer therapy based on plasmid DNA with the benefit of efficacy in a wide variety of cell types, ease of administration, lack of genome incorporation with the risk of malignancy, as well as the low potential for unintended immunogenicity (Yuan *et al.,* 2013). In many clinical trials, electroporation is currently being studied, especially in patients with malignant melanoma, prostate cancer, colorectal cancer and leukaemia (Moris, D. et al., 2019; Dong, S., et al., 2018).

In Vivo Electroporation Approaches Using DNA

In the effort to devise gene-based therapies for a range of cancers, a number of candidate genes have been the focus of preclinical trials. Such candidate genes are tumour suppressors, inhibitors of cell growth, pro-apoptotic agents, tumour antigens, and immunotherapy genes. The therapy can be given by intradermal, intramuscular, or intratumoral DNA injection accompanied by EP. Clinical applications of EP-based gene therapy have great potential (Zabner *et al.,* 1995). The use for gene silencing in the delivery of DNA vaccines and RNA is currently being studied in preclinical models and clinical trials.

A number of recent advances have been made regarding the gene transfer technique using in vivo EP. Most involve tumour microenvironment modification to improve transfection efficiency and the distribution of DNA. One research outlined the use of a three-dimensional melanoma model in vitro to predict the efficiency of in vivo transfection while using EP to transfer DNA (Tagami *et al.,* 2012; Marrero B et al., 2012). This is a valuable method to reduce the number of animals required to perform an experiment.

As regards solid tumours, when an external electric field is applied, the histological properties play an important role in the in vivo EP gene. Soft tumours with large spherical cells, low proteoglycan and collagen content, and low cell density have greater transfection efficiency than stiffer tumours with small spindle-shaped cells, high collagen and proteoglycan content, and high cell density (Mesojednik S et al., 2007). This study reinforces the idea that knowledge of target tissue characteristics is important for the selection of parameters for the electroporation. Researchers are looking to develop new ways of improving in vivo transfection efficiency. One way to increase the distribution of DNA and enhance gene transfer is direct modification of the tumour environment by hyaluronidase and collagenase (Cemazar M et al., 2012).

In tumours with high extracellular matrix content, pre-treatment with the enzymes increased the efficiency of gene transfer. This effect is less pronounced in small extracellular matrix tumours. Pre-treatment with a hyperosmotic mannitol solution increased the expression of reporter genes when EP

introduced DNA into a model of hind leg Tumour: Tumour (Fu *et al,*. 2012). The authors suggested this is a consequence of growing the extracellular tumour volume.

Improving muscle delivery for cancer therapy was also a focal point of several studies (Pereyra, A. S. et al., 2016; Winbanks, C. E et al., 2016; Rey-Rico, A et al., 2018). In transgenic tumour models, an oral TLR7 agonist was acting as a possible immunological adjuvant in DNA EP vaccination.

In a similar study, the antitumor effects of an HPV DNA vaccine were enhanced by TLR9 delivered by EP (Ohlschlager P et al., 2011). EP was used to examine the role that a specific gene plays in tumorigenesis, which can be very useful in the development of novel cancer therapies. In one study, the runt-related transcription factor-1 (Runx1) was overexpressed or knocked down to investigate the effects on leukemogenesis caused by BCR-ABL. In vitro and in vivo proliferation and migration were affected by alteration of Runx1 expression in BCR-ABL-transformed BaF3 cells. EP- administered DNA vaccines are effective in stabilizing cancers (Haller *et al,*. 2010). While this strategy has entered clinical trials, the efficacy of EP delivery is demonstrated by additional preclinical research with alternative genes.

In a recent study, heparinase DNA vaccine prophylactic administration in tumour-bearing mice using EP caused humoral immunity and cytoimmunity, and suppressed tumour growth. DNA EP vaccines also impede the growth of tumours in preclinical breast cancer studies (Goepfert *et al,*. 2011). A number of studies use vaccines with DNA to treat prostate cancer. Specific responses in vivo were produced by EP DNA vaccination with DNA-encoding prostate specific antigens. Another study used EP-disseminated diphtheria toxin A and prostate-specific antigen in xenografts of prostate cancer to slow cancer cell growth (Goepfert *et al,*. 2011). An important therapeutic approach is developing a vaccine that incorporates several epitopes to create a range of immune responses. A multi-epitope melanoma DNA vaccine produced by EP has generated prophylactic and therapeutic responses to antitumor. Angiogenesis is an important process in the progression of tumours; as such it is the focus of studies of cancer gene therapies (Yi, M et al., 2019; Markowska, A. et al., 2017; Salinas Vera, Y. M. et al., 2019). Strategies capable of inhibiting angiogenesis are of particular interest in anticancer therapy. A few studies have focused on an antiangiogenic strategy based on in vivo EP of a plasmid encoding soluble endothelial growth factor (VEGF) receptor, a negative angiogenesis regulator. In a mouse model of melanoma, a human surviving DNA vaccine administered via intradermal EP produced a specific cytotoxic T-cell response (Roos AK et al., 2006). It also suppressed angiogenesis, and protected against melanoma. EP of DNA encoding angiopoietin-like 4 (ANGPTL4) to mice did not prevent the growth of the introduced primary tumours but caused reduced metastases in the lungs of ANGPTL4 expressing mice.

EP's transfer of cytokine genes for cancer treatment has been the subject of preclinical and even clinical research. It is intended to generate strong immune responses from the host that inhibit tumour growth and lead to immunity for long term. Latest preclinical studies have focussed on interleukins and other genes associated with the immune system. Some studies have used EP to intramuscularly or directly deliver matrix metalloproteinases (MMP) to the tumours to modify them.

MMPs are involved in matrix degeneration, tissue remodelling, inflammation, and even the formation of metastases; as such, they represent an attractive option for gene therapy for cancer. In vivo EP can be combined with radiation therapy or electrochemotherapy as an alternative approach to cancer therapy of various plasmiden-coded genes (i.e., cytokines and inhibitory molecules)

Electroporation Approaches Using RNA

A more recent approach is the use of in vivo EP to deliver RNA to induce RNA interference (RNAi), the downregulation of gene expression produced by sequence-targeted double-stranded RNAs. Two types of RNAs endogenously generate RNAi: small interfering RNA (siRNA) and microRNA (miRNA). Those small RNAs are processed by a similar pathway in the cytoplasm (Tagami *et al.,* 2012). The mechanism through which they subsequently downregulate the expression of genes differs, however. Generally, siRNAs are 2030 nucleotides, and perfectly complement their target mRNA. These RNAs induce cleavage and degradation of a particular target mRNA after binding. In diseases or viral infections miRNAs are frequently misregulated. MiRNAs usually contain a mismatched base, bind target mRNAs 30 UTR, and suppress translation. MiRNAs with perfect base pairing to their target mRNA behave in a similar manner to siRNAs, causing mRNA cleavage and degradation. Small hairpin RNAs (shRNAs) have a double-stranded, stem-loop structure. These RNAs can be synthesized or delivered encoded in plasmids, in which the RNA polymerase III promoters U6 or H1 typically drive the expression.

Several applications of RNAi have reached clinical trials since 2004 for several indications, including cancer therapy. Small RNAs may be supplied therapeutically as siRNAs, shRNAs, or miRNAs. Synthetic oligonucleotides can be paired to form double-stranded RNA, but are unstable unless chemically modified on bases, sugars, or in the backbone. RNA delivered from extracellular sources can bind pattern recognition molecules on both immune and non-immune cells and induce Type I interferon production, resulting in immunostimulatory side effects and toxicity. The short half-life of siRNAs can be resolved by the direct delivery of plasmids encoding shRNAs using methods including in vivo electroporation.

Gene Silencing Using in vivo EP as a Delivery Method

It was first elucidated using reporter genes in preclinical models of tumours. An initial study showed that intratumor EP of plasmids expressing an anti-luciferase shRNA significantly reduced expression of luciferase protein in tumours generated with stably transfected mouse melanoma cells with the luciferase gene This reduction was also observed with synthetic siRNAs using in vivo EP. In a study on stably transfected B16 mouse melanomas with the enhanced green fluorescent protein (EGFP) gene, in vivo siRNA EP targeting EGFP significantly reduced its expression.

EP-mediated intramuscular delivery of siRNA constructs for the murine calf muscle has decreased exogenous luciferase expression for at least 100 days. A similar construct reduced the expression of endogenous TLR4 by at least 1 week.

Therapeutic in vivo EP of siRNAs or shRNA-expressing plasmids has been studied for many cancer therapy applications in several preclinical models. For such applications, transmission to the tumour is most usually performed directly. The expression of a melanocyte-specific transcription factor, Mitf, was down-regulated using siRNA in a therapeutic demonstration of silencing of a cancer-specific gene. Delivery of small mouse melanomas led to apoptosis and substantially delayed development of the tumours. This effect was improved by the introduction of interleukin (IL)-12 gene therapy (Kawakami K et al., 2001). SiRNA targeting multiple apoptotic genes used as adjuvants to the plasmid DNA vaccine encoding tumour antigens in the spontaneous mouse mammary tumour model demonstrated substantial tumour progression when administered intramuscularly.

The RNAi was therapeutically studied as a combined therapy with chemotherapy agents. In both subcutaneous and orthotopic models of pancreatic mouse cancer, intratumor EP of either siRNA oligo-

nucleotides or a plasmid expressing shRNA engineered to suppress tumour growth and increase survival significantly by the k-ras oncogene. These results were substantially enhanced by combination therapy with the cytosine arabinoside analog gemcitabine. In related combination experiments with subcutaneous human lung carcinoma tumors in nude mouse models, the combination of downregulation of the multidrug-associated protein using specific siRNAs with the drugs navelbine and epirubicin that are subject to drug efflux significantly reduced tumour development.

Three groups investigated the vascularisation of tumours. The hVEGF was attacked using modified siRNA oligonucleotides in four subcutaneous human tumour xenograft models in nude mice. An important impact on tumour growth was observed in the three strongly VEGF-expressed models. No major effect was observed in model with low VEGF expression. Injection of intravenous siRNA followed by intratumor EP also effectively slowed tumour development. The plasmids that express siRNAs were used to target the VEGF-A and VEGF-C isoforms in metastatic mammary cancer of the mouse A substantially lower number of tumours was found in groups in which the VEGF-A isoform was targeted. After delivery of siRNA, the lymph node metastases were reduced targeting either isotype; the combination allowed a reduction in lung metastases. In a short-term experiment, in a subcutaneous mouse neuroblastoma model In vivo EP, a GTPase induced by VEGF, Rac1, was controlled with controlled, but not control, siRNA significantly slowed tumour growth over a 7-day period.

One community therapy tested miRNA's in vivo EP. MiR-143 is associated with growth arrest, and rates of prostate cancer correlate inversely with the histopathologic grade of cancer. MiR-143 in vivo EP into subcutaneous human prostate tumours in nude mice significantly reduced tumour growth over a span of 6 days.

Although full tumour regression has not been achieved in either of these RNAi protocols, and while only short-term (100 days) tumour growth has been tracked, a slowing in tumour growth has been observed in each case, suggesting that this technique can hold therapeutic promise, either as a single or combination cancer treatment. No clinical trials for RNA transmission, however, use EP as of yet.

Chemical Mediated Gene Therapy

Non-viral chemical methods for cancer gene delivery systems use synthetic or natural compounds to shape certain particles which facilitate gene transfer into the cells. The synthetic vector has the ability to electrostatically interact with RNA or DNA and bind to compact the genetic information in accommodating greater genetic transfers. Therefore, endocytosis helps non-viral chemical vectors to enter the cells (Soltani *et al.,* 2013). There are usually two non-viral vectors like liposomes, and polymers. Liposome-based non-viral vectors use liposomes to assist gene transmission via lipoplex formation. When the negatively charged DNA interacts with positively charged liposomes, the lipoplexes form spontaneously. The non-viral polymer-based vectors use the polymers to bind with DNA to form polyplexes. Recently the application of the engineered polymeric nanoparticle has rendered non-viral gene delivery approaches method (DiMartino *et al.,* 2012).

Cationic liposomes are microscopic vesicles of synthetic phospholipids and cholesterol that that reach cells via endocytosis, with the ability to hold a number of molecules, such as drugs, nucleotides, proteins, plasmids and large genes (Soltani *et al.,* 2013; Zhao, Y. et al., 2016; Qian, Y. et al., 2018; Majzoub, R. N. et al., 2016). Combined with small interfering RNA (siRNA), cationic liposomes can contribute to tumour proliferation inhibition, apoptosis induction, and increased radiosensitivity to tumour cells.

Synthetic viruses were designed to leverage viral vector efficiency and the liposomal advantage. When they get into the target cell, the endosome releases DNA. In preclinical trials this approach has shown promising results (*Soliman et al.,* 2012). Transposons can also bear genetic material within the cell and into the nucleus (Nie *et al.,* 2011).

Bacterial Mediated Gene Therapy

Some bacteria have the ability to directly target tumour cells, resulting in RNA interference (RNAi) and gene silencing with RNA function blockage including cell metabolism and protein synthesis (Hackett *et al.,* 2013). Examples *include Escherichia coli, Clostridium, Salmonella typhimurium* and *Listeria*. Bacterial vectors can carry enzymes and cytotoxic agents that bind to drugs into tumour cells, and can mediate immune response to the host. They can be designed to carry magnetic or fluorescent material to enhance the usefulness of diagnostic approaches in tumour location, such as with magnetic resonance imaging (MRI) (Kwon and Min, 2013), and even in cancer vaccine development. The result, however, was far less pronounced compared with other techniques for silencing RNA interference (Benoit *et al.,* 2009). Overall, genetically engineered bacteria acting as RNA interference vectors are relatively safe, efficient, practical and cheaper to produce compared to viral vectors. They colonize and develop selectively inside the tumour. These can also be given by mouth, hence their use in treating gastrointestinal disorders (Baban *et al.,* 2010).

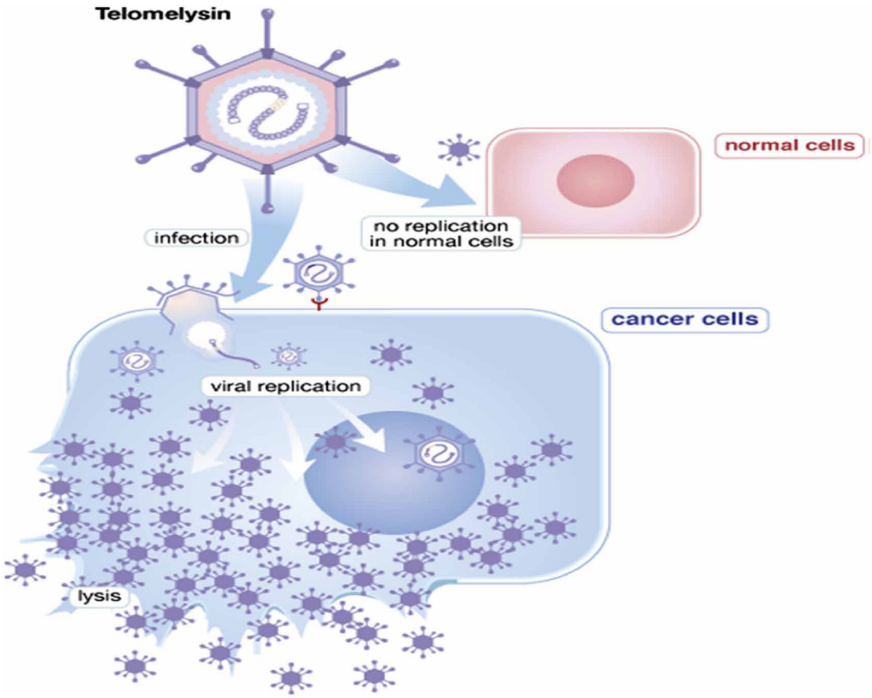
Delivery of RNAi Effectors by Invasive Bacteria

The successful delivery of appropriate doses of the RNAi-triggering molecules to the target tissue is a major challenge for the development of therapeutic RNAi effectors. Accordingly, principles for delivering RNAi-based drugs have been applied to the use of strains of therapeutic bacteria. An invasive bacteria strain containing a DNA vector which encodes the therapeutic RNA molecules must be designed in this framework (Jia *et al.,* 2012). Although it is in theory possible to use expression vectors encoding single siRNA strands, the RNAi-mediating agents would be shRNAs in the first place. In this context must be built an invasive bacteria strain containing a DNA vector that encodes the therapeutic RNA molecules. While expression vectors encoding single SiRNA strands can be used in principle, shRNAs will be the RNAi-mediating agents in the first place. (Fig. 1a)

In this approach, RNAi effectors are delivered by invasive bacteria conveying shRNA-encoding DNA constructs that will act as a matrix for transcription of the shRNA encoding DNA sequence in the target cell by the host cell's transcription machinery (Fig. 1b).

(A) By transkingdom RNAi (tkRNAi), bacteria are transformed with a shRNA-encoding vector capable of intrabacterial transcription. In turn, shRNAs are expressed inside the bacterial cells before the vectors are released into the target cell's cytoplasm. Following bacterial lysis, the shRNA molecules are processed by Dicer to the corresponding siRNAs, which are incorporated into the RISC complex. The guide (antisense) strand of the siRNA specifically hybridizes with its target mRNA, which is then degraded by the RISC complex leading to post-transcriptional gene silencing. (B) The bacteria mediated RNAi (bmRNAi) concept utilizes a shRNA-encoding plasmid for transformation of invasive bacteria. The microorganisms enter mammalian cells by endocytosis.

Figure 1. RNAi effectors delivery by invasive bacteria



Following bacterial lysis, the shRNA-encoding vectors are released into the target cell's cytoplasm, where transcription takes place. After Dicer-mediated procession of the shRNAs into siRNAs, the siRNAs are incorporated into the RISC complex, the sense strand is cleaved, and the target mRNA is degraded by the RISC complex. Source: Adapted with permission from Lage and Fruehauf.

Bacteria-Mediated RNAi

The concept of bmRNAi uses invasive bacteria to deliver therapeutic RNA molecules which encode DNA vectors into target cells (Ahmed, O. B. et al., 2019; Duong, M. T. Q. et al., 2019). The synthesis of the effectual therapeutic RNA molecules takes place inside the targeted cells via the transcription machinery of the host cell. Following translocation of the therapeutic RNAs from the host's cell nucleus to the cytoplasm, the RNA pathway can be triggered the attenuated, facultative anaerobic, invasive S in the first study which applies the bmRNAi strategy (Nguyen and Fruehauf, 2008). Use of S. *typhimurium* strain LH430 these bacteria delivered anti-Stat-3 (signal transduction and transcription-3 activator) shRNA encoding plasmids to the RM-1 in vitro and in vivo line of mouse prostate carcinoma cells. Stat-3 is a member of the latent, cytosolic transcription factors family of stat that specifically relate signals from the cytoplasm membrane to the nucleus. Stat-3 is constitutively activated in a wide range of human tumours by aberrant upstream tyrosine kinase activities and plays a significant role in promoting the cell cycle, proliferation, differentiation, angiogenesis and apoptosis inhibition. Thus Stat-3 was explored as a potential cancer therapy target molecule. In vitro, the approach to bmRNAi proved the *S. typhimurium*-administered RNAi effectors decreased the levels of cellular Stat-3 mRNA and protein expression to 13% or 18% of those of the control constructs. Accompanying the RNA-based Stat-3 inhibition was

downregulation of Stat-3 downstream effectors, i.e. Bcl-2, D1, c-Myc, VEGF, MMP-2 ... These effects resulted in induction of apoptosis, G1- arrest and decrease of cell viability. Additionally, RM-1 cells treated with *Salmonella typhimurium* containing the anti-Stat-3 shRNA vector did not develop tumours when they were injected into mice flakes. Further studies in mice with an orthotopic model of prostate cancer demonstrated the antitumor activity of the *S. typhimurium* LH430 strain It has been demonstrated, however, that treatment with *S. typhimurium* alone without anti-Stat-3 shRNA encoding plasmid exerted antitumor effects but with a single dose of bacteria transformed with bmRNAi construct a higher tumour suppressive effect was achieved (Jiang *et al*., 2007). Further tumour research also showed reduced rates of expression of Stat-3 proteins, followed by increased apoptosis and decreased proliferation. An antimetastatic effect of the silencing of the RNAi-mediated Stat-3 was also observed. Lastly, curves of long-term survival showed improved survival after treatment with *S. typhimurium* which contains plasmid anti-Stat-3 shRNA (Manuel *et al.,* 2011). In another study on bmRNAi, the attenuated Strain was treated with a mouse model of malignant melanoma. *S. typhimurium* strain LB5000 with plasmid encoding shRNAs directed toward Bcl-2. Bcl-2 family members are key apoptosis regulators and Bcl-2 was the first proto-oncogene to be identified as having anti-apoptotic function (Xiong *et al.,* 2010). Since Bcl-2 in various tumours, including melanoma, is over-expressed, it appears to be an ideal target molecule for cancer therapy.

Bcl-2-overexpressing mouse B16-F10 melanoma cells were treated with the therapeutic strain of bacteria containing shRNA vector in the bmRNAi method (Yang *et al.,* 2008). As a result, Bcl-2 was explicitly down-regulated at mRNA and protein level followed by an increased spontaneous apoptosis rate of the melanoma cells being bacteriatrized. In addition, B16-F10 cells were subcutaneously implanted in mice. The mice orally received attenuated S after tumour formation. *S. Typhimurium* which contains plasmid anti-Bcl-2 shRNA.

As a result, they observed delayed tumor growth and prolonged mice survival. The attenuated *S. typhimurium* was used for an extra investigation. MDR1/Pgp-encoding mRNA overexpressed in cisplatin-resistant human tongue squamous cell carcinoma cell line Tca8113/DDP *S. typhimurium* strain SL7207 for delivery of plasmid DNA-encoding shRNAs. MDR1/P-gp expression was down-regulated in mRNA and in vitro protein levels (Zhang *et al.,* 2007). Interestingly, cisplatin resistance has changed considerably. It is shocking as cisplatin is not usually a substrate of the MDR1/P-gp drug extrusion pump. However, the results have been verified in vivo by treatment of xenotransplants Tca8113/DDP derived developing on mice with orally administered *S. typhimurium* containing plasmid encoding anti-MDR1/P-gp shRNA, and cisplatin administration intraperitoneal. Oral administration of the bacteria suppressed tumour proliferation and increased the cisplatin anticancer effect. In a novel combination strategy of silencing immunosuppressive molecules followed by vaccination, the bmRNAi method was also applied, which can work synergistically to minimize tumour growth In this study, MVP728 or YS1646 encoding shRNA molecules directed against the Stat-3 encoding mRNA were initially treated with tumour-bearing mice generated by subcutaneous injection of the murine melanoma line B16F10 After this initial treatment, mice were treated with a vaccine — that is, a *S. typhimurium* clone MvP728 containing a vector for plasmid speech encoding human survivor cDNA. Tumour growth was attenuated in murine melanomas which overexpressed survivin using the therapeutic survivin-based vaccine alone. However, the vaccine was not effective under more immunosuppressive conditions, such as those linked to larger volumes of tumours. In mice infected with survivin, *S. typhimurium*-dependent downregulation Stat-3 increased the intratumoral CD41 and CD81 T lymphocyte proliferation and granzyme B levels. In tumours of double-treated mice, the combined approach also caused apoptosis.

For another study on bmRNAi, a combination therapy was applied. In this study, the attenuated *S. typhimurium* was treated with an orthotopic model of hepatocellular carcinoma in mice. *S. typhimurium* strain LH430 containing vector expression encoding shRNAs directed against Stat-3 encoding mRNA and endostatin angiogenesis inhibitor This combination therapy had more results than the clinical treatments themselves. In addition to Stat-3 downregulation, the downstream factor VEGF expression level was lowered, tumour cell proliferation decreased, apoptosis induced, and angiogenesis inhibited. In addition, it activated various immune cells and cytokines. Finally, in GFP-expressing murine bladder transitional cancer cell line BTT-T739-derived tumour-bearing mice with attenuated S, a small proof-of-concept study utilized the bmRNAi technology to target green fluorescence protein (GFP) expression. *S. typhimurium* strains LB5000 and SL3261 converted with vectors of plasmid expression encoding against GFP mRNA. Using this approach, the GFP expression level in tumour cells was inhibited by 73%.

The bmRNAi strategy, in accordance with the tkRNAi method, has shown less pronounced gene silencing effects than traditional application of classical RNAi effectors. Similarly, by developing new designed components of the bmRNAi system, including improved bacterial strains, vectors, and therapeutic RNAs, the efficacy of the bmRNAi concept may be increased.

Viral Mediated Gene Therapy

Gene therapy has the ability to treat a number of both inherited and acquired diseases, and viral vectors have emerged as a favoured gene delivery tool (Blind, J. E. et al., 2019; Tosolini, A. P. et al., 2016; Yoshimura, H. et al., 2018). If a therapeutic gene cassette replaces the viral genome, stripping the virus of replicative and pathogenic characteristics, these vectors are well suited as vehicles for gene transfer. An ideal gene therapy vector should hold and deliver a therapeutic gene for targeting cells and directing long term therapeutic expression reliably and efficiently. Viruses fulfil these conditions normally, except that they are vulnerable to host immune responses as the immune system in mammals has evolved to identify infectious agents. Several specific viral vectors have been used in past and current clinical trials, including adenovirus (Ad), adeno-associated virus (AAV), lentivirus (LV), ÿ-retrovirus murine, and herpes simplex virus (HSV). Two AAV-based therapies have been approved for the treatment of congenital blindness (Luxturna ®) and spinal muscle atrophy (Zolgensma ®), a primary immune deficiency ADA-SCID (Strimvelis ®), a CD19-based LV-based genetically engineered CAR-T cell immunotherapy for acute lymphoblastic leukaemia and non-Hodgkin lymphoma (Kymirah ® and non-Hodgkin lymphoma).

The suitability of a viral vector for a given application depends on multiple factors, including target cells or tissues, tropism, ex vivo vs. in vivo gene transfer use, packaging capacity, genome integration potential (and insertional mutagenesis), and also immunotoxicity propensity (Xue, K. et al., 2017; Gadalla, K. K.et al., 2017; Peccate, C. et al., 2016). Although LV vectors are now preferred for ex vivo gene correction (especially for gene transfer to hematopoietic stem cells, HSCs), AAV has emerged as the preferred vector for in vivo gene transfer because of its favourable safety profile compared to other vectors, ability to transduce a variety of tissues, and availability of a large number of viral capsids with different tropisms.

Although the use of virus-derived vectors takes advantage of their advanced evolutionary fitness to transduce human cells, these advantages have co-evolved with an equally sophisticated human immune system that seeks to defend host tissues by removing perceived as harmful foreign invaders (Thomas and Kay, 2003).

Certain viral vector components are indistinguishable to the immune system from their parent viruses (such as nucleic acids carried in a protein coat). Thus, these vectors undergo similar innate and adaptive immune responses as wild-type viruses. Innate immune receptors, or pattern recognition receptors (PRRs), detect viruses by recognizing retained molecular motifs, such as distinct nucleic acid conformations that cause antiviral immunity (Fig. 2). The virally derived capsid or envelope proteins are foreign proteins which can be the object of adaptive immune reactions (Fig. 3).

Furthermore, transgene-derived expression of a therapeutic protein that constitutes a neo-antigen that can be similarly targeted by both humoral and cellular immune responses (Fig. 3). Immune mediated rejection in viral gene therapy represents one of the most significant hurdles to human gene therapy (Kuroda and Fujiwara, 2013). A comprehensive understanding of the processes underlying these deleterious responses directed against both the viral vector and transgene product is critical for developing treatment modalities that mitigate immune-mediated rejection. A body of research has interrogated these mechanisms, which are reviewed herein. We will focus on 3 widely utilized and studied vector systems: Ad, AAV, and LV vectors (Fig. 3).

Figure 2. Innate immunity response against virus

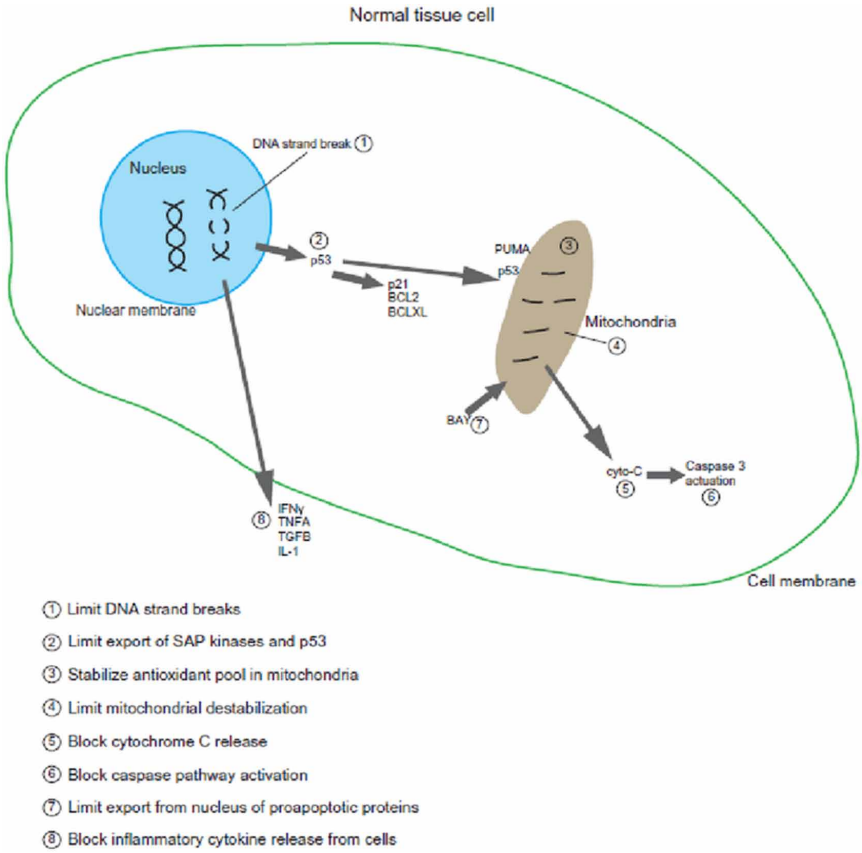
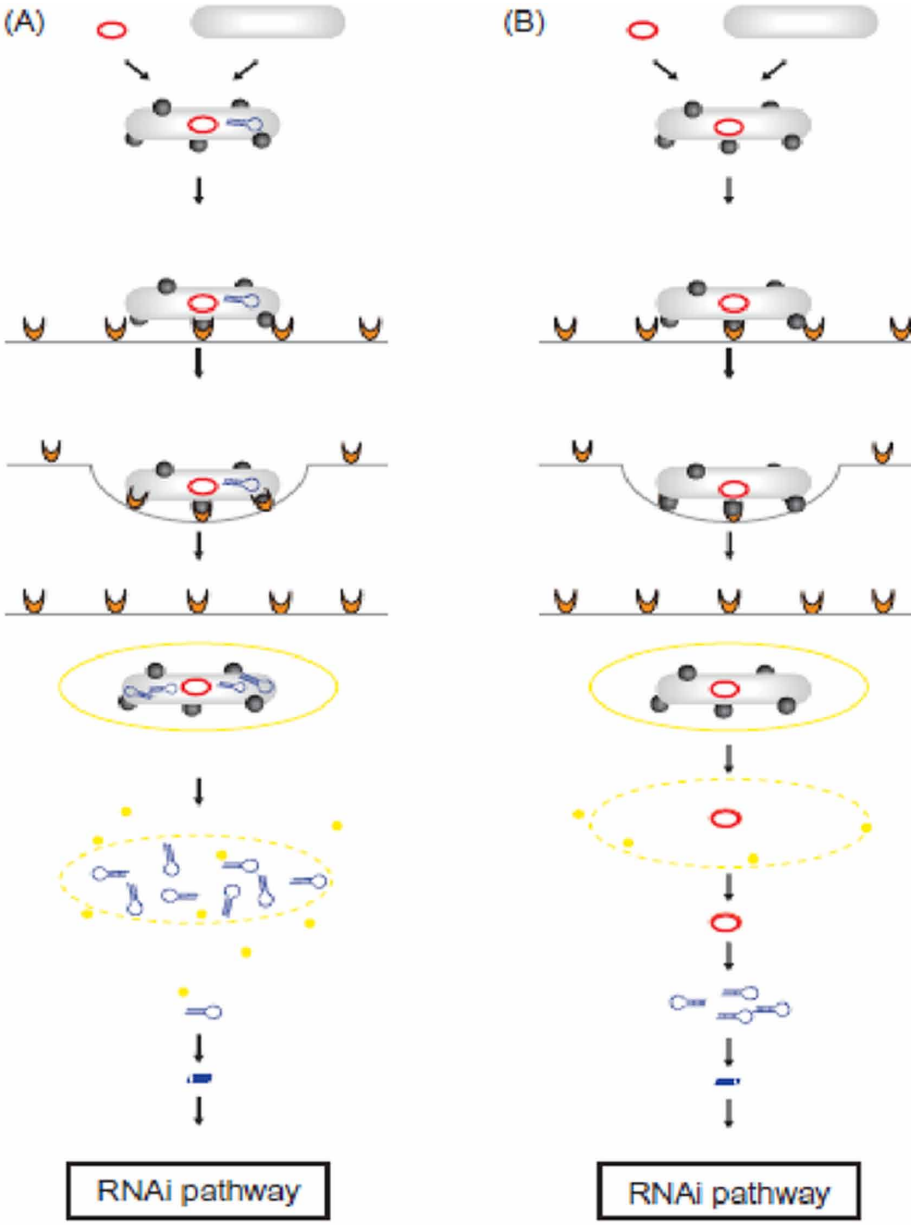


Figure 3. Viral vector systems: A) Adenovirus, B) Adeno Associate Virus, and C) lentivirus vectors



Overview of Immune Responses to Viral Vectors

The immune system consists of a complex interwoven network of several different types of cells that work together to protect host tissues from further infection and activate a collection of effectors specifically designed to remove the invading pathogen. In general, the cellular network may be broken up into innate and adaptive arms. Innate immune responses occur early, are not unique to the antigen, and do not induce immunological memory. Adaptive immune reactions are conditioned by the inflammatory

environment generated by innate immune sensing, rely on antigen specific (effector) B and T cell differentiation activation and clonal expansion, and generate immunological memory. Viral vectors share many commonalities with natural viruses but vary distinctly in that they are non-replicative, delivered in a single high-title bolus, and inserted at an unusual location. And while it is possible to apply canonical immunological principles, the unwanted immune response to viral vectors also has fundamentally specific aspects.

Due to pre-existing immunity, vector particles containing viral proteins that are identical or close to antigens to which humans are exposed as a result of normal infection may be neutralized by antibodies upon injection into certain humans. Recognition of viral structures (e.g., capsids or nucleic acids) by innate immune sensors can induce tissue infiltration by innate immune cells, can activate the development of IFNa / b (type 1 interferon, hereafter abbreviated as T1 IFN), thereby inducing an antiviral state in the tissue and reducing transduction, and provides activation signal for adaptive immune responses.

Activation of dendritic cells (DCs) and subsequent antigen presentation is a critical step in linking innate to adaptive immunity, leading to activation / differentiation and expansion of T cells. While MHC I restricted CD8 + T cells (cytotoxic T lymphocytes, CTL) are capable of lysing virally infected cells, MHC II restricted CD4 + T cells provide assistance for optimal CD8 + T cell activation and B cell activation, leading to the formation of an antibody. Also, T helper cells are critical for memory responses generation.

Adenovirus Vectors

Adenoviruses are double-stranded DNA viruses that typically cause human infection with moderate respiratory, digestive, and ocular (Houri N et al., 2013). Modified versions of the adenovirus and adeno-associated viral vectors were engineered in gene therapy. They are more active in infecting cells compared to wild-type, both dividing and non-dividing, replicating primarily in tumour cells, and specifically targeting certain cellular receptors or molecular defects (Wu and Nemerow, 2004). They present a very high efficiency of transduction that can exceed 100 per cent, with less propensity for viral shedding and latent infection.

They can be manufactured commercially easily in large amounts, and are capable of carrying both pro-drug genes and others (Mathis and Stoff-Khalili, 2005). We also have some drawbacks, however, including the propensity to establish genetic instability in transmitted genes. Subsequent chromosomal aberration may result in lymphoproliferative disorders developing. As nearly half of all humans have been exposed to these viruses throughout their lifetime, they may lead to high immunogenicity with the generation of neutralizing antibodies, with shorter duration of adenoviral-mediated transgene expression (Zemp *et al.,* 2010).

Modified oncolytic adenoviruses are currently being studied in various clinical trials, in particular in patients with brain astrocytoma, in conjunction with radiation and/or temozolomide chemotherapy (Balvers *et al.,* 2013). ONYX-015 (Onyx Pharmaceuticals) is a modified oncolytic adenovirus previously approved by the Chinese Food and Drug Administration (2005) in combination with cisplatinum for the treatment of refractory head and neck cancer. The treatment of other strong malignancies is currently under investigation. Other oncolytic adenoviruses are Ad5-D24, recombinant H103, Ad5-CD / TKrep, CG7870, KH901, and Telomelysin (OBP-301). The new adenoviral vector generation is the gutless adenovirus; it has an impressive safety profile, less immune response in vivo, and long-term sustained gene expression (Zemp *et al.,* 2010). Most clinical trials with oncolytic adenoviruses rarely produce a

dramatic response to tumours. However, good tumour regression has been reported when combined with other cancer treatment modalities.

Early Lessons From Adenovirus-Based Vectors

Adenovirus is one of the first viruses to be tested as a possible gene therapy vector and has also been the target of early in vivo gene transfer failures that illustrate the key inflammatory responses of the host to the reliability of therapeutic gene expression and the overall protection of this type of treatment. Early enthusiasm for Ad vectors has been largely focused on their high efficiency in transduction and packaging ability. Robust transgenic expression, however, was met with an equally high inflammatory response that resulted in transient expression and a potential for severe immunotoxicity leading to a patient's death. Due to their ability to activate CD8 + T cells effectively, subsequent efforts shifted towards their use as vaccine carriers and in gene therapy for the cancer.

Ad vectors contain a double-stranded DNA genome of ~36 kb packaged into a capsid of the viral protein. Different viral genes are removed to render defective virus replication. It is also possible to remove as viral coding sequences and to produce adenoviral vectors which are "gutted" or "helper-dependent." Some serotypes such as AdHu5 effectively transduce a variety of cell types in vivo (with especially high hepatocyte tropism), while serotypes that infect hematopoietic cells have been defined as well. The vector genome remains episomal when transduced. Ad vectors activate a wide spectrum of innate immune pathways and were therefore ideal tools for the study of innate viral immunity.

Early Innate Responses to Systemically Delivered Adenovirus

Transfer of the hepatic gene is achieved by intravenous adenoviral vector injection. Innate responses can, however, occur within minutes to hours, leading to changes in blood pressure, thrombocytopenia, inflammation, and fever. Coagulation dysregulation may spread to several organs, leading to DIC (disseminated intravascular coagulation). Activation of vascular endothelial cells by Ad vectors results in the release of the von Willebrand factor (vWF) ultra-large molecular weight multimers, a blood protein that is essential to platelet adhesion. Ad vectors also activate platelets and induce exposure of the adhesion molecule P-selectin and platelet-leukocyte formation, ultimately causing thrombocytopenia and thus a risk of bleeding.4 Important cellular interactions that occur early after systemic ad vector involve endothelial vascular and hepatic cells, platelets, Kupffer cells, hepatocytes, and splenic macrophages and DCs (Manickan, E et al., 2006).

If the virus is bloodborne, the adenoviral capsid hexon portion binds to coagulation factor X (FX), as shown by Shayakhmetov and colleagues (Doronin, K et al., 2012; Colella, P et al., 2018; Corti, M et al., 2017). Viral FX-decorated particles activate TLR4 on the surface of splenic macrophages and thereby cause IL-1b-dependent NF-kB activation, attracting polymorphonuclear leukocytes to the marginal spleen region (Ertl HC, 2016). Such processes, suggesting a coevolution of the immune and coagulation systems to protect against pathogens, help to clear the virus from spleen quickly. Upon delivery to a blood vessel, blood and immune organs molecules and cells that monitor systemic circulation will have a critical impact on the response to ad vectors. Besides binding proteins with gla domains to coagulation, adenoviral particles bind component C3 and natural IgM antibodies, resulting, for example, in activation of neutrophils. Antibody-virus complexes via the intracellular antibody receptor TRIM21 can activate inflammatory cytokine and chemokine responses in the macrophages (Fletcher, AJ & James, LC, 2016).

Interestingly, factor X binding tends to interfere with adenoviral interactions with complements and antibodies, protecting them from these components while fostering TLR4 signalling in spleen afterwards. Ad vectors also interact with shed cellular receptors, the effect of which is not yet to be studied in greater depth on immune responses.

Innate Sensing in Antigen Presenting Cells

Gene transfer with adenovirus results in innate inflammation at the transfer site and high doses at the death of the macrophage. Most of the intrinsic signalling was discovered from macrophage experiments in response to adenovirus. While primary receptors for adenovirus typically bind the fibre knob of the capsid, RGD loops in the penton bind to secondary receptors such as integrins. For examples, splenic MFs of the MARCO subset trap adenovirus. Binding to integrin b3 results in release of IL-1a, which in turn causes signalling through the IL-1 receptor, production of chemokines, and recruitment of other innate immune cells in order to kill virally infected MFs. At large vector doses, resident MFs in the liver (Kupffer cells) undergo necrotic cell death through a mechanism that is not completely understood but relies on IRF3.

Ad vectors also activate the NALP3 inflammasome, a process that involves intracellular DNA sensing (independent on TLR9 and therefore likely to reflect cytosolic sensing), leads to IL-1b expression, and also leads to necrotic cell death. Cytosolic sensing of adenoviral DNA via cGAS (see LV vector section for more information on this pathway) results in the development of T1 IFN, which promotes an antiviral state that can lead to transgenic silencing of transfer of hepatic genes. Nonetheless, the endosomal receptor TLR9 also senses adenoviral DNA, resulting, for example, in the development of IL-6 during hepatic gene transfer and T1 IFN in pDCs. Roles have also been proved for other TLRs. Finally, mechanisms of adenoviral DNA for nuclear sensing may increase or decrease immunity.

Adaptive Responses to Adenoviral Vectors and Transgenes

Pre-existing immunity to vectors derived from the human adenovirus has led to the development of alternative serotype vectors such as chimpanzee adenoviruses. Adenoviral vectors, as expected from viral vectors, elicit NAB responses that prevent re-administration. In addition, adenoviral vectors activate traditional and plasmacytoid DCs, and in vivo transduce DCs. Gene expression in DCs is considered an important contributor to the generation of adaptive immune responses to the transgenic product and to the products of the viral gene. Adenoviral vectors are especially effective in inducing responses to CD8 + T cells, facilitated by the potent induction of Th1 immunity. Elimination of all viral genes in high-capacity vectors and the use of tissue-specific promoters or promoters which are weak in professional APCs are strategies used to reduce T-cell responses. Pre-clinical studies have also been effective in blocking co-stimulatory pathways that are necessary for B and T cell activation. However, the potent innate response to adenoviral vectors greatly complicates the translation of these strategies, and the use of adenoviral vectors for in vivo gene transfer has been largely abandoned in the treatment of genetic disease.

Adeno-Associated Virus

It represents small, single-stranded DNA viruses, which typically do not cause infection without an aid virus being co-infected, such as adenovirus, or herpes simplex virus (Hu JC et al., 2006). They have the

advantage of wide host selection, low immune response rates and longer gene expression. One example is the Eukaryotic adeno-associated virus, a chimeric vector of the virus that combines parvovirus and adenovirus (Kaliberova *et al.,* 2009). It is capable of transfecting mitotic and quiescent cells, lacks human immunogenicity and pathogenicity, and integrates stably into the host DNA at a specific position in cell culture within a chromosome-19 but not in mammalian cells.

Immune Responses to AAV Vectors

AAV is a small non-enveloped parvovirus with an approximately 5 kb single-stranded genome that is naturally non-pathogenic, and defective in replication. AAV vectors do not contain any viral coding sequences and only give rise to mild inflammatory or T1 IFN responses when compared to other viruses, and their genome mainly persists in episomal form. Such features contribute to a favourable safety profile, although immunotoxicity may still occur after systemic delivery of very high doses.34,35 AAV vectors are widely tested for in vivo gene transfer in human gene therapy trials to a large number of different tissues and cell types, including CNS, liver, skeletal and cardiac muscle, skin, and lung. In addition to two drugs approved by the FDA for the treatment of Leber's congenital amaurosis (LCA) and spinal muscular atrophy (SMA), other medicines have entered Phase III trials as in haemophilia A and B hepatic-directed gene therapy.

Pre-Existing Immunity and Neutralizing Antibody Formation

Initial marketing approval was for ocular gene transfer using a subretinal injection route with limited vector doses. Likely encouraged by the eye's immune privilege, vector administration to one eye may later be accompanied by contralateral gene transfer to the second eye and it is also possible to repeat administration to the same eye. Many routes of in vivo vector administration are more commonly associated with neutralizing antibodies (NAB) formation that prevent vector re-administration. In addition, during infancy, humans grow NABs to different serotypes. Seroprevalence varies geographically, and some humans have NAB versus multiple serotypes, reflecting probably cross-reactivity. Some of the highest prevalence of NABs is against AAV2, the serotype identical to AAVs present in the human population, while some of the lowest prevalence is seen in the more complex AAV5. Overall, seroprevalence tends to vary, depending on capsid, from 5-60%. Human subjects are typically screened against the vector capsid for pre-existing NAB titers prior to enrolment in a clinical trial, and only those titled below a set threshold are enrolled. There is ongoing debate as to what level of pre-existing NAB, depending on the serotype, dose and route of administration, can prevent gene transfer. Methods such as adding "decoy" capsids to the vector product or plasmapheresis were explored to overcome or eliminate pre-existing NABs. Interestingly, pre-existing binding antibodies (which do not neutralize) do not obstruct the transfer of genes but may alter the vector's biodistribution. Theoretically it is possible to replicate AAV vector administration by flipping capsid series.

Such a technique, however, is complicated by people's ability to generate cross-reactive antibodies, and the need to create at least two products. An alternative solution is for the application of immune suppression. One protocol uses antibody-mediated B cell depletion combined with rapamycin, while animal models have been tested for rapamycin containing nanoparticles.

CD8+ T Cell Responses to Capsid

A loss of factor IX transgenic expression and temporary mild elevations of hepatic enzymes in circulation were associated with a CD8 + T cell response against the viral capsid in the first liver-directed gene therapy trial with AAV vectors (performed with an AAV2 vector in patients with haemophilia B). This came as a surprise since none of the animal models had shown such a response, including non-human primates, and because AAV vectors are engineered to not express capsid antigen. However, it was later shown that hepatocytes transduced by MHC I on their surface with AAV present capsid antigen, and can be attacked by capsid-specific CD8 + T cells in humans, several epitopes were described, some of which are conserved among several serotypes. In addition, AAV vector particles are known to be prone to proteasomal degradation following endosomal escape, which would result in capsid-derived peptide presentation of MHC I. Subsequent clinical trials used liver enzyme levels as a biomarker for the response of T cells and used immune suppression with steroid drugs (prednisolone) to counteract the response that appears to be vector-dependent. In some patients this strategy for immune suppression has been effective T-cell responses were not seen in all trials, however, raising concerns about the function of factors such as serotype, vector design, or method of manufacture. It is also possible that hepatotoxicity seen in some studies, such as expression for factor VIII, may not always be due to T-cell responses, but may be linked to the overexpression of certain transgenes.

It has been proposed that the lack of CD8 + T cell responses in animal models is due to the fact that they are not natural hosts for AAVs, while memory T cells originating from natural infection have been triggered in humans (and can occur even in the absence of NABs). While non-human primates harbour AAVs, their CD8 + T capsid cells have been found to be distinct from those in humans in terms of differentiation status and function. However, more recent data indicate that CD8 + T cell responses to capsid in patients after vector administration represent primary immune responses, whereas empty capsids (not containing vector DNA) or vectors with genomes largely depleted from immune stimulating CpG motifs primarily stimulate CD8 + T cells in memory. Studies in mice (and to some extent in human cells) showed that the development and priming of CD8 + T cells in AAV gene transfer depends on TLR9 (an endosomal DNA receptor particularly stimulated by unmethylated CpG sequences as found in viral or bacterial DNA, see below for further details) (Butterfield *et al.,* 2019). CpG depletion of expression cassettes is therefore one emerging method for attempting to "deimmunize" AAV vectors. Data from clinical trials show that the outcome of liver-directed gene therapy for haemophilia has been adversely affected by enrichment with CpG. Recent results indicate that long-term FIX expression may be correlated in the IL6-R gene given CpG enriched vector sequences, at least in some patients with polymorphism (Research and Practice in Thrombosis and Hemostasis, 2019). Another strategy is to remove phosphorylated tyrosine residues from the capsid, which in turn serves as a signal for ubiquitination and proteasomal degradation. As a result, these modified capsids may be less of the MHC I have presented. It should also be remembered that in animals such as mice or non-human primates CD8 + T cell responses to AAV capsid can be observed (Fitzpatrick, Z et al., 2018; Long, BR et al., 2019). And the nature of their peripheral blood distribution is somewhat different from humans. Responses in mice occur within 1-2 weeks while average in humans are one to several months. In addition, the reaction in the animals does not contribute to the destruction of transduced cells unless the T cells are further expanded ex vivo and then transferred adoptively. Those differences remain unresolved between animal and human responses (Louis Jeune, V et al., 2013).

CD8 + T cell responses to AAV capsid were also observed in gene transfer directed to the muscle. For example, in the muscle of patients who received AAV1 vector for treatment of a1-antitrypsin deficiency, a prolonged inflammatory response was seen (Ferreira, V et al., 2013; Flotte, TR et al., 2011). Interestingly, the expression of transgenes was somewhat reduced but not eliminated, and over time seemed to recover. In addition, regulatory T cells (Treg) CD4+CD25+FoxP3 + infiltrated the tissue and probably contributed to the resolution of this immune response while CD8 + T cells acquire a phenotype resembling exhausted T cells. Similar observation for lipoprotein lipase deficiency was made in muscle gene transfer.

Innate Immunity and Links to Adaptive Responses

Innate immune responses in tissues transmitted with AAV gene therapy are relatively mild compared to other viruses, resulting in rapid (within 1-2 hrs) but often minimal and highly transient (< 12 hrs) immune infiltration of macrophages, NK cells, and neutrophils and expression of pro-inflammatory cytokines and T1 IFN depending on the endosome in the mouse liver (Majowicz, A et al., 2013; Martinez-Navio, JM et al., 2019). The TLR2-dependent cytokine expression was observed in Kupffer cells in the culture of human liver cells. The viral genome and the capsid can therefore contribute to the innate recognition of AAV immune systems. A recent study shows that transduction of cells with AAV vectors through a mechanism that has yet to be identified leads to the formation of double-stranded RNA, which can be sensed by cytoplasmic dsRNA sensors such as MDA5, resulting in IFN-b expression (Manno, CS et al., 2006).

AAV vectors cause T1 IFN expression in human and murine dendritic plasmacytoid cells (pDCs) but not in traditional dendritic cells (cDCs) with CD11chi. T1 IFN development is the result of the signalling of TLR9-MyD88 in pDCs and promotes activation of CD8 + T cells. Consequently, CD8 + T cell responses to transgenic product or capsid are significantly reduced in mice deficient in TLR9, My88, or T1 IFN receptors. Cross-priming studies of AAV capsid-specific CD8 + T cells have shown that pDCs and cDCs cooperate to achieve CD8 + T cell activation (Fig. 4).

Sensing of the AAV genome by TLR9 occurs in pDCs, while cDCs carry the task of presenting antigen by MHC I. This process requires T1 IFN, which binds its receptor to cDCs, suggesting a direct effect of cytokine production by pDCs on the activation of cDC. Nevertheless, there is no need for NK cells that can indirectly mediate the effect of pDCs on cDCs. In addition to T1 IFN, CD40-CD40L co-stimulation is needed for the cross-priming of CD8 + T cells against AAV capsid, which is carried out by CD4 + T helper cells.

TLR9-MD88 signalling and T1 IFN development may have a modulating effect on the formation of antibodies against capsid or transgenic product but is not as strictly necessary as for CD8 + T cell priming (though MyD88 has an intrinsic role in B cells in Th1-dependent antibody class switching) (Herzog *et al.,* 2017). This is in contrast to CD4 + T aid and related co-stimulatory pathways, which are necessary for antibody formation and thus could potentially be targeted for both T cell and antibody response prevention. A recent study investigating the development of an antibody against AAV in healthy humans showed that IL-1b and IL-6 output activated B cells by circulating monocyte-derived DCs (moDCs) when pulsing with AAV particles or AAV capsid-derived peptides (Fig. 5).

Figure 4. Potential targets for normal tissue radioprotective gene therapy

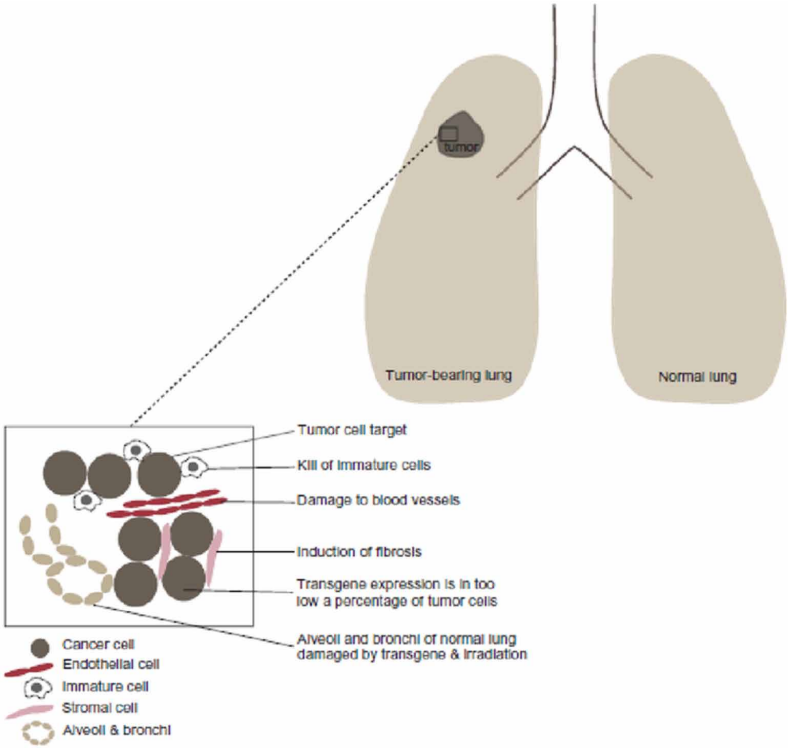
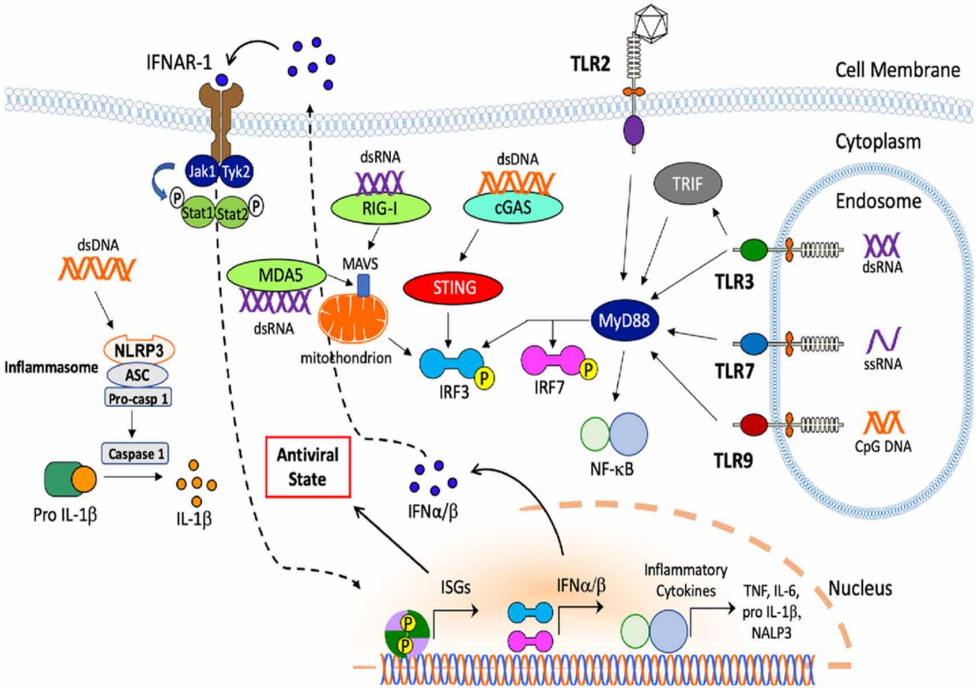


Figure 5. AAV capsid-derived peptides



Antibody formation inhibited by either cytokine is blocked against AAV in vitro and in vivo (in mice). While previous studies found no correlation between NAB presence and AAV-reactive IFN-g producing CD8 + T cells in humans, this new study found a good correlation between NAB presence and CD8 + T cells producing memory with TNFa.

Interestingly, when stimulated in vitro, NK cells from seronegative individuals seemed to react to both the capsid AAV and the capsid AAV peptide pools. Mice studies demonstrated a remarkable ability of TLR9 agonists to trigger antibody responses to the transgenic drug in muscle gene transfer, which occurred by inducing moDC responses that enhance T follicular helper T cells activation. MoDC activation is therefore a driver of T-cell responses which promote the formation of antibodies. Complement can also participate in creating NAB. AAV2 capsid, for example, was found to bind to iC3b complement protein (which, however, did not result in complement activation) and to supplement regulatory protein factor H, and C3 deficient mice had inhibited the response of an antibody to capsid.

Risks of Adaptive Responses to the Transgene Product

Many factors, including the underlying mutation in replacement therapy for genetic disease, the vector administration / target tissue route, specific vector design, AAV serotype, and vector dose, influence the risk of an antibody response to the transgene product. Additional host factors may include specific aspects of a disease such as inflammation of the tissue. For systemic delivery of proteins, muscle gene transfer bears an elevated risk of B cell activation, which for example complicates AAV-delivery of antibodies against viruses that cause infectious disease such as HIV. Multiple factors also affect the risks of CD8 + T cell responses. Although AAV vectors gained prominence in comparison with Ad vectors due to their inefficient activation of CTLs, thereby significantly increasing the probability of long-term transgenic expression, CD8 + T cell responses to dystrophin and, in rare cases, to a1-antitrypsin transgenic products were nevertheless observed in patients. Some patients with Duchenne muscle dystrophy may actually have pre-existing immunity to T-cells due to the occasional expression of endogenous dystrophin in reverting fibres. Because utrophin overexpression can partially compensate for lack of dystrophin, these immune complications may be avoided by AAV vectors which express this “self-gene” that is widely expressed in the muscle. Missense mutation typically causes a1-antitrypsin deficiency. Interestingly, in rare HLA forms, CD8 + T cell responses may be guided not against an epitope spanning the mutation but rather against a polymorphic sequence that may differ from the therapeutic transgene. Given the correlation between TLR9 signalling and CD8 + T cell activation, CpG depletion of the expression cassette has been implemented in the vector design to reduce this risk.84 Interestingly, CD8 + T cell responses to the transgenic product are often not completely functional when using traditional single-stranded DNA genome vectors in mice. They do not remove the transduced muscle, do not respond to vaccination boosting and upregulate immunoinhibitory molecules like PD-1.

However, self-complementary vectors (which can be produced by removing a nicking site in one of the ITRs) elicit more functional responses, likely due to enhanced TLR9 signalling and/or various transgene expression kinetics. These vectors do not need a second-stand synthesis, and can therefore express more easily, but can only be half the size of a traditional AAV genome to avoid reaching the packaging cap. Immune tolerance induction by hepatic gene transfer and in the case of gene deletion, the long-term expression of a secreted transgenic product without the development of an antibody can be accomplished through the transfer of hepatic genes with AAV vectors. These findings indicate the liver environment’s ability to help in the induction of tolerance. Tolerance is induced by merging mechanisms. The deletion

of effector T cells requires programmed cell death, while induction of FoxP3 + Treg is needed for both induction and tolerance maintenance.

Ability to induce Treg tolerance and extent depend on transgenic expression rates. Antigen presentation leading to Treg activation is likely to occur in liver-draining lymph nodes and in the liver environment itself, supplying immune suppressive cytokines and specific cell types capable of presenting antigen such as Kupffer cells, hepatic DCs, and endothelial liver sinusoidal cells (LSECs). Additional mechanisms include T cell anergy, T cell exhaustion and CD8 + T cell response suppression through IL-10 production. The dominant existence of mediated tolerance enables the addition of the therapeutic protein by gene transfer to other tissues or systemic protein delivery for storage disorders such as in enzyme replacement therapy. Hepatic mediated tolerance can also reverse pre-existing immune responses and can be synergistic with traditional immune suppression in autoimmune disease treatment. Lastly, hepatic gene transfer with miRNA regulated LV vectors can induce immune tolerance in a similar way.

Immunotoxicities

The development of systemic gene therapies for neuromuscular, neurodegenerative and storage disorders employs very large doses of vectors, ~1014 AAV vector genomes / kg. In this area, generally, the immune mechanisms mentioned above have not been studied and immunotoxicities are emerging in more recent pre-clinical and clinical studies. Some of these may be related to AAV itself, others to product impurities, others to transgenic product effects. In some patients treated with high-dose AAV9, complement activation has been reported, though it is unclear whether this follows the classical antibody-mediated pathway or direct virus binding to complement components. For example, at such high doses of vectors, it is conceivable that cell surfaces are decorated with viruses. Broader toxicity has also been identified, including thrombocytopenia, in a patient treated for muscular dystrophy. CD8 + T cell responses may give rise to wider toxicity when multiorgan or CNS are transduced. There is a strong need for further research to address those issues.

Lentivirus Vector

Lentiviruses are retroviruses which infect bovine, equine, nonhuman and human primates. Infection with the human immunodeficiency virus (HIV) is among the most harmful human pathogens (Breckpot and Thielemans, 2007; Comins C et al., 2013). It constitutes a class of enveloped viruses containing a single stranded RNA genome of 9.2 kb. The lentivirus bears a transcriptase reverse enzyme that transcribes RNA to double-stranded DNA once it reaches the cytoplasm. It then permanently incorporates the target cells into the nuclear genome. Types include immunodeficiency virus-derived lentiviral vectors such as HIV-1, HIV-2.

Researchers have eliminated the infectious parts of the virus through genetic manipulation, and inserted other parts from different viruses such as cytomegalovirus, producing a highly modified lentivirus (Liu *et al.,* 2006). Another genetic modification employed by previous research has created an integration-deficient lentivirus that has not been integrated into a host genome, and with slightly lower transduction efficiency (Bryson and Wang, 2013; Michelini Z et al., 2009). Such a modified lentivirus has the advantage of being fairly healthy, of having variable specificity to either a single cell or of being sufficiently large to infect all cells, and of having successful transduction of both dividing and non-dividing cells. Modified viruses have poor immunity to antiviruses, higher genotoxicity capacity due

to insertional mutagenesis, and the ability to bear genes within the nucleus (Breckpot and Thielemans, 2007; Cai Y et al., 2014). Major disadvantages include inadequate immune responses as well as antitumor response, the risk of viral transformation into pathogenic HIV infection, particularly in immunized individuals, and the risk of second malignancy with insertion mutagenesis of new cancer genes into the host genome (Hu *et al.,* 2009).

Immune Responses to Lentiviral Vector

LV vectors are derived from the human immunodeficiency virus (HIV) and are capable of both transmitting nondividing and dividing cells. They are enveloped viruses that contain a single-stranded RNA genome, transcribed backwards into DNA in the cytoplasm of the host cell upon infection. Stable incorporation into the host cell genome after transport to the nucleus will lead to long-term transgenic expression if the cell survives. LV vectors are common for transfer of ex vivo genes to hematopoietic stem cells in the treatment of genetic disease and to T cells for chimeric antigen receptor (CAR)-T cancer cell therapy. They are also being produced for the transfer of genes in vivo, e.g. to the liver, and integration-deficient LV vectors were also created. A more recent strategy in the development of vaccines is in vivo gene transfer to DCs. Pseudotyping with vesicular somatitis virus G (VSV) is common, since this protein envelope allows LVs to effectively infect a wide range of target cells. However, VSV pseutotyped LV vectors do not, for example, infect B cells, which prompted the creation of CD20-targeted envelopes containing a single variable fragment against CD20 fused to a protein enveloped by the measles virus. Similarly, envelopes were developed for precise targeting of different subsets of B and T.

Innate Immune Responses Against LV Vectors

While pre-existing immunity to LV is weak in humans, several factors, including phagocytosis, generally restrict the efficacy of in vivo hepatic gene transfer with LV vectors. To this end, the introduction of the human phagocytosis inhibitor CD47 into the LV membrane decreases phagocytic cell uptake and increases hepatocyte distribution. Another severely limiting factor is the production of T1 IFN, so mice deficient in T1 IFN signalling show considerably higher transduced hepatocyte numbers. Likewise, pharmacological suppression of the production of IFN (e.g. with dexamethasone) increases the efficiency of transduction. Although LV vectors elicit weaker pDC IFNa responses compared to the parent HIV-I virus, pDCs are thought to play a key role in the T1 IFN response against LVs. TLR-7, the PRR that senses endosomal single-stranded RNA molecules and TLR9 contributes to T1 IFN induction. It has been suggested that LV vectors with protein-pseudotyped VSV-G may contain tubulovesicular structures with DNA fragments that promote TLR9 signalsThe intensity of downstream signalling for TLR7 and TLR9 is partly regulated by the mTOR pathway. Interestingly, a miRNA (miR126, considered to have a vital role in vascular endothelial cells during angiogenesis) was found by Brown and colleagues to be expressed in pDCs within the immune system in a specific way. In pDCs miR126 aims for degradation to a negative mTOR regulator. For generating pDCs and for the TLR-mediated innate responses to nucleic acids and LV vectors in pDCs, an overactive mTOR pathway is important. In addition, this observation can be taken advantage of in vector design to avoid transgenic expression in pDCs by incorporating target sequences into the transcript. However, blocking the TLR7 or TLR9 signalling is insufficient to prevent IFN-a response from being induced by LV vectors. This is possibly due to the sensing of genomes of viral DNA (which results from reverse transcription) via a cytoplasmic sensing process involving the

cGAS-STING pathway. Cyclic GMP-AMP Synthase (cGAS) is a cytosanitary DNA sensor. The enzyme catalyzes the cyclic GMP-AMP (cGAMP) generation, which binds to the Interferon Genes Stimulator (STING), when binding to DNA. STING primarily locates endoplasmic reticulum (ER) and is a signaling adapter for T1 IFN development. Re-location to the Golgi system activates IRF3 phosphorylation via TBK1, leading to T1 IFN expression.

Adaptive responses to LV gene transfer, miRNA control strategy, and LV vectors for tolerance induction effectively transduce qualified APCs such as MFs and DCs, which have been recognized as strongly promoting immune responses to the transgenic drug. In fact, DCs transduction in vivo and ex vivo, e.g. using integration-deficient LV vectors, is being exploited in the development of the vaccines. Use of a hepatocyte-specific promoter in hepatic gene transfer has not been sufficient to fully prevent expression in APCs and activation of immune responses. Naldini and Brown took advantage of miRNA regulation of transgenic expression in an elegant approach to this problem and integrated several copies of a target for a miRNA (miR142) that is highly expressed in hematopoietic cells, including competent APCs. Combined with the use of a hepatocyte-specific promoter, it has prevented CTL and antibody responses to the transgene drug.

In addition, it induced immune tolerance to transgenic products such as GFP or factor IX. Activation of CD8 + T cells was abortive, while transgenic product-specific CD4+CD25+FoxP3 + Treg was induced to actively suppress immune response. The development of type 1 diabetes in nonbase diabetic (NOD) mice could be prevented using such an engineered hepatocyte-restricted LV vector expression insulin B line. Reversal of the autoimmune disorder was achieved when combined with monoclonal antibody immunomodulation directed against CD3.

Interestingly, when transgenic expression was restricted to hepatic endothelial cells (the normal site of FVIII biosynthesis), Follenzi and his colleagues showed an improved success rate of tolerance induction to factor VIII in hemophilia A mice, which merely needed an endothelial cell-specific promoter but not miRNA target sequences. In addition, this research line established transgenic expression as a major driver of immune responses in pDCs, whereas expression limited to myeloid cells can not result in immune responses. The producer of cell-derived polymorphic MHC I molecules is another potential source of T cell response against LV vectors. The producer of cell-derived polymorphic MHC I molecules is another potential source of T cell response against LV vectors. However, this can be eliminated by disrupting the beta-2 macroglobulin gene in producer cells, which results in MHC-free LV generation.

Transfer of ex vivo genes has been commonly used as a way of avoiding immune responses. Adaptive responses to gene-modified cells may, however, occur but can be mitigated by combining myeloablation and immune suppression regimens. In addition, a recent study has shown that enzyme replacement therapy for the treatment of lysosomal storage disorders (LSDs) may induce CD8 + T cells which may target lentiviral gene therapy with HSC. Hence ex vivo gene therapy for LSDs may require adjunct immunotherapy in patients with prior protein therapy. Others use ex vivo transfer of the HC gene with LV vectors to target transgenic expression to megakaryocytes for platelet protein delivery. This strategy is intended to release proteins such as FVIII upon platelet activation when the protein antigen is otherwise "hidden" from the immune system. Over time, platelet-targeted expression may also promote immune tolerance.

Herpes Simplex Virus

This is a big, enveloped double-stranded DNA virus (150 kb), naturally neurotropic (preferably nerve cells), which infects humans particularly at the oral and genital mucosa, but ultimately spreads to the sensory nerves to replicate or dormant in the sensory ganglia. Viral reactivation can cause oral or genital ulceration, rashes in the skin, or even encephalitis. The virus is seropositive to up to 80 per cent of the population (White and Gill, 2013). A modified oncolytic recombinant replication-selective herpes simplex virus has been developed with genetic manipulation and has had many advantages: it has large tropism, is powerful in causing tumour cell lysis, is non-integrative in targeting the cell genome (apart from non-essential genes), may escape the host immune system; and many successful antiviral therapies in the case of toxicity Another benefit is its viral capacity to bear a large load of transgenes, such as a pro-drug-activating gene thymidine kinase enzyme that improves tumour lysis when intravenously administered ganciclovir medication (suicide gene) (Hu *et al.,* 2006); Therapeutic immunomodulatory transgenes that improve the immune response of antitumor (such as alipogene laherparepvec) (OncoVEX GM-CSF) (Sharp, 2002: White and Gill, 2013); and antiangiogenic genes that inhibit the vasculature of tumours. Modified oncolytic herpes simplex viruses such as Talimogene laherparepvec (TVEC) and others are currently being tested as a monotherapy or in combination with surgery, radiation therapy or chemotherapy in several clinical trials, especially in patients with high-grade glioma. A few successes have been recorded at present (White and Gill, 2013).

Reovirus

This is an oncolytic virus primarily infecting animal. In humans, except for respiratory and gastrointestinal signs, very rarely causes serious illness. The virus is seropositive to nearly 100 per cent of human adults. It is non-enveloped, double-stranded RNA (dsRNA), and its oncolytic activities are primarily by stimulating the immune system, in particular by activating the bystander immune systems (). Innate immunity against tumour cells is further increased by the release of tumour-associated antigens following cell lysis.

The virus is known to be fairly stable, with good records of protection, does not require genetic modifications to become an oncolytic virus, and is less costly for commercial development. Because of its relative health, the virus is commonly used in many clinical trials, in conjunction with radiation therapy or chemotherapy, as oncolytic reovirus monotherapy, delivered intratumorally, intravenously or intraperitoneally; or as polytherapy (Comins *et al.,* 2013).

Poxvirus

Poxvirus is something of a complex virus of DNA. The genome is linear double-stranded DNA, and the size of the different poxvirus species ranges between 130 kb and 160 kb. In the terminal regions of the genome, the dsDNA molecule comprises the open reading frame (ORF) and the inverted terminal repeats (ITR) (Mullen and Tanabe, 2002). Poxvirus has been used in cancer therapy as a transgenic vector with tumour-associated antigens or tumour-specific antigens, which may enhance the ability of the hose to anti-cancer (Fenner *et al.,* 1988).

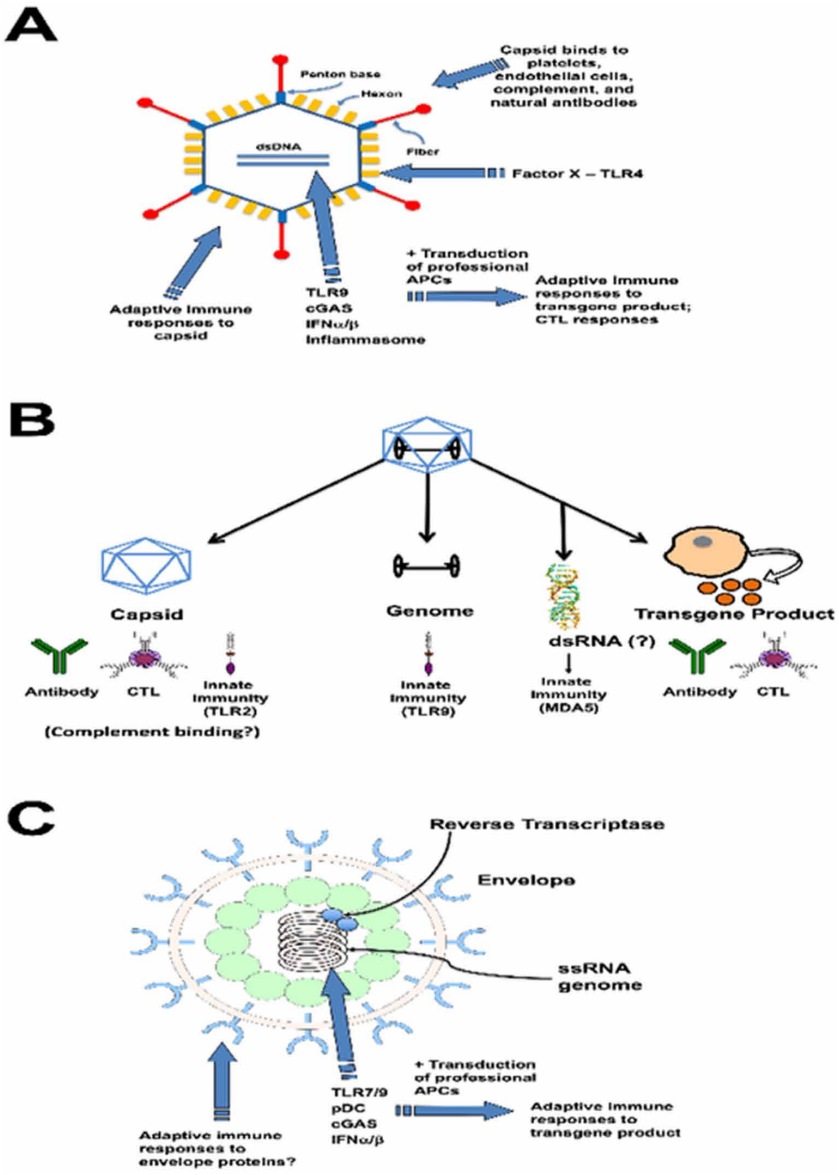
Gene Therapy Approaches to Enhance Management of the Radiotherapy Patient

The discovery of oncogenes and tumour suppressor genes, and the detection of gene translocation directly within tumour cells, present a challenge for patients with cancer to adapt gene therapy techniques. Accordingly, the first gene therapy strategies targeted tumour cells with "normal" gene expression in an attempt to counterbalance or overload abnormal cancer-specific gene expression. In the case of p53, gene deletion or blocked gene product expression, patients with lung cancer received a reasonable target for wild-type p53 transgene insertion (Epperly *et al.,* 2009). In addition, the localization of lung cancer tumours by diagnostic radiographs and the insertion of transgenes through stereotactic thoracic surgical techniques facilitated successful clinical trials. The same approach held for head and neck cancers found to contain mutated receptors of the epidermal growth factor (EGFRs), encouraging a clinical trial in which targeted surgical injection techniques of the wild form EGFR were introduced. Application of the therapeutic transgene to tumour targets often included approaches whereby a targeted surgical technique may inject an additive or synergistic cytotoxic agent. The target volume of prostate cancer treated with herpes simplex virus-activated cytotoxin in combination with external beam radiotherapy was found to be of therapeutic benefit. Ionizing transgenic-associated promoters offered an ideal method for locating transgenic activation in tumour volumes within the radiotherapy beam. While all tumour-specific gene therapy approaches offered attractive model systems, and many were successfully implemented into the clinic, the difficulty in finding a vector to induce transgenic expression in a sufficient number of tumour cells within a target volume was a common issue.

Other issues were the function of the tumour vasculature, the function of protective stromal cells, and the impact of infiltrating immune cells in both the reaction to and interaction with the transgenic product with ionizing irradiation. The success of gene therapy in targeting vascular structures in other noncancer diseases has given the field encouragement.

Localized transgene injection into tumour volume included tumour localisation, targeting with insertion tools, and the trauma associated with localized gene delivery. With head and neck and lung cancer and brain tumours, this technique was most apparent. Hemorrhage was a risk and could have been the result of inserting needles to allow for localized transgenic expression. Late effects of irradiation, primarily fibrosis or scarring, were a concern for potential deleterious additive toxic effects of gene delivery on tumors. An alternative approach to systemic (intravenous) delivery of transgene, while avoiding localized traumatic insertion techniques, created the new problem of transgenic expression within the target volume or distant sites of radiotherapy in surrounding tissues. A major concern was the potential higher level of expression of a radiosensitizing transgenic drug in normal tissues compared to a tumour — a condition that could tip the therapeutic ratio in the wrong direction, creating toxicity for radiotherapy later. Lastly, the use of fractionated irradiation in external beam approaches and the restricted degree of transgenic expression needed several transgene administrations that were impractical in the case of surgical operating injection sites to maintain gene product bioavailability. Despite all the concerns about potentially important toxicity, new methods have centred on finding transgenic products that are more harmful to cancer cells than normal tissues. For example, in the case of enhanced tumour cell survival under hypoxic conditions, the delivery of directly toxic transgenic products to hypoxic cells (HIF1 inhibitor), potentially non-toxic to normal tissues, may be a tumour-specific transgene targeting strategy (Post DE et al., 2003). Fig. 6 indicates different normal lung tissue from a lung cancer. The insert reveals tumour cells, endothelial cells in the blood vessel, stromal cells, immune cells and the question of transgenic expression targeting the tumour.

Figure 6. Potential problems with transgene radiosensitization for lung cancer patients undergoing radiotherapy



Normal Tissue Radioprotective Gene Therapy: The Logic of this Reverse Strategy

Since the initial use of radiotherapy to treat patients with cancer, typical tissue side effects have been of great importance in choosing the dosage of radiotherapy, beam strength, and daily fraction size. Symptoms of radiation poisoning are treated with palliative steps before a therapeutic (tumoricidal) dose is met or the severity of the side effects of radiotherapy precludes completion of the treatment plan. The best solution to normal tissue safety was, and continues to be, the blockage of normal tissues by specific columnisation of radiotherapy beams. 10 half-value layer lead blocks have been replaced with movable

multileaf collimators. The idea of radioprotective gene therapy for human tissues was followed by experiments developing natural tissue radioprotectors for the small molecule (Belikova *et al*., 2009; Bernard *et al.,* 2011; Goff JP et al., 2011). Studies at the Walter Reed Research Institute during the 1970s led to the discovery of WR2721, a free radical scavenger compound now known as amifostine therapy. Systemic and organ-specific delivery of the amifostine radioprotector showed some significant therapeutic effects. Most notable was the direct proof of salivary gland defence and reduction of xerostomia in patients with amifostin receiving head and neck cancer, due to the high concentration of medication in salivary glands. Attempts to use locally administered amifostin to protect rectal tissue to improve the effectiveness of prostate cancer radiation therapy, or swallowed amifostin to treat the oesophagus, or inhaled or systemic medication to protect normal lung, have been less effective in treatment (Kim H et al., 2011). The strategy of normal tissue radioprotection using small molecules led to the idea that gene therapy may also be used for normal tissue defence and gave the additional potential advantage of transient — but perhaps lasting several days — continuous development of transgene transcript and protein, which could be more advantageous and long enough for clinical radioprotection.

Identification of targets for normal radioprotection of the tissue using gene therapy resulted from an analysis of the components of the normal irradiation response. Fig. 4 shows potential targets for transgene therapy production for normal tissue radioprotection (DNA strand breaks, nucleus-to-mitochondrial targeting, apoptosis, cell death, cytokine growth, and secondary cell death). Due to the non-specificity of transgenic intake in normal compared to tumour tissue, strategies for preparing transgenes for DNA repair and minimizing DNA strand breaks were difficult. Strategies to target the production of cytokines by irradiation-damaged cells to prevent secondary normal apoptosis of tissue were also difficult because they had to avoid radioprotection of tumours. A promising strategy was that associated with gene therapy strategies to target normal tissue's oxidative stress response to ionizing irradiation relative to cancer cells, specifically the apoptotic cellular pathways.

Future Directions in and Applications of Gene Therapy in Radiation Oncology

What would be the perfect application of gene therapy in radiation oncology? Several investigators have proposed one which would incorporate the following five gene therapy principles:

1. Usage of targeting transgene with gene expression to the tissue / organ of interest in a sufficient number of cells to have therapeutic impact in setting ionizing irradiation
2. Minimal toxicity to normal tissues
3. Safety of the methodology, meaning rapid elimination of the transgene product from normal tissues with no delayed deleterious effect on somatic cells or germ cells, no transmission of gene product through the germline, and effective elimination of the gene product with death of tumour cells
4. In the setting of tumour radiosensitization, activation of the transgene product specifically in tumours by ionizing irradiation directly or indirectly through the radiation-induced bystander effect
5. In the setting of normal tissue radioprotective gene therapy, utilization of a transgene product that would have no protective effect on tumour cells and ideally could provide simultaneous tumour radiosensitization.

p53 GENE THERAPY

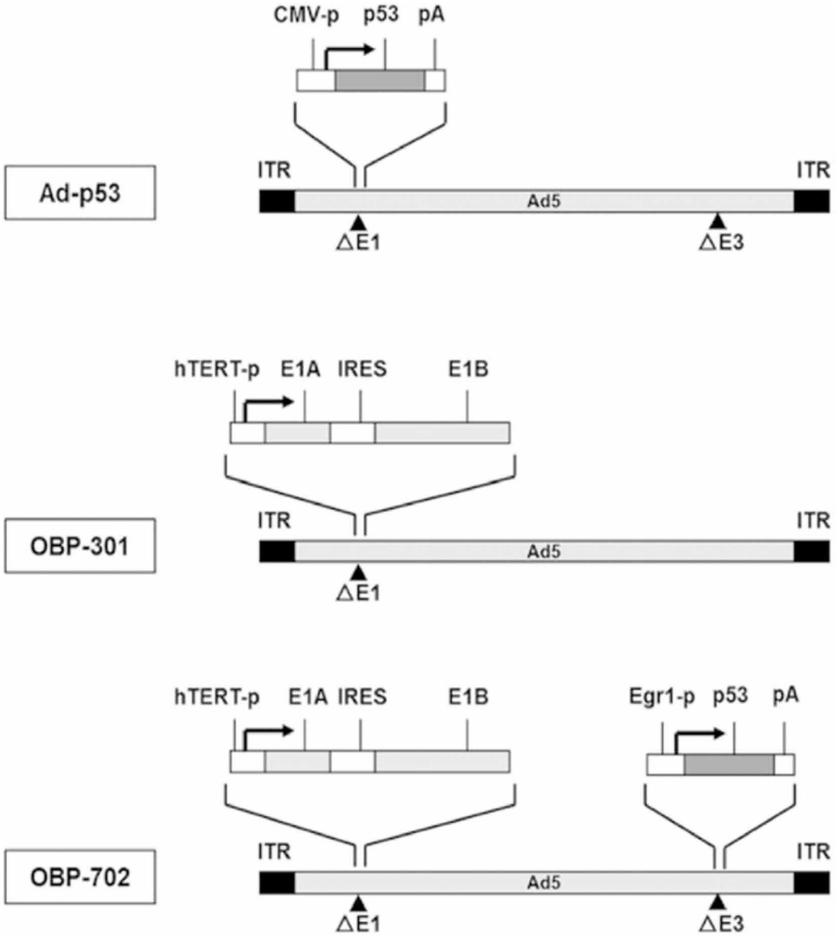
Replacement therapy for the tumour suppressor gene (TSG) is emerging as a successful antitumor treatment for inducing programmed cell death through the addition of a therapeutic TSG. Among the therapeutic TSGs used to induce tumour suppression, the most potent TSG is the p53 gene which functions as a multifunctional transcription factor for regulating various cellular phenomena such as cell cycle arrest, senescence, apoptosis, and autophagy (Robson T et al., 2003). The IARC TP53 database (http:/www-p53.iarc.fr/) shows that there are somatic mutations in the p53 gene in different forms of malignant tumours. The p53 gene is frequently inactivated in human cancers by aberrant genetic regulation, suggesting that the p53 gene plays a critical role in the network of tumour suppressants. The restoration of wild-type p53 function would therefore be a promising antitumor strategy to strongly suppress the growth of inactivated p53 tumours. P53 Replacement therapy is commonly and regularly used as a potent antitumor strategy to induce p53 gene expression and subsequent cell death in several types in p53-inactivated malignant tumours. There are four forms of p53 transfer systems for inducing ectopic expression of an exogenous p53 gene or p53 protein: cationic liposome – DNA plasmid complexes, a replication-deficient adenovirus vector, a replication-competent adenovirus vector, and a protein transduction mechanism. Exogenous p53 expression activation effectively induces p53-mediated signalling pathways for cell death in the tumour cells that are inactivated by p53. By contrast, reactivating endogenous p53 expression through treatment with chemical compounds such as Nutlin-3 or PRIMA-1 is another type of strategy for restoring wild-type p53 functionality. In tumour cells, Nutlin-3 induces p53 stabilization that overexpresses p53-suppressive mouse dual minute 2 (MDM2) by inhibiting MDM2–p53 interaction. PRIMA-1 induces apoptosis in human cancer cells with p53 gene mutation by restoring DNA-binding activity and functional conformation to a mutant p53 protein. However, since Nutlin-3 and PRIMA-1's therapeutic potential is restricted to tumours with MDM2 overexpression and unique p53 gene mutations, p53 transfer systems for inducing exogenous p53 expression should provide useful antitumor strategies that could be used more extensively and more frequently in replacement therapy.

p53-Mediated Cell Survival and Cell Death Signalling Pathways

There are typically many forms of signalling pathways for the p53-mediated cell death, including senescence, apoptosis, and autophagy (Fig. 7). When tumour cells with intact p53 function are subjected to genotoxic stress, p53 is activated to transcriptionally induce many types of p53-downstream target genes, such as p21WAF1 (p21) BAX or damage-regulated autophagy modulator (DRAM). Under mild genotoxic stress, p53 mainly upregulates p21 expression for the induction of cell cycle arrest, which enables DNA damage to be repaired and contributes to cease. Conversely, severe genotoxic stress induces higher p53 accumulation, which activates BAX- and DRAM-related signalling pathways that lead to apoptosis and autophagy, respectively, and leads to cell death induction. However, when the target gene MDM2 p53-downstream, which is a negative regulator of p53 via the ubiquitin – proteasome pathway, is upregulated after p53 activation, MDM2 activation inhibits the signalling pathway p53-mediated as a p53-negative feedback loop. Thus, the p53-mediated pathways of cell survival and cell death are strictly regulated by many kinds of target genes p53-downstream.

Genotoxic stress induces cell cycle arrest, apoptosis, or autophagy through the activation of the p53-target genes p21, BAX, or DRAM, respectively. Mild genotoxic stress induces accumulation of a small amount of p53, which contributes to p21-dependent cell cycle arrest and cell survival. However, severe

Figure 7. Scheme of cell survival and cell death pathways induced by genotoxic stress or p53replacement therapy



genotoxic stress induces a large accumulation of p53, which results in the activation of three distinct cell death pathways: senescence, apoptosis, and autophagy. Moreover, p53-induced MDM2 activation functions as a p53-negative feedback loop via ubiquitin-mediated p53 degradation. In contrast, p53 replacement therapy induces p53-mediated cell death signalling pathways via the ectopic expression of exogenous p53 gene or p53 protein.

p53 Replacement Therapy

An effective strategy in preclinical and clinical settings is to restore wild-type p53 function in a variety of p53-inactivated tumour cells, the overexpression of an exogenous p53 gene or p53 protein using one of several transfer methods (Fig. 7).

Liposome-based p53 DNA plasmid delivery or virus-based p53 gene delivery transcriptionally stimulates ectopic expression of the exogenous p53 gene, while membrane-permeable p53 protein delivery specifically induces exogenous p53 protein expression. In the following parts, we demonstrate

the therapeutic potential of p53 replacement therapy that includes in preclinical and clinical settings a liposome – DNA plasmid complex, a replication-deficient virus vector, a replication-competent virus vector, or a protein transduction system.

Cationic Liposome Complex with a DNA Plasmid

Cationic liposomes are useful delivery systems for the transfection of plasmid DNA vectors which in vitro encode ectopic p53 into human cancer cells. Recently an antibody-conjugated immunoliposome has been developed for cancer to improve the efficiency of transfection and the tumour-specific delivery of plasmid vectors. Transfection efficiencies using either liposome-based process, however, are still poor, and insufficient to induce cell death, particularly in tumour tissues in vivo. Improvement of liposome-based delivery systems is therefore necessary to effectively induce p53-mediated cell death within tumour tissues.

Replication-Deficient Adenovirus Vector

Compared to the poor transfection efficiency of exogenous p53 induction with a plasmid DNA vector, it has been shown that a replication-deficient adenovirus Ad-p53 vector effectively induces the expression of an exogenous p53 gene and subsequently exerts an antitumor effect in preclinical in vitro and in vivo experiments.(Fig. 8) The tumour suppressive mechanism regulated by Ad-p53 involves three mechanisms of death for cells: senescence, apoptosis, and autophagy. These cell death pathways are determined by inducing several target genes p53-downstream, such as p21 (Fig. 9).

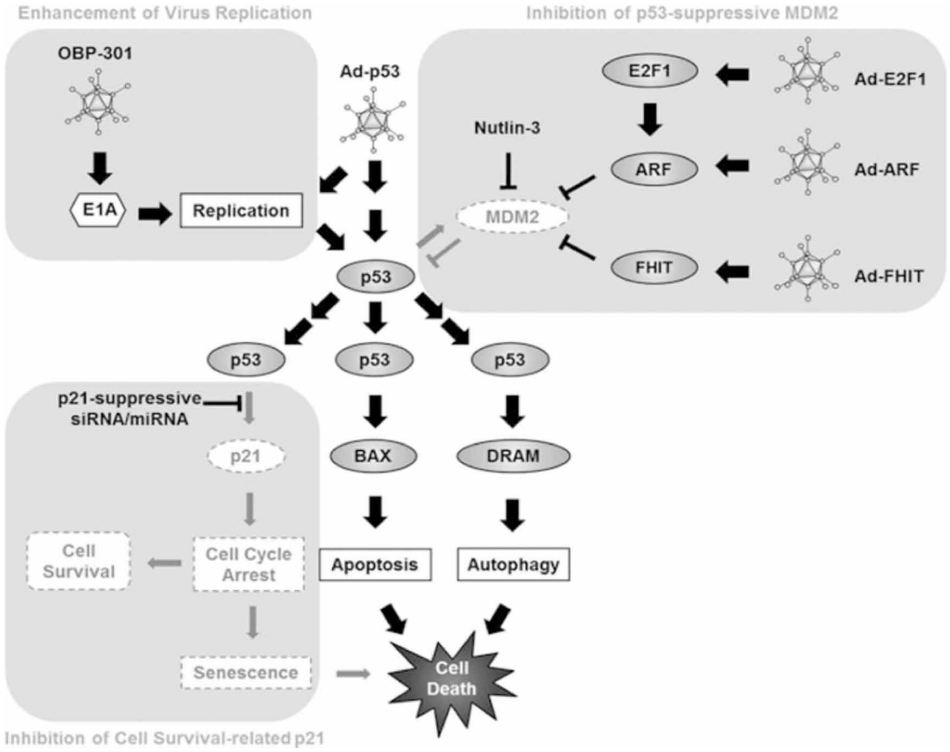
There are several combination strategies for enhancing viral replication, p53 expression, and p53-mediated cell death in the tumour cells infected with Ad-p53 to further encourage Ad-p53-mediated cell death pathways (Fig. 9).

The Ad-p53 vector is a p53-expressing replication-deficient adenovirus; a p53 gene expression cassette that is under the regulation of the cytomegalovirus promoter (CMV-p) is inserted into the E1 region; the E3 region is deleted. The OBP-301 vector is a telomerase-specific replication-competent oncolytic adenovirus; the hTERT gene promoter (hTERT-p) element drives the expression of two adenoviral E1A and E1B genes that are linked to an internal ribosome entry site (IRES). The OBP-702 vector is a p53-expressing conditionally replicating adenovirus. In OBP-702, the p53 gene cassette controlled by the Egr1 promoter (Egr1-p) is inserted into the E3 region of OBP-301.

The Ad-p53 vector induces BAX- and DRAM-mediated apoptosis and autophagy, respectively, resulting in cell death, rather than in p21-dependent cell cycle arrest and cell survival, when combined with E1A-expressing replication-competent OBP-301, several replication-deficient adenovirus vectors (Ad-E2F1, Ad-ARF, Ad-FHIT), Nutlin-3, or p21-suppressive siRNA/miRNA.

The first approach is to use an oncolytic adenovirus expressing E1A in combination therapy since Ad-p53 is a replication-deficient adenovirus vector suppressed by E1A. For example, we have previously produced a telomerase-specific replication-competent oncolytic adenovirus OBP-301 (Telomelysin), which induces telomerase dependent tumour-selective lysis of cells (Komata T et al., 2001). OBP-301 enhanced expression of Ad-p53-induced p53 in combination therapy which resulted in a stronger antitumor effect and increased apoptotic cell death compared to Ad-p53 monotherapy. Adenoviral E1A accumulation induced by OBP-301 has been used to replicate Ad-p53 which enhances the expression of Ad-p53-mediated p53. A second strategy is to suppress MDM2 expression, because MDM2 p53-

Figure 8. DNA structures of Ad-p53, OBP-301, and OBP-702 vectors



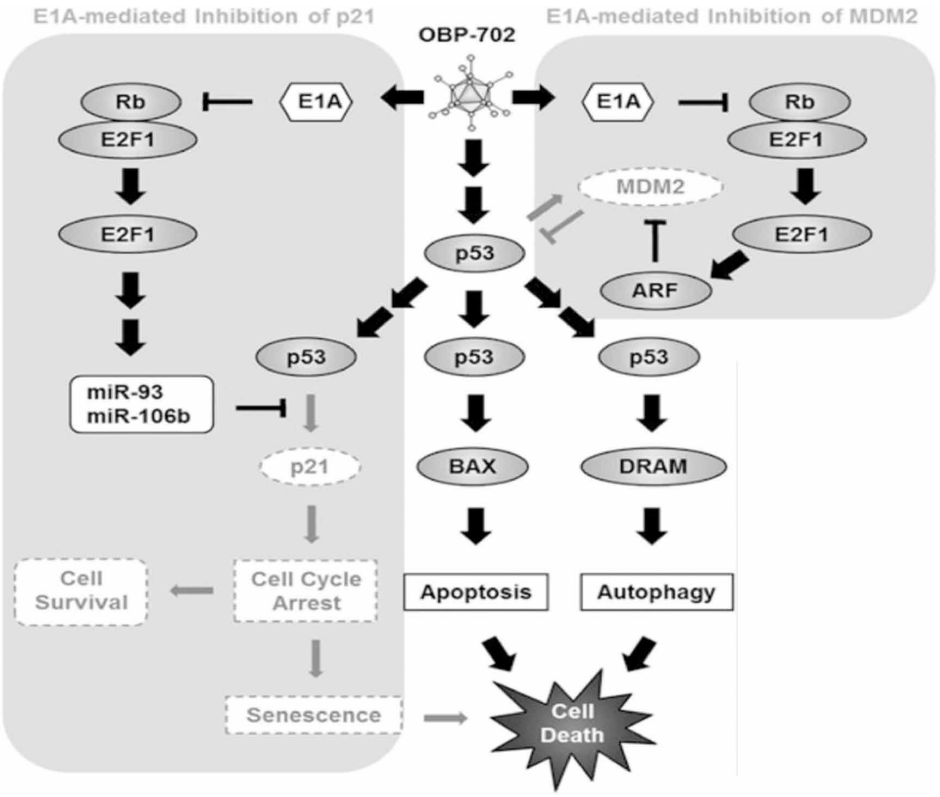
downstream activation inhibits p53 function via ubiquitin-mediated degradation of p53. Treatment with the small molecule drug, Nutlin-3 or tumour suppressor fragile histidine triad (FHIT) gene improves the expression of Ad-p53-mediated p53 and the death of apoptotic cells by suppressing MDM2 in human cancer cells. In addition, the overexpression of the ARF gene by Ad-ARF or Ad-E2F1 infections improves the expression of p53 and the antitumor effect caused by Ad-p53 via the suppression of ARF-mediated MDM2.

A third approach is to suppress the expression of p21, since activation of p53-downstream p21 causes cell cycle arrest and subsequent cell survival. Deletion of p21 expression by genetic deletion or an exogenous p21-targetedvsiRNA improves apoptosis induced by Ad-p53. In addition, p21-targeting miRNAs, miR-93 and miR-106b that enhance ad-p53-mediated apoptosis and autophagy because p21 acts as a suppressor of apoptosis and autophagy Thus, these three strategies for enhancing the Ad-p53-mediated cell death pathway are useful for enhancing the therapeutic potential of Ad-p53-based p53 gene replacement therapy.

Replication-Competent Adenovirus Vector

While in several clinical trials a replication-deficient Ad-p53 vector has been shown to be healthy, feasible, and well tolerated in patients with different types of cancers, it may be difficult to induce high exogenous p53 expression in all tumour cells through treatment with Ad-p53 because it is a virus deficient in replication. The low transduction rate of p53 gene transfer through this replication-deficient

Figure 9. A scheme for Ad-p53-mediated induction of cell death pathways and enhancement of Ad-p53-based p53 replacement therapy



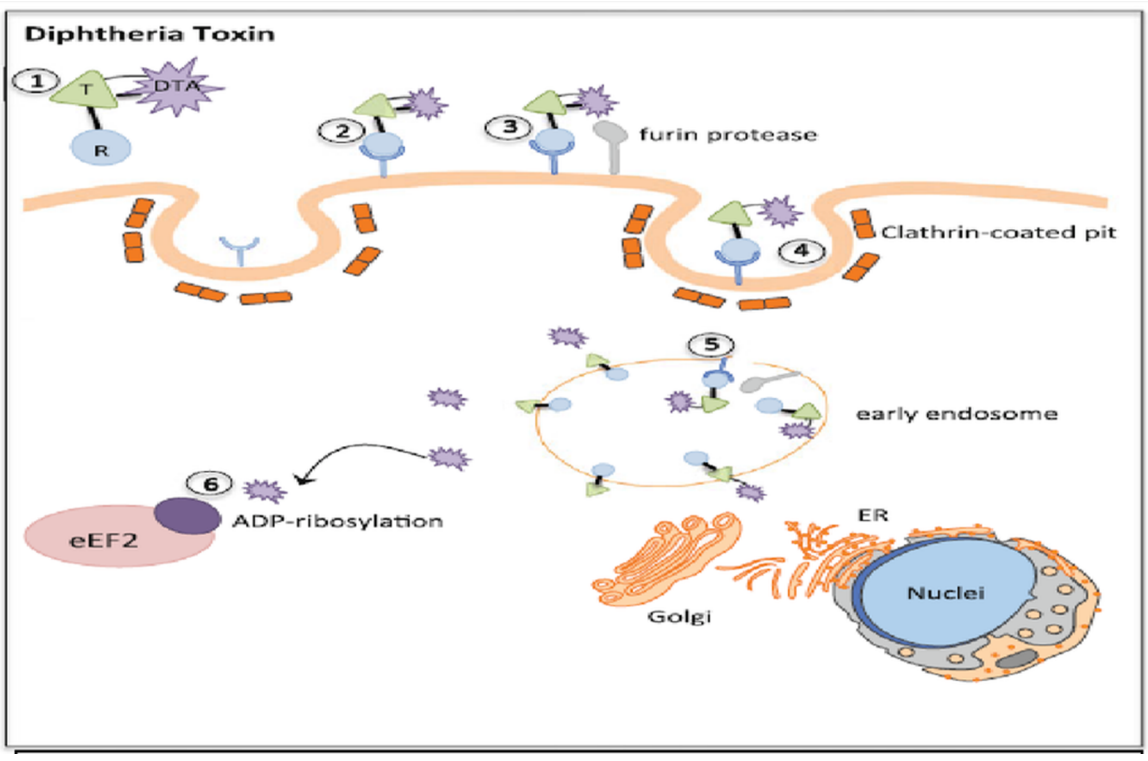
Ad-p53 vector is therefore a major problem for improving clinical outcome in advanced cancer patients. Tumour-specific, replication-competent oncolytic adenoviruses are being produced as novel vectors for anticancer gene therapies to enhance the transduction efficacy of p53 gene replacement therapy. For example, cancer-related gene promoters are utilized to regulate tumour-dependent virus replication. We have previously established a telomerase-specific oncolytic adenovirus OBP-301 replication-competent in which the human telomerase reverse transcriptase promoter (hTERT) drives the expression of two adenoviral genes, E1A and E1B, which are connected to an internal ribosome entry site. In telomerase-dependent, OBP-301 can induce tumour-specific lysis of the cells. Patients with advanced solid tumours in the USA were well-tolerated in a phase I clinical trial of OBP-301. When Ad-p53 was coupled with OBP-301, the expression p53 and the Ad-p53 mediated antitumor effect were enhanced (Fig. 3). On the basis of these facts, we produced an armed variant OBP-301 (OBP-702) which expresses the wild-type p53 gene under the control of the promoter Egr1. OBP-702 in the epithelial and mesenchymal types of malignant tumour cells suppressed the viability of both OBP-301-sensitive and OBP-301-resistant tumour cells more effectively than Ad-p53 or OBP-301. Ad-p53 and OBP-301 primarily induced apoptotic and autophagic cell death, while OBP-702 caused both apoptotic and autophagic cell death through exogenous p53 overexpression in tumour cells, respectively. These findings indicate that OBP-702 causes cell death by high p53 overexpression, both apoptotic and autophagic. With regard to the molecular mechanism by which OBP-702 is superior to Ad-p53 in the induction of cell death, we have recently found that the

potent OBP-702-induced antitumor effect involved E1A-dependent enhancement of the p53-mediated cell death signalling pathway (Fig. 10). When a similar dose of Ad-p53 or OBP-702 invaded tumour cells, OBP-702 induced a higher level of p53 expression than Ad-p53. This higher expression of p53 is due to OBP-702 viral replication, because Ad-p53 is a type of virus that is deficient in replication. However, in the OBP-702-infected tumour cells the expression rates of the p53-downstream targets p21 and MDM2 were lower than in the Ad-p53-infected tumour cells, despite their higher expression p53. This difference between the levels of p53 expression and those of the p53-downstream targets p21 and MDM2 was due to accumulation of adenoviral E1A.

The OBP-702 vector induces BAX- and DRAM-mediated apoptosis and autophagy, respectively, resulting in cell death; these effects are dependent on E1A-mediated suppression of p21 expression via E2F1-inducible miR-93 and miR-106b activation. Moreover, E1A-mediated suppression of MDM2, probably via E2F1-induced ARF activation, also enhances p53-mediated cell death.

Accumulation of E1A resulted in the upregulation of E2F1-inducible miR-93 and miR-106b, which suppressed p21 expression and increased p53-mediated apoptosis and autophagy (Hasei et al., 2013). By contrast, upregulation of E1A-mediated E2F1 results in the suppression of expression of MDM2 via ARF activation. These evidences indicate that OBP-702 induces an antitumor effect more efficiently than Ad-p53, through the enhancement of p53-mediated cell death signalling pathways based on E1A.

Figure 10. Scheme for OBP-702-mediated induction of cell death pathways



Protein Transduction Therapy

p53 replacement therapy with adenovirus vectors will more strongly induce ectopic expression of an exogenous p53 gene in various types of human cancers than is caused by a plasmid-based delivery system. As adenovirus can enter human cancer cells through direct interaction with virus particles and receptors for coxsackievirus-adenovirus receptors (CAR), CAR-expressing tumour cells are the main target cells for gene replacement therapy based on adenovirus p53. CAR-negative tumour cells may, however, escape eradication through adenovirus-based p53 replacement therapy.

Protein transduction therapy using membrane-permeable peptides may be useful for the direct introduction of exogenous p53 protein into tumour cells to target CAR-negative tumour cells. For example, 11 polyarginine peptides fused to the p53 protein have been shown to introduce the p53 protein into the cells, which subsequently induces p21 gene promoter activity similar to the induction that occurs with p53 gene replacement therapy based on Ad-p53.

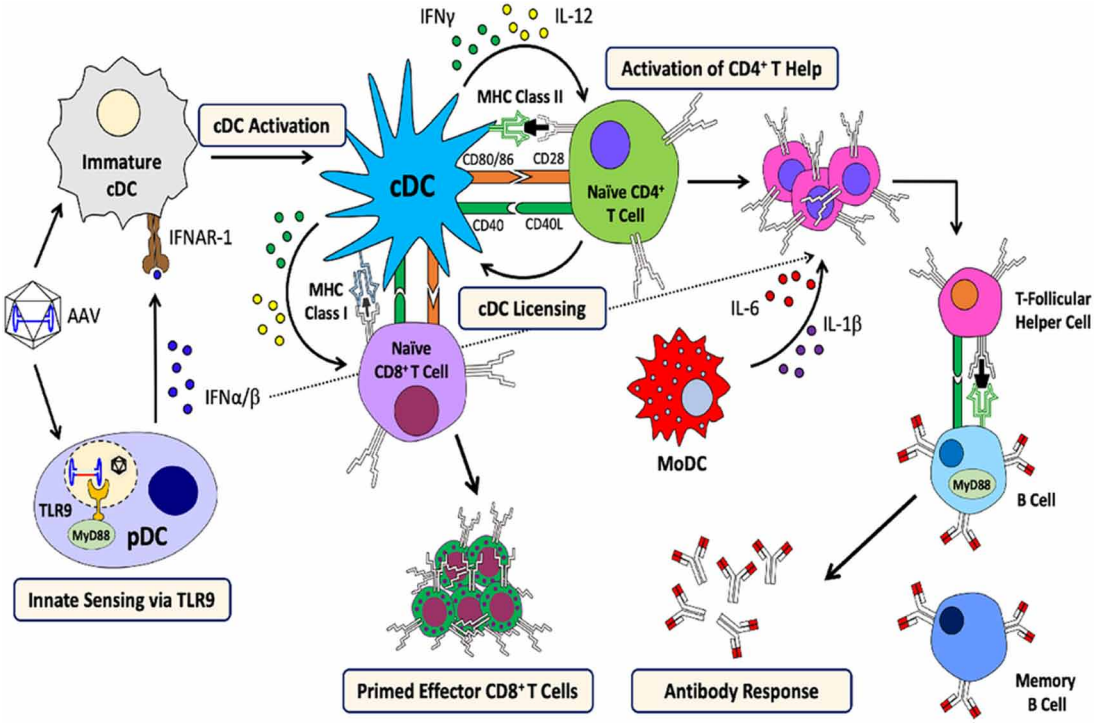
Genetically modified p53 proteins immune to MDM2-mediated ubiquitination are more effective at activating the transcription of target genes p53-downstream, resulting in a more potent antitumor effect compared to the wild-type p53 protein. In comparison, the introduction of wild-type p53 protein fused to three polyarginine peptides into pyrenebutyrate-cotreated cells was useful in inducing transcriptional activation of the target p53-downstream. In addition, the carboxy-terminal region of the p53 protein has been shown to effectively induce apoptosis and autophagy in human cancer cells by the use of this protein transduction system. These accumulating evidences suggest that the use of polyarginine peptides in this protein transduction therapy is a promising replacement therapy for CAR-negative tumour cells especially.

Bystander Effect of p53 Replacement Therapy

Replacement therapy p53 tends to cause cell death not only in tumour cells introduced by p53 but also in surrounding tumour cells by triggering the bystander effect. The bystander effect is a biological phenomenon in which untreated tumour cells display antitumor effects close to those of the tumour cells being treated. A radiation-induced bystander effect is caused in conventional antitumor therapy by activating the immune system, free radicals and inflammatory response. In replacement therapy with p53, it has been shown that Ad-p53 treatment induces a bystander effect on neighbouring tumour cells via multiple mechanisms in preclinical in vivo situations (Fig 11).

For example, Ad-p53 infection decreased the expression of angiogenic factors including vascular endothelial growth factor, and increased the expression of antiangiogenic factors, resulting in tumour tissue suppression of angiogenesis. Conversely, the activation of the immune response is also involved in the ad-p53-induced bystander effect (Fig. 5). Ad-p53 infection induced overexpression of the CD95 ligand, which triggered both apoptosis in infected tumour cells through the Fas receptor / ligand system and widespread invasion of neutrophils into tumour tissues that include infected and non-infected tumour cells. When bone marrow-derived dendritic cells (DCs) were used as carrier cells for Ad-p53 delivery, intratumoral injection with Ad-p53-integrated DCs induced an antitumor effect in subcutaneous xenograft tumour models in both DC-injected and non-injected tumour tissues (Murakami et al., 2004; Cotter, MJ et al., 2005; Crystal RG, 2014; Di Paolo et al., 2014). It has been shown that the natural killer cells are the immunological mediators of the ad-p53-induced bystander effect. Such accumulating evidence indicates that p53 replacement therapy represents a promising antitumor strategy for inducing high cell death through the tumour microenvironment's bystander-mediated impact modulation.

Figure 11. A scheme for Ad-p53-mediated induction of bystander effects within tumour tissue



When tumour cells are infected with the Ad-p53 vector, ectopic expression of p53 induces programmed cell death in the Ad-p53-infected tumour cells. In addition, surrounding uninfected tumour cells are also eradicated via induction of bystander effects, which include suppression of angiogenesis and activation of immune responses, in the tumour microenvironment.

CONCLUSION

p53 replacement therapy emerges as a successful antitumor strategy for fast activation in tumour cells of the p53-mediated cell death signalling pathways. Though a liposome-based delivery system is a useful method for in vitro studies, the efficiency of transduction in in vivo studies is still lower than that of a virus-based delivery system. Many clinical trials using replication-deficient Ad-p53 vectors have shown that administering an Ad-p53 vector via one of many strategies, including intratumoral, intraperitoneal, and intravesic injection, is a safe, feasible, and efficient antitumor strategy for patients with many types of cancer. However, while an Ad-p53 vector causes a bystander effect within tumour tissues, Ad-p53-mediated p53 activation may be insufficient to induce cell death throughout the tumour tissue, as this virus is a replication-deficient virus. Recently OBP-702 was developed to increase the low transduction efficiency of p53 replacement therapy based on adenovirus, a replication-competent oncolytic adenovirus that expresses p53. Conversely, a protein transduction therapy utilizing membrane permeable polyarginine peptides will also be a useful technique for integrating p53 into tumour cells that are immune to virus

delivery. Therefore, given the underlying molecular mechanisms of p53-mediated cell death signalling pathways that are triggered by different p53 transfer strategies, potential p53 replacement therapy should be built safer and more reliable.

GENETIC ENGINEERING TO EXPRESS THERAPEUTIC GENES

Oncolytic viruses are extremely versatile therapies, as they can be genetically engineered with elements that enhance their two primary action mechanisms: lytic function and antitumor immunity stimulation. Acting as a vector for the transmission of therapeutic genes, T-VEC secretes colony-stimulating factor (GM-CSF) granulocyte macrophage, which recruits and activates dendritic cells for optimal antigen presentation to T cells. Other approaches aimed at improving the immune response include cytokine delivery (IL-2, IL-12, TNF), expression of tumour-associated antigens, and incorporating costimulative signalling. To enhance tumour lysis, transgenes have been incorporated (1) to activate chemotherapy prodrugs, and (2) to express thymidine kinase (which converts administered ganciclovir into the toxic ganciclovir monophosphate).

GENE THERAPY IMPLEMENTATION

Once genetic materials are transferred to target cells and incorporated into the nuclear genetic DNA, they can induce the target cell genes to be silenced, down-regulated, modified or repaired. It may result in cell death and tumour necrosis (as with the suicide gene), or impaired cell growth with tumour regression (as with the silencing gene), depending on the severity of the gene expression.

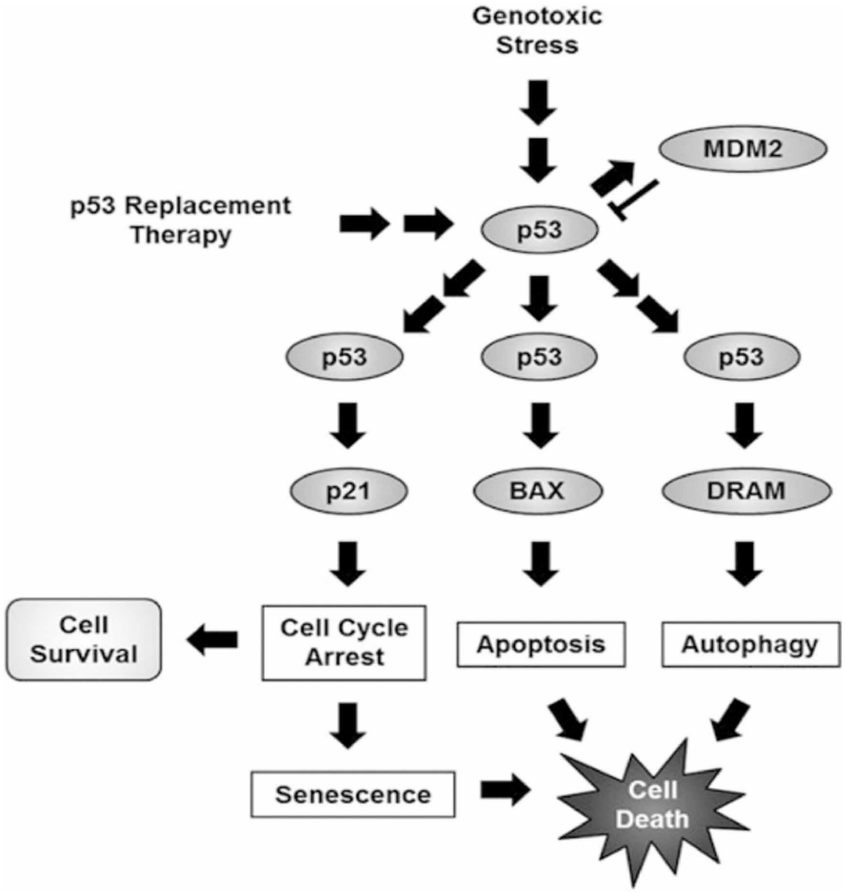
The gene modification may improve the response of subsequent cancer therapy, such as chemotherapy, immunotherapy, or radiation. Objective gene may help repair to prevent subsequent malignancy or cancer-related complications such as thrombosis. In the future, they might also be helpful in preventing hereditary cancer syndromes.

Suicide Gene

These are transgenes which constitute products that can cause a cell to kill itself by apoptosis. Such gene products are usually transcribed by different factors (promoters) leading to death and necrosis of the cells. One example of such promoters is the human gene H19 RNA which is highly expressed in most fatal organs but quickly cleared immediately after birth (Pachnis and Tilghman, 1988), This gene has an abnormal expression in different types of cancer cells and plays an important role in the proliferation of cancer cells, genetic instability, vascular angiogenesis, tumour metastases, multidrug resistance and cell survival despite hypoxia, with secondary tumour progression and spread (Matouk *et al*., 2013).

Blocking the function of the gene H19 results in marked regression of tumours, cell death and necrosis. Another important factor for cell immortalization and tumorigenesis is human telomerase reverse transcriptase (hTERT) (Ohana *et al.,* 2013). Its blockage with agents such as OBP-301 (Telomelysin) (Oncolys BioPharma) results in cell necrosis and regression of the tumours (Fig 12). Many strategies to cause tumour cell death include the use of small molecule drugs, monoclonal antibodies, and toxin gene therapy with agents such as the toxin-A chain Corynebacterium diphtheria (DTA-H19 therapy) (Li *et al.,* 2002).

Figure 12. Genetically-modified adenovirus acting as a suicide gene



The above mode of action represents an example of a modified virus acting as a suicide gene, namely OBP-301.

Suicide Gene Therapy Enzyme Prodrug Systems

The gene therapy approaches to chemotherapeutic suicide are known as gene-directed enzyme prodrug therapy. Suicide gene therapy methods are known as gene-directed enzyme prodrug therapy (GDEPT) or (GPAT), using deactivated medicines GDEPT uses a gene that encodes a foreign enzyme transmitted to the tumour and then administers a prodrug and activates a cytotoxic drug that has been released in the tumour (Marais *et al*., 1996). Three of the most promising suicide gene / prodrug combinations are (1) herpes simplex virus thymidine kinase (HSV1-TK) with ganciclovir (GCV), (2) cytosine deaminase (CD) with 5-fluorocytodine (5-FC), and (3) 5-(azaridin-1-yl)-2,4-dinitrobenzamide (CB1954) bacterial nitroreductase (NTR).

Enzyme prodrug systems destroy the targeted cancer cells by intervening in the processes of DNA replication or transcription (Harrington et al., 2000). In addition, the toxic substances created by the above-mentioned combinations can spread to neighbouring cancer cells and cause consequent cell death

(the bystander effect) (Karjoo and Hatefi, 2013). The two potential disadvantages of these enzyme prodrug systems are that there is a popular bystander effect and they appear to be less successful against non-actively dividing cancer cells.

The CPG2CMDA program is just another example of GDEPT. Kirn et al. developed a gene therapy for suicides based on the carboxypeptidase G2 (CPG2) bacterial enzyme. CPG2 has the advantage over the well-studied suicide genes HSV-TK and CD in that it activates both quiescent and proliferating cells that are able to kill prodrugs. CPG2 cleaves the prodrug CMDA such that its cytotoxic drug is directly released and has the advantage that no further enzymatic processing is required for drug activation.

As described earlier, the bystander effect, defined as the secondary effects on adjacent cells and tissues caused by treatment of a primary target with a therapeutic agent, is an essential aspect of toxins / suicide gene therapy. Such adverse effect can be seen in the plasmid and virotherapy of this prodrug, which can affect the immune system. Studies were thus conducted using a polymer / biomaterial that mimics a virus but is more stable as a delivery agent. It is important to note, however, that while these plasmid prodrugs have a major bystander effect, DT-A is unable to re-enter a neighbouring cell due to its properties as a toxin, has no bystander effect, and hence its role in gene therapy is considerable.

Targeting toxins and suicide genes in gene therapy

A suicide gene has, by definition, a product which causes a cell to kill itself via apoptosis. A number of promoters can transcribe this substance (e.g., constitutive promoters, and tissue-specific promoters), but tumour selectivity can be achieved with the use of tumour-specific promoters. The specificity of the promoter-targeted therapy can also be derived from conditions common to cancer such as hypoxia (Kim *et al.,* 2002). There have also been reports of inductive promoters affected, for example, by radiation, heat and drugs.

It is of great importance to find a promoter that specifically directs expression in cancer cells such as for H19. Another example is human telomerase reverse transcriptase (hTERT), the telomerase catalytic subunit — a crucial factor in the immortalization of cells and in tumorigenesis. The use of the hTERT promoter has given targeted preclinical therapeutic results in bladder and hepatocellular carcinoma cells, si already (Abdul-Ghani R et al., 2000).

Often the key safety benefit of unique promoter therapy is followed by the downside of poor action, which leads to a reduction in therapeutic efficacy. Enhancers can be added, and negative regulatory factors can be eliminated, to boost unique but ineffective promoters. For example, a nearly 20-fold enhancement in activity over the native PSA promoter and enhancer (PSE) was achieved by inserting four tandem copies of the synthetic androgen sensitive factor. In the case of H19, this promoter has the great advantage of being unique to cancer cells and also displays high promoter activity close to SV40. Targeted therapy also involves small-molecule drugs and monoclonal antibodies. Targeted treatment also contains medications for the small molecules and monoclonal antibodies. For example, monoclonal antibodies recognizing molecules CD20 (tositumomab and 131I-tositumomab (Bexxar), and ibritumomab tiuxetan (Zevalin)) and CD30 (brentuximab vedotin (Adcetris)) linked to toxic molecules have been approved by the United States Food and Drug Administration (FDA).

Toxin Gene Therapy: Toxins have the ability to efficiently destroy cells; thus, several toxins were investigated as possible anticancer agents (Ohana *et al.,* 2013). Targeted fusion toxins consist of a targeted protein such as a bacterial toxin-fused growth factor such as diphtheria toxin. Through the targeting

molecule, the fused toxin is directed to the tumour cells, guided into the cells via receptor endocytosis, and then released, resulting in tumour cell death.

Diphtheria Toxin A Chain: Diphtheria toxin (DT) is one of the most studied molecules, showing compelling activity as a therapeutic reagent of the gene for suicides. It effectively blocks the translational machinery of target cells by ADPribosylating the elongation factor-2 (EF-2). A single molecule of diphtheria toxin is estimated to destroy target cells, and several studies have successfully used its toxicity to remove target cancer cells (Rodriguez *et al*., 1998). Diphtheria toxin is secreted as a single polypeptide chain from *Corynebacterium diphtheriae* containing two main domains: DT-A, which carries the active site for ADP ribosylation of EF-2, and DT-B, which facilitates the binding of toxin to cells and the entry of the A chain into the cytosolic compartment (Li *et al.,* 2002). Though a very low level of DT-A expression is adequate for cell killing, in the absence of the DT-B chain, DT-A released from the lysed cells cannot reach the neighbouring cells.

The advantages of DT-A in gene therapy are defined in the literature and include (1) high potency, with one molecule capable of killing a cell; (2) cell cycle independence and p53 status; (3) localized toxic effect to transfected cells because the DT-B chain responsible for cell penetration is absent; (4) bypassing anti-DT immunity due to the endogenous production of DT-A protein via an expression cassette within tumour cells (to prevent neutralization by anti-DT antibodies that are ubiquitous in most people during systemic administration); and (5) the absence of cellular toxin resistance. All of these characteristics make the DT-A chain a very effective and therefore frequently used component of targeted cancer therapeutic approaches including immunotoxins (DT-A protein conjugates combined with either an antibody or a cytokine to specifically target cancer cell delivery) and gene therapy studies.

Other Toxins: To kill target cells, plant-derived ricin and pseudomonas exotoxin use a mechanism close to that of diphtheria toxin, and they have been tested as successful anticancer reagents. As a preferential approach to suicide genes for pancreatic cancer, the use of the suicide gene *Escherichia coli* purine nucleoside phosphorylase (ePNP) under either CEA or MUC1 promoter sequences showed preferential killing of the pancreatic tumour cells CEA- and MUC1-producing.

Rodriguez et al. revealed the relative potencies of the following eight recombinant cell toxins for irreparable death from prostate cancer cells in a comparative survival study: *Pseudomonas* exotoxin A, ricin, tumour necrosis factor-α (TNF-α), diphtheria toxin (DT), *Crotalus durissus terrificus* toxin, *Crotalus adamenteus* toxin, *Naja naja* toxin and *Naja mocambique.* Dose-dependent cytotoxic activity was only identified as highly potent for ricin and DT against all of the human prostate cancer cell lines tested. TNF-α had modest cytostatic activity on the screen; however, the combination of TNF-α and DT led to marked acceleration of the time for death of prostate cancer cells (Wu L et al., 2001). In the treatment of ovarian cancer, our group demonstrated the cytotoxic activity of TNF-α cytokine, along with the diphtheria toxin. Intratumoral injection of the toxin vector into tumours formed ectopically caused tumour growth to be inhibited by 40 per cent.

Gene Silencing

This was accomplished by precise delivery of a small interfering double-stranded RNA (siRNA) into target cells, and subsequent duplex formation of the RNA-induced silencing complex (RISC) that destroys messenger-RNA (mRNA), resulting in interference with RNA functions and protein synthesis within the target cells (Fung and Gersson, 2013). Through the correct nature of siRNA, it is theoretically possible to use the technology to silence any gene in the body, offering greater therapeutic potential in cancer

therapy as well as in the treatment of other medical conditions such as the hepatitis B virus, human papilloma virus, hypercholesterolemia and liver cirrhosis (Gujrati and Lu, 2013). Since siRNA does not interfere with chromosomal DNA, it has a lower risk of causing alterations in the target cell gene and potential mutagenesis. It is highly selective against target genes, with low systemic toxicity and does not cause resistance to multidrug. In addition, these genes that induce effective silencing of many cancer-related genes, leading to regression of tumours, but do not abolish abnormal genes. SiRNA therapy can be administered directly to tumours; however, it is somewhat difficult for systemic administration, as a naked siRNA protein is responsible for host-mediated clearance via enzymatic degradation, renal filtration and host cell phagocytosis (Sato *et al.,* 2008). In order to protect them from enzymatic degradation, several delivery systems for siRNA have been developed to promote their effect on silencing-specific genes (). CALAA-01 (Calando Pharmaceuticals) for patients with malignant melanoma and ALN-VSPOI (Alnylam Pharmaceuticals) for liver cancer and solid tumours are examples of the systemic delivery method currently in clinical trials (). Limited success, however, was achieved mainly due to relatively high toxicity and low efficiency of transfections.

Gene Modification

This may be useful in improving the results of cancer therapy, for example with radiation therapy. Radiosensitizing gene therapy promotes the expression of transgenes in tumour tissue, increasing tumour sensitivity to radiation with better control of the tumour (Geng *et al.,* 2014; Pan *et al.,* 2009). In comparison, radioprotective gene therapy distributes transgenes and their components to the natural tissue surrounding, thus reducing radiation-induced exposure of normal tissue (Lai *et al.,* 2009). Many preclinical studies are currently exploring the idea of integrating the two methods (Greenberger, 2009).

Gene Repair

The zinc finger nuclease attached to the lentiviral vector can be used to accomplish that. If the viral vector reaches the nucleus, to establish a newly edited double-stranded DNA, it binds to a specific position in the double-stranded DNA, splitting it at a specific location, with subsequent endogenous repair mechanisms (Baranyi *et al.,* 2013). Many technical methods include transcription-like effector nucleases (TALENs) and frequently clustered short palindromic repeats (CRISPR) interspaced (Cai, Bak and Mikkelsen, 2014; Uhde-Stone *et al.,* 2014; Sakuma and Aoltjen, 2014).

Gene Therapy for Mitochondria

Gene therapy can also target cytoplasmic organelles including mitochondria. The mitochondria in mammals are responsible for the metabolic functions. Nearly 300 of the known mutations that cause metabolic diseases are secondary to mitochondrial genome-influencing disorders. Several methods were employed to successfully transfer genes into cell mitochondria (Belikova *et al.,* 2007; epperly *et al.,* 2003; Kanai *et al.,* 2004; Kagan *et al.,* 2006).

Immunomodulation

It has become clear that the immune system is a key factor in the reversal or development of cancer. Immune responses are of two types: humoral immunity and cellular immunity. The tumour microenvironment also plays a significant role in the effects of the host immune system on cancer cells.

Humoral immunity is mediated by B-cell-released antibodies with a highly binding affinity to specific tumour antigens. Many antibodies against malignant cells were approved by the United States Food and Drug Administration (FDA), including trastuzumab for breast cancer (Cobleigh *et al.,* 1999), rituximab for indolent lymphoma (McLaughin *et al.,* 1998), cetuximab for lung cancer (Foon *et al.,* 2004), and bevacizumab for multiple solid tumours and several more (Hurwitz *et al.,* 2004).

Cellular immunity is mediated by cell-to-cell communication, which results in the identification of antigen and the destruction of a target cell. These are recognized by the host immune system based on the presence of tumour-associated antigens (TAAs) on the surface of tumour cells (Glassman and Balthasar, 2014). Dendritic cells are specialized in the identification of antigens and the initiation of immune responses to infectious agents or malignant cells by direct stimulation or inhibition of immune-effector cells such as T-cells, B-cells and natural killer cells (NK). Dendritic cells are produced from the bone marrow, migrating to lymph nodes and distant tissue in search of certain foreign antigens (Boon and Bruggen, 1996).

Cancer cells can escape the immune system by secreting immunosuppressive cytokines capable of down-regulating large molecules of histocompatibility, recruiting regulatory T-cells, and killing reactive cytotoxic cells. The tumour microenvironment is thus highly immunosuppressive, enabling a tumour to grow and metastasise (Steinman and Banchereau, 2007; DeSouza and Bonorino, 2009). There were numerous attempts to control the tumour microenvironment to cause regression of tumours. In cancer therapy immunomodulatory strategies Immunotherapy in cancer can be categorized into four main groups. Effective immunotherapy involves approaches that specifically sensitize tumour-specific antigens to the host immune system, exemplified by cancer vaccines.

Passive immunotherapy makes use of humanized or chimeric antibodies to precisely target tumour antigens without overt immune system activation. Adoptive immunotherapy uses immune cells of patients, whether T-cells or dendritic cells, stimulated or manipulated ex vivo, then infused backwards, to better respond to tumour antigens. Immune stimulation therapy aims at increasing co-stimulatory molecules or blocking molecules that inhibit them. Immune-based therapy may involve one or more of the above methods, either as a separate treatment for immunotherapy or in conjunction with other cancer therapy modalities.

ALTERNATIVES: OTHER GENE THERAPY APPROACHES IN CANCER MANAGEMENT

Multimodality treatment also provides greater outcomes compared to monotherapy as in other types of cancer therapy. This is similarly true for gene therapy, and is evident when gene therapy is administered following radical surgery or successful chemotherapy following maximum reduction in tumour load. Once paired with chemotherapy, gene therapy has a synergistic effect, with higher tumour responses and lower toxicity linked to treatment.

Gene Directed Enzyme Prodrug Therapy (GDEPT)

It is a new cancer treatment strategy which aims to reduce the side effects of chemotherapy. With such an approach, a gene that expresses a non-toxic enzyme into cancer cells is first delivered to the cells, followed by the systemic administration of a pro-drug that can be converted by the enzyme into a toxic compound, resulting in selective tumour cell death, with less adverse effects on normal tissues (Sakuma and Woltjen, 2014). Diffusion of toxic metabolites from cell to cell can damage nearby and adjacent tumour cells (bystander effect) (Karjoo and Hatefi, 2013). The release in the circulation of tumour cell necrotic material can activate the immune system in response to tumour antigen, with subsequent regression of distant tumour cells, such as metastatic nodules (distant bystander effect) (Nicholas *et al*., 2003). Examples include the use of a retroviral vector such as gene therapy for suicide and herpes simplex virus carrying the thymidine kinase enzyme to the tumour cell interior. The enzyme has a 1000-fold greater efficiency to selectively phosphorylate the pro-drug ganciclovir obtained from acyclovir (Agard *et al.,* 2001). When ganciclovir is given systemically, the drug is metabolized in tumour cells leading to cell death. Because the effectiveness of such a method is only around 10 per cent of tumour cells, the degree of tumour regression is largely mediated by the effects of bystanders (Agard *et al.,* 2001). Several research trials have tested out the program. Replacing ganciclovir with penciclovir, modified to generate radiolabelled analog, will also enable closer follow-up of the results of therapy, using high-quality positron emission tomography imaging studies (Edelstein *et al*., 2004).

Cancer Drug-resistance Gene Transfer

Several studies have used a gene transfer strategy aimed at improving the effectiveness of chemotherapy and radiation on cancer cells, while protecting normal tissue against toxicity caused by therapy. Such gene transfer can also be used to guard against HIV by rendering normal cells immune to viral invasion, or by correcting genetic defects such as sickle cell anaemia or metabolic disoders. However, introducing a new gene into the genome of a host stem cell for an individual's existence can cause other oncogenes to develop malignant disorders, and may alter other neighbouring genes, thus producing other medical conditions. Hence, gene therapy is a dangerous strategy. Recently, few clinical trials have been carried out in this regard. One example is the multidrug-resistant protein-1, encoded as MDR1 gene by the human gene ABCBI (Lin and Gerson, 2008). It activates the cell pump to expel cytotoxic drugs from the cytoplasm of cells to the outside, thereby shielding normal cells from side effects of chemotherapy, such as vinca alkaloids, taxanes, epipodophyllotoxins and anthracyclines (Maier *et al.,* 2008). The gene MDR1 is minimally expressed in malignant cells; thus, chemotherapeutic drugs entering the cytoplasm should remain at a higher concentration, leading to death of the cells (Maier *et al.,*2010). Other genes that are resistant to drugs include methyl guanine methyltransferase (MGMT) for alkylating chemotherapy, and cisplatin, doxorubicin and cyclophosphamide glutathione transferase (GSTP1) (Maier *et al.,* 2008; Biaglow JE et al., 2003).

Theragnostic Approach

Gene therapy can also be paired with other diagnostic interventions in an integrated diagnostic and therapeutic program (theragnostic), to help identify, treat and control the response to therapy, for example, a small interfering double-stranded RNA (siRNA) delivery system can be labelled using magnetic

resonance imaging (MRI) with imaging agents such as dextran-coated superparamagnetic nanoparticles for simultaneous non-invasive imaging of the delivery to tumours (Oguri *et al.,* 2000). Also, the siRNA delivery system can be branded with other imaging agents to track therapy closely, and can even predict the therapy outcome long before any anatomical changes (Oguri *et al.,* 2000). Such molecular diagnostic approaches have evolved relatively quickly in the last few years, and in the near future sometimes can become an important avenue for cancer diagnosis.

Nanomedicine

In this context, because of its interesting physicochemical properties (small size and high surface area) at nanoscale, nanomedicine plays an important role in overcoming the existing limitations of present antiangiogenic therapy recently, several researchers as well as our community have exhibited multiple, multifunctional nanomedicine applications in various diseases, including cancer, diabetes, neurodegenerative disease, cardiovascular disease, antibacterial, spinal cord injury, etc (Oguri *et al.,* 2000). It is possible to use nanoparticles combined with different targeting ligands to actively target antiangiogenic drugs for better therapeutic efficacy (Mukherjee and Patra, 2016; Mukherjee, 2018, Mukherjee *et al.,* 2014, Mukherjee and Mukherjee, 2019). In addition, numerous studies showed the non- and proangiogenic properties of many inorganic nanoparticles (NPs), including silver NPs (AgNPs), gold NPs (AuNPs), copper nanoparticles (CuNPs), carbon nanotubes (CNT), european hydroxide nanorods (EHNs), graphene oxides (GOs), zinc oxide nanoflowers, and cerium oxide nanoparticles (NCe) (Nethi *et al.,* 2019; Meka *et al.,* 2019). In addition, numerous other nanomaterials such as liposomes, lipid NPs, protein NPs, polymer NPs, viral and bio-inspired NPs are used for targeted tumour suppression of antiangiogenic agents (Muthuraj *et al*., 2016, Gaddam *et al.,* 2017; Mukherjee *et al.,* 2005; Barui *et al.,* 2012; Afsharzadeh *et al.,* 2019). Antiangiogenic successful targeting also helps to reduce harmful side effects and toxicity.

FUTURE PROSPECTIVES OF GENE THERAPY

Gene therapy has had its ups and downs. Indeed, this one is the most unpredictable, more than any other modern technology in biology. It had great success with the case DeSilva. Even worse, in 2002, a French trial to cure SCIDs, headed by Dr. Alain Fischer and initially considered to be the most promising ever, two of the participants developed vector-induced leukaemia and experienced a setback. The Fischer trial's trouble does not mark the end of gene therapy, but it has allowed scientists to identify the problems that need to be overcome to improve the efficacy of this procedure: Safer vehicles have to be identified or planned, techniques have to be developed to mitigate vector immune rejection and more focus has to be put on risk assessment. The last of these three problem areas would be fairly straightforward to tackle, but the first two are highly complex biological challenges which are likely to take several years to overcome.

Safer Vehicles

A safe vehicle is one that only reaches the target cells and positions itself inside the genome in a secure location — that is, far away from any genes. Retroviruses, for most gene therapy trials, are the vector of choice but none of them are capable of this specificity. Fischer trial failure which used a modified

retrovirus (MuLV) occurred because the vector inserted itself into or near a stretch of DNA in a gene called LMO2 known to be involved in cancer induction, particularly leukaemia.

Scientists have calculated that one in 100,000 cells may have inserted the MuLV vector, used in the Fischer trial, into the LMO2 gene. Each patient received around 1 million genetically modified cells in the study, and it is possible that at least one cell containing a vector-mutated LMO2 gene was obtained in some of the patients. This calculation however is based on the assumption that the addition of vectors is random, which may not always be the case. Some viruses can be introduced into favoured locations within the genome non-randomly. In this way, a leukaemia virus like MuLV can usually insert itself into or near cancer-causing genes, in which case the number of cells damaged in the Fischer trial could have been much higher than one in 100,000. So, a virus like MuLV may never be a safe vehicle. Non-random insertion presents a significant challenge to gene therapy based on viruses, but it also provides a way to enhance the protection of all insertion vectors, even if they are non-random, a vector may be constructed that will only be inserted into different genome areas.

Sequence-Specific Vector Insertion

Insertion of viral DNA into the chromosome is regulated by an integrase called a viral protein. During a retrovirus' life cycle, conversion of the RNA genome to DNA is accompanied by translocation of the viral DNA to the nucleus of the cell, where it is transcribed into messenger RNAs (mRNAs). The viral mRNAs pass out into the cytoplasm where they turn into protein. Integrase belongs to this group of viral proteins; because it bears a signal of nuclear localization, cellular enzymes escort it back into the nucleus, where it catalyses the incorporation of viral DNA into host chromosomes. The specifics of this event include attaching the integrase to the viral DNA, cutting the host DNA via integrase, and eventually inserting the viral DNA into the chromosome of the cell. So, it is integrase that determines where to inject the viral DNA. It is this protein that will make sequence-specific insertion possible, and others like it.

The year 2003 saw the completion of the human genome project, an undertaking that gave the world the full nucleotide sequence of the human genome. Scientists would be able to trace the precise position of all human genes and non-coding regions with this knowledge at hand (Venter JC et al., 2001). Careful analysis of this data will allow a family of integrase molecules to be built which will position a vector in a genome non-coding region. Indeed, it could be possible to map entire genetic communities that could be designated as secure insertion sites that could be used by all gene therapies without fear of cellular genes being impaired. This method would give gene therapy the sort of logical foundation it lacks today.

Improved Targeting of Cells and Organs

The very first is still the most successful gene therapy trial to date: the DeSilva trial. This trial's success is primarily attributed to the fact that the target cells were lymphocytes, cells that can be easily separated and removed from the blood, modified with the corrected gene, and then returned to the circulatory system of the patient. There is no targeting issue here and although certain things can still go wrong, as shown by the Fischer trial, the gene therapists know that the vector carrying the therapeutic gene is confined to a single type of cell.

This method is important to combat infectious diseases, but it does not understand the difference between a vector for gene therapy and an influenza virus. Gene therapy must therefore be planned to reduce the number of types of cells that will get infected with the vector. Improved targeting needs

matching to cell-surface receptors either viral capsid proteins, such as the retroviral gp 120, or adenovirus fibre proteins. It will be extremely difficult to achieve such a matching scheme, since detailed sequence information is needed for both the viral proteins and the cell surface receptors. Any of this knowledge is currently available for the vectors, and with the human sequence data now available, accurate targeting for gene therapy trials is likely to be possible over the next five to ten years.

Reducing Immune Rejection of the Vector

Gene therapy tests to target solid organ cells require the vehicle to be injected into general circulation. This is equivalent to tossing a sheep into a den of wolves with great hunger. Sentinels of the immune system launch an immediate assault on the invader and its pursuit is relentless. Most of the vector particles will never hit the target cells, and those that do will end up being killed along with any cells that they have reached by natural killer cells. Therefore, it is not shocking that the gene therapy's efficacy is exceedingly low, often too low to be of clinical benefit. Gene therapists therefore face problems similar to those that occur from organ transplants. They have to communicate with the immune system of the patient, or there is no chance of success. There are only two ways to deal with the immune system for gene therapists as for transplant surgeons: send the patient immunosuppressants to deactivate the system, or disguise the vector in some way to make it invisible to the lymphocytes and other members of the immune system, or at least appropriate to them.

Immunosuppressants

Cyclosporine and tacrolimus are two drugs widely used for organ transplants. Both of these compounds are isolated from the fungus and exert their influence by blocking the adaptive immune response, thus inactivating monocyte recruitment of T lymphocytes and natural killer cells. Either or both of these medications may be administered to a patient immediately prior to administering the vector and then slowly removed once it has reached the target cells. To date, however, immunosuppressants have not been used, but are being tested for use in gene therapy trials for the in future.

Improved Risk Assessment

There are now legal provisions that compel witnesses that record chemical reactions. The problem is to determine the parameters to be checked and the cut-off point which distinguishes a beneficial response from a toxic reaction. Important elements of good risk assessment include: initial vector titer and subsequent concentration of vectors in the blood of the patients, vector insertion and proliferation, and immune system response status. Obvious errors like this occur more frequently than scientists like to admit, but can be reduced by a three-tiered evaluation protocol (that is, three independent determinations of the titer).

As has already been pointed out, potential vectors are planned to be inserted within the genome at different locations. Yet you can't trust a vector configured for better targeting to do what it is programmed to do. Tests must be integrated into a standard gene therapy procedure that specifies the vector's specific insertion site, and if it is not where it should be, steps must be taken to resolve the potential side effects. Such tests pertain to any evidence indicating that the virus replicates. Most of the viruses used for gene therapy are altered such that they cannot reproduce, although it is still probable that the vector

will encounter a wild virus that already infects the patient, and that the two will genetically recombine to create a replica-competent vector.

Work to enhance targeting of cells and organs is also under way. Clinical trials will include testing to validate these vectors' targeting, and if vectors penetrate a number of non-target cells, these details should be taken as a possible toxic reaction. The final field of risk evaluation, which includes the reaction of the immune system, is the simplest to determine, but also offers the most valuable details. A growing number of white blood cells after administration, combined with a high vector titer, is a deadly mixture and must be the subject of every effort to improve safety of gene therapy.

PROBLEMS WITH GENE THERAPY

Transient fever and flu-like symptoms are among the most frequent side effects following gene therapy. Following intravenous administration, a grade 3 hypersensitivity reaction is usually transient and managed with the usual support measures. Leukocytopenia, and lymphopenia in particular, may represent a cellular redistribution of white blood cells to target tissues such as tumours. It also documented mild transient anaemia. However, there has been considerable concern about the toxicity, mutagenicity and immunogenicity associated with viral vector therapy.

Retroviral therapy (such as lentiviruses) mediated gene therapy leads to viral penetration into the host genome, triggering mutagenic events with a second malignancy. This was reported in earlier studies of the murine leukaemia retrovirus vector in the treatment of patients with severe combined immunodeficiency and leukaemia developed in five out of 30 cases, although no second malignancy has been reported in gene therapy for cancer so far (Min *et al.,* 2019). Such mutagenicity depends on the location where the virus is inserted. For this reason, the FDA has required reporting and analysis of viral vector insertion sites in all clinical trials involving genomic integrated viral vectors. Initial technique was linear amplification mediated polymerase chain reaction, but recently methods of high-throughput DNA sequencing were used (Howe *et al.,* 2008). Clinical trials which initially or subsequently demonstrate evidence of increased mutagenicity are usually discontinued. Information obtained from such studies is of great importance for the design of new and far safer therapeutic approaches.

Another important issue with cancer gene therapy is resistance to treatment with subsequent tumour recurrences and shorter survival. A potential mechanism is intrinsic to, and possibly acquired, tumour cell resistance to therapy-induced cell death (apoptosis) by dysregulation and release of apoptosis protein or Bcl-2 protein anti-apoptotic inhibitor. Some pharmaceutical companies have recently produced a range of drugs such as Novartis-LBH589, cIAP1, and cIAP2 that inhibit the Bcl-2 protein, thereby promoting cell death (apoptosis) and tumour regression, preventing or delaying tumour resistance, and prolonging post-gene therapy remission (Schmidt *et al*., 2002; Bartholomae *et al.,* 2012). Currently these drugs are in clinical trials.

ETHICAL ISSUES IN CANCER GENE THERAPY

Here, we discuss key ethical issues related to conducting research on translational gene transfer of anticancer drugs. Our emphasis is on ethics and not on rehearsing a litany of current regulatory research

standards. Although the two share common similarities and overlap in content, there is no assurance that ethical concerns will be addressed adequately.

Present research regulations evolved out of a collection of ethical principles outlined in seminal papers that together form our international research ethics heritage. Although they vary in content, form and intended audiences, various statements of ethical consensus (notably the Nuremberg Code, the Helsinki Declaration, and the Belmont Report) implicitly or explicitly set forth four important values underlying the ethical conduct of research: scientific integrity, charity, respect for persons, and justice. Without scientific integrity (understood here in the classical sense as "completeness" or "fullness" derived from the Latin root integritas of the term), there would be no ethical justification for conducting research especially research that would waste resources and impose unnecessary risks because of its lack of scientific rigour. Scientific integrity is also an important ethical value, because it relates to the duty of a scientist to be a spokesman for truth. In other words, a scientist must not only seek the truth using sound, reproducible methods but also truthfully communicate its results to the public, funders, publishers, and the larger scientific community. Scientific integrity is an ideal in this sense, not only for conducting research, but also for the character of the scientist herself — a type of personal wholeness between her scientific commitments and actions. Profit is the social obligation to contribute to the welfare of others. Researchers' benefit may be directed towards society as a whole or towards the individuals involved in research. When charity is aimed at individual subjects of human studies, it obliges researchers and science regulators to seek to reduce potential harms and optimize potential benefits for study participants. As described in the Belmont Study, "In the case of particular projects, researchers and members of their institutions are obliged to give preliminary consideration to maximizing the benefits and the risk that may emerge from the research investigation."

Perhaps the most well-known of all the moral standards associated with research is "respect for people." This requirement is typically understood to entail, on the one hand, a requirement for informed consent before undertaking research on human subjects with decision-making capacity, and, on the other hand, a limitation on permissible non-therapeutic risk for human subjects lacking decision-making capacity. The Nuremberg Code begins with the statement that "the voluntary consent of the human subject is absolutely essential." The Helsinki Declaration states that "subjects must be volunteers and informed participants in the research project." Respecting individuals in this way is honouring their right to make decisions about their own lives and bodies. Normally this right is called the right to personal autonomy or the right to self-determination. Justice is another ethical value which bears on science behaviour. The definition of justice, in the most abstract sense, demands impartially that persons obtain what is due them. Distributive justice is the principle of justice that concerns contemporary research ethics, involving the fair distribution of burdens and benefits among individuals or groups. Distributive justice in research involves an equal distribution of burdens and rewards, both during the course of research among human subjects and subsequently when research findings are accessible to wider segments of society. The Belmont Report, for example, states that human subjects must be selected fairly to volunteer for research, not systematically selected from targeted populations because they are easy to manipulate. Neither should subjects be chosen from groups which are unlikely to benefit from later research applications. Similarly, the Helsinki Declaration notes that "scientific work is justified only if there is a fair possibility that the communities in which the study is performed will benefit from the research findings."

Historically, the broad ethical values previously described provided the basis for U.S. institutional ethical research standards — both the U.S. Federal Policy for the Protection of Human Subjects (45 CFR 46), issued by the Department of Health and Human Services (DHHS) and overseen by the Of-

fice of Human Research Protections, and the U.S. Equivalent to Food and Drug Administration (FDA), Such federal laws establish the American national standard for work on human subjects. Both DHHS and FDA policies require that an Institutional Review Board (IRB) review research on human subjects. IRBs are tasked with upholding the values of respect for individuals, charity and justice in the Belmont Report, each by evaluating aspects of a proposed study related to informed consent, risk / benefit evaluation and equal selection of subjects. As these two brief examples indicate, abstract ethical principles provide the benchmarks required to legitimize ground level regulatory research requirements. Without the navigational star of having a collection of general ethical principles at first, there is no way for us to answer the question, "Why use this realistic standard for controlling research and not some other? "Only by appealing to the ability of each to realize a presupposed set of philosophical moral values can we justifiably choose one regulatory requirement over another. However, this is much easier said than done, as there are no standardized ways to decide how best to fulfil all of these abstract ethical principles within a collection of regulatory criteria, nor how best to reconcile these principles when they come into conflict with one another. At the regulatory level, the ethical values of scientific integrity, beneficence, respect for individuals and justice are open to a fairly wide range of interpretation, and reasonable people may disagree with what each of these values requires in concrete situations. Perhaps these above points can best be demonstrated if we turn our attention to ethical concerns arising from each specific stage of the clinical translation process:

(1) When to start clinical trials,
(2) Design of clinical trials, and
(3) Human research subjects being enrolled.

OVERVIEW OF GENE EDITING

Gene editing or genome editing with engineered nucleases is a form of genetic engineering in which DNA is inserted, removed or substituted into an organism's genome using engineered nucleases, or "molecular scissors," which create site-specific double-strand breaks (DSBs) at desired genome locations. The induced double-strand breaks are repaired by nonhomologous end-joining (NHEJ) or homologous recombination (HR) which results in targeted mutations ('edits'). We can broadly characterize these two edits specifically by their mechanism.

DNA Double Stranded Break (DSB) Repair Mechanisms

To understand these principles, you need to understand the mechanisms for restoring DNA double stranded break (DSB). The non-homologous end joining (NHEJ) and homology directed repair (HDR) are two of the known DSB repair pathways, which are essentially functional in all organisms. NHEJ uses a number of enzymes to attach a double-strand break directly to the DNA ends (Yeh et al., 2019). In comparison, in HDR, a homologous sequence is used at break point as a reference for the reconstruction of missing DNA sequence. These pathways' natural properties form the very basis of nuclease-based editing of genomes.

NHEJ is prone to error, and the repair site has been shown to cause mutations. Thus, if one is able to create a DSB in multiple samples at a desired gene, it is very likely that mutations will be generated at that

site in some of the treatments due to NHEJ infidelity-caused errors. On the other hand, HDR's reliance on a homologous sequence for restoring DSBs can be exploited by inserting a desired sequence into a sequence that is homologous to the flanking sequences of a DSB which, when used as a prototype by the HDR system, would lead to the formation of the desired change within the genomic region of concern.

Despite the distinct mechanisms, the concept of gene editing based on the HDR is similar to that of targeting homologous recombination-based genes. However, when DSBs are created, the rate of recombination is increased by at least three orders of magnitude and HDR is at work thus making the recombination based on HDR much more efficient and eliminating the need for stringent positive and negative selection steps. And if one is able to generate a DSB at a particular position within the genome based on these concepts, then the cell's own repair mechanisms can help to produce the required mutations (Yeh et al., 2019).

Site-Specific Double Stranded Breaks

Using restriction enzymes it is easy to create a DSB in DNA. However, many DSBs will be created if the genomic DNA is treated with a specific endonuclease restriction. This is due to the fact that a few base pairs on the DNA are known by most restriction enzymes as their target and very likely that a similar combination of base pairs would be present at several locations around the genome. Three distinct classes of nucleases have been discovered and bioengineered to date to solve this obstacle and build site-specific DSBs. Which are the nucleases of the zinc finger (ZFNs), transcription-activators such as effector nucleases (TALEN), and meganucleases (A. Nemudryi et al., 2014).

The concept behind ZFNs and TALEN technology is based on a non-specific DNA cutting enzyme, which can then be linked to specific DNA sequences that recognize peptides like zinc fingers and transcription activator-like effectors (TALEs). The secret to this was to find an endonuclease whose DNA recognition site and cleaving site were separate from each other, a situation unusual among enzymes that are restrictive. If this enzyme has been detected, it could isolate its cleaving component which would be rather non-specific because it would have no recognition capability. This section could then be connected to the sequence of peptide recognition, which could contribute to very high specificity.

There are basically four families of engineered nucleases being used:

1. Zinc finger nucleases (ZFNs)
2. Transcription Activator-Like Effector-based Nucleases (TALENs)
3. CRISPR-Cas system
4. Meganuleases

a. Zinc Finger Nucleases

Zinc-finger nucleases (ZFNs) are artificial restriction enzymes that are created by fusing a DNA-binding domain of the zinc finger to a DNA-cleavage. Zinc finger domains can be programmed to target different sequences of desired DNA and this helps zinc-finger nucleases to target unique sequences within complex genomes (Paschon, D. E. et al., 2019; Paschon, D. E. et al., 2019; Dunham, R. A. et al., 2018). These reagents may be used to precisely modify the genomes of higher species by using the endogenous DNA repair machinery. In addition to the Cas9 and TALEN proteins, ZFN is becoming a popular genome editing method.

A zinc finger nuclease is an endonuclease unique to the site designed to bind and cleave DNA at different positions (see Figure 15). There are two types of protein there. The first domain is the binding DNA domain that is made up of eukaryotic transcription factors and includes the zinc finger. The second domain is the nuclease domain, which consists of the enzyme FokI restriction and is responsible for DNA's catalytic cleavage.

DNA-binding domain: Typically, individual ZFNs' DNA-binding domains contain between three and six individual zinc finger repeats, and can recognize between 9 and 18 base pairs each. In principle, even a pair of 3-finger ZFNs that recognize a total of 18 base pairs can target a single locus in a mammalian genome, if the zinc finger domains are perfectly appropriate for their intended target location. The easiest method of producing new zinc-finger arrays is to combine smaller "packages" with known specificity with the zinc-finger. The most common modular assembly process involves combining three separate zinc fingers that can each recognize a 3 base pair DNA sequence to generate a 3-finger array that can recognize a 9 base pair target site.

DNA-cleavage domain: Usually, the non-specific cleavage domain from the endonuclease FokI restriction of type IIs is used as the cleavage domain inside ZFNs. In order to cleave DNA, this cleavage domain must dimerise and thus a pair of ZFNs are needed to target non-palindromic DNA sites. Normal ZFNs attach the cleavage domain of each zinc finger domain to the C-terminus. To allow the two cleavage domains to dimerize and cleave the DNA, the two individual ZFNs must connect a certain distance apart to opposite DNA strands with their C-termini.

a (i). Potential side effects of ZFN: Off-target cleavage may occur if the zinc finger domains are not precise enough for their target site or they do not target a unique site within the interest genome. Such off-target cleavage may result in the production of ample double-strand breaks to overpower the repair machinery, resulting in chromosome rearrangements and/or cell death. Off-target cleavage events can also facilitate random Donor DNA incorporation. As with other foreign proteins introduced into the human body, the therapeutic agent and the cells in which it is active are at risk for an immunological response. Since the protein will need to be expressed only transiently, however, the time over which a response may develop is short.

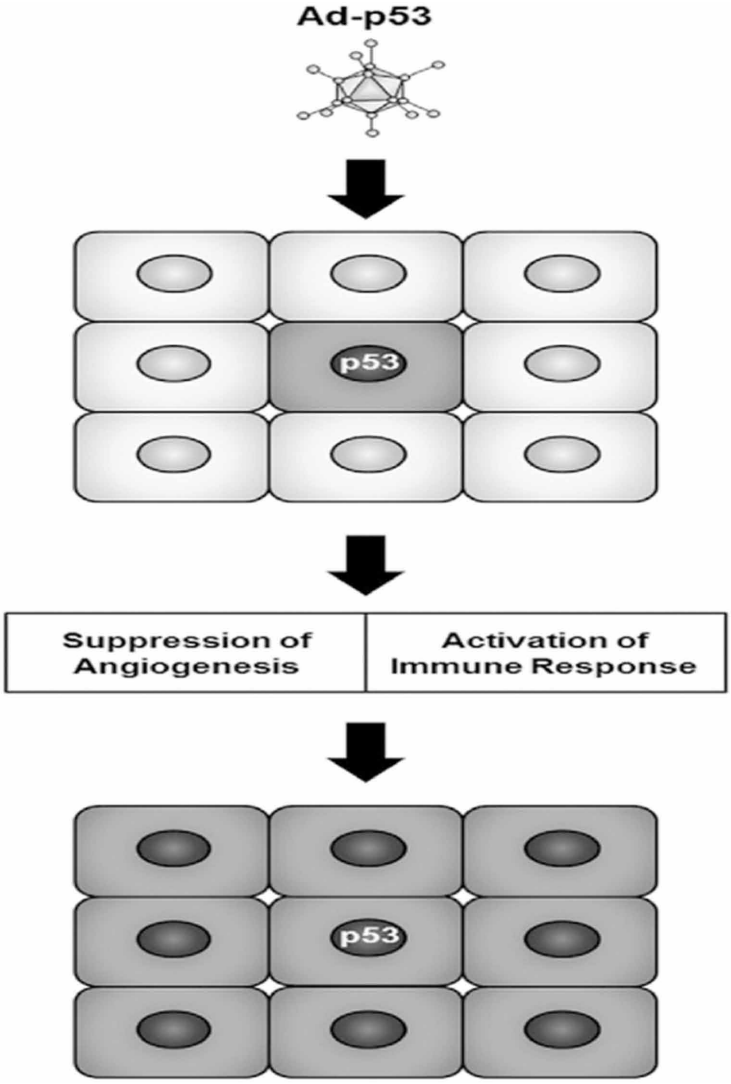
b. TALEN - Transcription activator-like effector nuclease

Transcription activator-like effector nuclease (TALEN®) technology leverages artificial restriction enzymes generated by fusing a TAL effector DNA-binding domain to a DNA cleavage domain (A. Nemudryi et al,. 2014).

Restriction enzymes are enzymes which cut strands of DNA at a particular sequence. Transcription activator-like effectors (TALEs) can be engineered quickly to bind virtually any desired sequence of DNA. By combining such an engineered TALE with a DNA cleavage domain (which cuts DNA strands), enzymes that precisely cut any desired DNA sequence can be engineered restricting (Nakano, C. et al., 2019; Zhao, X. et al., 2016; Jin, L. et al., 2018). When introducing these restriction enzymes into cells, they can be used for gene editing or for in situ genome editing, a technique known as genome editing with engineered nucleases. TALEN is becoming a leading method in the field of genome editing alongside the zinc finger nucleases and Cas9 proteins (A. Nemudryi et al,. 2014).

TAL effector DNA-binding domain: TAL effectors are proteins secreted by the bacteria Xanthomonas. The DNA binding domain comprises a strongly conserved, repeated sequence of 33–34 amino acids with divergent 12th and 13th amino acids. These two positions, known as the Repeat Variable Diresidue

Figure 13. Schematic Showing Step by Step Zinc-Finger Nuclease-Induced Genome Editing
Adapted from Dr. Sikandar Hayat Khan Molecular Therapy: Nucleic Acids Vol. 16 June 2019



(RVD), are highly variable and show a strong correlation with the recognition of specific nucleotides. This relationship between amino acid sequence and DNA recognition has allowed specific DNA-binding domains to be engineered by selecting a combination of repeat segments that contain the appropriate RVDs (Carroll, 2017).

DNA cleavage domain: The non-specific DNA cleavage domain from the end of the FokI endonuclease can be used to create hybrid nucleases active in several different types of cells. The FokI domain acts as a dimer, requiring two constructs with separate DNA binding domains with proper orientation and spacing for sites in the target genome. Both the number of amino acid residues between the binding domain of TALE DNA and the cleavage domain of FokI and the number of bases between the two individual binding sites of TALEN seem to be important parameters for achieving high activity (Carroll, 2017).

TALEN mechanism: The clear relationship between the sequence of amino acids and the TALE binding domain DNA recognition allows for effective protein engineering. When the TALEN constructs are assembled, they are inserted into plasmids; then the target cells are transfected with the plasmids, and the gene products are expressed and incorporated into the nucleus for entry to the genome. Alternatively, TALEN constructs can be administered to cells as mRNAs, which eliminates the risk of the TALEN-expressing protein being incorporated genomically (Khan, 2019). Using an mRNA vector can also significantly increase the level of Guided Homology Repair (HDR) and introgression performance during gene editing.

TALEN technology can be used to rewrite genomes through the induction of double-strand breaks (DSB), to which cells react with repair mechanisms. Non-homologous end joining (NHEJ) reconnects DNA from either side of a double-strand break, where the sequence overlap for annealing is very small or no. This repair mechanism causes genome errors by insertion or deletion, or chromosomal rearrangement; any such errors that make the coded gene products non-functional at that place. Since this behaviour can differ depending on the species, type of cell, target gene, and nuclease used, the design of new systems should be controlled. Alternatively, in the presence of exogenous double-stranded DNA fragments, DNA may be inserted into a genome through the NHEJ. Homology-driven repair may also introduce foreign DNA at the DSB, since the transfected double-stranded sequences are used as templates for repair enzymes.

2.1.1c CRISPR-Cas9

Clustered regularly-interspaced short palindromic repeats (abbreviated as CRISPR, pronounced crisper) are prokaryotic DNA segments containing short base sequence repetitions. CRISPR is used as a tool which enables scientists to edit genomes with unparalleled accuracy, efficiency and versatility. CRISPR is much better than older gene-splicing and editing techniques (Haapaniemi, E. et al., 2018; Thavalingam, A. et al., 2018; Zhu, Y. et al., 2019).

The CRISPR / Cas system is a prokaryotic immune system that gives resistance to foreign genetic elements, such as plasmids and phages, and provides an acquired form of immunity (Cui, Y. et al., 2018; Lino, C. A. et al., 2018; Fajrial, A. K. et al., 2020). These exogenous genetic elements are recognized and cut by CRISPR spacers in a manner similar to RNA interference in eukaryotic organisms. A set of genes was found to be associated with CRISPR repeats, and was called the genes, or the genes associated with CRISPR. The case genes encode putative nuclease or helicase proteins, which are enzymes capable of cutting or relaxing DNA. The Cas genes are still near the sequences of the CRISPRs (Singh et al., 2017). There are a variety of Cas enzymes, but the best known is called Cas9, which originates from pyogenes of Streptococcus.

The technique of CRISPR interference has tremendous potential use, including altering the germline of humans, livestock and other species and manipulating food crop genes (Barman, N. C.K. et al., 2020; han, M. N. M. et al., 2020; Minet, C. et al., 2018). The genome of the organism can be cut at any desired position by delivering the Cas9 protein and proper RNAs guide into a cell. CRISPRs is used in combination with different endonuclease enzymes in the tree of life for genome editing and for gene regulation in animals. Ethical questions about this new biotechnology and the possibility of editing the human germline have been raised.

Mechanism

The genome editing of CRISPR / Cas9 is performed using a Type II CRISPR method. Cas9 is a DNA-cutting enzyme (nuclease), and CRISPR is a set of DNA sequences which tells Cas9 exactly where to cut. An RNA guide is required to feed the right sequence on Cas9, anywhere you want to cut and paste bits of DNA sequence into the genome. This system includes Cas9, CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA) and an optional portion of the DNA repair template used in either Non-Homologous End Joining (NHEJ) or Homology Guided Repair (HDR) applications when used for genome editing. The crRNA contains the RNA used by Cas9 to guide it to the correct host DNA section along with a region that binds to tracrRNA (usually in the form of a hairpin loop) forming an active Cas9 complex. The tracrRNA binds to crRNA and forms with Cas9, an active complex (Tian et al., 2019).

CRISPR / Cas9 commonly utilizes a plasmid to transfect the target cells. The crRNA must be programmed for each sample, since this is the sequence that Cas9 uses to classify and bind directly to the DNA of the cell. The crRNA shall only bind where editing is needed. The repair prototype must also be built for each procedure, as the sequences on each side of the cut and code for the insertion sequence need to overlap. This sgRNA can be combined with the Cas9 gene, and converted into a plasmid for transfection into cells. With the help of crRNA the Cas9 protein finds the correct sequence in the DNA of the host cell and creates a single or double strand break in the DNA. Properly spaced single strand breaks in the host DNA that cause guided repair homology, which is less prone to error than non-homologous end joining, usually following a double strand break. Providing a portion of the DNA repair template enables a different DNA sequence to be inserted at an exact position within the genome. The repair template should extend 40 to 90 base pairs beyond the DNA break that was induced by Cas9. The aim is to use the provided repair template for the cell's HDR process and thus incorporate the new sequence into the genome. This new sequence, once incorporated, is now part of the cell's genetic material and passes into its daughter cells.

Human Germline Modification: Case Study of CRISPER

In April 2015, scientists from China published a paper (Protein Cell. 2015 May; 6(5): 363–372) reporting results of an attempt to alter the DNA of non-viable human embryos using CRISPR to correct a mutation that causes beta thalassemia, a lethal heritable disorder (Gordon & hall, 2010). The experiments resulted in changing only some of the genes, and had off-target effects on other genes. The researchers who performed the research said that CRISPR is not ready for clinical use in reproductive medicine. This paper raised serious concerns about gene editing in embryos (see Chinese Scientists Edit Human Embryo Genes, Raising Concerns.

In December 2015 Washington hosted the International Conference on Human Gene Editing. Leaders of the American, British, and Chinese national scientific academies discussed the ethics of germline alteration. In conclusion, they decided to continue basic and clinical work according to acceptable legal and ethical guidelines. A clear distinction has been made between clinical uses of somatic cells, where editing effects are restricted to one organism, versus germline cells, where genome changes will be inherited from future generations. This could have unintended and far-reaching consequences for human evolution, genetically (e.g. interactions between gene and environment) and culturally (e.g. social Darwinism), so it was claimed irresponsible to alter gametocytes and embryos to generate inheritable changes in humans. In addition, they agreed to establish an international forum to discuss these issues on

an ongoing basis and to harmonize research regulations across countries. In February 2016, regulators gave British scientists permission to genetically alter human embryos using CRISPR-Cas9 and related techniques.

On February 8, 2015, the U.S. director of national intelligence added gene editing to a list of threats posed by "weapons of mass destruction and proliferation" in the U.S. intelligence community's annual worldwide threat assessment report. According to the evaluation, it is the relative ease of use of gene editing that concerns the U.S. intelligence community. "Given the broad distribution, low cost, and rapid speed of development of this dual-use technology, its deliberate or unintentional misuse could lead to far-reaching consequences for economic and national security," the report said. Though CRISPR is not mentioned by name in the report, Clapper clearly had in mind the newest and most versatile gene-editing systems. The low cost and relative ease of use of the CRISPR technique — the basic ingredients can be bought for $60 online — seems to have spooked intelligence agencies.

Researcher Jiankui He reported in November 2018 that he had produced the first human genetically modified infants, known by their pseudonyms, Lulu and Nana.

d The Meganuclease Family of Endonucleases

Properties and Use

Meganucleases have been used to trigger gene targeting for more than 15 years. Recent developments in the precision of meganuclease re-engineering have further expanded its breadth of application. The scores of publications and contributions to meganuclease-related scientific meetings show a major evolution in acceptance and applicability, with a increasing group taking an interest in these proteins' exceptional properties.

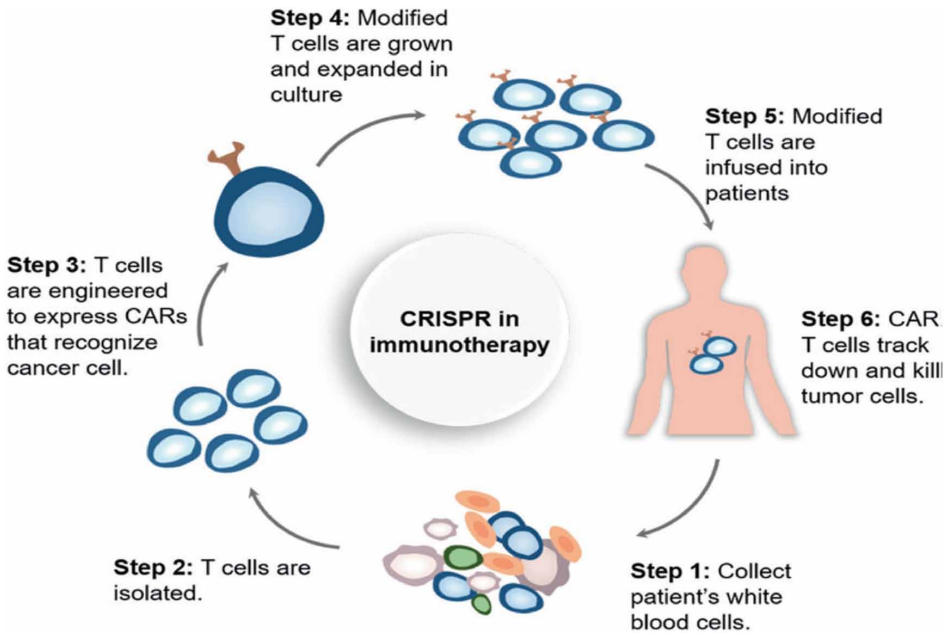
The Meganuclease Family

Meganucleases, also known as homing endonucleases, can be classified into five families based on patterns of sequence and structure: LAGLIDADG, GIY-YIG, HNH, His-Cys and PD-(D/E) XK (Silva et al., 2011). The well-studied family is that of the LAGLIDADG proteins found in all kingdoms of life, which are generally encoded within introns or inteins although there are also freestanding members. To date, these proteins have not been recognized as having a purposeful function within the host and appear to be labeled as "selfish genetic elements." LAGLIDADG proteins exhibit one of two primary behaviours, with few exceptions: (a) they serve as RNA maturases involved in facilitating the splicing of their own intron, or; (B) act as highly specialized endonucleases capable of recognizing and cleaving the exonexon junction sequence in which their intron resides, thereby giving rise to the "homing endonuclease" name. Homing endonucleases have been hypothesized to have a so-called "life-cycle," as illustrated in Fig. (14) I start as invasive endonucleases capable of mobilizing their coding sequence; (ii) acquire a concomitant RNA maturase activity to help ensure proper splicing of their intron; (iii) lose the "invasive" nuclease activity over time, leaving only the RNA maturase function, and (iii) lose the RNA maturase function. (iv) At last, when the maturase operation is lost, the propagation of the intron is unviable and the intron is lost. It is thus inferred that a given LAGLIDADG protein's functionality (endonuclease, maturase, or both) represents a snapshot into the current state of its lifecycle.

GENE EDITING IN CANCER THERAPEUTICS – APPLICATION OF GENE EDITING

Oncogenes and mutant tumor suppressor genes provide exceptional opportunities for using modulating approaches to the genome (Navarro, S. A. et al., 2016; Siddique, N. et al., 2016). Genome editing technology has accomplished essential targeted cleavage events in a number of fundamental studies, ranging from its initial proof of successful gene editing in eukaryotes to its recent applications in the development of hematopoietic stem cells (HSCs) and tumor-targeted T cells; this technology has established novel gene modification concepts and expanded to a border field of cancer research. ZFN controlled targeting has been successfully applied as an archetypal interface for programmable DNA cleavage to alter several genes in human cells and a variety of model organisms, thereby opening the door to the creation and application of genome editing technologies. ZFN-driven gene disruption was demonstrated primarily in 1994 when a three-finger protein was built to specifically block the expression of human oncogene BCR-ABL that was transformed into a mouse cell lineage. Afterwards, a study used a modified line of human lymphoblast cells (Singh et al., 2017).

Figure 14. Proposed life-cycle of a homing endonuclease
Adapted from (Gogarten JP et al BMC 2006).



This cell line was used by chronic myeloid leukemia (CML) patients and a custom-designed ZFN to deliver site-specific DSBs to the telomeric portion of the breakpoint cluster region of the mixed lineage leukemia (MLL) gene and to examine chromosomal rearrangements associated with MLL leukemogenesis through DSB error repair. Successful targeted modulation was also achieved using designed ZFNs, which promoted disruption of the β- and α-chain endogenous T-cell receptor (TCR) genes (Gonzalez et al., 2018). ZFN-treated lymphocytes lacked CD3-TCR surface expression and expanded with an increase

in interleukin-7 (IL-7) and IL-15. By targeting the long terminal repeat (LTR) promoter feature from human T cell leukemia virus type 1 (HTLV-1), a novel therapeutic ZFN directly killed HTLV-1-infected cells in an adult T cell leukemia (ATL) model in vivo. In addition, successful cleavage of the BCR-ABL fusion gene by highly specific ZFNs has been reported to end the translation of the BCR-ABL protein and to induce apoptosis in imatinib resistant CML cells. In addition, cancer-relevant translocations in human Ewing sarcoma and anaplastic large cell lymphoma (ALCL) cells induced by ZFNs have shown that custom nucleases can achieve precise genomic rearrangements in the relevant cell typesIn addition, the use of HER2-positive cell-penetrating peptide (CPP) in conjugation with mammalian mTOR-specific ZFN has made the mTOR locus non-functional and inhibited relevant cancer signaling pathways, providing insight into the design of new molecular targeted therapies (in particular) for breast cancer and other types of cancer. In addition, because the tumor suppressor gene p53 plays a crucial role in preventing the development of cancer, genome editing strategies have been investigated to restore wild-type p53 activity. A four-finger yeast-one-hybrid (Y1H) ZFN was designed to replace mutant p53 with wild-type p53 in several cancer cell lines (from glioblastoma, leukemia, and breast cancer) through ZFN-induced HR. Although the HR events were not particularly effective in this case, changes at p53 loci still provided a framework for further investigation. Researchers have applied ZFNs in addition to altering the viral genes associated with tumorigenesis to improve T cell-mediated antitumor therapy. For example, glioblastoma-specific cytolytic T lymphocytes (CTLs) may be generated by importing a chimeric TCR comprising an extracellular IL-13 domain (zetakine) and a cytoplasmic CD3 domain into CD8 + T cells. To this end, Reik and his team knocked down the glucocorticoid receptor with ZFNs in the modified CTLs. Accordingly, the cytolytic efficacy of "zetakine" transgenic CTLs against glioblastomas was maintained independent of glucocorticoid treatment presence. Recently, this technology has been effective in knocking out transported glucose genes (MCT4 or BSG) in two models of glycolytic tumors: colon adenocarcinoma and glioblastoma.

A landmark of TALENs was achieved when they were introduced specifically to effectively disrupt the endogenous genes NTF3 and CCR5 in human leukemia cells through the insertion of NHEJ- or HDR-induced modification into a coding sequence, showing that TALENs could be engineered for selective endogenous gene cleavage (Khan, 2019). Interestingly, when two human loci (CCR5 and IL2RG) compared TALENs and ZFNs abreast, TALENs displayed substantial cytotoxicity decreases. In addition, when compared with ZFN technology, the CCR5-specific TALEN was able to differentiate between the CCR5 target locus and a very similar site in CCR2Precise disruptions were also introduced into the T cell receptor α constant (TRAC) gene and the CD52 gene in allogeneic T cells through TALEN-induced HDR by the adoption of TALEN gene editing technology. The TALEN used in this study was engineered by a retroviral vector that expressed a chimeric antigen receptor (CAR) targeting CD19 + leukemic B cells, which helped to grow the "universal" CAR T cells. Alternatively, a site-specific TALEN was used to inhibit one single Fms-related tyrosine kinase 3 (FLT3) gene allele and to produce isogenic cell leukemia clones. In vitro, TALEN-mediated FLT3 haplo-insufficiency impaired proliferation of cells and colony formation. In vitro, TALEN-mediated FLT3 haplo-insufficiency impaired proliferation of cells and colony formation. These suppressive effects were preserved in vivo, and the survival rate of NOD / SCID mice transplanted with K562 mutant clones was increased. The use of engineered TALENs in prostate cancer cells functionally classifies target gene rearrangements to the androgen receptor (AR) as resistance drivers. Piganeau and his work team have induced cancer-related translocations into anaplastic large cell lymphoma (ALCL) using TALENs to precisely cut the necessary translocation breakpoints. The reversal of the ALCL translocation in a patient cell line was achieved through an analogous strat-

egy, restoring the integrity of the two involved chromosomes. Recent studies have also shown that the TALEN gene editing technology used to knock down genes in cancer cells (including prostate cancer cells, breast cancer and hepatocellular carcinoma (HCC) is an effective and widely available tool for exploring gene mutations at the molecular level.

The CRISPR / Cas9 method has attracted significant attention because of its various advantages in genome editing and scientists slowly find it a important therapeutic tool for treating diseases associated with genome mutations. The main aim of CRISPR / Cas9 cancer therapy is to eliminate malignant mutations and replace them with regular sequences of DNA (Georg Hausner et al, 2017). In a recent study the leukemia model was created by reviving several inactivated oncogenes in primary hematopoietic stem and progenitor cells (HSPCs) through the lentiviral delivery of the Cas9-sgRNA system. The pooled lentiviruses in this study have targeted genes, including Tet2, Runx1, Dnmt3a, Nf1, Ezh2, and Smc3. The objective HSPCs were selected through a fluorescent marker; those HSPCs are engaged in myeloid neoplasia growth. In addition, CRISPR / Cas9 technology was adopted to develop models of organoid tumors. For example, models of organoid colon cancer were constructed in vitro using CRISPR technology by introducing tumor suppressor gene mutations (APC, TP53, SMAD4, etc.) and oncogene modification genes (KRAS, PI3 K, etc.) (Shankar et al., 2018). In addition, driven by colonoscopy, Roper et al. developed CRISPR engineered mouse tumor organoids via mucosal injection, delivering viral vectors carrying CRISPR / Cas9 components to the distal mice colon. In a research modeling tumor progression with a series of adenoma-carcinoma-metastasis such an method has already been applied. The use of CRISPR / Cas9 technology to create precise models of cancer would greatly encourage functional cancer research in future.

IMMUNE SYSTEM IN CANCER PATHOGENESIS

It has always been an interesting question: How cancer cells avoid immune-attack destruction? Tumor growth can, in general, be controlled by cytotoxic innate and adaptive immune cells; however, as the tumor transforms from neoplastic tissue to clinically detectable tumors, cancer cells evolve different mechanisms that imitate peripheral immune tolerance to escape tumoricidal attacks. Here, we will emphasize on the role of the immune system and challenges of the future throughout the study of inflammatory cells associated with cancer, with emphasis on metastatic carcinomas.

The connection between the immune system and cancer has been widely appreciated for over a century and was first emphasized more than 150 years ago by Rudolph Virchow. The fundamental basis of this relationship between cancer and immunity involves three basic concepts of how the immune system works to defend and protect an individual: it detects ‘non-self’ antigens from pathogens or infected/malignant cells; it involves effector functions to precisely target and kill pathogen or infected/malignant cells while protecting the host; And it builds immunological memory via adaptive immune responses to subsequent mechanisms of defense following injury or host attack. Through this phase, the immune system has acquired characteristics that give rise to the paradigm known as immunoediting which provides a balance between the oncology domain of immune surveillance and cancer progression. This multifaceted process consists of the three main phases: elimination, equilibrium, and escape, respectively, which lead to cancer elimination, dormancy, and progression. Interestingly, this ability of cancers to avoid the immune response is now recognized as one of the most esteemed hallmarks of cancer, and provides the basis for immunotherapy treatments.

While the initial use of immunotherapy for cancer therapies dates back to the early 19th century, indicating research performed by William B. Coley and colleagues, recent scientific advancements have helped to elucidate novel methods to incorporate immunotherapy to prevent and/or treat various cancers. Such discoveries made the notion of immunooncology and immunotherapy for cancer more clinically important. Here the new and developing results that contribute to the understanding of immunooncology will be discussed and the importance of appropriate immunotherapies for future therapeutic approaches in cancer treatments will be emphasised.

Cancer Immunoediting

For several decades, the role of the immune system in cancer pathogenesis has been a topic of great interest and debate due to its ability to mediate cancer defense and advance cancer progression. In the context of the cancer biology, the function of immune responses is commonly referred to as immunosurveillance. Although Paul Ehrlich is regarded as the scientific leader behind the concept of immunosurveillance, conflicting reports on this concept based on studies performed by the oncology community Burnet and Thomas and Stutman brought the concept of immunosurveillance to the forefront. The definition was widely dismissed because of these and other incoherent findings from research illustrating the mechanisms of immunosurveillance in cancer. Creation of studies to investigate immunosurveillance, however, was feasible with the scientific advancement of genetically modified animal models. Consequently, in the 1990s the role of immunity in cancer was again re-evaluated. Several counterarguments against the cancer immunosurveillance hypotheses have been dismissed with the manipulation of mice models deficient in adaptive immunity (RAG2 knockout mice) or mice missing interferon-gamma (IFN-α) signaling cascade components. In particular, studies of these and other animal models deficient in some type of immune response were highly predictive of carcinogenesis and tumor formation immunity defense. In addition, immune-mediated cancer protection is not only limited to animal models but it has become increasingly clear that immunosurveillance is also clinically observed in humans. Interestingly, recent reports suggest that there is a delicate balance between cancer dormancy and progression, and that balance is the foundation of the principle known as immunoediting in oncology. The removal, equilibrium, and escape are three major phases which constitute the immunoediting process in cancer pathogenesis. Such immune responses underlying the immunoediting help form the immunogenicity of different cancers. Immunoediting findings can be due to factors that include the cancer's temporal or spatial location, molecular processes involved in transformation from normal to transformed cells, and the immune system's underlying genetic factors.

The immunoediting process of elimination is a component of the theory of cancer immunosurveillance and refers to the capacity of the innate and adaptive immune system to identify and eliminate cancer cells. Mechanisms through which cancer cell lysis occurs are through the secretion of perforin from cytolytic immune cells (i.e. NK cells, NKTcells, πTcells, and CD8 + Tcells), ADCC, or CDC (Pandya et al., 2016). The equilibrium phase focuses on the complex state of the cancer cells in order to negatively regulate the immune system leading to a blockage in the immunoediting phase and a transition to the equilibrium phase.

Immune responses against the tumor are still involved in the equilibrium phase; immune cells help to regulate and monitor cancer growth or metastasis while preserving it in the latent dormant state. The equilibrium phase is known to be the longest step in the immunoediting cycle. Given these control points, which are modulated by the immune system, the cancer's heterogeneity and genetic differences cause

them to develop the ability to become immune-evasive and escape the host's equilibrium state to spread and become detrimental. This escape process is mediated by multiple immunosuppressive mechanisms one of which involves down regulation or aberrant expression of MHC class I on the surface of the cancer cell protecting it from cytotoxic effector functions of immune cells in the innate and adaptive immune system. Multiple mechanisms such as suppression of the expression of tumor antigen, activation of antiapoptotic pathways to avoid cytotoxicity, and cancer-induced immunosuppression help in the escape of cancer cells from the phases of immunity elimination and balance. Notably, it is this escape from immunity from cancer cells and the processes involved in this escape that has been the driving force of immune-oncology paradigm-focused study. Gaining a thorough understanding of the immunoediting cycle in cancers would be key to the advancement of cancer treatment immunotherapies.

Precision Medicine

Precision medicine, a new approach to patient-specific treatments, is revolutionizing clinical outcomes and care quality. Therefore, medical advances have been made in developing treatments that stimulate the immune system in attempts to combat various types of cancer. These particular types of cancer treatments based on the use of innate and adaptive immunity are called immunotherapies for cancer. Because of the paradigm shift in health care that focuses on precision medicine, more effort is being geared towards developing tailored therapies focused on immunotherapy for individual patients with cancer. These immunotherapies for cancer can be divided into various categories: vaccines, monoclonal antibodies, recombinant cytokines, small molecules, and autologous T-cells. The site, form, and stage where the specific cancer is in determine the type of therapy best suited for the patient.

Several FDA approved immunotherapies were developed and clinical trials were conducted to treat various forms of cancer. In spite of these therapies' initial promising success rates, the vast majority of patients are relapsing. This can be due to numerous factors that differentiate individual patients such as age, gender, chemotherapy regimen, and cancer site / type, all of which play a functional role in the genomics of cancer and serve as the cornerstones of precise medicine. These cancers harbor a subset of genetic mutations that may result in distinct molecular characteristics, giving rise to the potential for future therapies to be predictive biomarkers. Interestingly, the term "genetic mutations" is not only limited to the primary tumor / cancer in the area of cancer genomics. "Genetic mutations" also include gene mutations that can vary between new and relapse cancers, primary and metastatic cancers, as well as genetic mutations caused by therapy in patients with cancer. Both of these factors may lead to resistance and/or relapse of the patients to anticancer treatments. Identifying these genetic mutations can lead to the detection of predictive molecular immune signatures or immune biomarkers (cancer-related neoantigens) among individual patients with cancer, which is a crucial step in developing new immunotherapies for patients. Interestingly, the patient-specific cancer which causes determinants can be identified and treated appropriately by evaluating specific genetic mutations.

To date, it has developed many immunotherapies to treat cancers. Although some of the therapies are already on the market or have been accepted for clinical phase trials, multiple genetic mutations can be detected by the use of high-throughput sequencing technologies to create customized therapies

Case Study 1

Currently, patients with cancer are treated with therapy regimens that include chemotherapy / radiation therapy along with targeted drugs that can affect various factors in the progression of cancer, such as immune responses, DNA damage or growth. Interestingly, due to the idea that cancers "breaking" the immune responses during the immune-editing process, there was also interest in trying to combine immunotherapies. Dr. Wolchok's group performed a Phase I trial in which immunotherapies of Ipilimumab (anti-CTLA4) plus Nivolumab (anti-PD1) were used together for advanced melanoma patients. Ipilimumab promotes the activation and priming of T cells, and Nivolumab prevents the interaction of PDL1 with PD-1 in cancer cells. Through these studies, Wolchok and colleagues reported that the desired effect, efficacy and outcome of Ipilimumab is better when given with Nivolumab simultaneously. Such trials provided the basis for testing certain new combinations of immunotherapy in tandem with chemotherapy / radiation therapy in order to further increase survival rates for patients with cancer.

Genome Editing in Cancer Immunotherapy

Cancer immunotherapy has sparked considerable interest in harnessing the patient's own immune system against tumor cells. One promising field of immunotherapy is the application of genetically engineered T cells, known as chimeric antigen receptor (CAR) T cells, which activate antigens associated with the targeted tumor and may improve the response to the therapy. The preparation of functional CAR T cells involves several main steps: first, the white blood cells of the patient are extracted and the T cells of the patient are isolated through leukapheresis, after which T cells are re-engineered and modified with tumorantigen-specific receptors and costimulating molecules; second, a viral vector containing CAR is transformed into the modified T cells, followed by the amplification. CARs are synthetic receptors usually containing the following parts: an antibody-derived targeting ectodomain that recognizes tumor antigens; a costimulative molecular region that can bind to receptors such as CD28, 4-1BB, or CD278; and a T-cell signaling domain (Qian et al., 2014). The CAR can transmit signals after binding to a specific antigen, and activate modified T cells. By avoiding the restriction historically imposed by the major histocompatibility complex (MHC), the freedom of CAR recognition endows genetically engineered CAR T cells with a fundamental antitumor advantage. However, the applicability of this transformative drug is highly limited due to the difficulty of the manufacturing process, the limited range of target antigens and the inadequate response of antitumors to solid tumours (Pankita H. Pandya et al,.2016). Flexible gene editing techniques have become important engineering tools in recent years to overcome these limitations and further develop CAR T designs.

The creation of allogeneic CAR T cell therapy would simplify the manufacturing process of autologous CAR T cells and overcome some challenges. The endogenous αβ T cell receptor (TCR) is responsible for the identification of the major and minor antigen histocompatibility. By genetically disrupting specific parts of the αβ TCR complex and/or the human leukocyte antigen (HLA) class I loci of allogeneic T cells, a universal cell therapy drug can be developed that confers a wider range of application capabilities with minimally associated adverse effects, including graft-versus-host disease (GVHD). Prof Torikai used engineered ZFNs in 2012 to suppress the expression of α or β chains in endogenous TCRs, resulting in the loss of TCR function in CD19 CAR T-cells. These modified T cells did not respond to specific TCR stimuli but retained the ability to recognize and target CD19, leading to the generation of antigen specific CAR T cells associated with universal allogeneic tumors. The selective removal of HLA expression in

CD19-specific T cells and in embryonic stem cells was accomplished with the same method, which increased the applicability of this strategy by preventing infusion of HLA disparate immune cells (Philip et al., 2015). have carried out similar work in 2015, using TALEN-mediated editing. The expression of αβ TCR was inactivated by the application of TALEN-mediated gene editing, thereby removing the risk of T-cell responses to allogeneic antigens and GVHD. In two infant patients with relapsed refractory CD19 + B cell acute lymphoblastic leukemia, the beneficial function of TCRdepleted CD19 CAR T cells in evading GVHD was recently validated, resulting in positive molecular remissions within 4 weeks. In addition, TALENs simultaneously inhibited the target of the lymphocytic depleting monoclonal antibody alemtuzumab, CD52, a human glycoprotein located on the surface of lymphocytes, in order to suppress the ability of any residual alloreactive T cells and to facilitate the engraftment of cell therapies. TCR / CD52-deficient CAR T cells were administered concurrently with alemtuzumab as proof of application of this platform and demonstrated antitumor activity in a lymphoma murine model similar to unmodified anti-CD19 CAR T cells, with resistance to alemtuzumab destruction.

The widespread use of ZFN- and TALEN-based gene editing techniques has been hindered by the need to design different nuclease pairs for each new gene target. Production of the CRISPR / Cas9 method has succeeded in supporting multiple gene editing in CAR T cells more rapidly and easily. Using this technology, Liu et al. effectively produced CAR T cells in which two (TRAC and B2 M) or three genes (TRAC, B2 M, and PD-1) were disrupted simultaneously and their antitumor function was tested in vitro and in vivo. They had planned four sgRNAs to target the first exon of TRAC and B2M. Two sgRNAs were designed to target the first exon of PD-1, and one reported sgRNA was tested. Lastly, double-knockout (B2 M and TRAC) T cells were induced with high efficiency, but in triple-knockout (B2 M, TRAC and PD-1) T cells, only 64.7 percent of the PD-1 PCR product clones were mutants, suggesting that PD-1 expression could be down-regulated during T cell development. More specifically, in a model of xenograft mouse lymphoma, the CRISPR / Cas9-mediated multiplex gene-edited CAR T cells retained CD19-specific antitumor activity, indicating that they are promising cancer treatment reagents (Tian et al., 2019). In another interesting study, the efficient double knockout of endogenous TCR and HLA class I molecules was achieved by a one-shot CRISPR protocol that incorporated multiple gRNAs into a CAR lentiviral vector to generate allogeneic universal CAR T cells. In this study, CRISPR / Cas9 mediated the simultaneous knockout of four T-cell surface receptors PD-1 and CTLA-4 loci, and successfully produced universal allogeneic T-cells. More recently, the CRISPR / Cas9-mediated generation of CAR T cells that specifically disrupts inhibitory immune receptors such as T cell membrane protein-3 (TIM-3), adenosine 2areceptor (A2aR) and lymphocyte-activation protein 3 (LAG-3) showed a better percentage of full remission in xenograft mouse models by increasing the secretion of antitumor-related cytokines (such as IFN-g, GM-CSF a). These factors may be associated with CAR T cell fatigue and acute myeloid leukemia (AML) dysfunction, because the combination of checkpoint inhibitors with CAR T cells may contribute to increased antitumor efficacy of AML and other hematological malignancies. Taken together, these findings indicate that genome editing can serve as a good platform to generate "normal" CAR T cells, and can be applied against multiple targets to large-scale development of stable "off-the-shelf" T cells.

APPLICATION OF GENE EDITING IN CLINICAL TRIALS

As an enticing and daunting therapeutic approach, genome editing will modify or remove mutations that occur in the development of cancer and other genetically induced diseases. Ex vivo genome editing has been the most commonly used up to now, i.e. genetic modification of in vitro cells and then re-engrafting the modified cells back to patients. In recent years, teams led by China and the United States have performed a series of gene editing clinical trials, such as developing more effective CAR T cells for cancer treatment and knockout the BCL11A erythroid-specific enhancer to upregulate gamma globulin in autologous erythroid HSCs as a possible sickle cell disease and β-thalassemia therapy.

Anticancer Clinical Trials

In 2010, the gene editing clinical trial using the ZFN drug GRm13Z40-2 for the treatment of stage III or IV patients with malignant glioma (NCT01082926) began. ZFN-mediated GRm13Z40-2, a genetically engineered allogeneic CD8 + cytolytic T cell line expressing the glucocorticoid-resistant IL13-zetakine, was administered to tumor cells via intratumoral injection. In another phase I clinical trial (NCT02800369), ZFN agents (ZFN-603 and ZFN-758) were transfected into cervical epithelial cells infected with HPV to determine whether these agents could prevent malignant progression of cervical intraepithelial neoplasia and decrease incidence of cervical cancer. This study has concluded the phase of data collection to date. Only two studies were documented using the TALENs in CAR T cells. One research (NCT02808442) established a portfolio of allogeneic, universal CAR T cells (UCART19) that target CD19-positive Bacute lymphoblastic leukemia, either relapsed or refractory. Alloreactivity and vulnerability to alemtuzumab were removed in this study by disrupting the loci encoding TRAC and CD52. In AMLs and blastic plasmacytoid dendritic cell neoplasms (NCT03190278), a similar concept is used to generate allogeneic TALEN-edited CAR T cells which target CD123 (UCART123).

The CRISPR / Cas9 method has become an important instrument in the advancement of cancer therapy due to the simple design process and the ability to make multiple gene edits at one time.

To date, 11 clinical trials have been performed to determine the effectiveness of the CRISPR method in cancer therapy, seven of which are immunotherapies that regulate the expression of protein PD-1. In 2016, the first clinical trial using the groundbreaking CRISPR / Cas9 cancer treatment technique enrolled the first patient at West China Hospital, Sichuan University. In this non-randomised, open-label Phase I trial (NCT02793856), after all standard therapies, the protection of ex vivo modified PD-1 knockout T cells has been tested with progression in the treatment of metastatic non-small cell lung cancer. In this trial, PD-1 expression in peripheral blood lymphocytes obtained from the enrolled patients was disabled by CRISPR / Cas9. The modified lymphocytes have been separated, multiplied and then re-infused into the patients. The same principle of PD-1 knockout autologous T cells is used in current clinical trials to treat other forms of cancer, including prostate cancer (NCT02867345), oesophageal cancer (NCT03081715) and renal cell cancer (NCT02867332). These trials can be considered as the first proof-of - concept studies to apply the technique of knockout of the in vitro CRISPR / Cas9 gene in cancer treatment. Studies now exist in the development of therapy that combine PD-1 knockout with other targeted editing, which may lead to improved efficacy for clinical application. One example is the addition of PD-1 knockout in phase I / II clinical trials (NCT03044743) to Epstein – Barr virus (EBV)-specific autologous T cells for the treatment of EBV-positive cancers.

CRISPR's removal of endogenous TCR and PD-1 may enhance activity in tumor rejection. The US National Institutes of Health's (NIH) Recombinant DNA Advisory Committee (RAC) recently approved a clinical trial to be piloted at the University of Pennsylvania. In this trial, CRISPR / Cas9 in HLA-A*0201 restricted NY-ESO-1 TCR redirected autologous T cells will abolish PD-1 and the endogenous TCR. These redirected, engineered T cells can be added to a number of forms of cancer including relapsed multiple myeloma refractory, melanoma, synovial sarcoma, and myxoid / round liposarcoma (NCT03399448).

The use of CRISPR / Cas9 technology to produce CAR T cells for the attack of malignant cells has become a clinical trial research hotspot. A phase I / II clinical trial (NCT 03166878) evaluated the health and resistance of patients with recurrent or refractory CD19 + leukemia and lymphoma to multiple doses of universal CD19-specific CAR T cells (UCART 019). UCART019 cells were obtained in this study by combining lentiviral delivery of CAR receptors and CRISPR RNA electroporation to inhibit the endogenous TCR and B2 M genes simultaneously. These cells are derived from one or more stable, unrelated donors but can help prevent graft-versus-host-disease (GVHD) and improve host-mediated immunity, thereby providing patients with reasonably safe anti-leukemic effects. Unfortunately, the loss of CD19 expression in tumor cells caused a small number of patients to relapse. Hence, another clinical trial (NCT03398967) that is more applicable to a wide range of patients based on allogeneic CRISPR-edited bispecific CD19+CD20 + or CD19+CD22 + CART cells, which may identify and destroy the CD19-negative malignant cells by CD20 or CD22 recognition. In another study, a new clinical trial (NCT03057912) proposed evaluating the safety and efficacy of TALENs and CRISPR / Cas9 combination genome editing by targeting HPV16 and HPV18 E6 / E7 DNA in the treatment of HPV-associated cervical intraepithelial neoplasia. CAR T cells modified by both techniques were administered for 4 weeks twice a week in this trial to inhibit target gene expression, and expected to reduce off-target results.

The mutation rate of the type 1 (NF1) gene for neurofibromatosis is one of the highest in the human genome, and is likely to cause multiple benign or malignant tumors. The CRISPR / Cas9 technology was developed to test and classify NF1-specific drugs in one trial (NCT03332030). First, the creation of a human iPSC library from NF1 patients with good phenotypic characteristics and the development of various cell lines (NF1+/+, NF1+/− and NF1−/−) using CRISPR / Cas9. Then, after repeated drug use, it may classify possible therapeutic agents by analysing the phenotypes of reversal or remission. While the outcomes of clinical trials of genome editing seem encouraging, further research needs to be done to ensure this tool's protection and efficacy of treating human cancers.

CHALLENGES IN THERAPEUTIC TARGETING

In addition to the many advantages of genome editing, the translation of such therapies into clinical disease therapy poses several technological challenges, mainly in terms of the obstacles of accuracy, efficacy and delivery (You et al., 2019). To meet these challenges, scientists will need a deep knowledge of the molecular nature of cancers, especially heterogeneous solid tumors, as well as carefully developed platforms for genome editing in preclinical studies.

2.6 OUR GENOME-EDITING FUTURE: FURTHER READING

As a scientist using genome-editing technology, Carlson hopes it will be applied by researchers for the good of humanity and the planet. He hopes the public can realize that having a finished product is in reality a lengthy process. Biotechnology Company Recombinetics has gained media attention for using TALENs to breed polled (hornless) cows — which saves farmers the trouble of dehorning them. The project started in 2012 and Carlson says the company is continuing to work to make the editing more successful.

CRISPR dominates genome-editing predictions, due to its popularity and availability. CRISPR-based systems will keep improving incrementally, says Carlson. Researchers routinely post higher efficiency or precision on improved gRNAs. Multiple Cas-type enzymes with various PAMs or activities have been discovered or engineered (6). For example, Cas13 targets RNA and is the basis for baseline editing of RNA. Beam Therapeutics, whose co-founders include Liu and Feng Zhang, who developed CRISPR for mammalian cells, are licensed for this method and Liu's DNA base editing.

Methodological improvements to CRISPR include treatment of small molecular cells during editing to nudge DSB repair away from NHEJ and toward HDR. Controllable systems use light or small molecules to turn Cas9 on, reducing its operation to minimize off-target effects. Researchers are scouring the microbial environment for new enzymes of the Cas type and entirely new genome editing systems. "We are still finding new molecules with editing capabilities and we don't completely appreciate the editing methods that we have," says Hennebold. "We still have a lot to learn." There is growing practice of using CRISPR to correct disease-causing mutations: for an inherited blindness, Editas Medicine and Allergan announced human in vivo CRISPR-therapy trials.

The probability of human immune responses to its bacterial components is a possible barrier to therapeutic CRISPR. For example, most of the blood samples tested showed established immune responses to Cas9, usually taken from the bacteria Staphylococcus or Streptococcus.

The wish list for genome editing involves improved multiplexing methods — editing more than one gene at a time. Multiplexing, for example, will accelerate advances in T-cell-based immunotherapy, which works for several patients but involves multiple gene alterations. And plant scientists also want to build "stacks" of related genes that are inherited together as a kit for disease resistance, pests and other threats to agriculture. Multiplexing will hasten the production of these goods.

Multiplexing with CRISPR is, in theory, simple, requiring only the insertion of one single Cas enzyme and gRNAs and template DNAs for each target gene. Dr. Gunawardane has tried CRISPR multiplexing to tag multiple genes in the same cell and says it's feasible, but in reality, each added gene becomes increasingly complicated. Systems that use SSRs, ZFNs, or meganucleases that offer advantages such as smaller components that make introductions easier.

Ask scientists about the complexities of genome editing, and discuss the introduction of components into cells. They state that they are looking for transient systems that transmit editing enzymes as proteins instead of their genes so that the proteins are destroyed after functioning instead of being expressed continually. Limiting activity that way might reduce off-target effects. Gao observes that DNA-independent delivery of genome-editing systems may alleviate concerns about GMOs. "Proteins cannot incorporate into the genome," she says, "and the resulting plants should be considered non-GMO if no foreign DNA is provided at all."

CRISPR is already very strong and there are so many researchers working on it and other genome-editing technologies that they will probably keep developing, says Gao. "Researchers want to develop new methods and new technologies," she says, "and every day we are still seeing change. Now, in principle we say we can edit any target, but it will be true in five years.

SUMMARY

Over the last two decades, gene therapy for cancer has progressed fairly quickly, and currently few drugs are available commercially, although many are still in clinical trials. Most studies on gene therapy have shown strong safety profiles with tolerable transient toxicity. The lack of success in several clinical trials may be attributed partly to the selection of patients. Patients with advanced and treatment-resistant malignancies are now participating in gene therapy trials, close to the original chemotherapy results thirty years ago. Maybe gene therapy in patients with earlier stages of malignancies or those with lower tumour burden could be much more effective. Alternatively, after successful cancer treatment with full reduction of tumour load, such as after radical surgery, after radiation therapy, or after successful chemotherapy, gene therapy can be best used. Future widespread use of patient and tumour genomic analysis as well as host humoral and cellular immunity assessment should enable better selection of the most appropriate gene therapy per patient.

ABBREVIATION

AAV: Adeno Associate Virus
ADA: Adenosine Deaminase
Adv: Adeno Virus
APC: Adenomatous Polyposis Coli
ALCL: Anaplastic Large Cell Lymphoma
AML: Acute Myeloid Leukaemia
AR: Androgen Receptor
CAR: Coxsackievirus-Adenovirus Receptor or Chimeric Antigen Receptor
cDNA: Complementary Deoxyribonucleic Acid
CEA: Carcinoembryonic Antigen
CML: Chronic Myeloid Leukaemia
CNS: Central Nervous System
CRISPR: Clustered Regularly-Interspaced Short Palindromic Repeats
DC: Dendratic Cells
DIC: Disseminated Intravascular Coagulation
DNA: Deoxyribonucleic Acid
DSB: Double-Strand Breaks
DT: Diphtheria Toxin
EGFP: Enhanced Green Fluorescent Protein
EGFR: Epidermal Growth Factor
EMA: The European Medicines Agency

EP: Electroporation
FDA: Food and Drug Administration
FHIT: Fragile Histidine Triad
GDEPT: Gene-Directed Enzyme Prodrug Therapy
GM-CSF: Granulocyte Macrophage-Colony Stimulating Factor
GMO: Genetically Modified Organisms
GPAT: Gene-Prodrug Activation Therapy
GVHD: Graft-Versus-Host Disease
HCC: Hepatocellular Carcinoma
HDR: Homology Directed Repair
HSC: Hematopoietic Stem Cells
HIV: Human Immunodeficiency Virus
HSV: Herpes Simpex Virus
hTERT: Human Telomerase Reverse Transcriptase Promoter
ITR: Inverted Terminal Repeats
LCA: Leber's Congenital Amaurosis
LV: Lentivirus
LSEC: Liver Sinusoidal Cells
LTR: Long Terminal Repeat
MDM: Mouse Double Minute
MFs: Mycosis Fungoides
MHC: Major Histocompatibility complex
MLL: Mixed Lineage Leukaemia
MMP: Matrix Metalloproteinases
moDC: Monocyte-Derived Dendratic Cell
MRI: Magnetic Resonance Imaging
NHEJ: Non-Homologous End Joining
NIH: National Institutes of Health's
NK: Natural Killer cells
NOD: Nonbase Diabetic
NP: Nanoparticles
ORF: Open Reading Frame
PNP: Purine Nucleoside Phosphorylase
PRR: Pattern Recognition Receptors
RAC: Recombinant DNA Advisory Committee
RISC: RNA-Induced Silencing Complex
RNA: Ribonucleic Acid
RVD: Repeat Variable Diresidue
SCID: Extreme Combined Immunodeficiency
TALE: Transcription Activator-Like Effectors
TALEN: Transcription-Activators Such as Effector Nucleases
TCR: T-cell receptor
TLR: Toll Like receptors
TNF: Tumor Necrosis Factor

TRAC: T cell receptor α constant
TSG: Tumor Suppressor Gene
VEGF: Vascular Endothelial Growth factor
ZFN: Zinc Finger Nucleases

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Chapter 5
Gene Editing and Gene Therapies in Cancer Treatment

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ABSTRACT

In past years, several novel treatments have been given by gene therapies for the treatment of cancer. Gene-based therapeutic approaches include gene transfer, oncolytic virotherapy, and immunotherapy. Gene Transfer or gene editing is the most recent treatment method that allows the insertion of new genes into the cancer cell to mediate the slow growth or death of the cancerous cell. Gene transfer is a very flexible technique, and a wide range of genes and vectors are being used in clinical trials with positive results. CRISPR/Cas9 is found to be a promising technology in cancer research. It helps to dissect the mechanism of tumorigenesis, identify the target for drug development, and helps in the cell-based therapies. Oncology virotherapy uses viral particles that are capable of replicating within the cancer cell and results in cell death. Oncology virotherapy has shown great efficiency in metastatic cancer. In immunotherapy, cells and viral particles are genetically modified before being introduced within the patient's body to trigger the host immune response to destroy cancer cells.

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INTRODUCTION

World Health Organisation (WHO) defines cancer as a large group of diseases characterized by the uncontrollable growth of abnormal cells in the body. Such cells gain the ability to go beyond their usual boundaries and invade neighbouring cells/tissues/parts of the body. When the cancerous cell becomes malignant, the process called metastasizing starts which are considered as the major reason for death from cancer. Neoplasm and malignant tumours are other names to refer to cancer. Globally, in 2018, cancer accounts for an estimated death of about 9.6 million i.e., one in every 6th person dies off due to cancer. Men are found more prone to lung, prostate, and colorectal cancer being the top 3, apart from stomach and liver cancer while in women breast, colorectal, lung, cervical, and thyroid cancer is found more prevalent (Bray et al., 2018). Considered as an important barrier to increasing life expectancy, researchers, scientists, and biotechnologists are trying hard to make early detection accessible, provisions for quality treatment, and survivorship care to the sufferer (Lagergren, 2019). Amongst other highly successful and reliable methods/processes to overcome this global burden, gene editing, and gene therapy are gaining high importance. Gene therapy uses gene(s) as pharmaceutical agents to treat genetic disorders. It promises to prevent the mortality rate by providing innovative treatment options (Cross & Burmester, 2006). On the other hand, Gene editing offers a way to rewrite the genome of the species wherein the mutated genes are revised, spliced, or substituted with the functional and healthy gene at the DNA level. This nullifies the effect of the existing mutated gene by giving its healthy version. Owing to its importance in treating diseases, science personnel approach this by identifying the precise location of mutated genes and exchange them with the functional one (Ormond et al., 2017). With no permanent and clear-cut cure for cancer, gene editing is thought of as a cutting-edge tool to restore the effectiveness of treatments and gene therapy as one of the most recent and best approaches to reduce the mortality rate by targeting gene expression of the genome (Das et al., 2015). Gene editing and gene therapy have revolutionized the world of biotechnology by giving and understanding an insight into cancer born as a result of the genetic defect. Cancer cell genome exhibits multiple genetic and epigenetic variations such as the ineffective working of enzymes during DNA methylation, histone acetylation, methylation of histone proteins amongst many other factors which are responsible for the pathogenesis of the disease (Cheng et al., 2019). Further development of such variation introduces disturbance in cell signalling, cell division, and growth, cell motility, etc. that transforms normal cell to tumour resulting in malignancy at later stages (Sever & Brugge, 2015). Considered as one of the most serious diseases, cancer offers challenges to both human lives as well as public health. The majority of the on-going clinical trials based on gene therapy are focused to come up with a solution that has a cure for cancer (Wirth & Yla-Herttuala, 2014). Finding novel ways to treat cancer has become important because the present therapies are not sufficient because of the toxicity they offer. RNAi strategies, prodrug activating suicide gene therapy, oncolytic virotherapy, immunomodulation based on gene therapy, anti-angiogenic gene therapy, gene defect correction/compensation, manipulation of the genes involved in the pathways leading to apoptosis and invasion by tumorigenic factors, and antisense therapies are a few gene modulation methods that are reported to be employed for cancer treatment. Gene therapy has been used to target various types of cancer such as the brain, breast, lung, pancreatic, liver, skin, ovarian, colorectal, bladder, prostate, head and neck, and renal cancer (Roma-Rodrigues et al., 2020). Treating cancer with therapeutic approaches has gained much importance because of its ability to activate the host's immune response against cancer cells by killing it. Currently, recombinant vaccines for cancer are being developed that unlike other vaccines works by boosting the patient's immune system to identify

cancer affected cells and show up to the antigenic determinants and another immunomodulatory cell/ debris (Cross & Burmester, 2006). The success of gene therapeutic approaches is based on safe, effective, and controllable vector delivery into the human cells. Multiple clinical trials have been made and drugs were approved clinically (Lundstrom, 2018) that used viruses for gene (RNA/ DNA) delivery but offered several constraints such as immunogenicity, increased risk of cancer due to the therapeutic payload insertion near the genes controlling cell growth and limited production of viral vectors on a mass scale. With these outcomes, the development and engineering of non-viral vectors came into play with the support of nanomedicine (Sun et al., 2019).

With considerable advancement in human genomics over the past few decades, it was known that somatic aberrations play an important role to provoke normal cells to turn into cancerous cells. This led cancer researchers to find potential cures by playing with the genes involved in the disease thereby finding therapeutic approaches that can overcome it effectively, reducing the mortality rate to a minimum number. Previous researches to treat cancer using gene therapy approach includes the transfer of genetic material into a host cell with the help of virus (oncolyticvirus) or bacteria and non-viral vectors, altering the immune response of tumour cells or the host immune system to the desired level (immunomodulation) and manipulating the microenvironment around tumour cells to reduce vascular tumours or to enhance the antigenic effect of tumour cells for better response by the host immune system. With the application of gene therapy, modest successful attempts have been made with minimal side effects in treating cancer (Amer, 2014).

Gene editing stands as a promising tool to treat cancer as it offers an opportunity to play with the tumour cells at the molecular level and find the way out by manipulating the existing aberrations leading to this disease. A study on the well-characterized molecular alterations will enable the scientists to edit genes *in vivo* hence providing a platform allowing direct manipulation at the gene level and correcting it to reduce the cancer risk. One such example already reported in a study is the feasibility of the abovementioned process in the correction of TERT mutations in glioblastoma (Troike & Lathia, 2020).

Another tool of gene editing is CRISPR/ Cas9 that has evoked a revolution in the field of biological science offering tremendous potential to treat deadly diseases and cancer is one of them. When tumour cells are left unidentified, they invade the surrounding cells and progress in terms of growth. This happens due to the suppression of the immune system by tumours or when tumours find other mechanisms to evade immune surveillance. Researchers have used CRISPR and CRISPR- associated protein 9 (cas9) endonuclease to target multiple genes present in T cells and engineer them for successful cancer immunotherapy and concluded it a promising tool to be implemented in improving the condition of the cancer patient. Edward A. Stadtmauer and his team carried out research to treat tumour cells with the application of CRISPR/ Cas9 in which they targeted three genes *viz.* TRAC, TRBC, and PDCD1. TRAC and TRBC are genes encoding T cell receptors (TCR). T cells were engineered by deleting these two genes which were responsible for mispairing of TCR and this deletion led to the enhanced expression of TCR transgene which was specific to cancer i.e., NY-ESO-1. The third gene PDCD1 consisting of protein programmed cell death protein-1 and encoding the programmed cell death function was removed which has facilitated the antitumor immunity, the final aim of the research. T cells modified in this way persisted in the body for a good time indicating the feasibility of the technique and lower immunogenic risk (Stadtmauer et al., 2020).

GENE THERAPY

What is Gene Therapy?

Gene therapy targets the delivery of genetic material into hosts' target tissue or cell, to increase a therapeutic impact after its expression. Gene therapy has an advantage over traditional treatments it can be delivered locally. This results in a high therapeutic dose without creating negative systematic antagonistic impacts. Moreover, most of the gene treatments have one-time applications, in the long term, they are expensive. In the coming years, this practice may help doctors for the treatment of many diseases by inserting the gene into the host (patient's) cell as a replacement for surgery and drugs. Scientists are working to test some other aspects of gene therapy, like;

- Knocking out or Deactivation of a gene that is either not functioning properly or mutated.
- Insertion of a new gene into the cell that will help the body to generate a more effective immune response to fight against disease (Pagliarini et al., 2015)
- Deletion of a mutated gene that is causing disease and replacing it with a healthy copy of the gene (Hong et al., 2014).

In several diseases like cancer, certain viral infections, and inherited disorders treatment, gene therapy is a promising technique. But the safety and effectiveness of this treatment process are still under study to overcome the risk associated with it. Currently, gene therapy is only tested against the diseases that have no treatment or having less survival rate like cancer. For successful treatment of cancer by gene therapy some fundamentals have to be considered like an appropriate and specific target to be modified or replaced, a vector to take gene of interest to the cell, and appropriate expression of a gene of interest having a therapeutic effect in the target cells. Apart from high therapeutic efficacy, safety is essential in the treatment.

Gene Therapy for Cancer

Cancer is a genetic disease that has been evolved from the transformation of a single cell by chemicals, and physical environmental factors and virus influence. These effects ultimately result in a mutation in which a lot of gene change is observed. The deactivation of tumour suppressor genes and activation of oncogenes is the major transformation a cell goes through in cancer.

Some biological activities are observed in transformed cancer cells. These activities include inhibition of apoptosis, uncontrolled proliferation, replicative immortality, proliferative signals, angiogenesis, metastasis, and invasion (Hanahan & Weinberg, 2011). Traditional chemotherapy mainly focuses on the killing of tumour cells directly. But now a majority of target-specific therapies are directed to eliminate one or more above-mentioned cancer cell activities. Gene therapy to be used as a treatment for cancer can be mainly divided into three categories: Immunotherapy, gene transfer, and oncology virotherapy.

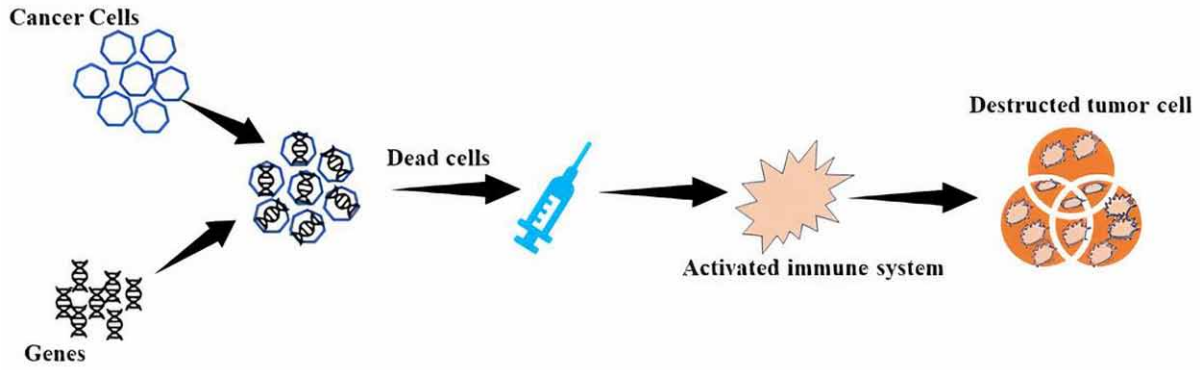
Immunotherapy

Immunotherapy is a technique that is used to enhance the immune system to target and destroy the cancer cells. This technique is in practice for the last 100 years for the treatment of cancer. Though traditional

immunotherapy has shown limited success, as cancer cells have a special mechanism that suppresses the immune system. But now a wide range of gene therapy techniques are being to overcome the previous limitation (Armstrong et al., 2001).

Presently gene therapy is used for the development of recombinant vaccines for cancer. These vaccines are not same as that for an infectious agent, the motive of this vaccine is not to prevent disease, but to cure or contain it by training or enhance the memory of the patient's immune system to recognize the cancer cells by presenting it with highly immunostimulatory and antigenic cellular debris. Firstly, the cancer cells are collected from the patients or the cancer cell lines maintained in the laboratory (*in-vitro*). These cells are engineered with the addition of one or more genes, in a way that they can be more easily recognized by the immune system. In most of the cases, the gene is cytokine genes that produce highly antigenic protein genes or molecules that stimulate a pro-inflammatory immune response. These engineered cells are maintained *in vitro* and killed, and then the cellular contents are incorporated into a vaccine (Figure 1) (Kowalczyk et al., 2003).

Figure 1. Immunotherapy with altered cancer cells

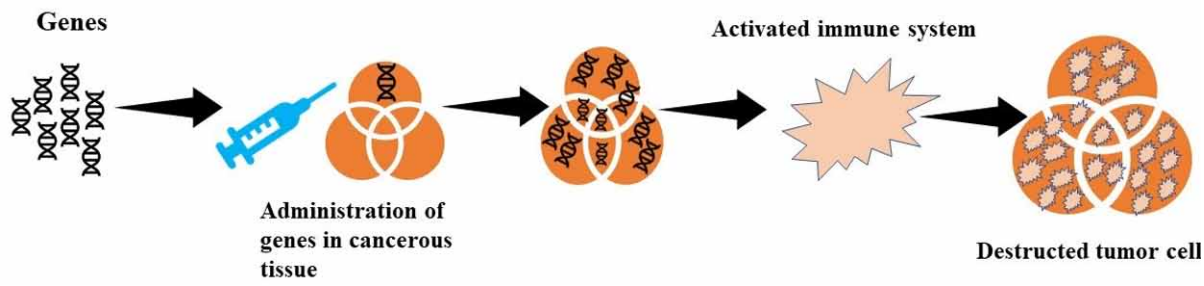


Another way of Immunotherapy is by inducing immunostimulatory genes, (cytokines), into the tumour *in vivo* (Figure 2). A wide range of cytokines have been tested to do far like, GM-CSF, IL-2, IL-4, IL-12, IL-18, IL-24, IFN-a, and IFN-g (Dranoff, 2002; Choi et al., 2011; Shashkova et al., 2007).

The main objective of immunotherapy is to enhance the presentation or recognition of tumour-associated antigen (TAA's). Immunotherapy has faced some common challenges like natural tolerance towards the highly immunosuppressive tumour microenvironment and tumour-associated antigen. Intense research has been carried out on the genetic engineering of T cells. Against a known TAA, the introduction of the T-cell receptor (TCR) is a perfect example of genetic engineering (Kershaw et al., 2013). *Morgan et al.* reported that the transduction of normal peripheral blood lymphocytes (PBLs) by a retroviral vector with an anti-MART1 TCR gene isolated from tumour-infiltrating lymphocytes (TILs) of cancer infected patient. After two months of cell infusion in 15 patients, durable engraftment of T-cells exceeding a level of 10% of peripheral blood lymphocytes was observed. They also observed a high level of circulating sustained engineered PBLs even after one year of injection into patients.

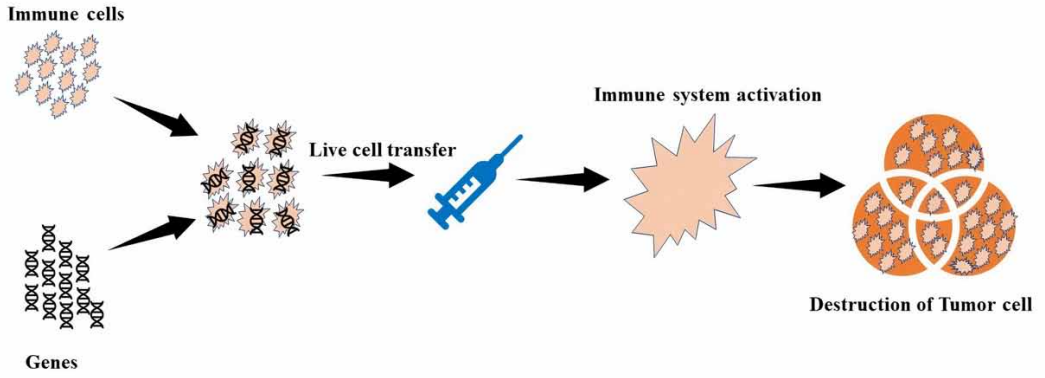
One more report of a clinical trial in which, against an antigen NY-ESO-1, transduction of T cells with a T-cell receptor resulted in the expression of cancer/testis (CT) in different cancer (Robbins et al., 2011). However, in this trial patients showed evidence that targeting a TAA by TCR represents a promising option for cancer treatment.

Figure 2. Immunotherapy with genes in vivo



Recently, immunotherapy has gained much popularity in which T cells that are genetically modified with chimeric antigen receptors (CAR) are found to be successful in advanced cancer (Figure 3) (Barrett et al., 2014). CAR is a combination of the receptor of a T-cell signalling domain and an antibody (Ab) derived targeting domain expressed on T-cells using a retroviral vector (Sun et al., 2014).

Figure 3. Immunotherapy using altered immune cells



GENE TRANSFER

Gene transfer is one of the most exciting treatment categories of gene therapy. In this treatment technique, a foreign gene is introduced into the cancer cell or tissue surrounding it. Genes introduced into the cancer cell have functions including antiangiogenetic gene, cellular stasis gene, and suicide genes. In clinical trials for the delivery of these genes, several viral vectors have been used. Replication incom-

petent adenovirus is the most commonly used viral vector. Oligodendromer and naked DNA transfer are non-viral gene delivery modes whereas electroporation and DNA coating viable gene delivery modes (Patil et al., 2005). The type of vector selected for the delivery of the gene for therapy depends upon the specificity, and time under which the gene must be expressed to show therapeutic effect. For example, antiangiogenetic genes such as statin-AE, and sFLT-1 needs to be continuously expressed in a way to perform the therapeutic effect and are delivered using a plasmid containing a transposon for the insertion of a gene into cellular DNA (Ohlfest et al., 2005).

However, an adenoviral vector which is replication-incompetent and is containing the gene of herpes simplex virus thymidine kinase (HSVtk) just needs transient expression to accomplish cell death and is generally delivered via an adenoviral vector (Sadeghi & Hitt, 2005).

Table 1. Immunomodulation in gene therapy for cancer treatment

Predominant action	Examples	Commercially available
Immune enhancement	Antibodies blocking cytostatic T-lymphocyte antigen 4(CTLA-4) Inhibitors for malignant melanoma	Ipilimumab
Passive immunotherapy	Antibodies against • human epidermal growth factor receptor-2 (HER/2) receptor protein in breast cancer • CD20 Protein on lymphoma cell • epidermal growth factor receptor (EGFR) Receptor on squamous cancer • CD20 Protein on chronic lymphocytic leukaemia (CLL) • human epidermal growth factor receptor-2 (HER/2) receptor protein in breast cancer • CD20 Protein on chronic lymphocytic leukaemia (CLL) • CD20 Protein on lymphoma cells • CD52 Protein on chronic lymphocytic leukaemia (CLL) • CD20 Protein on lymphoma cells • epidermal growth factor receptor (EGFR) Receptor on colorectal Cancer • CD30 Protein on Hodgkin lymphoma cells • human epidermal growth factor receptor-2 (HER/2) receptor protein in breast cancer	• Trastuzumab • Ibritumomab • Cetuximab • Ofatumumab • Pertuzumab • Pertuzumab • Rituximab • Alemtuzumab • Tositumomab • Panitumumab • Brentuximab • Ado-Trastuzumab
Active immunotherapy	• Antigen-specific plasmid-based vaccine: (human epidermal growth factor receptor-2) HER/2, prostatic acid phosphatase antigen (PSA), Modified carcino embryonic antigen (CEA) vaccine. • vaccine for Single Tumourcell surface antigen. • Vaccine for genetically modified tumour cells: Using Recombinant fowlpox virus, Vaccinia virus, Poxvirus. • Tumour cells, irradiated as vaccine	
Adoptive immunotherapy	• Genetically modified activated T-lymphocytes • Activated dendritic cells • Autologous activated T- lymphocytes • Chimeric antigen receptor integrated T-lymphocytes • Genetically modified dendritic cells	Sipuleucel-T

[#]Commercially approved medication by FDA (till 1st July 2014).

Viral Vector: Retroviral vectors, Lentiviral vectors, Adenoviral vectors, Adeno-associated viruses (AAV), Baculoviruses, Herpes simplex virus (HSV), Poxviruses, and Vaccinia viruses are the most commonly used viral vectors for gene transfer. These vectors differ from each other regarding their cell expression profiles, tropisms, immunogenicity, transgene capacities, and indifferent duration of transgene expression.

Viral vectors can also be divided into non-integrating and integrating vectors. Examples of non-integrating vectors are baculoviruses and adenoviruses. These vectors are not able to integrate their genome into the host genome. Whereas, retroviral, lentiviral as well as adeno-associated viruses have the potential to integrate into the host genome. Integrating vectors most often results in long- term expression. The safety of such vectors is of concern because of the fact of integration of the transgene into the host genome. This is due to its occasional observation of integration with retroviral vector at an actively expressed site (Montini, 2011; Matrai et al., 2010).

Ex vivo approach of gene transfer is also possible. In this, the gene is induced into the cell ex vivo (outside the patient), into autologous cells that were previously isolated, which are then reintroduced back into the patient.

Presently, *adenoviruses* are one of the most dominant vectors for gene delivery used in gene therapy. Around 50 different serotypes of adenoviruses have been identified, which are divided into subgroups of six (A–F) (Sharma et al., 2009). Out of it, commonly used in gene therapy are serotypes 2 and 5. A limiting factor of adenoviruses is that its detectable level by pre-existing antibodies is found to be in 97% individuals, which most often will affect the efficiency of transduction and its therapeutic outcome.

Adeno-associated viruses are ssDNA viruses. These viruses are unable to cause infection until they are co-infected by the helper virus, like herpes simplex virus or adenovirus. Broad host range, longer gene expression, and low immune response are the advantageous features of these viruses. Eukaryotic adeno-associated virus is an example of such viruses, it contains adenovirus and parvovirus that's why it is also known as chimeric virus vector (Nuesch et al., 2012). It has a potential of transfecting quiescent and mitotic cells, pathogenicity and immunogenicity in humans, and stably integrates into DNA of the host at the desired location within chromosome-19 in cell culture.

Retroviral vectors are derived from retroviruses 7-10 kb single-stranded linear RNA having a lipid envelop. Specific receptors for retroviruses are expressed on mammalian cells as soon as the viral particle enters it (Overbaugh et al., 2001). As the viral enters the mammalian cell the virus RNA is transcribed into double-stranded DNA (dsDNA) by the action reverse transcriptase enzyme. By utilizing binding proteins, the pre-integration complex (PIC) a nucleoprotein, is formed by the dsDNA in the cytoplasm (Yi et al., 2011).PIC integrated the genome of the host by migrating into the cell nucleus. The main advantage of a retroviral vector to be used in gene therapy for cancer is the only expression of its transgene in the dividing cells that prevents undesired expression in nondividing cells of tissues. The long-term expression of the transgene is because of the integration of the retroviral gene in the desired cell of the host.

Herpes simplex virus is naturally neurotropic, dsDNA viruses that are enveloped and have a size of around 150 kb. At first, they infect humans at the genital and oral mucosa, then they spread to nerves (sensory) to replicate at the ganglions (sensory). Reactivation of viral may leads to encephalitis, oral ulcerations, or even skin rashes. Around 80% of the human population is seropositive towards the virus (Sharp et al., 2002). With the advancement of genetic engineering a new genetically engineered an oncolytic recombinant replication-selective herpes simplex virus. This virus has many advantages like it has broad potent and tropism in cell lysis of tumour, it can escape the host immune system. The major advantage is its capacity to transfer a large amount of transgene.

Lentiviral vectors are derived from a retrovirus. These vectors can integrate the transgene into the genome of the host that can result in long term expression of the gene. Transduction of both nondividing and dividing cells is the most advantageous feature of these vectors and thus makes it more suitable and efficient than retrovirus. At the entry of the cell, the improved targeting strategy and transcription of the

transgene has made it to be more appropriate in gene therapy trials (Wold & Toth, 2013). But, accidental integration into the genome of the host as of retrovirus is the limitation of lentiviral to be used as a vector.

Poxviruses vectors were the first ones to be employed in gene therapy. Previously they were used in the in-vitro creation of live vaccines and proteins. In genetic cancer trials, the attenuated form of Poxviruses has been used (Moss et al., 1996). The poxviruses have a feature to act as an immunostimulatory that makes them more appropriate to induce immunity against tumours.

Baculoviruses are viral particles having dsDNA of 80-180 kb in size. Naturally, they can infect the insect cells. No disease in humans related to baculoviruses has been reported yet. They have the potential to carry around 40 kb of the transgene, with easy manipulation, production, and multiple inserts (Airenne et al., 2013). *Autographa California* has a circular dsDNA of 135kb in size, it is multiple nucleopolyhedroviruses (AcMNPV) that is one of the most use baculoviruses in studies related to gene therapy. In the production of Cervarix (GlaxoSmithKline), a human vaccine component, AcMNPV is already approved in gene therapy related to cervical cancer (Zhang et al., 2014).

Viral vectors have emerged to be effective as a gene transfer tool. However, the drawbacks associated with them are, their inflammatory potential, their immunogenic potential has commended the development and exploration of new vectors for gene delivery i.e. non-viral vectors.

Non-viral vectors: The naked plasmid DNA (pDNA) is the simplest form of non-viral vectors. The naked pDNA is advantageous in a way that it possesses less toxicity and other unwanted reactions. The formulation and production of naked plasmid are easy and cheap. But it also holds a disadvantage of less efficiency for transfection compared to viral-mediated vectors. To overcome the disadvantages like enhancement of uptake and transfection of plasmids i.e. transfection efficiency, lipid formulation, and cationic polymers have been developed (Heyde et al., 2007). These lipids or polymer formulations are advantageous in a way that they are comparatively easy to be designed to attain specific properties. A report suggests that a non-viral vector can target a cell or tissue by linking cell-specific targeting moieties or tissue on the carrier. Moreover, the cellular internalization, biodistribution, and intercellular trafficking of the nano or microparticle can be affected by determining the size (Pathak et al., 2009).

Cationic lipids are the most commonly used gene delivery agent (Legendre & Szoka et al., 1992). In cationic lipids, the head group binds to DNA and the collapsing of DNA lipid complex is enabled by the lipid tail (Paul et al., 2003). Through an endosomal pathway, the lipoplexes (cationic lipid DNA complex) enters the target cell. The efficiency of transgene expression is low with this complex. Results stated that only a minor part of the DNA injected can reach the tumour tissue. The gene delivery with the lipid-based formulation is found to be limited with the intra-tumoral application. A potential risk of adverse immune reaction is associated with this vector administration. In cancer gene therapy trials, it can act as a potential technique by reducing systemic toxicity by developing a systemic lipid delivery system. When folate targeted LPD complexes is administered systemically in a study on an animal model of breast cancer have shown a positive result in the tumour size and has increased the survival (Bruckheimer et al., 2003).

In gene therapy, the easiest method for DNA delivery is the delivery without using synthetic or virus vector. This includes mechanical methods of gene delivery like, gene gun method and microinjection in which DNA is coated with the gold nanoparticles. Naked DNA is injected into the muscle that led to in vivo expression of DNA (Cheng et al., 1993). In current researches, this technology has shown a promising application in the development of vaccines for cancer. Studies related to this have successfully helped in the development of antitumor immunity towards melanoma and colon cancer (Sobol & Scanlon, 1995). This DNA delivery method is restricted to cells around the injection point and it doesn't target tissue.

Another method of gene therapy approach is electroporation. The mechanism of action behind this is the disruption of the cell membranes with high power electric pulses that results in nanopores formation on the surface through which chemotherapeutic agents, the gene of interest (GOI), and naked DNA moves into it (Kelly & Russell, 2007).The major advantages of this approach include its effectiveness in different cell types, lack of genome integration, less immunogenicity, and easy administration. This makes it potential for plasmid DNA based gene therapy. This technique is used in gene therapy clinical trials related to prostate cancer, malignant melanoma, leukaemia, and colorectal cancer (Shirely et al., 2013). The success and efficiency of non-viral methods of gene therapy rely on numerous intracellular and extracellular barriers. Gene delivery systems efficacy is also affected by it, like an endosomal escape, cellular uptake, gene expression, and nuclear uptake (Heyde et al., 2007; Escoffre et al., 2010).

Table 2. Gene transfer in gene therapy for cancer treatment

Principle action	Examples	Commercially existing
Viral • ssDNA viruses • ssRNA viruses • dsDNA viruses • dsRNA viruses • dsDNA viruses	• Adeno-Associated: Parvovirus • Lentiviruses: human immunodeficiency virus-1 (HIV-1), HIV human immunodeficiency virus-2 (HIV-2), Simian IV, Feline IV • Adenoviruses: CG870, Ad5-CD/TKrep, Ad5-D24, Gutless adenovirus, OBP-30 Recombinant H103, • Reoviruses • Herpetic viruses: TVEC, Herpes simplex 1,	ONYX-015
Non-Viral	Nanoparticles, Electroporation, transposon, cationic liposomes, synthetic viruses, hydrodynamics	
Bacterial	Salmonella, Escherichia coli, CEQ508, Listeria, Clostridium,	

#Commercially approved medication by FDA till 1st July 2014.

Oncolytic Virotherapy

In the wake of the achievements in the technology to eliminate cancer, oncolytic virotherapy (OV) has gained considerable attention and is seen as a good option for the patients who either don't respond to chemotherapy or are unable to exhibit expected responses to the treatment inhibiting the immune checkpoints with the aid of oncolytic viruses (OVs). OVs offers the following advantages:

a. Prefer to replicate in cancerous cells
b. Once engineered, possess the ability to express transgene to supplement the cytotoxic effect and enhance the immune-based activities
c. Holds the ability to regulate tumour microenvironment, working towards eradicating tumour cells (locoregional metastasis or systemic) based on immunostimulatory actions
d. Offers various mechanisms to destroy tumour cells
e. Does not interfere with the standard therapies

OVs work by entering the tumour cells and releasing tumour-specific antigens that evoke innate as well as adaptive immune responses (Harrington et al., 2019; Motalleb, 2013). This method has been used to get tumour vaccines for human gene therapy trials (Motalleb et al., 2009). OVs have a unique mechanism of action i.e., different from the conventional ones to kill cancer cells and hence are not affected by the normal drug or radiation therapy. Therefore, it becomes necessary to know if cancer-initiating cells (CICs) are affected with OVs or not. OVs showing replication competency are gaining interest to be used as a therapeutic agent to treat cancer (Motalleb et al., 2009; Cripe et al., 2009)

Genetic engineered vectors that can act as a lytic agent have an improved therapeutic index have shown great potential in OVs. Herpes simplex virus (HSV), parvovirus, adenovirus, Newcastle disease virus, and retrovirus are the most used modified OVs. Adenoviruses have the potential to infect a broad range of tumour cells, non-dividing, and dividing normal cells. These viruses have the potential to be genetically engineered for specific types of gene therapy that can be conditionally replicative (CRAds) or tumour-selective oncotropic nature.

Raihan J. and their team show the possible tumoricidal activity of Newcastle disease virus (NDV). NDV-AF2240 which is a Malaysian strain to treat breast cancer in mice. Production of cytokine from the virus was elucidated, which was responsible to carry out the tumorigenic effect by suppressing the tumour growth in the breast region when injected into the tumour cells (Raihan et al., 2019).

CRISPR-CAS9: A CUTTING-EDGE TOOL TO COMBAT CANCER

Despite the advent of modern medicine and attainments made in treating cancers, still, the number of people dies due to cancer consequently, more considerable and efficient efforts are in need to treat cancer (Thun et al., 2010). CRISPR/Cas9-mediated genome editing holds the potential to exhibit a role in cancer therapy (Yao et al., 2015). Genome or gene-editing technologies are based on editing genetic material on particular positions in the genome by inserting, removing, or modifying the genetic material (Maeder & Gersbach 2016). Numerous approaches to genome editing have been developed and the modern one is known as CRISPR-Cas9 (Clustered regularly interspaced short palindromic repeats-associated protein 9).

CRISPR-Cas9 is found in bacteria and archaea and categorized as an RNA mediated adaptive immune system. These are used to identify, destroy, and eradicate viral genetic material thus provide immunity against subsequent infections. Cas9 is an enzyme that utilizes CRISPR sequences as a guide RNA (sgRNA) to cleave the complementary DNA strands to the CRISPR sequence (Khadempar et al., 2019). CRISPR sequences along with the Cas9 enzyme constitute a technology identified as CRISPR-Cas9. It is a fast-developing editing technology and is emerging as a promising technology for genome editing. There are three distinct types of categorizations of the CRISPR/Cas9 system. These are types I, II, and III, though the presence of type I and III are observed in archaea and bacteria both, while type II is distinctive to only bacteria (Makarova et al., 2011). CRISPR/Cas9 system regulates endogenous gene expression in combating cancer (Yi & Li, 2016). The specific target gene can activate or repress by the dCas9. The dCas9 is taken on to the specific target DNA sites by gRNAs (Hilton et al., 2015). An additional approach to gene editing is epigenome editing which is based upon tethering of dCas9 protein and histone modifiers need to change DNA methylation. Due to the involvement of numerous epigenetic factors in cancer, targeting epigenetic machinery can be a safe and efficient approach to treat cancer (Enríquez, 2016).

Table 3. Advantages and disadvantages of common vectors used in gene therapy of cancer patients

Vector	Advantages	Disadvantages
Adenovirus (35 kb)	• Transduction efficiency is high • Capable of infecting different type of cells • cell division is not necessary for infection	• Doesn't integrate • Require packaging cell line • Replication competence • Toxicity • Immunogenicity • Insert size, 4-5 k • No targeting
Adeno-associated virus (5kb)	• Doesn't contain the viral gene • cell division is not necessary for infection	• Requires packaging cell line • Low safety • No targeting • Insertion capacity is 5kb
Retrovirus (10 kb)	• Able to integrate • Cell division is required for transduction	• Requires packaging cell line • Transduction efficiency is low • No targeting • Insertion capacity of 9-12 kb • Replication competence
Avipox virus (260 kb)	• cell division is not necessary for infection • Insertion capacity is less than 4 kb	• Toxicity • Immunogenicity • No targeting • Low efficiency • Less safe
Vaccinia virus (260 kb)	• Large insertion capacity of 25 kb	• Toxicity • Immunogenicity • No targeting • Low efficiency • Less safe
Baculovirus (80-230 kb)	• Protein expression is at a high level • Gene can be transferred to the liver	• Toxicity • Immunogenicity • No targeting • Low efficiency • Less safe
Herpes simplex virus (152 kb)	• Potential expression • Large (40-50 kb) insertion capacity • Neuronal tropism	• Requires packaging cell line • Toxic
Liposome	• No size and nucleic acid type limitation • Completely synthetic	• No targeting • Less efficient
Protein/DNA complex	• Cell-specific targeting • No size and nucleic acid type limitation	• Doesn't integrate • Toxic • Less safe • Less efficient in vivo • Immunogenic
Mechanical administration	• No nucleic acid size limitation	• Surgical procedure requirement • Less efficient • No targeting

CAR T- cell is an emerging technology in treating various cancers. This technology is based upon modifying the primary human T-cells by CRISPR-cas9 by introducing exogenous chimeric antigen receptors (CARs). These CAR T-cells are grown ex-vivo through genetic engineering and thus transfuse into the patient (Figure 4) (June et al., 2018; Mollanoori et al., 2018). This strategy may bring anti-cancer effects to patients by assisting host-mediated immunity by killing cancer cells and thus relieving from

the adverse outcome of conventional cancer treatment. Another clinical trial is focusing on killing the CD19-negative malignant cells by CRISPR-edited dual specificity of CD19 and CD20 or CD22 CAR T-cells (Schubert et al., 2016). The availability of CAR T-cell therapy is lessened to a large section of society due to its high cost, conversely, additional progress in CRISPR-Cas9 technology might be efficient in lowering its cost thus making it available to a larger group of patients (June et al., 2018).

Another approach to anti-cancer therapy is the genome editing of oncolytic viruses using CRISPR/Cas9 in killing cancer cells. The genetic modifications in these viruses made it to lack its virulence against normal cells but preserve its ability to attack and destroy cancerous cells with deficient antiviral defences. This strategy was found to be more effective when used in the onset of cancer when the immune system is more intact (Stanziale & Fong, 2003).

Adoptive cell therapy (ACT) can be employed in cancer therapy by ex vivo *PD-1* gene deletion in T-cells by CRISPR/Cas9 technology and the reintroduction of *PD-1* gene deleted in T-cells into patients, where these cells will trigger the immune response by addressing to the tumour with the chance to suppress tumour (Martinez-Lage et al., 2018). Anti-PD-1/PD-L1 and anti-CTLA-4 antibodies are used in blocking immune checkpoint by stopping checkpoint molecule triggered exhaustion and signifies an influential tool for tumour management. The PD-1 knockout T-cells approach has been tested in six clinical trials with lymphoma, lung, prostate, gastric, renal cell, bladder cancer, and carcinoma (Rao et al., 2017).

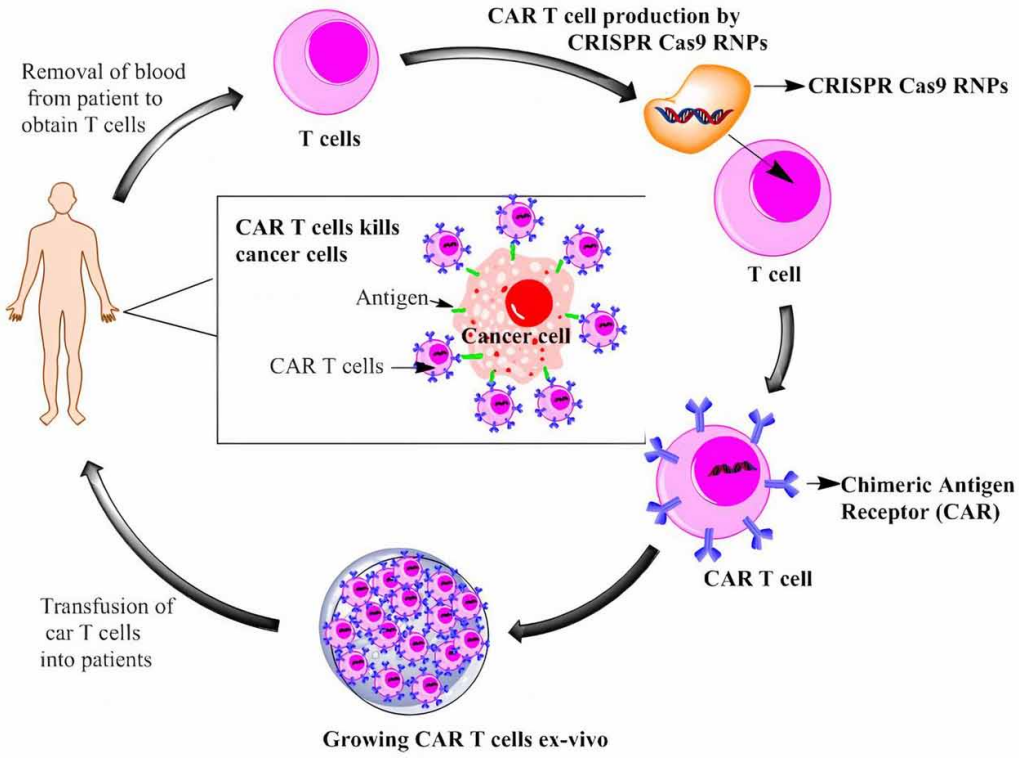
Even though in clinical trials ACT therapies have revealed promising outcomes on leukemia and lymphoma, still, during trial stages several patients died because of neurotoxicity and cytokine release syndrome (Mellman et al., 2011). Meanwhile, the only approved therapy from the FDA is CAR T-cell therapy for the treatment of relapsed and refractory B-cell acute lymphoblastic leukemia in pediatric and young adults (Rosenbaum, 2017).

FUTURE PROSPECTS OF GENE EDITING/THERAPY

Genome editing is one of the most important contributory ways of biological research that expands the range of gene therapy applications. Though existing gene therapy to fight against cancer is succeeding much better than earlier ones, there still needs improvement in some areas. At specific sites in random genome editing would encounter low applicability and efficiency barriers. Genome editing tools have been established to develop the therapeutic approach by directly targeting the gene loci to treat different diseases. The most effective strategy of gene therapy is the CRISPR/Cas9 system, with the development of these gene-editing technologies, more establishment of the clinical phenotype disease model occurs (Strong & Musunuru, 2017).

The exploration of novel drug targets will be achieved in the future by CRISPR/Cas9 technology which enables the screening of lethal interactions of genes in cancer lines on a genetic and epigenetic level. Further, CRISPR/Cas9 technology enables to alter one of the noncoding region of the genome of cancer cells. This approach provides a new tool for practical analysis of the feature of cancer cells that are poorly characterized. The clinical practice of genome editing has been eased by the development of engineered nucleases such as CRISPR/Cas9, ZFNs, and TALENs. The development of such more engineered nucleases and the combination of genome editing technology with tumour immunotherapy in the future will provide a much better and easier way for clinical practices of genome editing (Li et al., 2020). The effective effect of CAR T-therapy in treating the B-cell malignancies brings its way to pharmaceutical companies and clinics in 2017 and got approved officially for human disease treatment. But its high

Figure 4. Outline of the CRISPR-cas9 technology for gene therapy through Cas9 ribonucleoproteins (RNPs) for the treatment of cancer



cost and its capacity in killing solid tumours are still unclear which is making it unaffordable to common people. Thus, more efforts are needed to lower the cost of CAR T-cell therapy to make it affordable to cancer patients. Recently viral vector loaded with therapeutic elements exhibits a potential role in gene therapy in combating cancer. The exposure of adeno-associated virus (AAV) increases organ specificities of genome editing elements in different tissues. Regardless of the significant advancement of viruses mediated genome editing, there are still a few limitations that obstruct clinical applications (Mingozzi & High, 2011). The risk of oncogenesis and mutagenesis can be increased due to the random integration of the lentiviral vector (Baum, et al., 2006). On the other hand, the AAV virus due to its packing capacity limitations causes the death of patients in clinical trials (Zetsche et al., 2015). Thus, an effective and safer therapeutic approach is needed to treat cancer by adenoviral-mediated gene therapy. The combination of nonviral viral and vectors may be an ideal strategy for viral-mediated genome editing for treating cancer.

Simultaneously, gene-editing technology provides a tool in the development of epigenetic modifications, gene expression regulation, and therapeutic drug development in cancer therapy. Ongoing trials of gene therapy cancer vaccines are going to be fulfilled and that might be incorporated into cancer therapeutic treatment. Despite tremendous development in gene therapy there still needs additional innovative genome editing complexes, reduced toxicity, and more specificity during the deliverance procedure that will make genome editing technology more efficient and effective to patients. Gene editing technology might play an efficient and novel role in eliminating cancer due to its potential to illuminate the biological mechanism in cancer expansion.

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Chapter 6
Epigenetic Regulation of Breast Cancer

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ABSTRACT

Breast cancer is a carcinoma of mammary glands, which starts off as abnormal proliferation of ductal cells. This could, then, become either benign tumours or metastatic carcinomas. It is one of the most common causes of deaths because of cancer, and is one of the most common types of cancer in women in the whole world. India along with the US and China accounts for one-third of the breast cancer burden. The breast cancer carcinogenesis is attributed to epigenetics, which is the study of the reversible changes in the phenotype without any change in the DNA sequence. Genes, which are concerned with proliferation, anti-apoptosis, invasion, and metastasis, have been seen undergoing epigenetic changes in breast cancer. Cancer can be caused either by global hypomethylation (causing activation of oncogenes and leading to chromosomal instability) or by locus-specific hypermethylation (causing repression of gene expression and genetic instability due to inactivation of DNA repair genes). Other epigenetic mechanisms involved in carcinogenesis are histone modification and nucleosomal remodeling.

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INTRODUCTION

Cancer has profound socio-economic consequences on people, which causes family impoverishment and social inequality. In 2012, 6,00,000–7,00,000 deaths were caused by cancer alone (Mallath. et. al., 2014). It is one of the most growing diseases in the world today, everyday more and more people are succumbing to cancer; therefore, it is the need of the hour to invent effective treatment strategies for the same. Cancer occurs when cells lose the ability of contact inhibition, which causes the cells to divide without any control. In normal cells, the timing of the cell division is under strict constraints, and many signals are involved which dictate when a cell can divide and how often a cell can divide. In cancer cells, this restraint is lost and hence cells grow uncontrollably. Cancer develops when any kind of cell in our body proliferates abnormally, and since we have hundreds of types of cells, there are a variety of types of cancer. The two main types of tumors that all the cancers are classified into are Benign Tumors and Malignant Tumors. Benign tumors are localized to a particular location, and neither do they invade surrounding tumors nor do they spread to distant organs. Skin tumors usually fall in this category. Malignant tumors on the other hand invade the underlying basement membrane and intravaste the blood vessels and spread to distant organs, this phenomenon is known as Metastasis. For instance, the usual metastatic sites for breast cancer cells are bone, lung, liver and brain. This process by which particular cancer cells metastasize from their primary location to the locations of their preference is called Tropism. The extent of severity of cancer is deduced by the extent of metastasis that had happened in a cancer patient.

The type of cancer which is behind highest death rate among females in the world is breast cancer. Breast cancer starts when a few cells in the breasts become abnormal and start dividing uncontrollably and ultimately form a tumour. Surprisingly, breast cancer can also develop in males, where it starts off in the lining of the ducts (ductal cancer). In women cancer can also develop in the milk producing glands (lobular cancer). Most inherited cases of breast cancer are due to BRCA1and BRCA2 genes whose function is to repair the DNA damage and keep the breast cells growing normally. The alterations in these genes is the cause of developing breast cancer. BRCA gene mutations account for only 10% of all the breast cancers, this low incidence of BRCA-mediated breast cancer is because these mutations are passed on from generation to generation. Women with BRCA gene mutations often have the family history of breast cancer (Genetics, Breastcancer.org). Other genes involved in development of breast cancer are HER2, EGFR and c-Myc genes. Aging is one of the biggest risk factor of breast cancer, the cases of breast cancer increase with increasing age. Family history of breast cancer also a risk factor. Endogenous and exogenous estrogen is a risk factor too. The main source of exogenous estrogen is oral contraceptives and hormone replacement therapy (HRT).

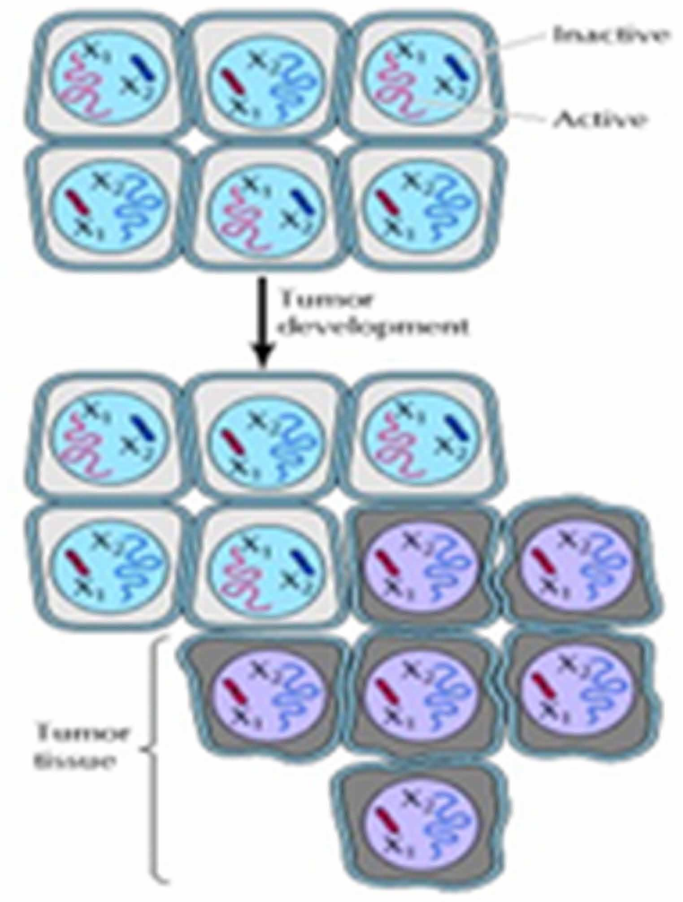
Among many strategies in place to treat cancer, one of the most promising one is the epigenetic therapy. Epigenetic changes are the changes in the gene activity/phenotype without the any alteration in DNA sequence. These changes can be transmitted to daughter cells, although experiments have shown that these changes can be reversed. These are the changes that can lead to development of cancers, and these changes are being exploited so that they can be reversed using natural and synthetic agents. Epigenetic changes are natural and essential at times, but if they occur improperly, they can be fatal. The major identified epigenetic changes are methylation, acetylation, deacetylation, phosphorylation, and ubiquitylation. Chromatin re-modeling and imprinting are other types of epigenetic changes that are identified. There are two kinds of genes involved in tumour development – Tumour Suppressor Genes (TSGs) and Oncogenes. TSGs are the ones that have anti-cancer effects and oncogenes have pro-cancer effects. Epigenetic changes in the tumour suppressor or oncogenes is the reason why cancer develops.

There is extensive research going on to find naturally occurring or synthetic agents that can be used to reverse epigenetic changes and hence prevent cancer development. Inhibitors of various enzymes involved in epigenetic process, like DNA Methyltransferases and Histone Deacetylases, are being tested to gauge their anti-cancer effects.

CANCER

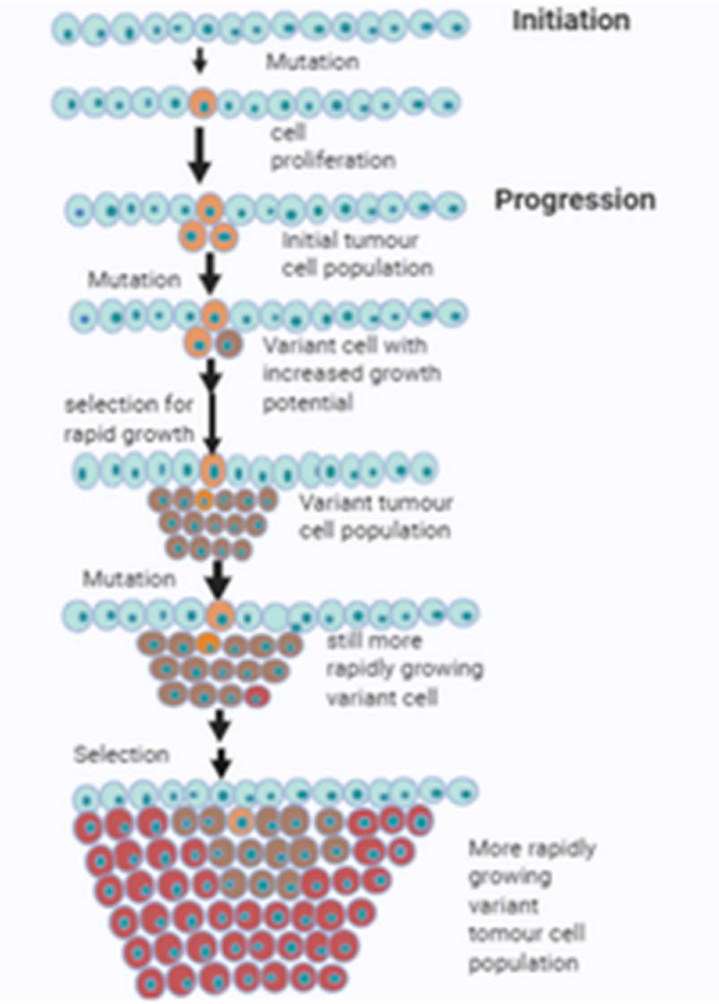
Tumor clonality is the origin of tumors from the single cells which proliferate abnormally. The single-cell origin of tumors is backed by the study of X chromosome inactivation, where one member of the X chromosome pair is inactivated by being converted to heterochromatin in female cells. X-inactivation happens at random during embryonic development, therefore one X chromosome is inactivated in some cells while the other X chromosome is inactivated in rest of the cells. In normal tissues, cells with different inactive X chromosomes are present, consequently, expression of both alleles is seen in a heterozygous female. On the other hand, tumor tissues generally express only one allele of a heterozygous X chromosome gene. This suggests that all of the cells of a tumor are derived from the single cell of origin, in which the X inactivation pattern is determined before the tumor developed (Fig. 1).

Figure 1. Tumour clonality



At cellular level, carcinogenesis is a multistep process, and the first step is tumour initiation. It happens because of genetic changes leading to abnormal proliferation of a single cell. This is followed by the outgrowth of clonally derived tumor cells (Fig. 2). Cancer progression continues as more mutations occur in cells. Some of these mutations have an advantage like more rapid growth, and hence the descendants of the cell with such a mutation will become dominant in the tumor population. This is called Clonal selection, since a new clone of tumor cells has evolved because of its enhanced growth rate or other properties (such as survival, invasion, or metastasis) that have a selective advantage (Cooper, G. M., & Hausman, R. E., 2000).

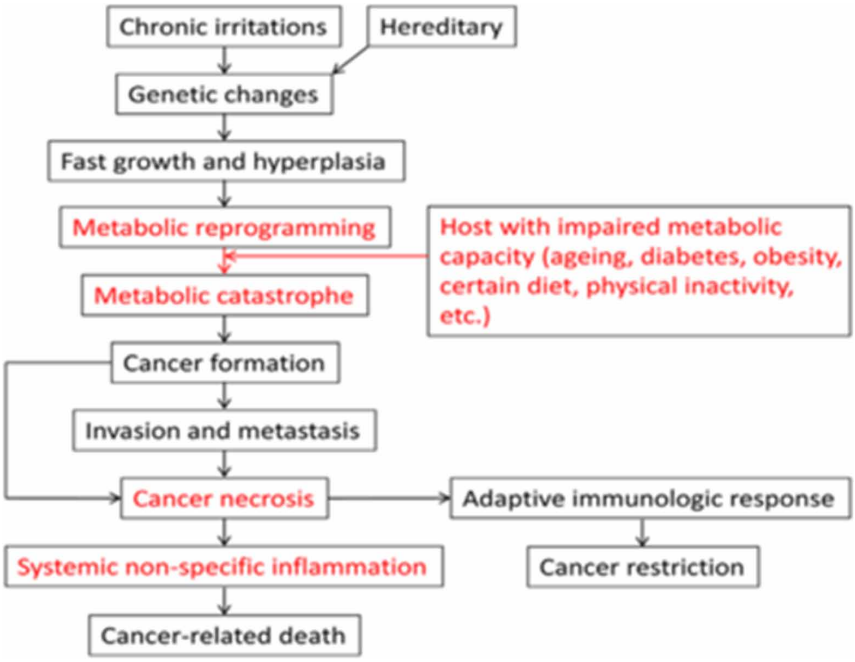
Figure 2. Stages of tumour development



The mechanism of cancers can also be viewed by the help of Integrative theory of cancer. In this, chronic irritations cause tumors with genetic mutations and rapid proliferative ability. Tumor cells adapt the metabolism and exercise aerobic glycolysis to grow rapidly. The patient provides the nutrients to

support tumor growth via the tumor microenvironment. In conditions such as aging, diabetes, obesity and a high-fat diet, the exhaust system is impaired, triggering a metabolic imbalance between the tumor and host, which results in metabolic catastrophe, making tumor cells reside in a hostile environment and forcing them to invade, metastasize and undergo necrosis (Fig. 3). This theory views cancer in integrative manner and suggests that genetic alterations and tumor-host interaction decide the destiny of tumor and host. Although cancer is a genetic disease, tumor biology is basically the nature of the host (Luo, G., & Liu, N., 2019).

Figure 3. Integrative theory for Cancer



Classification of Cancer

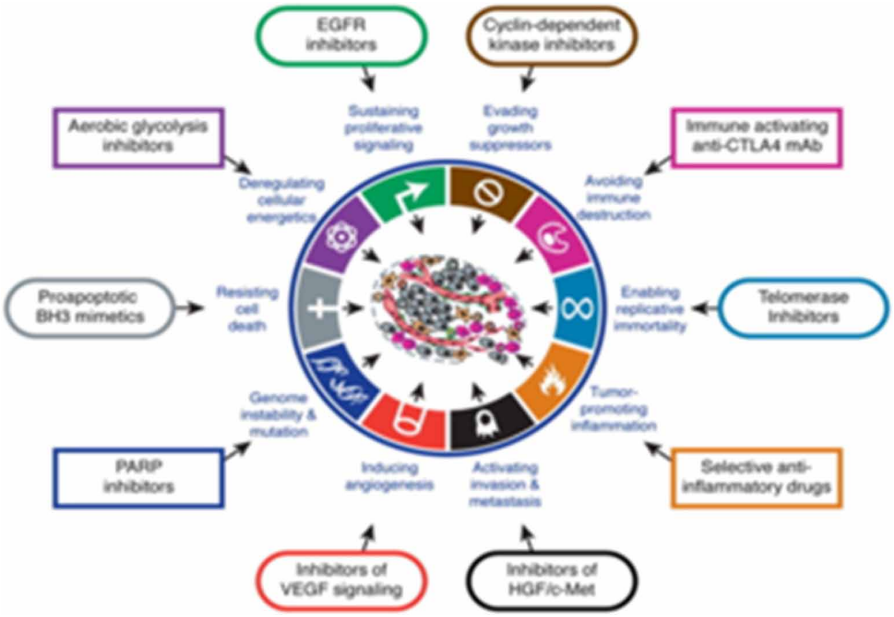
Cancer is classified on the basis of the type of cell it arises from. Typically, cancers belong to either one of the classes – Carcinomas, Sarcomas, Leukemias, Lymphomas. Carcinomas are amount to 90% of the human cancers (Cooper, GM., 2000). They are the type of cancer which arise from the inner or outer lining of organs, i.e. from epithelial cells. This cancer develops from either of the ectodermal, mesodermal and endodermal layers during the time of embryogenesis. The most common examples of carcinoma are lung and breast cancer. Sarcomas are the type of cancer which arise from the connective tissue, and are rare in human beings. The example of sarcoma is bone cancer. Leukemias and Lymphomas are somewhat related in the sense that they both arise from immune cells. Leukemia arise from hematopoietic stem cells in the bone marrow, it usually results in abnormal high number of white blood cells in the patient. Leukemias are classified into 4 broad subtypes – Acute Lymphoblastic, Acute Myelogenous, Chronic lymphocytic and Chronic Myelogenous (Davis, AS., 2014). Lymphoma arises from infection-fighting

cells on immune cells, which are called Lymphocytes. These cells are present in all the lymph nodes and lymphoid organs like spleen, thymus and bone marrow.

Hallmarks of Cancer

Robert Weinberg and Douglas Hanahan described 10 hallmarks on cancer (Fig. 4). These are the properties which distinguish cancer cells from normal cells.

Figure 4. Hallmarks of cancer given by Weinberg and Hanahan
(Source – cell.com)



Sustained Proliferative Signaling. Normal cells require external stimuli to proliferate, but cancer cells produce their own proliferative signals. This decreases their dependency on outside growth signals and hence they become independent. This is brought about by 3 ways: they synthesize their own growth factors and stimulate themselves via autocrine signaling, or they overexpress growth factor receptor which makes them hypersensitive to the ambient levels of growth factors, or they alter the cytoplasmic circuitry which transmits the information from receptors and trick the cells into synthesizing abundant growth factors.

Evading Growth Suppressors. Normal cells are subjected to growth suppressors which turn off cell division, but cancer cells manage to overrule them and continue proliferating. A normal cell stops dividing when it comes in contact with other cells, phenomena called Contact Inhibition, but a cancer cell would not stop. Retinoblastoma (Rb) is a tumour suppressor protein and is a direct regulator of cell cycle. Rb traduces growth inhibitory signals and decides whether a cell should divide or not. Defects in Rb pathway can lead to sustained proliferation.

Resisting Cell Death. Normal cells undergo programmed cell death when they grow old or get damaged, but cancer cells escape apoptosis and accumulate in the body. Cancer cells have developed various strategies to escape cell death, like loss of P53 (a tumour suppressor protein) function, hiking up the expression of antiapoptotic regulators (like Bcl-2, Bcl-Xl) and downregulating some pro-apoptotic Bcl-2 related factors (like Bax, Apaf-1). Cancer cells can also resort to autophagy to become (reversibly) dormant when stressed, this way cancer cells escape the anticancer therapy.

Enabling Replicative Immortality. In normal cells, the continuous loss of telomeres causes the cells to undergo apoptosis or p53-dependent cell cycle arrest in order to maintain a healthy number or normal cells. Cancer cells however recruit Telomerase which extends the telomeres by adding a short stretch of nucleotides at the 3' end of chromosomes, and hence cancer cells keep on replicating.

Inducing Angiogenesis. Like all cells cancer cells also need oxygen and nutrients to grow, hence they induce the formation of new blood vessels, a phenomenon called Angiogenesis, by secreting growth factors like basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Tumour associated angiogenic factors can be produced either by the tumour cells or by inflammation. This hallmark is one of the bases of imaging the location of tumors during screening or post-treatment checkups.

Invasion and Metastasis. Invasion is the spreading of cancer cells to their immediate surrounding and metastasis is when cancel cells travel to a distant location and gets anchored there. It is believed that cancer cells spread from their primary location because the environment in the tumour gets stressful for cancer cells. Epithelial to Mesenchymal Transition (EMT) is an essential step of invasion and metastasis. The cells invade the underlying basement membrane, and eventually enter blood stream through leaky openings of the newly formed blood vessels and they can metastasize to other organs.

Deregulating Cellular Energetics. Cancer cells consume more glucose than normal cells, yet they reduce it to lactate (anaerobic respiration), whether oxygen is present or not, instead of $C0_2$ and save energy, this is called Warburg Effect. Lactate causes acidification of cells which promotes invasion and metastasis. The energy and the molecules are redirected towards synthesizing more proteins, lipids and DNA required by growing cells.

Avoiding immune destruction. Cancer cells evade immune response by various strategies like paralyzing cytotoxic T-cells and natural killer cells by secreting transforming growth factor (TGF) beta or other immunosuppressive agents, or expressing immunosuppressive cell surface ligands like programmed death-ligand 1 which inhibit the cytotoxic effects of T-cells.

Genome Instability and Mutation. This property acts as an enabling hallmark of cancer. Normal cells, when genetically damaged, are subjected to either DNA repair or apoptosis, but cancer cells do not undergo DNA repair, and hence remain mutated. DNA damage can occur because of external agents or impaired DNA repair system due to epigenetic changes. The types of gene instability are – Nucleotide Instability (e.g. Xerodermapigmentosum), Microsatellite Instability (e.g. Lynch Syndrome) and Chromosomal Instability (e.g. Breast cancer).

Tumour promoting Inflammation. This is another enabling hallmark of cancer. Inflammation has paradoxical effects on tumour in the sense that it enhances tumorigenesis by supplying bioactive molecules, such as growth factors, to the tumour microenvironment. Chronic infections, smoking, alcohol consumption, obesity are the risk factors for cancer and all these factors are linked to cancer through inflammation. There are some similarities between tumour growth and wound healing, i.e. they both require survival and migration of cells and both require the formation of new blood vessels. (Hanahan, D., & Weinberg, R. A., 2011)

Risk Factors of Cancer

Cancer can be prevented and avoided to a great extent if its causes and risk factors are known. If we know the reason behind development of cancer, we can deduce if the cancer is preventable or not. These factors increase the chances of developing cancer:

- Tobacco is the single most leading cause of deaths due to cancers. It is the cause of lung cancer, the most prevalent of all cancers. Second hand smoking also increases the risk of cancer by 5%. Many of the chemicals present in tobacco smoke are carcinogenic, such as arsenic, benzene, chromium, nickel, formaldehyde, toluene, etc.
- Ageing is another risk factor in development of cancer. The chances of cancer occurring increases manifold in people with age more than 55.
- Alcohol consumption causes 3.6% of all cancers. Cancers of mouth, throat, esophagus, stomach, colon, prostate, kidneys, breast, ovaries, etc due to excessive alcohol usage. Liver metabolises around 7 gms of alcohol per hour, and upon its metabolism, acetaldehyde is produced which is a carcinogen. Exposure to acetaldehyde might cause a defect in alcohol dehydrogenase gene, and tumour suppressor gene (like BRCA) is inactivated which can cause cancers of upper GI tract, breast and liver cancers.
- Infections cause around 18% of all cancer cases.
    - Human Papillomavirus (HPV) is the leading cause of cervical cancers. Infection with oncogenic strain of HPV is essential for it to develop cancer.
    - *H. pylori* is the leading cause of stomach which can lead to MALT-lymphoma in stomach and esophagus cancers. Stomach cancer responds poorly to treatments.
    - Hepatitis B and C viruses both can cause liver cancer.
    - HIV infected people are at greater risk of developing lymphoma and kaposi's sarcoma.
    - Human T cell lymphoma virus – 1 (HTLV1) can cause lymphomas.
    - Epstein Barr Virus can cause Burkitt's lymphoma.
    - Human herpesvirus – 8 (HHV8) is the leading cause of kaposi's sarcoma.
    - *S. typhi*can cause gallbladder virus.
    - *S. bovis*can cause colon cancer.
- Sexual steroid hormones, like androgens, estrogen and progesterone, can cause the growth of certain cancers of breast, ovaries and endometrium. Diethylstilbestrol is a form of estrogen which has shown to increase the risk for breast and ovarian cancers. In men, prostate gland is controlled by the hormone Testosterone which is associated with increased prostate cancer risks.
- Sunlight has UV radiation which can cause the early ageing of skin and can lead to skin cancer. The UV radiation can also come from sunlamps and tanning booths. The UV rays can cause skin damage like benign tumors, freckles, pigmentation, discoloration, destruction of elastin and collagen proteins and cancers such as basal cell, squamous cell carcinoma, and Melanoma.
- Pollution of air, water and soil cause 1.4% of all cancers. Even if the individual chances of getting cancer because of pollution is low, yet the exposure of large population to the carcinogens is high. The worst affected people are the ones who are poor and who do not have the financial means to improve their quality of life.

- Food carcinogens could be natural or manmade. The example of a natural carcinogen present in food is Aflatoxins, which is a mycotoxin produced by *Aspergillus* fungi which lives on grains and groundnuts. Aflatoxins in association with Hepatitis B virus can cause liver cancer. Food can also be contaminated by synthetic carcinogens like insecticides and pesticides. Carcinogenic compounds in food can also be accumulated while cooking for example, polycyclic aromatic hydrocarbons.
- Genetic susceptibility of causing cancers amount to approximately 4% of all cancers. Women with BRCA1 mutation, although rare, have 70% chance of developing breast and ovarian cancers. Around 20 cancer syndromes are caused by single gene mutations (Sloan, FA., Gelband, H., et al 2007; Parsa, N., 2012).

Breast Cancer

The breast has mainly two types of tissues i.e., glandular and stromal (supporting) tissues. Glandular tissues contain the milk-producing glands (lobules) and the ducts (the milk passages), whereas Stromal ones entail fatty and fibrous connective tissues of the breast (Fig. 5). It also consists of lymphatic tissue, which is an immune component that gets rid off of cellular fluids and waste. Most breast tumors are benign, for example, fibrocystic tumour is a non-cancerous condition in which cysts (accumulated packets of fluid) are developed, fibrosis (formation of scar-like connective tissue), lumpiness, thickening, tenderness, or breast pain. Most of the breast cancers origin in the cells which line the ducts (ductal cancers). Some begin in the cells that line the lobules (lobular cancers), etc. (Sharma, G. N., et al., 2010).

This cancer is a heterogenous type which includes different entities with specific biological and pathological features. It is the main cause of mortality due to cancer among women in Indian metropolitan cities like Delhi, Mumbai, Bangalore, Pune, Kolkata, etc. in northeast and rural India it still holds the second position. Approximately 1.67 million cancer cases were diagnosed in 2012 which made up around 25% of all cancers. The numbers are more in less developed regions (883,000) than in developed regions (794,000). The mortality rate is between 6 in Easter Asia to 20 per 100,000 in Western Africa. The approximate number of breast cancer patients in India during 2012 was 145,000 cases with age standardized incidence rate of 25.8 per 100,000 women. The estimated number of deaths in India in the year 2012 was 70,000 (Manoharan, N., 2017).

Types of Breast Cancer

According to site:

- **Noninvasive breast cancer.** The cells remain confined to their primary location instead of spreading to the other sites. For example, Ductal Carcinoma in Situ is the common non invasive cancer and amounts to 90% of all breast cancer, and Lobular Carcinoma in Situ is less common and its presence marks increase risk.
- **Invasive breast cancer.** These cancer cells spread through the ducts and lobes of the breast and spread to surrounding tissues, such as fatty and connective tissues. It may or may not be metastatic (Fig. 6).

Figure 5. Diagram of a human breast

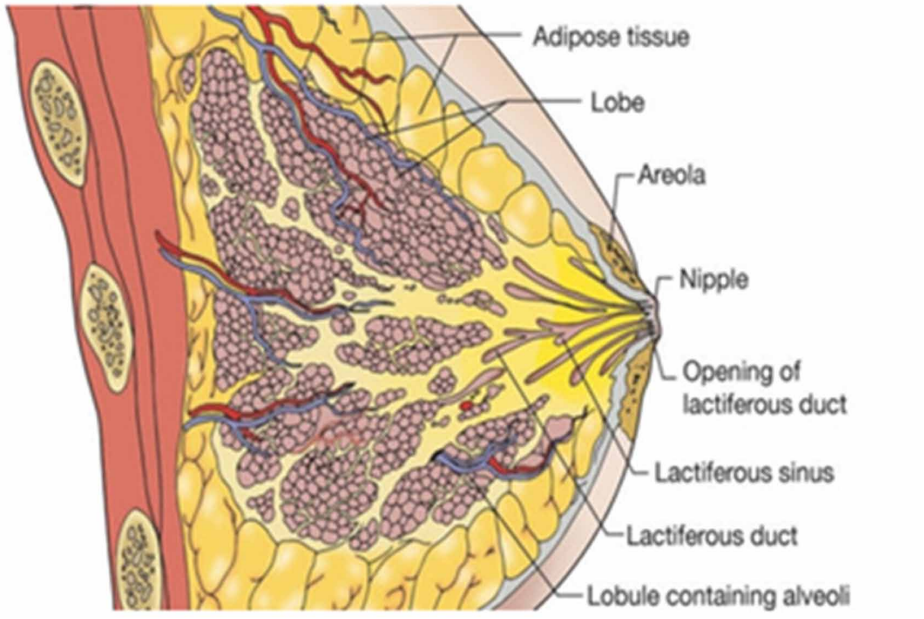
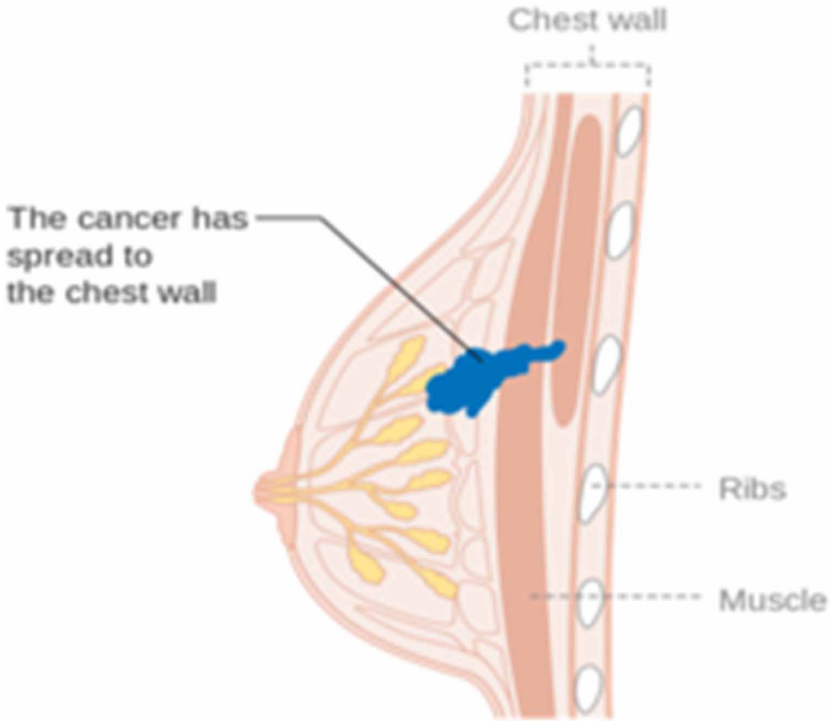


Figure 6. Invasive breast cancer



Frequently occurring breast cancer:

- **Lobular Carcinoma in Situ.** It occurs in the milk glands/lobules.
- **Ductal Carcinoma in Situ.** It occurs in ducts, for example, Ductal Carcinoma.

Infiltrating cancers:

- **Infiltrating Lobular Carcinoma.** This happens when LCIS starts metastasizing to other body parts. It accounts for 10-15% of breast cancers.
- **Infiltrating Ductal Carcinoma.** This happens when DCIS metastasizes to other body parts.

Less commonly occurring breast cancer:

- **Medullary carcinoma.** It is an invasive cancer that makes a boundary between normal and tumour tissues.
- **Mutinous/Colloid carcinoma.** It is rare and formed by mucus producing cells.
- **Tubular carcinoma.** It is a type of invasive carcinoma which has better prognosis than other types. It accounts for 2% of the breast cancer.

Inflammatory breast cancer:

In this type of cancer, breasts appear inflamed (red and warm) with thick ridges, on skin, due to the blockage of lymph vessels by cancer cells. Though this cancer is rare, yet it is fast growing.

Paget's disease of nipple.

It usually appears in the milk ducts and then spreads to the skin of nipple and areola. It accounts for 1% of breast cancers.

Phylloides tumour.

It can either be benign or malignant, and it begins in the connective tissues of the breast. It is very rare, and it can be treated with surgery (Sharma GN., 2010).

Molecular Subtyping of Breast Cancer

Gene Expression Profiling (GEP) has allowed us to characterize breast cancer tumors which has lead to advent of cancer prognosis in terms of identifying patients with good prognosis so as to regulate the chemotherapy given to the patients. Sorli et al. has given 5 molecular subtypes of breast tumors – Luminal A, Luminal B, Her2 over expression, Basal like and normal tumors. The difference between these subtypes is seen at the molecular level with respect to gene expression of all the types of tumour cells.

1. Luminal A tumors
    - They represent ER+PR+HER2-, but this is not always true.
    - Their expression profile is similar of normal luminal breast epithelium.
    - These tumors have high ER regulated genes' expression.
    - They show lower expression of proliferation related genes.
    - They show under expression of Her2 gene cluster.
    - These tumors are more in common.
    - They are sensitive to endocrine manipulation.
    - They are a bit resistant to cytotoxic agents in anti-cancer therapy.
    - They make up for 40% of all breast tumors.
    - They are associated with favorable prognosis.

2. Luminal B tumors
    - They have low ER related genes' expression.
    - They show variable expression of HER2 cluster of genes.
    - They have a high expression of proliferation related genes.
    - They have genetic instability and TP53 mutations are observed in these tumors.
    - They make up for 20% of breast cancer.
    - These tumors have a higher risk of relapse.
    - Like luminal A, these tumors are also less sensitive to cytotoxic chemotherapy.
    - They are less common than luminal A and have poorer prognosis.

3. HER2 overexpression tumors
    - They represent 20-30% of all breast tumors.
    - They show high expression of Her2/*neu* genes and less expression of luminal cluster genes, like cytokeratins CK7,8,18 and 19, other luminal-related markers such as human endogenous retrovirus envelope PL1, X-box-binding protein 1, hepatocyte nuclear factor 3, GATA-binding protein 3, Annexin XXXI, and estrogen receptor 1.
    - They are HER2 + and ER/PR negative, but not always.
    - They have poorer prognosis than luminal A.

4. Basal like tumors
    - They represent around 15% of invasive ductal cancers.
    - Its name is comes from the fact that it shares expression profile of normal basal epithelial cells.
    - They express genes like keratin 5,6, and 17, integrin-β4, laminin, and fatty-acid binding protein 7.
    - They are triple negative tumors due to less expression of luminal and HER2 neu gene clusters.
    - They show increased BRCA1 mutations, genomic instability, high expression of proliferation cluster genes and a high histologic grade (Kittaneh, M., et al., 2013).

Hallmarks of Breast Cancer

1. Sustaining Proliferative Signaling

The immunohistochemistry (IHC) biomarkers in breast cancer include Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2), and these help in the subtyping of this cancer. Estrogen is behind the progression of cancer and ER is the biomarker that is helpful in prognosis of cancer. ER is a nuclear hormone receptor and it acts as a transcription factor. ER has 2 isoforms – ER alpha and ER beta. PR is a receptor for the hormone progesterone and it plays a role in downward signaling of ER. PR also has 2 isoforms – PR-A and PR-B. HER2 is a transmembrane tyrosine kinase receptor that controls cell growth, proliferation and survival through various signaling pathways. HER2 gene amplification is seen in 15-30% of tumors and it is a powerful prognostic biomarker. These hormonal and growth factor receptors mediate cellular signaling in breast cancer cells, for example estrogen enables the multiplication of breast cancer cells which have the receptors for estrogen. Breast cancer tumors have been divided into 4 subtypes on the basis of these biomarkers:

- ER+ PR+ HER2- - These cells have receptors for ER and PR, but not for HER2.
- ER+ PR+ HER2+ - These cells have receptors for all three biomarkers.
- ER- PR- HER2+ - They have receptors for only HER2.
- ER- PR- HER2- They do not have any kind of receptors. They are also called triple negative.

ER is an essential biomarker in breast tumors classification, and it is involved in breast cancer carcinogenesis. Inhibition of ER is the basis of anti-cancer endocrine therapy. ER+ tumors are less aggressive, well differentiated and respond well after the surgery than ER- ones. Patients with this kind of tumor have shown positive response to endocrine therapy targeting ER (e.g. Tamoxifen and Aromatase Inhibitors) and local and distant recurrence is prevented. PR activation depicts that ER signaling is active because ER regulates PR expression and PR positivity indicates functional estrogen – ER axis. If in case PR+ tumour is diagnosed along with ER negativity, then it could be a false diagnoses in terms of ER state. PR+ tumors make up to 65-75% of total breast cancer tumors. PR and ER are normally used together in breast cancer subtyping, like ER+PR+, ER+PR-, ER-PR+, ER-PR-. Double positive group makes up to 55-65% of breast tumors, and the patients with this type of tumour respond to endocrine therapy, are usually aged and have small sized tumors and lower mortality rates. Double negative tumors make up to 18-25% of tumors, do not respond to endocrine therapy and have high recurrence rate and have lower overall survival. Single positive phenotype tumors, like ER+PR- and ER-PR+, make up to 12-17% and 0.2-10% of all breast tumors, respectively. These tumors are usually large in size, usually aneuploid and show high expression of genes related to proliferation like EGFR and HER2. Single + tumors respond less well to endocrine therapy and their properties are somewhere lie between those of ++ and – tumors.

HER2 gene expression is also an essential biomarker in prognosis of breast cancer. It is how we deduce if the tumors are reacting well or not to the treatment. HER2 over expression is seen in response to anti-HER2 therapy which involves (1) monoclonal antibodies against HER2 (e.g. Trastuzumab and Pertuzumab), (2) inhibitors of tyrosine kinase receptors like lapatanib, and (3) antibody-drug conjugate of trastuzumab like ado-trastuzumabemtansine (Fragomeni, SM. et al., 2018).

2. Activating Invasion and Metastasis

Basal, EMT and stem cell biomarkers taken together represent the properties of activating invasion and metastasis. Basal like cancer is an aggressive carcinoma which frequently happens in younger women. Basal like carcinomas are negative for hormone receptors and positive for genes linked with basal epithelial cells like basal cytokeratin, P-cadherin, Beta 4 integrin and nestin (Choo, J. R., & Nielsen, T. O., 2010).Basal biomarkers like cytokeratins (CK) 5/6, 8/18, 14 and 17, and EGFR are most widely used.

Epithelial to Mesenchymal Transition (EMT) is the conversion of epithelial characteristics to mesenchymalones, this is often associated with the aggressiveness of cancer. EMT causes the cancer cells to lose epithelial biomarkers like E-cadherin, alpha and gamma catenins, and the cells gain mesenchymal biomarkers like fibronectin, vimentin and N-cadherin (Elzamly, S. et al., 2018). Markers of EMT include VIM, SNAI1, SNAI2, TWIST1, TWIST2, ZEB1, ZEB2, CDH1, CLDN3 (claudin 3), CLDN4 (claudin 4), CLDN7 (claudin 7).

Breast cancer stem cells (BCSCs) are small number of breast cancer cells which have the capacity of self renewal and they propagate heterogeneous populations of breast cancer cells. They are considered to be responsible for resistance to treatment and cancer relapse (Zhou J. et al., 2019). Molecules conventionally considered as stem cell markers include CD44, CD24, EpCAM, CD10, CD49, CD29, MUC1, THY1 and ALDH1A1.

3. Evading Immune Destruction

ER-PR-HER2- (triple negative) tumors are interferon-rich tumors, and the genes for interferons are overexpressed in these tumors. Examples of these genes are STAT1 and SP110. STAT1 is a transcription factor which regulates interferon regulated genes, and SP110 has prognostic value.

4. Resisting Cell Death

Breast tumors are associated with increased level of B cell lymphoma 2(BCL2) protein, which is a suppressor of apoptosis. The function of BCL2 is inversely correlated to that of TP53. Increased expression of BCL2 is related to low mitotic count, low S phase fraction, low cathepsin D expression, high degree of differentiation and lack of p53 function and tumour necrosis.

5. Genome Instability and Mutation

Genome instability of breast tumors can cause drug resistance in any breast cancer subtype. TP53 protein is responsible for tumour drug resistance. The protein is responsible for controlling cell proliferation, survival, apoptosis and genome instability by acting as a gatekeeper when cells are stressed due to DNA damage, hypoxia and oncogene activation. Therefore, lack of TP53 function can cause genomic mutations and hyperproliferation of cancer cells. P53 mutations are responsible in developing resistance to estrogen in ER+ tumors.

6. Deregulating Cellular Energetics

High amount of vit. D metabolites is related with low breast cancer risk, and its receptors, Vit. D Receptors (VDR), androgen receptor (AR), and ER are associated with tumour differentiation. On the basis of these receptors, breast tumors are differentiated into 4 tumors - HR3 (ER+AR+VDR+), HR2 (ER+AR+, AR+VDR+, ER+VDR+), HR1 (ER+, VDR+, AR+), and HR0 (ER-AR-VDR-) [HR – Hormonal Receptors]. This classification is different from the ER-PR-HER2 classification. One of the applications of this classification is the targeting of VDR and AR along with ER in patients which are being treated by hormonal therapy (Dai X. et al., 2016).

Genes Involved in Breast Cancer

BRCA genes are breast cancer susceptibility genes, which are of two types – BRCA1 and BRCA2. The proteins synthesized from them work in a single pathway to protect the genome. These proteins work at different stages of DNA damage response (DDR) and DNA repair. BRCA1 is a pleiotropic DDR protein that regulates checkpoint activation and DNA repair, while BRCA2 regulates the process of homologous recombination (HR). These genes are the tumour suppressor genes, and if a mutation in one of the genes happens, it results in hereditary breast and ovarian cancer (HBOC) syndrome which is inherited in autosomal dominant manner. Both the proteins, in addition to having similar disease phenotype, have similar roles as well, i.e. they both work in HR, which is a DNA repair mechanism that uses undamaged sister chromatid to repair replication associated DNA double stranded breaks (DSBs). HR is the main genome repair mechanism because the other processes cause chromosome deletions and translocations. The tumors that develop because of germline heterozygous mutations in any one of the genes have defective HR-mediated repair mechanism, this indicates that BRCA1-BRCA2 pathway is essential in HR mediated repair.

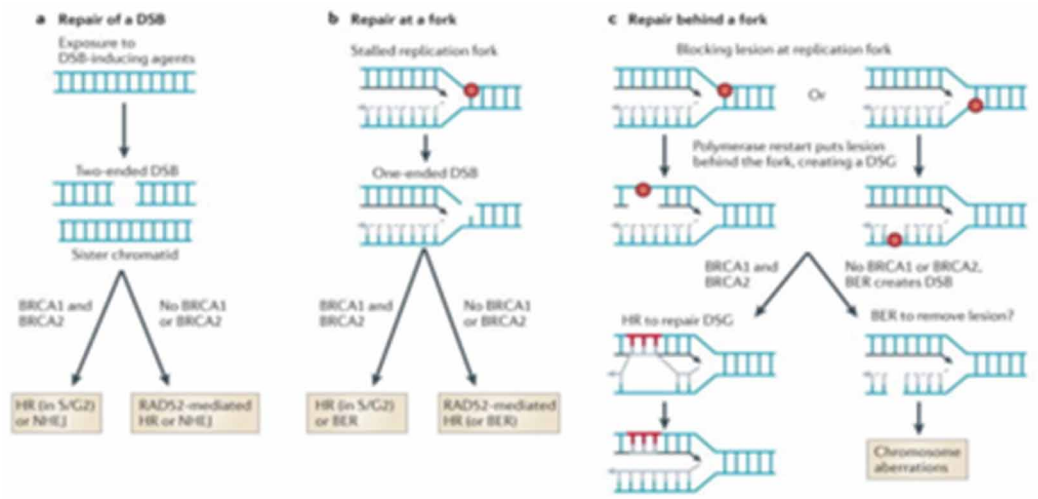
HR repair is prompted by ionization-induced one-ended or two-ended DSBs which are produced by the cleavage at a replication fork (Fig. 7). Single stranded lesions do not produce a strand break, and are overlooked by replication machinery and replication restarts downstream the lesion leaving behind as sDNA in which no double stranded break is present. Replication-associated one-ended DSBs or daughter strand gaps (DSGs) recruit BRCA1 and BRCA2, among other genes. BRCA1-mutants exhibit telomere dysfunction, chromosome translocations and chromatid aberrations, and BRCA2-mutants show chromatid breaks and abnormal exchanges, thus proving occurrence of BRCA1–BRCA2-mediated HR response to replication-associated DNA damage.

BRCA1 is involved in Non-Homologous End Joining (NHEJ) (a Double strand break repair pathway) and Single Strand Annealing (SSA) (an HR repair pathway to correct DSBs between two repeat sequences), other than in DDR signaling, checkpoint activation and HR. On the other hand, BRCA2 is mainly involved only in Homologous Recombination. The disorders related to *BRCA1*/*BRCA2* germline mutations are similar, and the common connection between the two BRCA proteins is the HR pathway. Hence, this pathway is important for protecting the genome and it is irregulated in cancers. In the BRCA1–BRCA2-mediated HR pathway, BRCA1 functions upstream of BRCA2, the function of which depends on BRCA1. In mammalian cells, Homologous Recombination can also occur via another option which is BRCA1–BRCA2-independent and is called RAD52-dependent pathway (a DSB repair protein). When BRCA2 does not function in a tumour, then RAD52 helps the cell to remain functional. Cells that are lack RAD52 and BRCA1 or RAD52 and BRCA2showlethality. Thus BRCA1 and BRCA2

are connected in a common HR pathway that repairs DSBs and damaged DNA replication forks. Heritable Mutations in HR pathway related genes lead to HBOC syndrome, suggesting that this pathway is crucial for tumour suppressing activity. Mutations in *BRCA1* and *BRCA2* are the main causes of HBOC syndrome (Roy R. et al., 2012).

There are many other genes involved in breast carcinogenesis. Increased cancer risks are associated with mutations of genes like HRAS1, GSTM1, GSTP1, CYP1B1 (codon 119), CYP2D6, CYP19, and VDR (ApaI and poly-A). Surprisingly, less breast cancer risks were seen in females homozygous for the variant allele for the intron 3, exon 4, and intron 6 polymorphisms of the Tp53 gene, the XbaI polymorphism in the ER gene, and PROGINS polymorphism in the PR gene. Polymorphisms of other genes like L-myc, NAT1, CYP1A1 (m2, m3 and m4), AR, UGT1A1, CYP1A1 (m1) and COMT are also associated with breast cancer risks (De Jong M. M. et al., 2002).

Figure 7. Homologous recombination at different types of DNA damage



Causes of Breast Cancer

The following are the causes of breast cancer:

- A woman who has had breast cancer before is more susceptible of getting cancer in the other breast.
- A woman has increased risk of developing breast cancer if other women in her immediate family have breast cancer. First degree relatives are more essential in estimating the risk.
- Genetic causes like mutations in BRCA1 and BRCA2 genes may also cause breast cancer.
- Hormonal replacement therapy, pregnancy at early age, use of oral contraceptives, and all other hormone related agents can cause breast cancer.
- Sedentary life style, diet abundant in fats, and obesity, especially in post menopausal women, are the main causes related to life style.

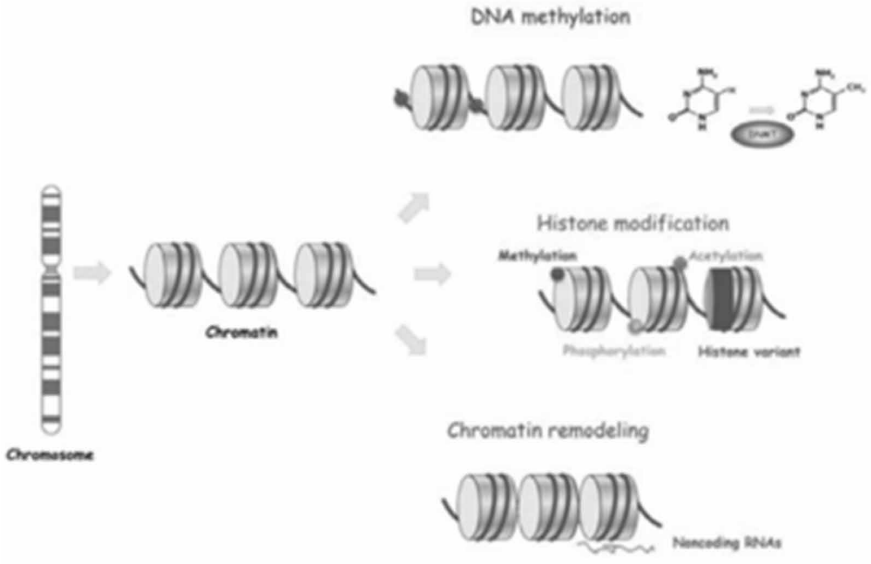
- Environmental causes, for example work atmosphere also leads to cancer development. For instance. Females working as X ray technicians who are exposed to low doses of X rays for a long time have increased risks (Sharma GN., 2010).

Epigenetics

For a long time, diseases have been explained just by genetic and environmental factors, but the epigenetic factors were started being considered nearly 50 years ago. Epigenetics has grown a lot in the past decade, and has attracted attention and holds promise of treating grave diseases like cancer. These modifications are working right from the beginning of life and continue regulating the functions of the body till the end. For example, right after the fertilization, DNA methylation (a type of epigenetic modification) patterns establish, reestablish and maintain themselves. These modifications are crucial for normal embryo and placenta development. All through life and on to the next generation, epigenetic events establish, maintain, erase and reestablish themselves. Cancer results from the accumulation of genetic and epigenetic alterations in the genome (Golbabapour S. et al., 2011).

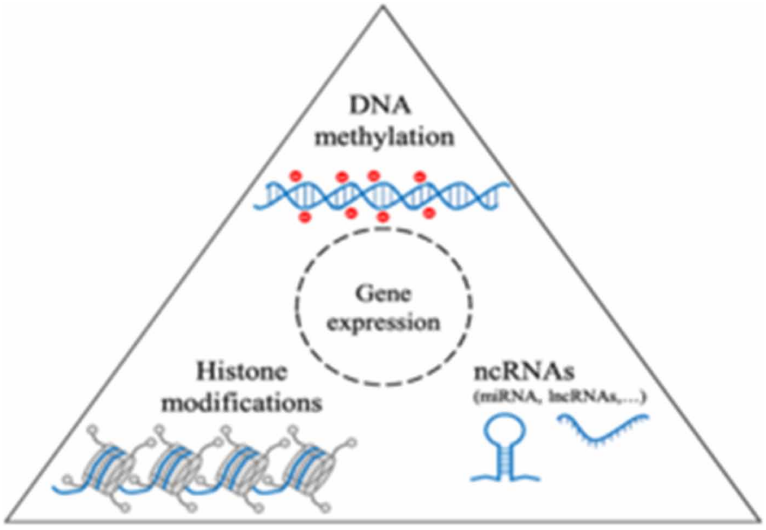
Epigenetics is the study of heritable changes in the gene expression without any alteration in the basic nucleotide sequence of DNA, this leads to the change in phenotype without any change in the genotype (Fig. 8). These changes are influenced by many factors like age, environment and a particular disease state. Epigenetics decides which genes should be turned off or on in a particular type of cells. This is done by chemically changing the chromosomal DNA without altering the DNA sequence. Every cell has identical DNA, yet every cell type has different functions and gene expression patterns which are determined by epigenetic changes, like the tagging of histones/nucleotides with certain chemical groups (like methyl group, acetyl group, ubiquitin, etc). If it had not been for epigenetic tags, there would have been chaos and the human body would not have developed such complex mechanisms. This is how muscle cells and liver cells have managed to turn only those genes on which are required for their respective activity, and have turned the other genes off, even though the genome is the same in both the cells.

Figure 8. Epigenetic modifications



The 3 main epigenetic mechanisms are DNA methylation, Histone modification and non-coding RNA (ncRNA)- associated gene silencing (Fig. 9). DNA methylation occurs when a methyl group is added to the cytosine residues present in the CG rich region of the promoters called CpG Islands (CGIs). This phenomenon causes the genes to turn off (gene silencing). It is a controlled mechanism and is directed by enzymes called DNA methyltransferases (DNMTs). Histone modification is usually the alterations that happen, post translationally, at the tails of the histone proteins which form nucleosomes. The tails stick outside and they are tagged by different chemical signals for which they are marked, which results in many kinds of modifications like acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation. For instance, Histone3K9 acetylation is related to transcription activation, while Histone3k27 trimethylation is related to transcription repression. Hence, histone modifications indulge in activation/inactivation of transcription, chromosomal packaging, and DNA repair. Non-coding RNA-associated gene silencing is the most recently studied epigenetic mechanism. A noncoding RNA is transcribed from DNA, but it is not translated into proteins. They include miRNA, siRNA, piRNA, and lncRNA. They regulate gene expression at the transcriptional and post-transcriptional level. These RNAs are involved in heterochromatin formation, histone modification, DNA methylation targeting, and gene silencing (Al Aboud N. M. et al., 2019).

Figure 9. Main epigenetic mechanisms



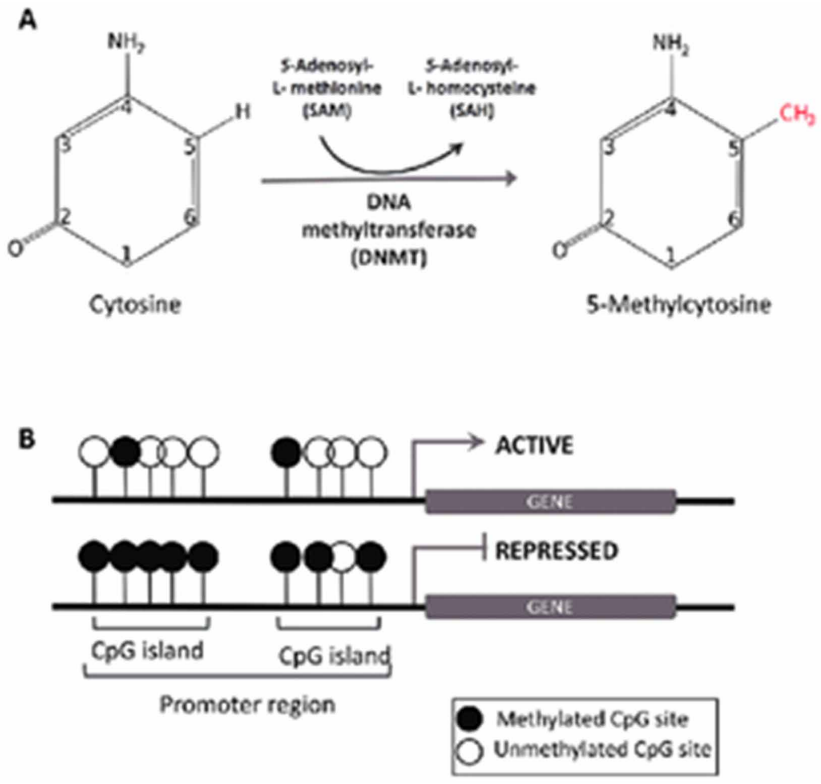
EPIGENETIC MECHANISMS

DNA Methylation

It is the adding of a methyl group to the fifth carbon atom of cytosine residue to produce 5-methyl Cytosine (5mC). The methyl group of 5mC lies in the major groove of DNA, and can interfere with the binding of transcription factors, hence preventing gene expression (Fig. 10). DNA Methylation can also happen outside the CpG sequences, which is important for regulation of expression of genes in embryonic stem

cells. Methylated DNA-binding proteins, like Methyl CpG Binding Protein 2 (MECP2) and the methyl CpG Binding Domain (MBD) family, bind to methylated cytosine and inactivate gene transcription by blocking transcription factors. The dinucleotide (CpG) has the ability to directly inactivate gene expression, especially tumor-suppressor genes. CpG sites can be found all through the genome, especially in repetitive sequences such as tandems and interspersed repeats, distal gene regulatory regions, and CpG Islands. Inside the cells, S-adenosyl methionine (SAM) acts as a methyl group donor. 5mC, produced after methyl group is transferred from SAM to cytosinse, can hinder transcription by blocking the attachment of transcription factors or by enabling the binding of transcriptional repressors, like histone deacetylases (HDACs). DNA methylation is mediated by DNA methyltransferases (DNMTs) which are of several subtypes, like the de novo methyltransferases DNMT3a and DNMT3b; and DNMT1, which identifies and methylates the non-methylated daughter strand during replication. Base-pairing allows the maintenance of reciprocal methylation during further replication cycles, which, in turn, offers a way to pass a non-genetic trait from cell to cell. In this way, DNA methylation is considered a long-term, stable epigenetic trait.

Figure 10. Basics of DNA Methylation



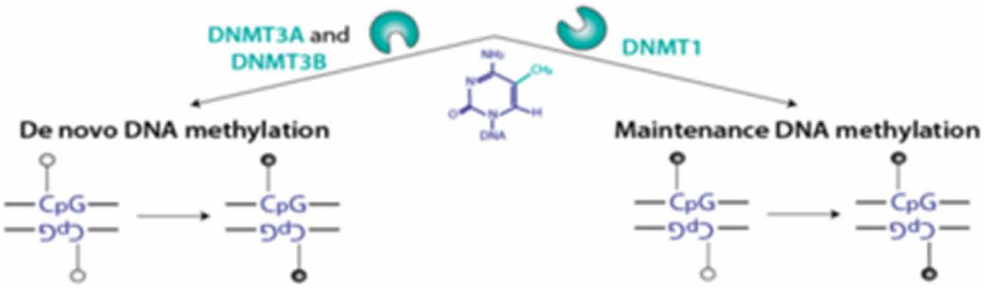
CpG dinucleotides in CGIs are usually un-methylated in normal cells. The CGIs are DNA stretches having high frequency of CpG dinucleotides. Although during development, as an exception to the rule that normally methylation is found only in X inactivation and in imprinted genes, a subset of promoter

CGIs is methylated in tissue-specific manner, Another method of spreading of DNA methylation is the genome-wide demethylation that starts right after fertilization, and Re-methylation, then, of most of the genome occurs after the blastocyst stage and continues during the rest of the developmental span. This phenomenon happens because of self-perpetuating interaction between chromatin-modifying proteins and DNA methylation.

The DNMT family of enzymes takes care of *de novo* DNA methylation and its maintenance. DNMT1 is mainly responsible for maintaining cellular levels of CpG methylation. It recognizes hemi-methylated DNA (when one of the two strands is methylated) and adds methyl groups to the non-methylated daughter strand formed during replication. DNMT1 identifies the replication fork where new hemimethylated DNA is synthesized, and binds to newly formed DNA and methylates it just like the original methylation pattern present before the replication. This enzyme can also repair DNA methylation, and thus, is called as the Maintenance DNMT, because it maintains the original DNA methylation pattern in a cell lineage. DNMT1 is pivotal in cellular differentiation and in dividing cells. Therefore, methylation can be a long-term and stable trait which maintains cellular phenotype.

During embryogenesis, *de novo* methylation is done by DNMT3A and DNMT3B. They both are similar in their structure and function (Fig. 11). Unlike *Dnmt1*, *Dnmt3a*and *Dnmt3b*, can methylate both native and synthetic DNA with no preference for hemimethylated DNA whenever they are overexpressed. This is why they are called *de novo* DNMTs, because they can methylate naked DNA. Primary difference between them is their gene expression patterns. *Dnmt3a* is expressed ubiquitously, while *Dnmt3b* is expressed poorly and in differentiated tissues other than thyroid, testes, and bone marrow. Like *Dnmt1*, knockout of *Dnmt3b* in mice is fatal, on the other hand, *Dnmt3a* knockout mice are runted but survive till approximately 4 weeks after birth. Thus, *Dnmt3b* is required during early development, while *Dnmt3a* is required for normal cell differentiation.

Figure 11. Differences between DNMT3A/B and DNMT1

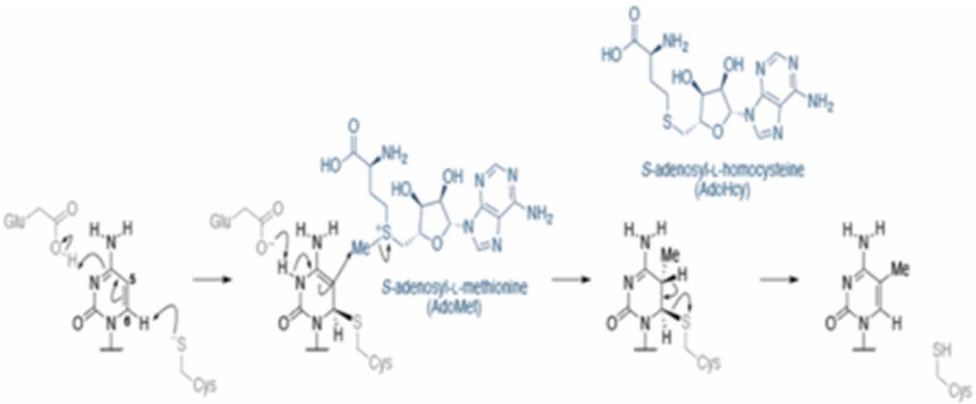


CpG methylation suppresses transcription in many ways. Firstly, methyl group at a specific CpG may hinder DNA recognition and binding of transcription factors to promoters. Alternatively, there are other factors which may bind to methylated DNA, and hence unable transcription factors to access the promoters. For example, Methyl CpG binding protein 2 (MeCP2) inactivates transcription by bind to methylated sites and recruiting histone-modifying proteins, like HDACs. (Moore L. D. et al., 2013; Virani S. et al., 2012; Handy D. E. et al., 2011).

The mechanism of DNA methylation involves an electrophilic attack by the cofactor *S*-adenosyl-l-methionine (AdoMet; SAM), which displaces a methyl group to C(5) of cytosine. DNMTs help by

activating and increasing the nucleophilicity of C5 atom of cytosine because it is not particularly nucleophilic (Fig. 12). DNA methyltransferases have a conserved cytosine residue which acts as a strong nucleophile on deprotonation to the thiolate anion. This cysteine thiolate attacks the C(6) atom of cytosine in a conjugate addition reaction, and a covalent bond is established between the cysteine sulfur atom and the cytosine C(6) atom. The negative charge on cytosine is stabilized by glutamate residue interaction. Nucleophilic attack then takes place on the methyl group of *S*-adenosyl-l-methionine, which is converted to *S*-adenosyl-l-homocysteine (AdoHcy). Finally, β-elimination occurs across the C(5)-C(6) bond, releasing the enzyme (Epigenetics).

Figure 12. Mechanism of DNA methylation



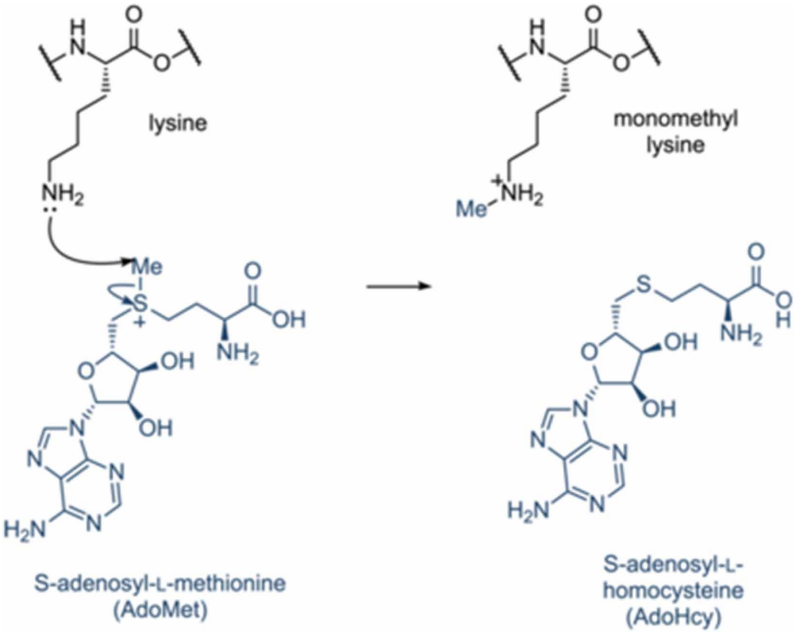
Histone Modification

Histones are the proteins that are involved in DNA packaging. They are post translationally modified, and these modifications are of various kinds, like acetylation, deacetylation, methylation, phosphorylation, and ubiquitylation. These cause the chromatin structure to change, which in turn have consequences on the gene expression. Histone acetylation is catalyzed by histone acetyl transferases (HATs), which are present at the acetylation sites via transcriptional cofactors. Deacetylation is looked after by a class of enzymes called Histone Deacetylases (HDACs), which are themselves controlled by posttranslational modifications. Histone lysine methylation is an essential modification which affects gene expression. Histone methylation, like histone acetylation, is reversible (Loscalzo J. et al., 2014).

Histone methylation is the addition of methyl groups to Arginine and Lysine residues present on tails of H3 and H4 histone proteins. Histone methylation is mediated by histone methyltransferases (HMTs), including lysine methyltransferases (KMTs) and arginine methyltransferases (PRMTs).

Lysine methylation, carried out by histone-lysine-*N*-methyltransferases (aka K-methyltransferases), includes the transfer of methyl groups from the cofactor S-adenosyl methionine (SAM) to lysine's ε-amino group (Loscalzo J. et al., 2014). Numerous HKMTs methylate lysine present within the N-terminal tails (Fig. 13). HKMTs that methylate N-terminal lysine's possess a SET domain that has the enzymatic activity. They are specific enzymes, for instance *Neurosporacrassa* DIM5 specifically methylates H3K9.

Figure 13. Lysine methylation



Arginine methylation is carried out by two classes of arginine methyltransferases (PRMTs), the type-I and type-II enzymes. The two types of PRMTs constitute a large protein family (11 members) (Fig. 14). All PRMTs transfer a methyl from SAM to the ω-guanidino group of arginine within different types of substrates. Examples of PRMTs are PRMT1, 4, 5 and 6.

Histone Methyltransferases have an extended catalytic active site which is their characteristic feature. The SAM-binding site is on one face of the enzyme and the peptidyl acceptor channel is on the opposite face. A molecule of SAM and histone substrate come together from opposing sides of the enzyme. This manner of entering the enzyme's active site might provide a chance to design drugs that can distinguish between histone arginine/lysine methyltransferases and other methyltransferases such as DNMTs (Handy E. et al., 2011).

Histone acetylation occurs on lysine residues and it is believed that it enhances transcription by charge neutralization of positive histones, hence lowering their interaction with negatively charged DNA (Fig. 15). This process is carried out by histone acetyltransferases (HATs), also known as K-acetyltransferases, and histone deacetylases (HDACs). HATs add acetyl groups to histone lysines using acetyl coenzyme A as a cofactor and form an open/permissive chromatin state of structural conformation, while HDACs remove acetyl groups and induce a closed/repressive state.

There are three different families of HATs, namely, Gcn5 family, p300/CBP family, and they all have a role in carcinogenesis. The Wnt signaling pathway is enhanced by HAT Gcn5 in breast cancer. CBP (cyclic AMP response element-binding [CREB] protein) and p300 can acetylate all four core histones along &other non-histonic proteins, like p53, Rb, E2F, and myb. Both p300 and CBP are tumor-suppressor genes that may undergo loss of heterozygosity in different types of cancers. MYST family HATs are important in hematopoiesis and, are dysregulated in acute myeloid leukemia.

Figure 14. Arginine Methylation

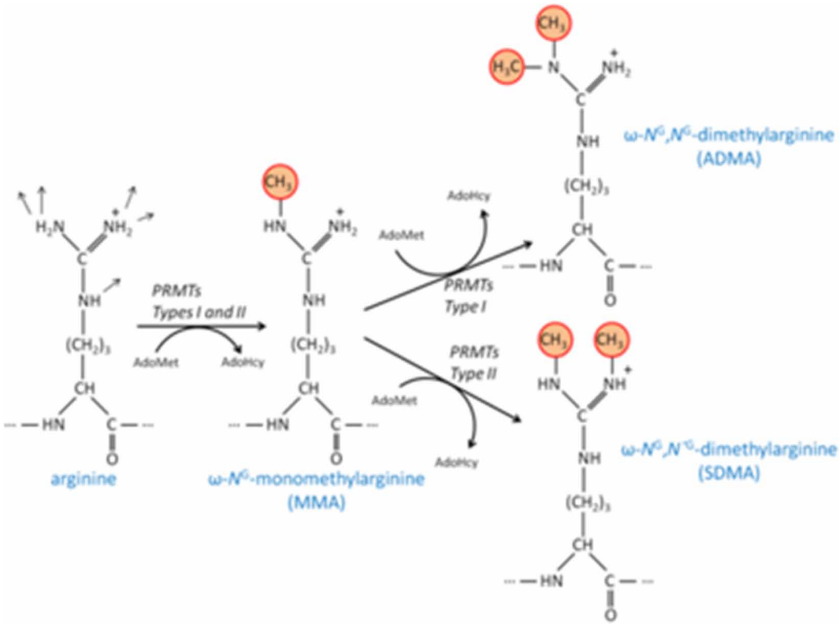
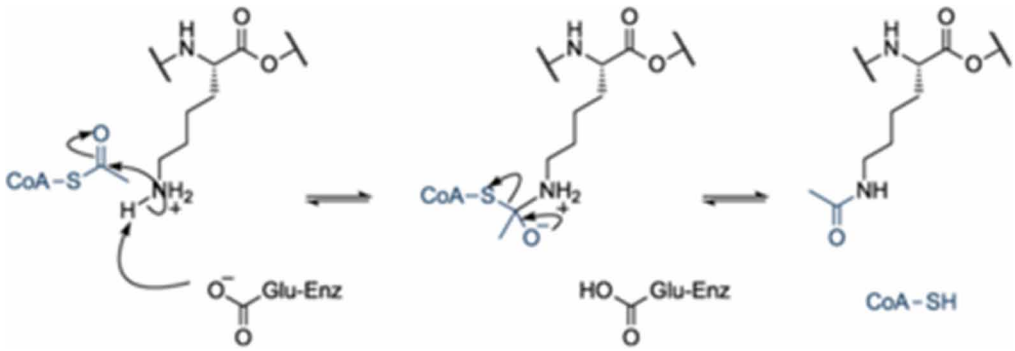


Figure 15. Histone acetylation mechanism

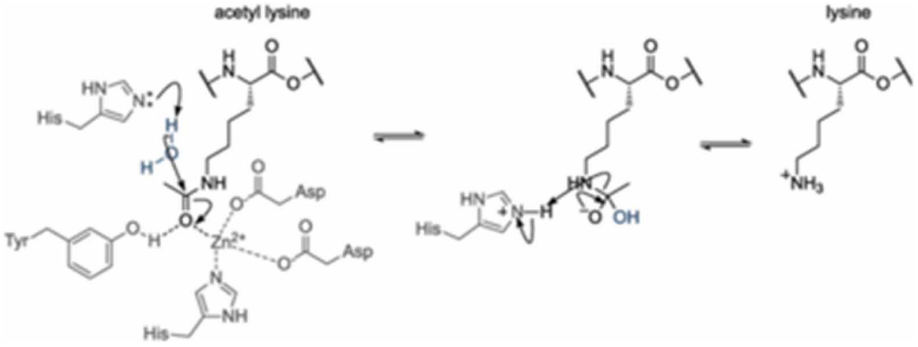


Lysine acetylation is carried out by histone lysine acetylases, and the source of acetyl group, Coenzyme A is converted to Coenzyme A.

Histone Deacetylation is catalyzed by HDACs which remove acetyl groups, and inactivate transcriptional repression (Fig. 16). HDACs have non-histone proteins as substrates and can deacetylate numerous proteins which are important in carcinogenesis, like p53, YY1, and STAT3. Studies have shown that histone deacetylation is an early step in the process of carcinogenesis. Many cancer cell lines, primary lymphomas and colorectal adenomas were hypo-acetylated, proving that histone deacetylation is a widespread event in cancer. HDAC deregulation in cancer cells has provided a target for chemotherapeutic intervention—the HDAC inhibitor. They have been widely used in cancer treatment and psychiatric diseases. There are two HDAC inhibitors which are currently in use for cutaneous T-cell lymphoma—suberoylanilidehydroxaminc acid (vorinostat) and romidepsin. (Virani, S., Justin A., 2012).Eighteen

enzymes belong to the HDAC superfamily, and they are further subdivided into four classes, class I (HDAC1, HDAC2, HDAC3, and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), class III, sirtuins (SIRTs; SIRT 1–7; enzymes evolutionally and mechanistically different from the other HDACs), and class IV (HDAC11). Class I HDACs are expressed in cell nucleus of all tissues, class IIb HDACs are present both in the nucleus and cytoplasm, and class IIa HDACs are present in cytoplasm (Alhamwe AA et al., 2018)

Figure 16. Histone deacetylation by class I, II and IV HDACs



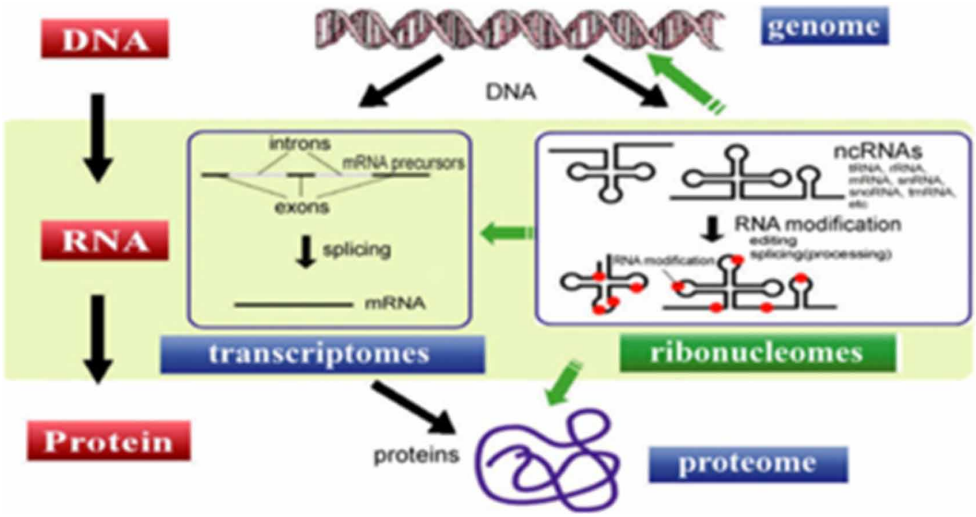
ncRNA Gene Silencing

Non-coding DNAs are the regions in the genome which do not code for proteins, also called as "junk DNA". They function as bookmarks, i.e. partitioning the genome to allow gene to compartmentalize. In eukaryotes, a high percentage of ncDNA exists in the genome, approximately 70%–90%. These regions were initially thought to be inert, but research has proved that these non-coding DNAs are transcribed. Since many of these ncRNAs are derived from non-coding DNAs, hence they are named non-coding RNAs (ncRNAs) and the majority of ncRNAs is not translated. These ncRNAs are divided into two main types on the basis of their length: short non-coding RNAs (such as microRNAs (miRNAs)) and long non-coding RNAs (lncRNAs). Other types of ncRNAs grouped according to their length, localization, and/or function are microRNAs (miRNAs), small interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), and PIWI-interacting RNAs (piRNAs). The lncRNAs have nucleotides more than 200 nucleotides and are subdivided according to their biogenesis loci: intergenic lncRNAs (lincRNAs), intronic lncRNAs, antisense lncRNAs (aslncRNA or natural antisense transcripts, NATs), bidirectional lncRNAs, and enhancer RNAs (eRNAs) (Fig. 17). They coordinate physiological processes in association of other molecules, and any damage to them impacts several pathologies, like cancer. They control the flow of genetic information, such as chromosome structure modulation, transcription, splicing, messenger RNA (mRNA) stability, mRNA availability, and post-translational modifications. lncRNAs, can interact with nucleic acids or proteins via base-pairing or structural recognition, respectively, hence a single lncRNA molecule can interact with variety of macromolecules. This interaction is carried out by ribonucleoprotein complexes in which lncRNAs are associated with proteins. A 100-ribonucleotide hairpin structure can interact with more proteins at the same time than peptide domains of 100 amino acids interacting with other proteins. Long non-coding RNAs can be

found in the nucleus, nucleolus, cytoplasm, and also in the mitochondria. It has been seen that mostly lncRNAs are localized in the nucleus, specifically in association with chromatin in several cell lines. Recent studies suggest that some of the expressed lncRNAs control stability and translation of mRNAs while being present in the cytoplasm. Localization of lncRNAs depends on motifs signatures like protein signal-peptides, nuclear-restricted lincRNA BMP/OP-responsive gene (BORG), and Alu-related sequences. The changes in the structure of lncRNA regulate the availability of recognition sites for RNA binding proteins via thermodynamic adjustments in the hairpin structure stability. The most common RNA chemical modifications are the exchange of adenosine with inosine, which is catalyzed by adenosine deaminases, and the reversible modifications by N6-methyl-adenosine (m6A) methylation. Other than regulating function, these modifications help in the recognition of RNAs as endogenous and non-pathogenic molecules, whereas non-modified RNAs are have the capability of activating the immune response which is mediated by toll-like receptors (TLRs).

The lncRNAs like mRNAs, are synthesized with the help of RNA Polymerase II, though they can appear without typical mRNA modifications like polyadenylation, alternate splicing, etc. The genes for lncRNAs share common features as that of mRNA genes, and they are regulated by same transcription factors like p53, nuclear factor kappa B and Sox4, to name a few. The antisense lncRNAs on the other hand are transcribed from the complementary strand of protein coding genes. Divergent transcription might occur when RNA polymerase II is recruited in the antisense strand upstream of the site of the protein-coding gene promoter, but only a few of them synthesize functional transcripts which are called bidirectional lncRNAs.

Figure 17. In vivo role of ncRNAs in cells



The ncRNAa, especially antisense ncRNAs, have been recently found to be involved in gene silencing via epigenetic remodeling. The non coding RNAs guide chromatin remodeling and hence contribute to chromatin structure and maintenance of epigenetic memory. Various ncRNAs regulate chromatin structure and gene expression. Antisense ncRNAs have been found to be silencing tumour suppressor genes, and the characterization of these RNAs which are involved in carcinogenesis may lead to the

use of ncRNAs as early detecting biomarkers in cancers. The siRNAs, a type of ncRNAs, mediate post translational gene silencing (PTGS) as a result of mRNA degradation. They can inhibit the transcription of many human and viral genes by targeting the promoters. Some of the genes that they have been known to silence are ubiquitin C, progesterone receptor gene, androgen receptor genes, cyclooxygenase 2, cadherin 1, oncogene cMYC, etc. The proteins involved in siRNA mediated gene silencing (TGS) have been yet been characterized, yet a protein called Ago1 (argonuate 1) is an RNA binding protein which is required for initiation of TSG in humans (Fig. 18). Other than this, Ago2 and TAR-RNA binding protein 2 (TRBP2) are also involved. Antisense RNA binds with the Ago1, and RNA-Ago1 complex targets nascent promoter-associated RNA, which was transcribed by RNA polymerase II (RNAPII) in the sense direction. Then, the silencing complex, which consists of the PRC2 Polycomb complex, HDAC1, the DNA methyltransferases Dnmt3A and Dnmt1 and the histone methyltransferases KMT1C (G9a) and/or KMT1A (Suv39h1), is recruited at the promoter, with the help of interaction of Ago1 with Dnmt3a, or directly through promoter-associated RNA. HDAC1-mediated histone deacetylation may precede histone H3 methylation at Lys9 and Lys27 of the nucleosomes proximal to the promoter target site. Histone methylation is carried out by lysine methyltransferases KMT6 (for H3K27) and KMT1C (for H3K9) and/or KMT1A, resulting in the formation of heterochromatin at the target promoter (Fernandes et al., 2019; Malecova et al., 2010).

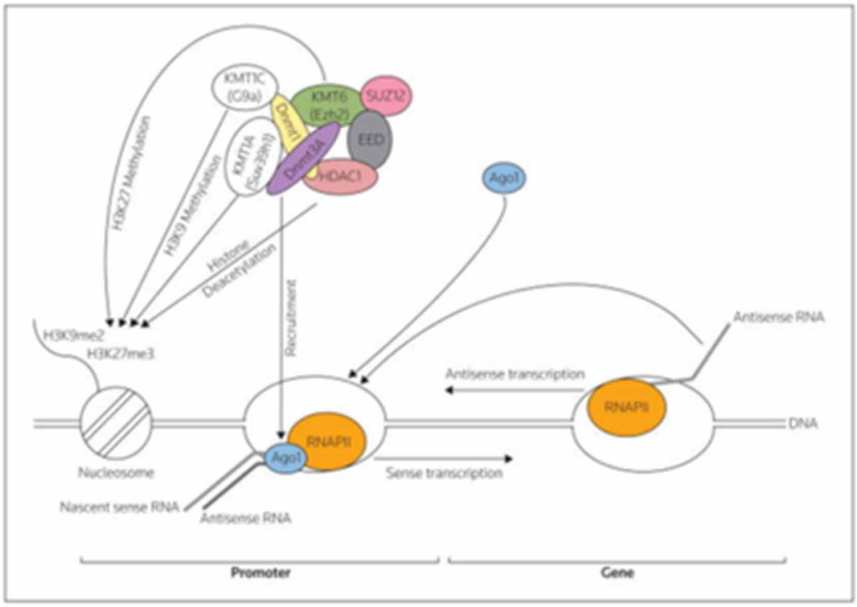
Cancer and Epigenetics

Cancer initiation and progression, other than being controlled genetically, are also regulated by epigenetic mechanisms. The epigenetic landscape of normal cells is completely distorted in cancer cells. These mechanisms are heritable and reversible. Disruption of epigenetic processes leads to dysregulation of gene functions, which can in turn lead to carcinogenesis. They occur at early neoplastic stages and are the key players of cancer development. These 'Epimutations' cause the silencing of tumor suppressor genes independently, and also in association with genetic mutations, serving as the second hit required for cancer initiation according to the 'two-hit' model proposed by Alfred Knudson.

The classic hallmarks of cancer can be achieved "purely" by epigenomic dysregulation, suggesting that epigenetic deregulation may alone cause cancer without genetic contribution. Epigenetic mutations can also be used in predicting the clinical outcomes of a disease, for example, low levels of H3K4me2 are linked to poor prognosis in prostate, lung and kidney cancers. Low levels of H3K18ac and H3K9me predict worse prognosis in kidney and lung cancer. High levels of H3K9ac in patients with lung cancer is suggests a lower survival.

DNA Methylation patterns, when disrupted profoundly, cause cancer initiation and progression. Cancer genome is marked by genome-wide hypomethylation and site-specific CpG island promoter hypermethylation. Global DNA hypomethylation has an imperative role in tumorigenesis, and it occurs at many genomic sequences like repetitive elements, retrotransposons, CpG poor promoters and introns. DNA hypomethylation at repeat sequences results in increased genomic instability by enhancing chromosomal rearrangements. Hypomethylation of retrotransposons results in their activation and, hence, their translocation to other genomic regions, increasing genomic instability. DNA hypomethylation may lead to the activation of growth-promoting genes, like *S-100* in colon cancer and *MAGE* (melanoma-associated antigen) in melanoma. On the other hand, site-specific hypermethylation promotes to tumorigenesis by silencing tumor suppressor genes (TSGs), for instance, CGIs hypermethylation of *Rb* promoter (a tumor suppressor gene associated with retinoblastoma), *p16*, MutL Homolog 1 (*MLH1)* and *BRCA1*. These are

Figure 18. Antisense ncRNA mediated TSG



the genes are involved in cellular processes, which are crucial for cancer development and progression, including DNA repair, cell cycle, cell adhesion, apoptosis and angiogenesis. This epigenetic silencing of such TSGs serves as the second hit in the Knudson's two-hit model. Other than activating TSGs, DNA hypermethylation also indirectly silences additional genes by silencing the transcription factors and DNA repair genes. Hypermethylation-induced promoter silencing of genes of transcription factors, such as *RUNX3* in esophageal cancer, and *GATA-4* and *GATA-5* in colorectal and gastric cancers, causes inactivation of their downstream targets. To add on to this, silencing of DNA repair genes (e.g. *MLH1*, *BRCA1* etc.) enables cells to accumulate further genetic mistakes leading to the rapid progression of cancer.

Histone modifications like loss of acetylated H4-lysine 16 (H4K16ac) and H4-lysine 20 trimethylation (H4K20me3), mediated by histone deacetylases (HDACs), result in gene repression. HDACs are overexpressed in various types of cancers, and thus, are a major target for epigenetic therapy. Histone acetyltransferases (HATs), which work antagonistically and in concert with HDACs to maintain histone acetylation levels, are also altered in cancer. In addition to alteration of histone acetylation, cancer cells also display magnificent changes in histone methylation patterns. Changes in H3K9 and H3K27 methylation patterns are associated with erratic gene silencing in various types of cancer. Deregulation of histone methyltranferases (HMTs) results in altered histone methylation patterns in cancers, and leads to aberrant silencing of TSGs. For example, EZH2, which is the H3K27 HMT, is overexpressed in breast and prostate cancer. High levels of G9a, the H3K9 HMT, is found in liver cancer and is responsible for perpetuating malignant phenotype by modulating chromatin structure. Chromosomal translocations of Mixed Lineage Leukemia (MLL), the H3K4 HMT, lead to ectopic expression of homeotic (Hox) genes and play an important role in progression of leukemia. Lysine specific-demethylases also work in coordination with HMTs to maintain global histone methylation patterns, and hence they are also implicated in cancer progression. For example, LSD1, a type of lysine demethylase, removes activating and repressing marks (H3K4 and H3K9 methylation, respectively) depending on particular binding partners

that it binds to, hence, acting as either a corepressor or a co-activator. Another example of histone lysine demethylase that have been discovered is Jumonji C domain proteins. Such histone demethylases (HDMs) are up-regulated in various types of cancer, thus, making them potential targets for anticancer therapy.

Non-coding RNAs, especially lncRNAs, are associated with several diseases, most notably cancer. For instance, a lncRNA, *PCAT-1*, enables cell proliferation and is targeted for Polycomb Repressive Complex 2 (PRC2) regulation. *ANRIL*, another type of lncRNA, is up-regulated in prostate cancer and it represses the tumor suppressors INK4a/p16 and INK4b/p15. HOX (homeobox) transcript antisense intergenic RNA (*HOTAIR)* overexpression is associated with worse prognosis in breast, liver, colorectal, gastrointestinal, and pancreatic cancers, and is supposed to increase tumor invasiveness and metastasis. *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1), an lncRNA related to various cancers and metastasis, affects the transcriptional and post-transcriptional regulation of cytoskeletal and extracellular matrix genes. *lincRNA-p21* (named for its vicinity to the *CDKN1A/p21* locus) is up-regulated by p53 upon DNA damage, and is implicated in repressive consequences of the p53 pathway on genes regulating apoptosis. In some cancer types, p53 mutations maintain the protein's ability to induce the *PANDA* (P21 Associated NcRNA DNA damage Activated) pathway (and its antiapoptotic effects) while abolishing its ability to induce p21 and its promotion of cell-cycle arrest, thus increasing tumor cell survival. The above examples show that lncRNAs can be used as diagnostic markers or therapeutic targets in the treatment of cancer.

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Chapter 7
Lipids, Peptides, and Polymers as Targeted Drug Delivery Vectors in Cancer Therapy

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ABSTRACT

The authors aim to describe valuable information and experimental reviews that may help to develop and design different formulation, which can boost up the overall efficiency of the final product. Further, they explained the overall efficiency, method of preparation, target delivery approaches, drawbacks, and other characteristics in relation to lipids, peptides, polymers, and vaccines. In addition, they also propose to uncover the physico-chemical properties, in-process manufacturing issues, and external factors that influence the fate of a medicine. That major includes the excipients, method of preparation, dose, delivery route, chemical and biological properties, drug-drug interaction, drug-body interaction, patient compliance, modifications in lipid based nano-vectors, polymer-mediated delivery systems, conjugate delivery systems, and others. In conclusion, by the end of this chapter, the authors are able to explain a robust mode of delivering active constituents more safely and economically to the target site by showing maximum bioavailability.

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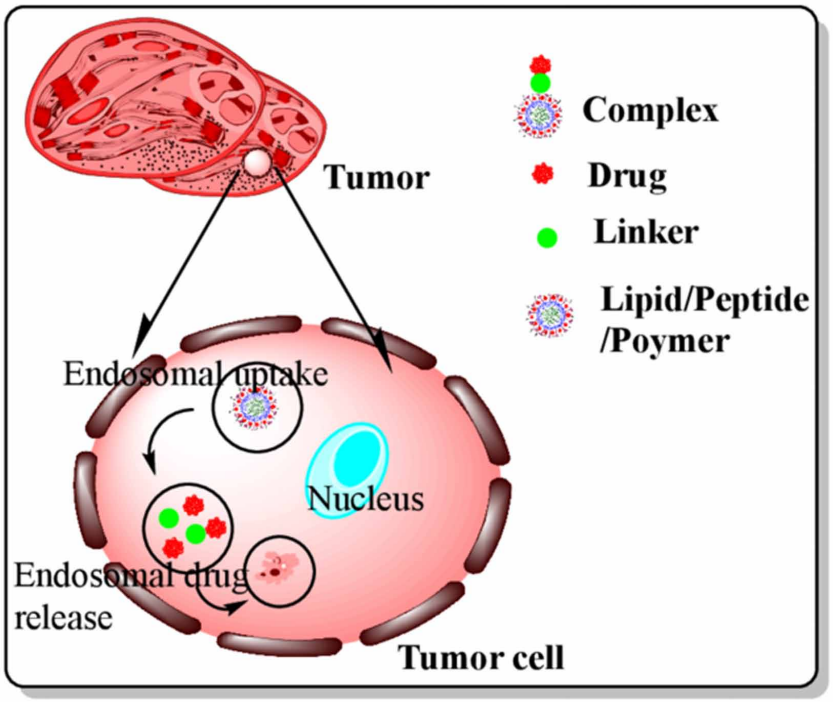
INTRODUCTION

As we know cancer is among one of the most leading causes of death worldwide, one in 4 deaths in the US is due to cancer (Yassin et al.,2013). So, our main objective is to elaborate and modulate drug delivery system constituents (lipids, peptides, and polymers) using different carriers and mode of mechanisms as a targeted drug delivery approach against cancer (Fig. 1). Thus, synergizing the therapeutic action by replacing the conventional approaches with a new and advanced set of therapeutics, for example where conventional synthetic lipids like 1,2-Dioleoyl-3-trimethylammonium propane (DOTAP), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) are now replaced with functional lipids showing their own anti-cancer properties. There are thousands of drug delivery system that comprises of lipids and excipients to develop the desired formulation. Notable aspects of development in the application of drug delivery systems have occurred in cancer therapy over few decades.(Alavi& Hamidi, 2019).There is a constant increase in the number of cancer cases that not only requires the development of new effective chemotherapeutic drugs but also unique and different ideas to deliver drug formulations adequately by modulating different physio-chemical properties of delivery systems(Jampílek & Kráľová, 2019).In addition to such properties, these cancer nano-medicines with different carrier systems are capable of delivering chemotherapeutic agents while providing lower systemic toxicity(Bor et zl., 2019). We do not consider the fate or metabolism of these structures which later results in severe cell toxicities causing major disorders. Here, we emphasize on exposing the astonishing effects of lipids, polymers, peptides and other excipients as different carrier moieties in achieving target specifications. Alsoin gene mediated delivery nucleic acid requires a sophisticated delivery vehicle because of its rapid degradation in the circulation, thus these carriers in the form of lipids hold up the bottleneck in RNA delivery systems. (Tam et al., 2013)There are many techniques to reduce the overall load on such excipients as like, the dose for an anticancer drug can be reduced as one Active Pharmaceutical Ingredients (API) will be used to carry another pharmaceutically active ingredient giving synergism and reducing the overall ratio of core active pharmaceutical ingredient and avoiding the unnecessary burden of excipients to the molecule, and manufacturing cost. Different techniques have been generated that potentiates the controlled release of drug into the active site. (Hardenia et al., 2019). Thus, exempting body from unnecessary load of un-metabolized constituents and ensures the controlled drug release systematically. However, conventional liposomal formulations offer a minimal degree of protection to the normal cells because they are made up of conventional lipids. A practical step forward to this end would be to substitute such lipids with bioactive or molecularly targeted lipids using conjugated Polyethylene glycol (PEGylated) and poly(lactic-co-glycolic acid) (PLGA nanoparticles) (Li et al., 2015). The development of anti-ligase lipid-based liposomes for administrating DNA alkylating drugs using polymers such as timazolamide and doxorubicin gained immense importance in cancer therapeutics (Prasad et al.,2016). These novel dosage forms will not only deliver the drug but also sensitizes cancer cells by inhibiting the elevated ligases. Together, this reduces the dose of the chemotherapeutic agent and also exemplifies conceptually new combinatorial approach for the effective treatment of cancer. In the beginning stage of liposomes as an anti-cancer therapeutics different lipid were used to develop liposomal formulation, as the encapsulation of drugs in liposomes enhances the therapeutic index. However, the above formulations are made up of conventional lipids with very less on no positive charge. Theses formulations were made from conventional lipids (DOTAP, DOTMA etc.) holds success up to some level, till the gene therapy came into existence. In 2010, Davis et al. reported the delivery of genetic material siRNA with the help of cationic lipids or the lipids that possess permanent positive charge (Ozpolat et al.,2014). As the charge on nucleic acids

is negative hence a positive carrier is required to obtain the deliver siRNA to the target site for obtaining maximum bioavailability (Barry et al.,1996). In this regard, mimicking the concept of molecularly targeted drugs that successfully solved the toxicity related issues of the conventional chemotherapeutic agents can offer a solution. Researchers all over the globe came up with an idea of using transiently charged cationic lipids so as to avoid the harmful effects caused by permanently charged cationic lipids.

siRNA-therapeutics came into existence through a widespread clinical application of nanotechnology. The broad therapeutic applications of siRNA-based therapeutics in cancer largely depend on the development of rationally designed systemic delivery systems. Among the existing delivery systems, cationic liposomes are promising nonviral vectors because of their safety, biocompatibility, and scalability (Fig. 2). However, after delivering genetic materials, fate of the constituents' cationic lipids (CLPs) is a relatively unexplored aspect in gene therapy. In general, constituents of CLPs are disassembled after internalization and freely available to interact with cellular proteins; hence there is a high chance of developing unwarranted biological responses. Although some studies have reported the interactions of Cationic liposomes CLs with cellular pathways, they are restricted to few lipids and limited pathways. Since structure governs the cellular functions of a molecule, it is not possible to generalize the cellular interactions of lipids based on the few reports. A practicable solution for the aforesaid intricacy is to develop a therapeutic cationic lipid that can be formulated to therapeutic CLPs i.e. functional liposomes. Developing a functional CLP that can deliver genetic medicines is not only challenging but also has great potential for the development of safe synergistic cancer therapy of nucleic acid drugs.

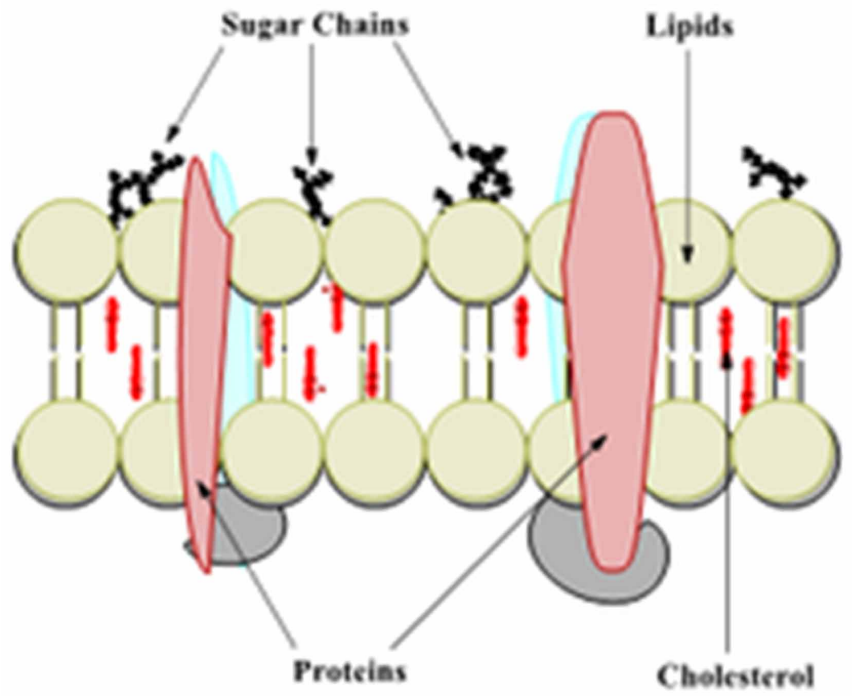
Figure 1. General hypothetical mechanism for different carriers to deliver drugs in cancer therapy



Genomic Based Strategies

RNA interference (RNAi), an evolutionarily conserved ubiquitous gene silencing mechanism, has the potential for clinical development of nucleic acid therapeutics. The delivery vector is crucial for the clinical success of therapeutic RNAi. To fully exploit the therapeutic potential of RNAi in cancer therapy, various small interfering RNA (siRNA) delivery strategies have been developed, including stable nucleic acid-lipid particles(SNALP) formulations that encapsulate small interfering RNA (siRNA) designed to silence polo-like kinase 1(PLK1), small interfering RNA (siRNA) lipoplexes made up of cationic lipid and siRNA. Naturally, RNAi is an important defense mechanism by which eukaryote cell can degrade exogenous genes.

Figure 2. Allocation of lipids with other constituents in the body



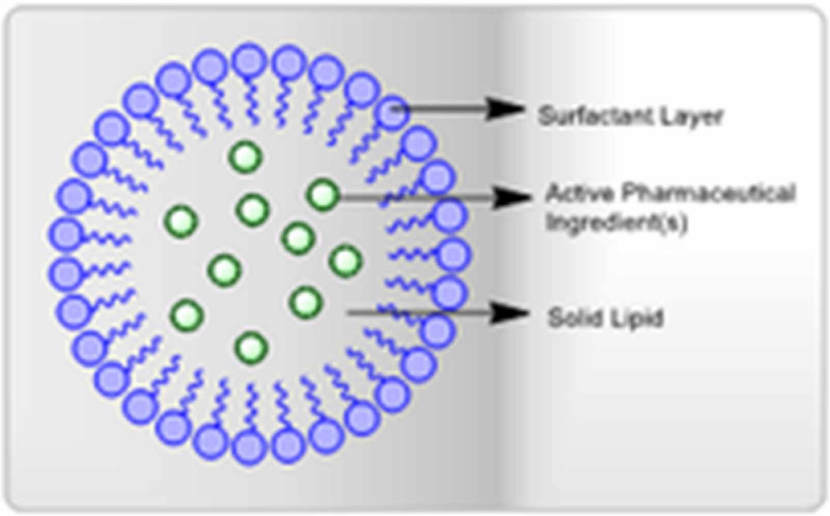
Most siRNAs were administered by local delivery, typically via intravitreal or intranasal routes. Small interfering RNA (siRNA) has been emerging as one of the most prominent agents for the treatment of various diseases, due to its specific silencing of targeted gene. However, due to its large molecular weight, negative charge, RNAase degradation and rapid elimination from the systemic circulation, naked siRNA are almost impossible to enter the target cells and silence the specific genes. Thus, Cationic lipids (e.g.,1,2 dioleyol-3-trimethylammonium-propane (DOTAP)) or cationic polymers (e.g.; polyetherimide (PEI), Poly-L-Lysine (PLL), Polyamidoamine (PAA) and chitosan have been commonly used for siRNA delivery. The mechanism involved is Gene Silencing “Interruption or suppression of the expression of a

gene at transcriptional or translational level". Gene silencing occur during either transcription or translation, gene silencing is offer considered as gene knockout.

LIPIDS

Lipid arrives from the Greek word "lipos" that means vegetable oil or fat. To be more simple lipids can be defined as substances such as wax, oil or fat that easily dissolves in alcohol but are insoluble in water as they are non-polar in nature. Lipids are made up of carbon, oxygen, and hydrogen. Solid lipid nanoparticles (SLNs) are the most widely used formulation to deliver a chemotherapeutic agent to the target site in cancer therapy. Where lipid's characteristics vary from solvent evaporation method to thin lipid hydration methods depending upon various physio-chemical properties (Fig. 3). Depending upon various physio- chemical properties lipids used that are used in the solid and liquid state are summarized below:

Figure 3. Solid Lipid Nanoparticle



Structure of Lipid

Lipids can be anionic or cationic depending upon the charge they have. Cationic lipids are majorly used to carry anti-cancer nucleic acids to the target site as they are positively charged and nucleic acids are negatively charged. Few of the synthesized marketed cationic lipids are shown below as DOTAP, DOTMA, DDAB, DC-6-14, etc. Cationic lipids are amphiphilic molecules and generally consist of three parts: a hydrophobic domain (for example, aliphatic chains), a hydrophilic head group (for example, quaternary ammonium), and a spacer including a linker bond (for example, ester bond) and backbone domain (for example, glycerol)) between these two parts (Fig. 4). A solution of cationic lipids, often formed with neutral helper lipids, can be mixed with siRNA to form a lipoplex Well-characterized and widely used commercial reagents for cationic lipid transfection include N-[1-(2,3-dioleyloxy) propyl]-N,N,N-tri

methyl ammonium chloride (DOTMA), [1,2-bis(oleoyloxy)-3-(trimethylammonio) propane] (DOTAP), and 3β[N-(N', N'-dimethyl aminoethane)-carbamoyl] cholesterol (DCCholHydrophobic Tail group: There are two major types of hydrophobic moieties, namely aliphatic chains and lipid-based derivatives. (Pinnaduwage, Schmitt, & Huang, 1989) elaborated why 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) is more efficient and less toxic than cetyltrimethyl ammonium bromide (CTAB) in the preparation of cationic liposomes. However, (Tang et al.,1999) demonstrated that 6-lauroxyhexyl ornithinate (LHON) with one tail was more efficient and of lower cytotoxicity compared with DOTAP. This result shows that we cannot completely abolish the possibility of one tail cationic lipids for gene therapy application. In any case, the influence of hydrophobic chain length on the parameter may well depend on the physicochemical features of the other two domains.

Table 1. Solid lipids used in the preparation of SLNs

Solid Lipid	Active Ingredient	Methods of Preparation	Reference
Palmitic acid	Tamoxifen	Microemulsion and precipitation method	Fontana et al. (2005)
Stearic acid/Glycerylmonostearate	Tamoxifen	Solvent injection method	Hashem et al. (2013)
Compritol 888 ATO Acevedo-)	Camptothecin	Modified solvent emulsification and ultra-sonication method	Morantes et al. (2013
Trimyristin	Paclitaxel	Homogenization method	Lee et al. (2007)
Stearic acid/Glycerylmonostearate	Emodin	High pressure Homogenization method	Wang et al. (2012)

Table 2. Liquid lipids used in the preparation of Nano-structured Lipid Carriers NLCs

Liquid Lipid	Active Ingredient	Methods of Preparation	Reference
Glyceryltridecanoate	Quercetin	Phase inversion method	Sun et al. (2014)
Olive oil Melt	Tamoxifen	emulsification method	How et al. (2013)
Labrafil WL 2609 BS	Tamoxifen	Solvent diffusion method	Shete et al. (2013)
Oleic acid	Doxorubicin/Paclitaxel	Solvent emulsification method	Zhang et al. (2008b)
Polyoxyl castor oil	Baicalein Doxorubicin	Emulsion-evaporation solidification method	Liu et al. (2015)

Hydrophilic Head group

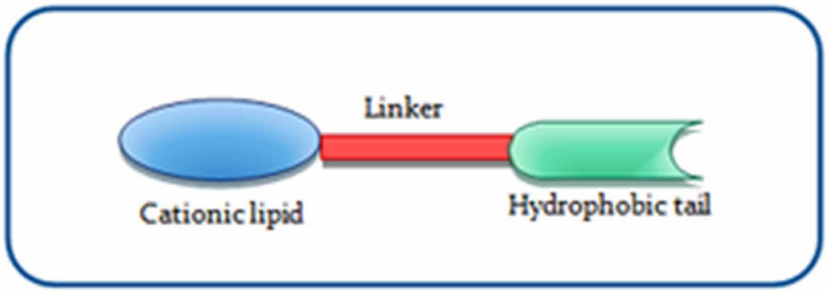
The cytotoxic effect is associated with the cationic nature of the vectors, which is mainly determined by the structure of its hydrophilic group. The head group often consists of primary, secondary, tertiary amines or quaternary ammonium salts, but guanidino and imidazole groups have also been trialed. The research shows that many derivatives of cholesterol that contain tertiary or quaternary nitrogen headgroups can inhibit PKC activity. A recent solution to circumvent these problems was to spread the positive charge of the cationic head by delocalizing it into a heterocyclic ring. Lies et al. reported that 1-(2, 3-dioleoyloxy propyl)-2, 4, 6-trimethyl pyridinium lipid, a kind of pyridinium lipid, was able to transfect several cancer cells lines with similar or better efficiency than DOTAP while producing lower

cytotoxicity. The import of a heterocyclic ring as the substitution of the liner amine headgroup, such as pyridinium and guanidine, can spread the positive charge of the cationic head, and then toxicity is decreased significantly.

Linker bonds

Most of the linker bonds in the above-mentioned synthesized lipids are ether, ester carbamate and amide bond. Although compounds with ether linker render better transfection efficiency, they are too stable to be biodegraded thus cause toxicity. Cationic lipids with ester bonds such as DOTAP in the linker zone are more biodegradable and associated with less cytotoxicity in cultured cells, but those with ester or amide linkers are liable to decompose in the circulation system (Fig. 5a). In recent years, carbamate-linked lipids which with lower toxicity as novel cationic lipids have been developed. It is familiar to chemists that compounds comprising carbamate bond is stable in the neutral circumstance and is liable to acid-catalyzed hydrolysis. As well known, the pH value in endosomes is 1–2 lower than that of the circulation system, and it is expected that these carbamate-linked lipids can keep stable in the circulation system while decompose to release siRNA after entering endosomes in cell because of the pH decreases. The lipids may be rapidly degraded into nontoxic low molecules in the cell. Aberle et al. proposed that cytotoxicity due to cationic lipids may occur at a stage before the lipoplexes were encapsulated into endosomes. These results show that the cytotoxicity is lowered while the linkage is degradable.

Figure 4. General structure of cationic lipid

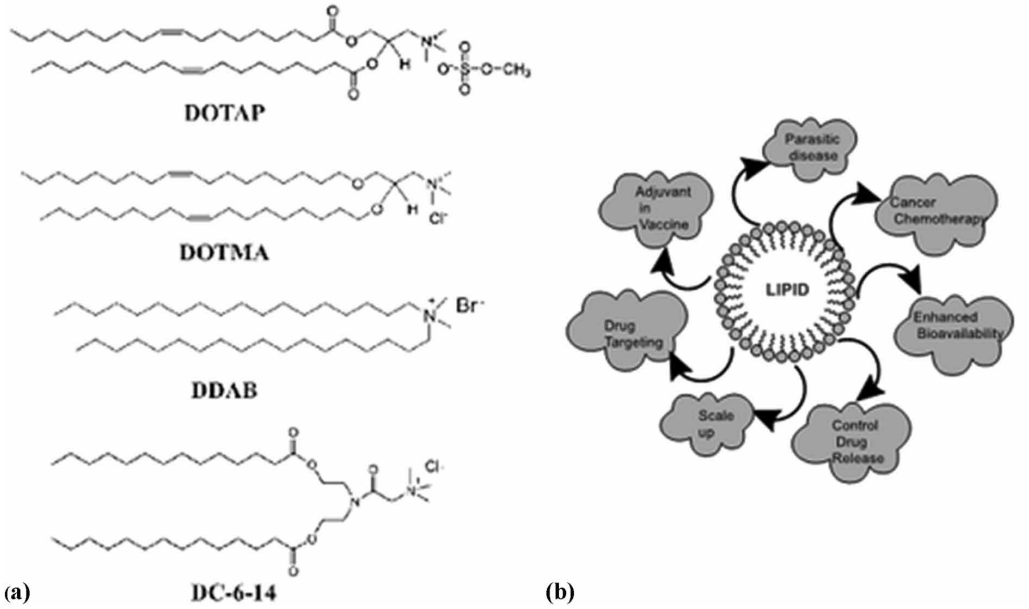


Why Cationic Lipids in Cancer Therapy?

Cationic liposomes are prepared from cationic lipids containing two hydrophobic aliphatic long chains and positively charged functionalities in their head-group region. Cationic lipids are generally formulated in combination with neutral lipids like DOPE or cholesterol for use as gene transfer vectors. Because of their opposite surface charge, cationic liposomes can form a charged complex with a negatively charged siRNA molecule. The resulting charged lipid-siRNA complexes (popularly known as “lipoplexes”) do not experience the electrostatic barrier faced by the naked siRNA in entering biological cells and get endocytosed by the cell plasma membrane. In addition, cationic liposomes also protect siRNA from attack by the en-route RNases. Broadly speaking, cationic transfection lipids are designed to protect siRNA so that favorable interactions with plasma membrane occur leading to efficient endocytosis

and subsequent destabilization of endosomes. In 1987, Felgner et al. for the first time, used chemically designed and synthesized cationic lipids in transfecting cultured cells with plasmid DNA. Since then, a large number of efficient cationic lipids having different molecular architectures have been reported till date. The main advantages associated with the use of cationic transfection lipids include there: (a) robust manufacture; (b) ease in handling & preparation techniques; (c) ability to inject large lipid: DNA complexes and (d) low immunogenic response, etc. (Fig. 5b).

Figure 5. (a) General structure of synthetic cationic lipids: DOTAP, DOTMA, DDAB, DC-6-14 (b) Schematic Representation of different advantages of lipids



Cationic Lipids as siRNA Delivery Vectors

1. Lipofectamine 2000

Lipofectamine 2000 provides high transfection efficiency and high levels of transgene expression as reported in many studies. The most commercially existing cationic liposome/lipid-based systems are Lipofectamine 2000 and CDAN based liposome composed of CDAN: DOPE at different molar ratios.

2. 1, 2-bis (oleoyloxy)-3-(trimethylammonio) Propane (DOTAP)

Leventis and Silvius were first synthesized the DOTAP in 1990. Its structure made up of glycerol in which two oleoyl chains bound by an ester bond as a spacer and one with a quaternary amine. By using cationic DOTAP liposome's Sorensen et al. injected anti- TNF- α siRNA in mice and they were successfully inhibited lipopolysaccharide-induced TNF- α gene expression. Ma et al. also reported that this cationic lipid, used with cholesterol in 55:45 ratios is able to deliver siRNA.

3. N-[1-(2, 3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTMA)

The first synthesized and commercially available cationic lipid to be designed for gene delivery is DOTMA. It is made up of glycerol in which two oleoyl chains bound by an ether bond as a spacer and one with a quaternary amine. When compared to other cationic lipids, DOTMA has better in vivo transfection efficiency.

4. 3β [N- (N', N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol):

Among the existing gene delivery systems, DC-Chol/DOPE liposome is one of the most effective systems in gene delivery (Fig. 6a). YajunGuo and Jianming Chen examined the effect on the siRNA and plasmid DNA (pDNA) transfection by using DC-Chol/DOPE liposomes with different molar ratios. These results disclose that the mechanism of siRNA and pDNA transfection efficiency depend on the DC-Chol/DOPE liposomes at a different molar ratio.

LIPIDS IN CANCER THERAPY

Different lipid agents are used in drug delivery (Fig. 6b).

1. Estradiol

Estradiol is a steroid and primary female sex hormone. Apart from its physiological functions, it is also involved in many disease-promoting processes notably in certain cancers, originating in estrogen sensitive tissue such as breast cancer. Estradiol, the endogenous ligand for Estrogen receptor (ER) is chemically modified to modulate the ER function, which is of high importance for treating variety of diseases including breast cancer and osteoporosis.

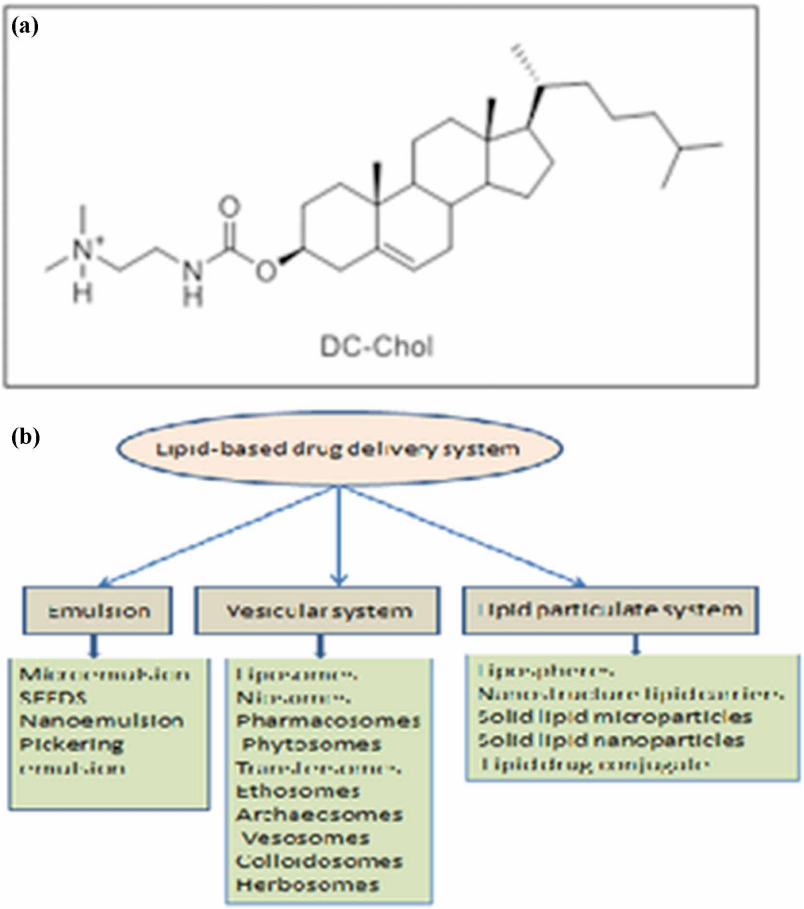
2. Haloperidol

Haloperidol is a neuroleptic drug that shows high affinity towards σ (sigma) receptors (SR) and it has been shown to induce apoptosis at higher concentrations in SR over-expressing melanoma and carcinoma (Fig. 7a). It has been shown that it exhibits anticancer activity and induces apoptosis in different tumor cell lines at moderate concentrations. Interestingly structural alteration of haloperidol by conjugation with a quaternary ammonium lipid moiety enhanced its anti-proliferative activity without hampering the targeting ability.

3. Dexamethasone

Dexamethasone (Dex) is a type of steroidal medicine that acts as a ligand for glucocorticoid receptors. It is used as an anti-inflammatory agent and also shows moderate anticancer activity. However, it exhibits certain side effects associated with its steroidal nature, while being used for antitumor treatments. For effective anticancer treatment, controlled and structural alteration of Dex is needed.

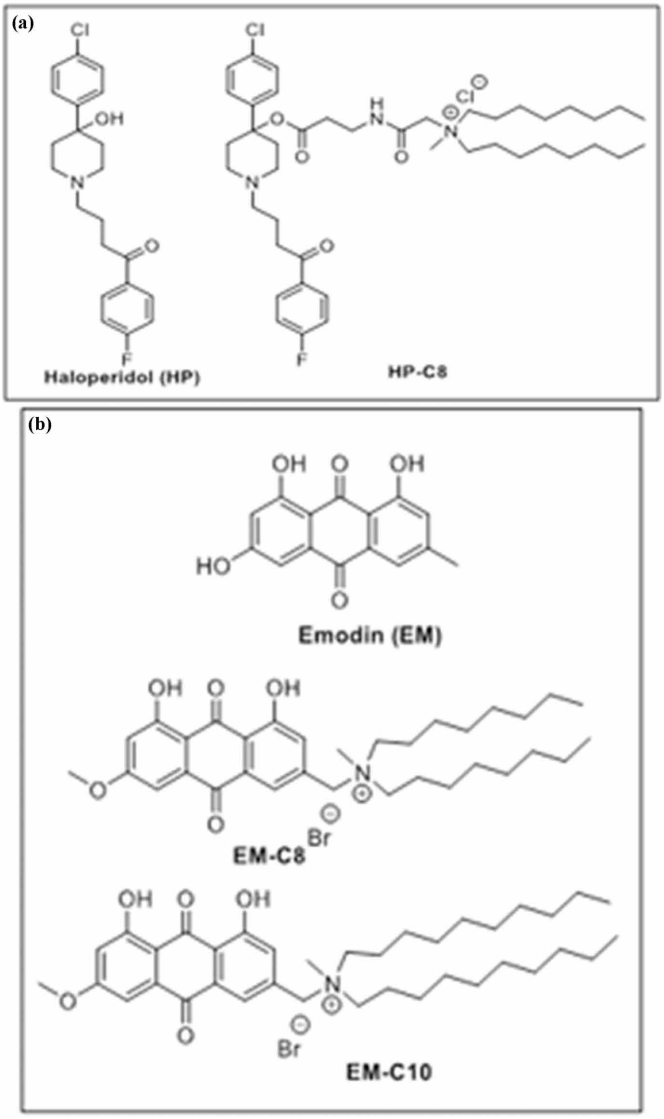
Figure 6. (a) Structure of 3β [N-(N', N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol), (b) Lipid-based drug delivery system



4. Emodin

Emodin (1, 3, 8-trihydroxy-6-methyl-9, 10-anthraquinone) is a natural chemical supplement found in Rhubarb (Fig. 7b). It possesses anti-tumor, anti-viral and anti-bacterial activities. Among them, only anti-cancer activity is more widely reported. However, to potentiate the anticancer effects of emodin molecule, certain chemical modifications are necessary. Teich and Gu both affirmed that the cationic side chain containing emodin derivatives showed stronger cytotoxic activity compared to simple emodin molecule. The molecule with eight and ten carbon chain-lengths (EM-C8, EM-C10) significantly inhibited proliferation of cancer cells via arresting the cell cycle predominantly in the G0/G1 phase, and at the same time, exhibited low cytotoxicity to non-cancerous cells. In vivo studies also revealed that the 10 mg/kg of EM-C8 derivative and 25 mg/kg of EM-C10 derivative showed significant anti-proliferative activity compared to emodin molecule.

Figure 7. (a) Structure of haloperidol and HP-C8, (b) Structure of Emodin (EM), EM-C8 and EM-C10



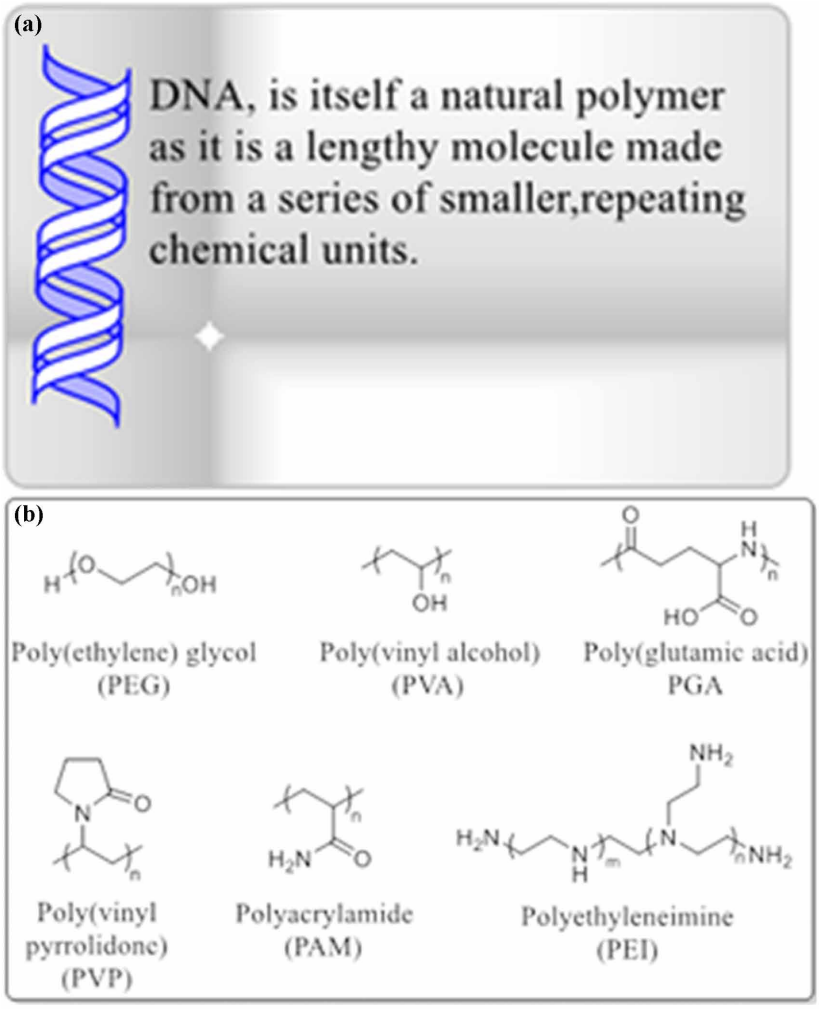
POLYMERS

Polymer is derived from polymerization. A century ago, in 1920, Professor Hermann Staudinger published his paper, "Uber Polymerization," which coins the term 'polymerization.' Polymers can be of natural or synthetic class (Fig. 8a).

Polymers in Drug Delivery

Since beginning polymers are playing very integral role in the establishment and advancement of drug delivery technology (Fig. 8b). Polymers provide controlled release of medicaments thus maintaining a cyclic dosage form. Polymers can also tune up the release of both hydrophilic and hydrophobic drugs. There are different desirable properties of polymers that boosted researchers to develop chemotherapeutic dosage forms using different polymers.

Figure 8. (a) DNA as a natural polymer, (b) hydrophilic polymers used in drug delivery



These polymers are successfully tested in controlled drug delivery formulations as they are chemically inert and does not show leaching properties among which few extensively used polymers are listed below:

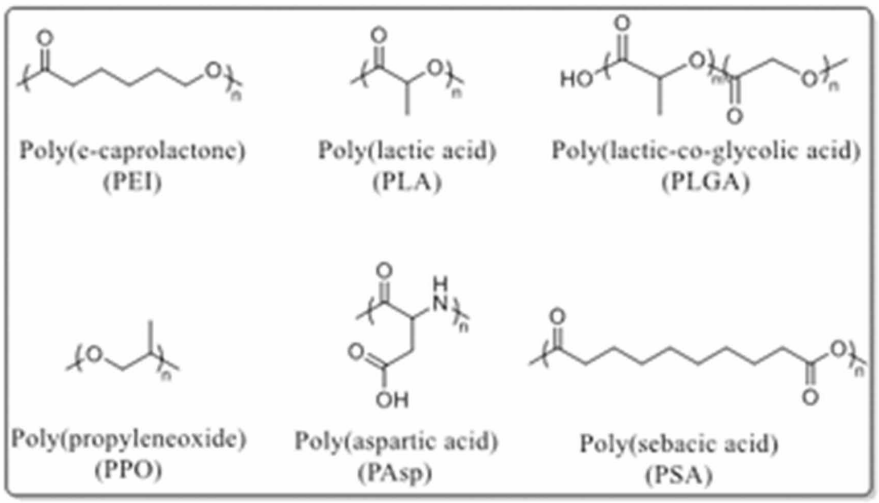
- Poly(urethanes) for elasticity.
- Poly(siloxanes) or silicones for insulating ability.

- Poly(methyl methacrylate) for physical strength and transparency.
- Poly(vinyl alcohol) for hydrophilicity and strength.
- Poly(ethylene) for toughness and lack of swelling.
- Poly(vinyl pyrrolidone) for suspension capabilities.
- Poly(2-hydroxy ethyl methacrylate).
- Poly(N-vinyl pyrrolidone).
- Poly(methyl methacrylate).
- Poly(vinyl alcohol).
- Poly(acrylic acid).
- Polyacrylamide.
- Poly(ethylene-co-vinyl acetate).
- Poly(ethylene glycol).
- Poly(methacrylic acid).

With the advancement in medical science, researchers came up with few polymers that not only helps in the controlled release (Fig. 9) but are also degradable within the body, that includes-

- Polylactides (PLA).
- Polyglycolides (PGA).
- Poly(lactide-co-glycolides) (PLGA).
- Polyanhydrides.
- Polyorthoesters.

Figure 9. hydrophobic polymers used in drug delivery



Polymer Types Used in Preparing Co-Delivery Systems

Depending upon different physiochemical characteristics of polymers, researchers have gained immense success in optimizing formulations using such properties.

Table 3. Different properties of polymers used in formulation development and delivery of drug

Types	Polymers	Dosage form	Drug 1	Drug 2	Cell Line	Ref.
pH-sensitive	PDEA-PDMA-PEG, Trimethyl Chitosan, PDP-PDHA	M	siBcl-2	DOX	Hep G2	Wang, Y et al.
Redox-sensitive	PEI-CD, PEG-PLG-PDMAPMA	NP	miRNA-34a	DTX	MCF7, HT1080	Fan, H et al.
Thermo-sensitive	PLGA–PEG–PLGA, PECT	HMM	IL15, DOX, MTX	DTX, CDDP	B16F0-RFP, Hep G2	Huang, P et al.
MMP-sensitive	PEG-pp-PEI-DOPE, PEG-PLA, G0–C14	NP	miRNA-34a, siSurvivin	PCT, DOX	MCF7, HT1080	Salzano, G et al.
Magnetic-responsive	ASA-MNPs-CDDP/ mPEG-PLL-FA	NP	CDDP, DOX	MTX	HNE-1, NP69, MCF7	0. Metin, E et al.

TARGETED DELIVERY IN CANCER THERAPY

To achieve targeted delivery system in cancer different polymers are used depending upon their properties (temperature, pH, light, redoxpotential, and other special factors) so as to show maximum bio-availability, adequate target specification and with lesser or no toxicity.

1. Block Co-Polymer Conjugates

These kinds of polymers are prepared after conjugating hydrophilic and hydrophobic polymers with diverse properties together. They are formed due to physical or chemical interaction between these polymers. Block co-polymers integrates the advantages of various blocks in a single stream. They are used for the co-delivery purpose as these synthetic block co-polymers self-assemble into polymeric nanoparticles or micellar nanoparticles. Major material used to reduce toxicity in these biodegradable polymers are-chitosan, poly (lactic acid), gelatin, poly[N-(2-hydroxypropyl) methacryl amide] (HPMA) and their copolymers, poly(lactide-co-glycolide-co-caprolactone) and poly (lactic-co-glycolic acid).

There are six general methods for preparing polymeric nanoparticles-

1 Emulsion-diffusion
2 Emulsification
3 Coacervation
4 Double emulsification
5 Surfacepolymerization and
6 Layer-by-layer methods.

The unique and definite properties of such polymers allow them to change their drug release profile and accumulation as per the need of the formulation. They are not only used to target drugs, but also to target genes and bioactive substances. The ability of these systems to selectively deliver therapeutic agents to target tissues, cells, and cell compartments makes then the priority choice to deliver chemotherapeutics in a precise and accurate way. By doing so, the overall, release profile, pharmacological properties, and therapeutic outcomes are improved compared to delivery as free drugs.

2. Thermo-Sensitive Polymers

As we know, thermos-sensitivity is one of the most commonly used characteristics of biomedical applications. There are two kinds of polymers that can be distinguished by their phase distribution. 1-UCST (upper temperature of the critical solution), passes between phases during cooling. In the second type LCST (lower critical temperature of the solution), this transition occurs with increasing temperature. Polymer systems that have with UCST are more prevalent in the solubility of polymers in an organic solvent, whereas the systems with LCST shows solubility in aqueous solvents as van der Waals interaction is responsible for the solubility of polymers in organic solvents. Hydrogen bonds are formed as per the solubility of polymers in water. These pH-sensitive polymers can be weak bases (more polar in an acid environment in a protonated form) or weak acids (more polar in a basic environment in the deprotonated form).

3. Redox-Sensitive Polymers

Changes in redox potential in cancer tissues are due to the production of reactive oxygen species by activated macrophages. Oxidatively degradable polymers, such as arylborone based on acid esters (which after oxidation become phenols and boric acid), or dialkylsulphide-based polymers (which after oxidation become more hydrophilic), have been used as delivery systems for drugs to inflamed tissues. These types of polymer are applied mainly for diagnostic purposes. It can be said that designing a polymeric drug system with micro-environmentally sensitive polymers is a “smart” strategy. Combining multiple therapeutic agents that inhibit tumor growth through different pathways into one system is also a “smart” strategy. Many polymeric systems have shown promising effects in cancer therapy based on these two ideas.

Polymer-Mediated Anti-Cancer siRNA Delivery

Polymer-mediated delivery systems, usually called polymeric nanoparticles, are solid, biodegradable, colloidal systems which have been widely studied as drug vesicles. According to the material used, polymer-mediated delivery systems can be divided into two categories: water-soluble cationic polymers and polymer nanoparticles. For anticancer siRNA delivery, water-soluble cationic polymers mainly include cyclodextrin or polyethyleneimine (PEI), while polymer nanoparticles are usually based on polycaprolactone (PCL), poly(D,L-lactide) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA). Cyclodextrin is the most promising natural polymer for siRNA delivery. It was first introduced for the delivery of plasmid DNA in 1999 and later reoptimized for siRNA delivery. Less than a decade later, cyclodextrin polymer (CDP)-based nanoparticles were moved into clinical trials for siRNA delivery. Cyclodextrin polymer nanoparticle was the first targeted siRNA delivery system that entered clinical

trials for cancer treatment. Cyclodextrin polymers are polycationic oligomers synthesized by a step-growth polymerization between diamine-bearing cyclodextrin monomers and dimethyl suberimidate, yielding oligomers with amidine functional groups. In cyclodextrin polymer-mediated siRNA delivery systems, adamantaneePEG (ADePEG) and adamantaneePEGetransferrin (ADePEGeTf) are usually used to improve delivery efficacy in vivo

Research in polymer therapeutics has enjoyed success over the past few decades in mediating safe and effective delivery of bioactive agents to treat an enormous variety of medical conditions. The research initiatives highlighted in this review show great promise in enhancing drug delivery so that drugs will be distributed only to locations where needed in therapeutically relevant quantities and will rely less on the dosing efforts of the patient. Looking ahead, research efforts should progress toward understanding more about how polymers and polymer products interface with biological systems. Many studies in recent years have reported on novel chemical roots for advanced drug delivery systems, but too often biocompatibility studies are overlooked until late in development. The result is that many new devices fail at a later stage of their development. Judicious cellular and animal studies early in device development will help to ensure that polymer-related breakthroughs and in vitro successes result in effective and safe drug delivery platforms.

Peptides

Peptides are a short, cyclic or linear chain of amino acids, linked together in a proper fashion by peptide bonds, also known as amide bonds (Fig. 10a). Peptides differ from proteins in terms of the number of amino acids present in chains, traditionally, peptides consist of between 2 - 50 amino acids, on while, proteins are made up of 50 or more amino acids(Snyder & Dowdy, 2004). Although, proteins are long molecules made up of multiple peptide subunits, termed as polypeptides, can be digested by enzymes (other proteins) into short peptide fragments. In our body, peptides perform several physiological functions. For example, some peptides act as hormones, which are molecules that when released from cells affect other areas of the body.

Classification

Best of our knowledge, peptides can be classified into two classes: on structure basis and on functionality basis, which further classifies into two sub-categories, like, linear and cyclic peptides, cell-penetrating and cell-targeting peptides respectively (Fig. 10b).

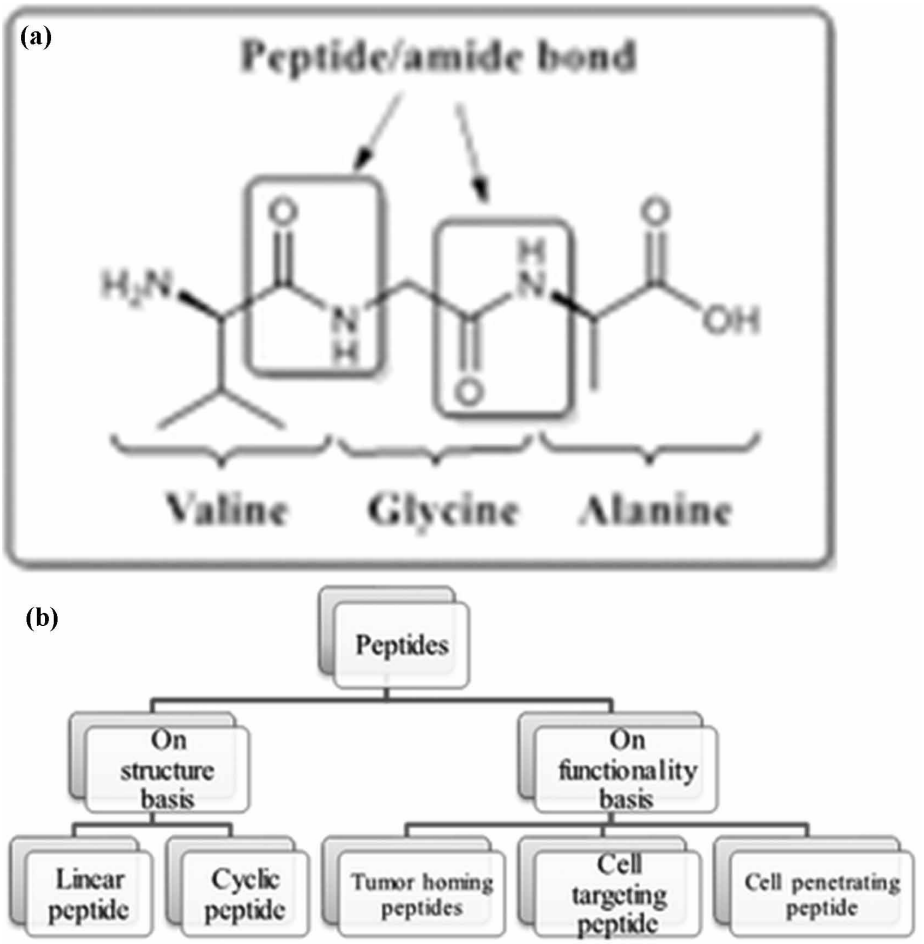
Linear Peptides

Linear peptides that contain 2–10 amino acid residues are especially flexible in solution. Once the length of linear peptides extends to between 10 and 20 amino acid residues, random linear peptide sequences can begin to obtain secondary structures, including a-helices, turns and b-strands (Roxin& Zheng, 2012).

Cyclic Peptides

Cyclic peptides have a higher selectivity for the receptor than do the parent linear peptides because cyclic peptides have more restricted conformations, and are thus more stable (Fig. 11a) (Roxin& Zheng, 2012).

Figure 10. (a) General structure of peptide, (b) Classification of peptides



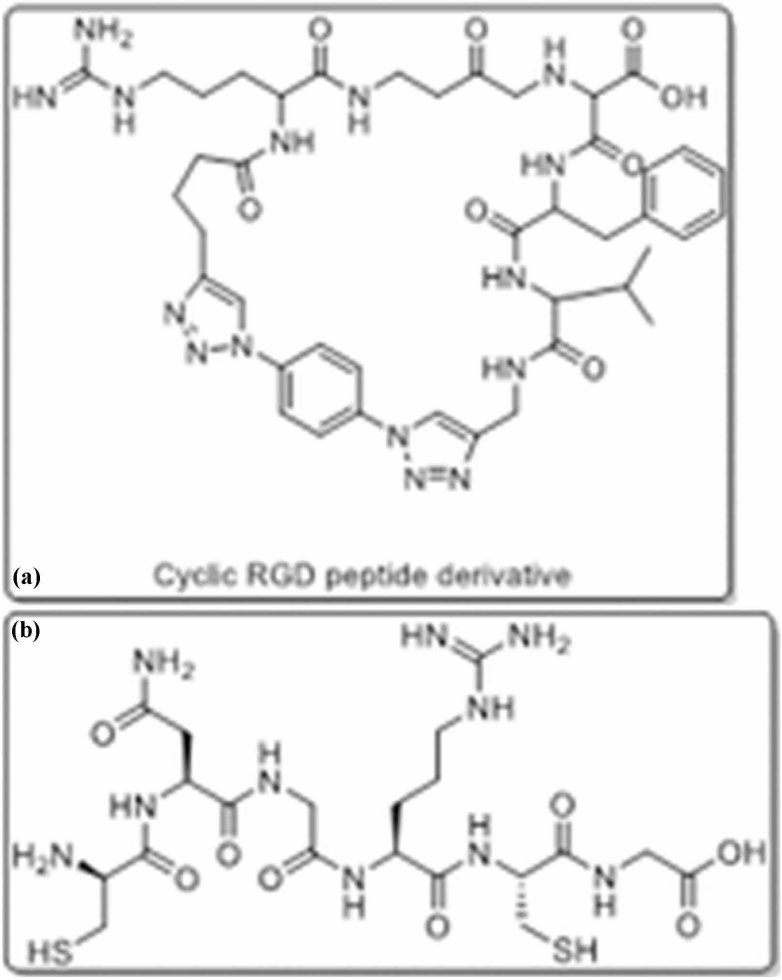
Tumor Homing Peptides

Tumor homing peptides have common sequence motifs such as the amino acid sequences RGD, or NGR (Fig. 11b). These peptide motifs specifically bind to a surface molecule on tumor cells or tumor vasculature.

Cell-Penetrating Peptides

CPPs, also known as protein transduction domains (PTDs), are positively charged short peptides with 5–30 amino acids long that can penetrate into the biological membrane and deliver a wide variety of cargos into cells. Transactivated-Transcription (TAT) and penetrating were first Cell-penetrating peptides CPPs derived from HIV-TAT and Antennapedia homeodomain, respectively. CPPs have received extensive attention in recent decades due to their high transduction efficiency (internalization efficiency of CPPs into cellular membrane) and also low cytotoxicity. Owing to the ability of these peptide sequences to transport across the cellular membrane, they found to be a promising candidate for intracellular delivery. Indeed, attachment of cargo molecules to the CPP results in penetration of the intact cargos and then internalization into cells.

Figure 11. (a) Structure of cyclic RGD peptide, (b) Structure of NGR peptide



Conjugation of CPP to cargo molecules could occur in two ways: covalent and non-covalent binding. In covalent conjugation, cargo molecules attach to CPP via covalent bonds in a time-consuming process. This method has a serious drawback when transporting various cargos because each type of cargo needs its own covalent conjugation. In second approach, CPPs through electrostatic interaction bind to cargo. This method due to its high flexibility is suitable for a wide range of cargo delivery applications. Overall, delivery efficiency of the CPPs may be depending on some parameters such size of complex of cargo-CPP, nature of CPP, the type of peptide sequence and so on. Foran instance, the CPP-cargo complex should be smaller than 200 nm in order to achieve the optimum endocytic uptake. Besides, amphiphilic CPPs and arginine-rich CPPs because of high electrostatic interaction with negatively charged cellular membrane can significantly internalize into biomembranes(MacEwan& Chilkoti, 2013).

Cell Targeting Peptides

Researchers have developed strategies in which chemotherapeutic drugs are conjugated to cancer targeting peptides (CTPs) that exploit the unique characteristics of the tumor microenvironment or cancer cells, thereby improving cancer cell specificity.

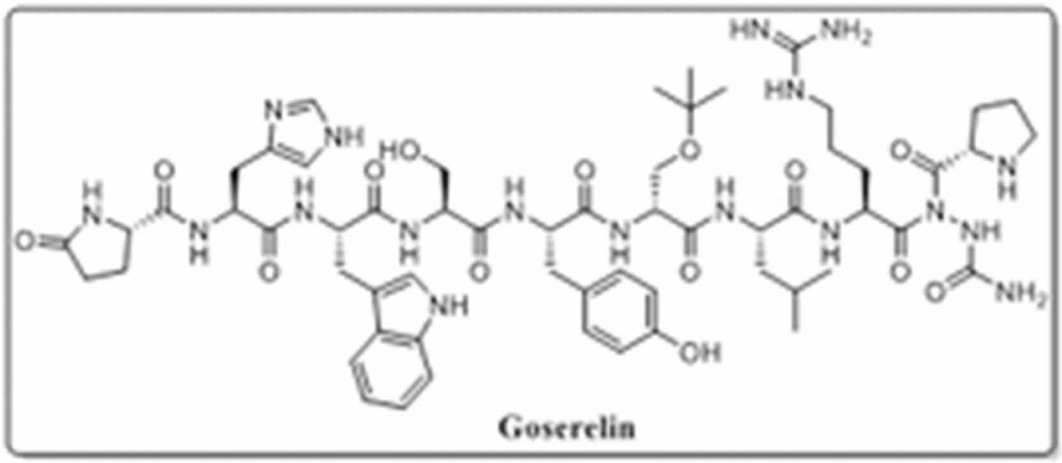
PEPTIDE VACCINES

In the last decade, this idea of vaccinations against cancer has transformed into clinical studies aiming to optimally deliver vaccines based on defined antigens to induce anticancer immunity. This method of treating cancerous cells relies on vaccines consisting of peptides derived from the protein sequence of candidate tumor-associated or specific antigens. Tumor cells express antigens known as tumor-associated antigens (TAAs) that can be recognized by the host's immune system (T cells). Many TAAs have already been identified and molecularly characterized.

Advantages of Peptides

Currently there are about 60 approved peptide drugs in the market generating an annual sale of more than $13 billion. Out of four peptide drugs in the market which have reached global sales over $1 billion, three peptides are used in treating cancer directly or in the treatment of episodes associated with certain tumors (leuprolide, goserelin, and octreotide) (Fig. 12).

Figure 12. Structure of goserelin



Another category of anti-cancer peptide is peptide antagonists which can preferentially bind to a known receptor. Moreover "pro-apoptotic" peptides mediate significant induction of apoptosis (programmed cell death) in tumors.

CONCLUSION

With advancement in research, different modifications and advancements occurs in the delivery of chemotherapeutic drugs. Our main emphasis was to uncover the most recent and smart technologies for different nano-materials that offers a unique way to deliver chemotherapy drugs to their intended target without affecting healthy cells. Various approaches to modulate and upgrade the drug delivery system in cancer were discussed with a particular emphasis on lipids-cationic& anionic, polymers-in response to pH, redox potential, enzymes, temperature, and light.

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Chapter 8
Use of Lipids, Polymers, and Peptides for Drug Delivery and Targeting to Cancer Cells or Specific Organs

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ABSTRACT

Cancer has been the most deleterious disease in recent times, and unfortunately its spread is increasing. Systemic treatment with chemotherapeutics remains the conventional way of treating many cancers, despite the serious damage long-term chemotherapy can cause in healthy tissues. Many therapeutic strategies have achieved popular practical applications, but drug delivery systems still face challenges associated with safety, and this has led to the development of safer drug delivery methods composed of biocompatible substances. In this respect, lipid-, polymer-, and peptide-based drug delivery systems have been proposed as safer candidates for cancer therapy. These delivery methods are expected to as biodegradable systems with low cytotoxicity for cancer therapy. Therefore, in this chapter, the authors discuss use of lipids, polymers, and peptides as delivery vehicles for chemotherapeutic agents and their structural characteristics.

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INTRODUCTION

Cancer is a major cause of mortality in the most of countries across the earth. However, the ability of existing standard treatments for a range of cancers is suboptimal. Basically, utmost cancer treatments deficient specificity which signify that these treatments imitate both cancer cells and their regular counterparts. Furthermore, several anticancer agents are extremely toxic and therefore constraint their use in treatment. Also, a number of cytotoxic chemotherapeutics are greatly hydrophobic, which restricts their efficacy in cancer therapy (Snaebjornsson et al., 2020). Conclusively, many chemotherapeutic agents show short half lives that restrain their efficiency. Radiation therapy, surgical exclusion, and combinatorial approaches have been also suggested as treatment options, still, these modalities cannot be used to kill malignant cells that have already spread through a body. As a result of these insufficiencies, many existing treatments lead to the massive side effects, failure, and cancer patients inconvenience due to complications in administration. These situations has led to the development of new drug delivery systems that can help to overcome the limitations of conventional treatment approaches (Bandopadhyay et al., 2020).

Lipids, polymers, and peptides can be incorporated in drug delivery systems for effective cancer chemotherapy with lowers side effects such as low cytotoxicity, enhanced solubility of hydrophobic drugs and controlled release of drugs. Targeted delivery combined with controlled drug release has a critical role in the future of personalized medicine. The significant benefit of using lipids, polymers and peptides as a drug carrier noticeably increases organ or cell-specific drug accumulation and opens up the opportunity of controlled release of the delivered drug where the remedial effect is required. Selective activation in this manner could inhibit the drug's toxicity from affecting normal tissues and cells, eliminating whichever harmful side effects it might otherwise have which indicates the current achievements in the development of polymers nanoparticles in cancer therapy. The objective of targeting specific cell surface receptors through structural compatibility has revived the use of these biomolecules as enormously specific carriers as short peptides are typically non-antigenic, are structurally simple and synthetically distinct. In recent years, many developments in the field of lipid, polymers and peptide-based nanoparticles principally for cancer therapy, as their use can bypass the side-effects and can reduce the damage common to conventional chemotherapy (Maghrebi et al., 2019; Deb et al., 2019). In this chapter, we describe the progression of these biomolecules for targeted delivery of chemotherapeutics and discuss the latest innovations in the field that must lead in the near prospect to their clinical application.

LIPIDS

Over the past few years, lipids and liposomes have gained attention as a carrier system for therapeutically active agents. This is due to their unique characteristics, including biocompatibility, low toxicity, biodegradability, lack of immune system activation, and capability to incorporate both hydrophobic and hydrophilic drugs. Lipids have shown tremendous therapeutic potential as carriers for payloads and delivery to targeted sites, which has led to several liposomal formulations designed for the clinic and clinical trials for cancer therapy. There are several types of lipid-based carriers that stem from manufacturing methods and the main components used. For example, liposomes, micelles, emulsions, solid lipid nanoparticles, core-shell-type lipid-polymer hybrids and biomimetic vesicles have been widely investigated for lipid-based drug delivery (Markovic et al., 2020).

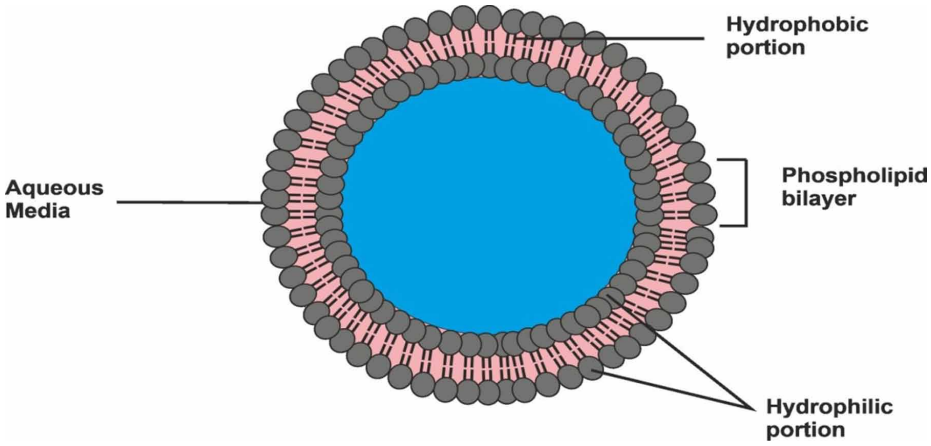
LIPOSOMES

One of the most efficient drug delivery systems for disease treatment, are Liposomes. Liposomes are spherical vesicles of phospholipid bilayer enclosing a water droplet (Figure 1), especially designed artificially to act as a vehicle for drugs or other substances transportation into the tissues. These systems have unique properties such as smaller size, biocompatibility, biodegradability, low toxicity hydrophobic and hydrophilic character, and immunogenicity that result in significant efficiency on cancer therapy (Zhang and Huang, 2020).

Niosomes

Preparation of liposomes by non-ionic surfactants such as alkyl esters or alkyl ethers results in niosome structures. An advantage of niosomes is storage and handling with biocompatible, biodegradable, and non-immunogenic properties without any specific conditional requirements. The oral bioavailability of drugs with low absorption efficiency can be increased by these delivery systems; also, they have a suitable impact on the clearance of the drug from reticulocyte endothelial system and lead to the therapeutic effect of drugs (Barde and Dighe, 2020).

Figure 1. The general structure of liposomes



Archaeosomes

Archeobacteria membranes have diether and/or tetraether linkages that are used for generation of lipid layers of archeosomes. Archeosomes, known to be present in archaeobacteria, are archaeol, macrocyclic archaeol, 3´-hydroxy archaeol, caldarchaeol, nanitol-caldarchaeol and cyclopentane-caldarchaeol. Although, some of archaeobacteria have various amounts of hydroxyl archaeols (hydroxyl diethers), their exact function remains unknown. For example, archeobacteria such as *Methanosarcina mazei* and *Halobacterium cutirubrum* contain archaeol lipids whereas thermophilic type *Thermoplasma acidophilum* are rich in caldarchaeol lipids. The higher efficiency of archeosomes in drug or gene delivery for is resulted from the biocompatibility and higher stability of these archeosomes (Sharadha et al., 2020).

Novasomes

Novasomes are produced by mixture of polyoxyethylene fatty acids (as monoester), free fatty acids and cholesterol. The diameter of novasomes generally range between is 0.1 up to 1 micron. Novasomes have 2-7 bilayers and a large amphipathic core with 80-85% of drug loading. Novasomes can capsulate both hydrophobic and hydrophilic drug molecules, and can be encapsulated by novasomes. Moreover, it is possible for entering drugs/compounds in bilayers and therefore prevents the incompatibility of drugs in surface charge properties. These systems can deliver a high amount of drug ingredients (Abd-Elal et al., 2016).

Emulsomes

Emulsomes are phospholipid bilayer with a solid fat core. Solid fat core is enclosed by ones or more phospholipid bilayers. Internal core of emulsomes is different and affect the hydrophobic drug loading. Emulsomes have properties of both emulsion and liposomes. In order to produce emulsomes of smaller size, the drug loading is followed by sonication. Many types of stabilizers, such as soya lecithin or cholesterol can be utilized for the improvement of oil-in-water emulsion formulation (Ucisik et al., 2015).

Virosomes

Virosomes offer the same versatility with regard to lipid composition as liposomes but in addition include viral membrane proteins, either virus-derived or recombinant. The main feature of pH-dependent influenza hemagglutinin mediated membrane fusion is also attractive for cytoplasmic drug delivery. Efficient encapsulation of the respective drug and specific targeting of the vehicle can be achieved (Rathor et al., 2019). A successful application of the virosomes formulation was conducted by Waelti et al., (2002) showing the inhibition of tumour progression in a mouse model after treatment. This formulation included phosphatidylethanolamine-polyethylene glycol -anchored antibodies for targeting and hemagglutinin for cytoplasmic delivery of the encapsulated doxorubicin.

Vesosomes

Vesosomes is a multi-compartmental structure of lipid vesicles, derived from liposomes, which are potentially powerful models used for drug delivery. These structures include membrane-bound vesicles, which encapsulate drugs in their core. The function of external bilayers is the protection of the drug from degradation by enzymes and other immune-defensive elements of human body. Considerable advantages of vesosomes are simple preparation and multiple drugs loading that are important in cancer treatment with resistance to special drugs (Arshad et al., 2020).

Cryptosomes

Cryptosomes are liposomal composition which comprises of poloxamer molecules (polymers) and liposomes embedded with one or more delivery agents. Cryptosomes decrease contact and uptake by mononuclear phagocytic system, through high amount of polyethylene glycol on their surface. The result of such a characteristic is the rise in the time of circulation. Also, active targeting of cryptosomes get

possible by application of various other ligands. Poloxamer have non-ionic triblock copolymers structure of a central hydrophobic chain of polyoxypropylene, surrounded by two hydrophilic chains of polyoxyethylene. These structures are used in cryptosomes preparation, and some of which can be incorporate into the bilayers and production of micelles (Sharadha et al., 2020).

MICELLES

Micelles are amphiphilic macromolecules that have distinct hydrophobic and hydrophilic block domains, with the structure of the copolymers. Recently, micelles formulations have gained considerable attention as a versatile platform with improved drug delivery and efficacious response in cancer treatment. The most commonly used hydrophilic blocks are polyethylene glycol with a molecular weight of 2–15 kDa, while the hydrophobic blocks typically are polyethers, polyesters, or polyamino acids, such as poly (L-aspartic acid), poly(ε-caprolactone) and poly (propylene oxide). The idea of stimuli-responsive drug delivery systems comes from the fact that the tumor tissue possesses a different characteristic feature as compared with the normal tissue such as altered redox potential, acidic pH, and over expressed proteins, enzymes and temperature. In addition, these stimuli responsive can be transported to the tumor site externally to allow micelles to bypass the biological barriers, reach their target sites, and release their loaded drugs (Biswas et al., 2016).

pH-responsive Micelles

The pH-responsive micelles have recently emerged as an important formulation for anticancer drug or imaging agent delivery. As a result of enhanced metabolic rates and increased aerobic glycolysis, the microenvironment of most solid tumors is intrinsically acidic (pH 6.5–7.2), while the pH value in the blood and healthy tissue is about 7.4. Moreover, an even lower acidic pH is found in endosomes (pH 6.5–5.5) and lysosomes (pH 4.5–5.0). Accordingly, the difference in pH has been widely exploited to achieve tumor site- or organelle-specific activation of pH-responsive PMs and on-demand drug release. The pH sensitivity of polymers mainly results from the protonation of ionizable groups or the degradation of pH-sensitive linkages. Therefore, one possible approach to impart pH sensitivity to micelles is to incorporate the ionizable groups into the micellar polymers to ionize at the tumor extracellular or intracellular acidic pH. After the micelle decomposition or destabilization, their payloads are released at the tumor tissues (Kocak et al., 2017). For example, a pH-sensitive mixed micelle was prepared by Wu et al., 2013 for cytosolic delivery of doxorubicin, which was composed of two block polymers, 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine- polyethylene glycol and pH-responsive poly(histidine) - polyethylene glycol.

Redox-responsive Micelles

In the past few years, a tremendous work was done in the development of redox-responsive micelles for targeted intracellular drug or gene delivery. Redox potential has been regarded as a viable biomarker to distinguish between the extracellular and intracellular environments, as well as between the tumor and normal tissues due to the difference in the glutathione concentration (Zhang et al., 2019). In study conducted by Shi et al., 2014 used four-arm poly(ε-caprolactone) - polyethylene glycol copolymers to

develop a multifunctional star shaped micellar system by combination of active targeting ability and redox-responsive behavior. The redox-responsive behavior is developed by connecting poly(ε-caprolactone) and polyethylene glycol via disulfide bonds, and active targeting ability was achieved by the introduction of folate ligands to the end groups of the hydrophilic segment. Chemotherapy cancer drug doxorubicin was trapped into the micelles during the self-assembly of the star-shaped poly(ε-caprolactone) - polyethylene glycol copolymers. The prepared redox-responsive micelles could be specifically internalized by tumor cells through the folate receptor-mediated endocytosis, and the disulfide bonds could be immediately cleaved in response to the intracellular high level of glutathione, resulting in quick doxorubicin release.

Enzyme-responsive Micelles

Dysregulated enzymes have been considered as biomarkers for diagnosis and prognosis in different types and stages of cancer These are also emerging as promising biological triggers for targeted cancer therapy. Expression of several types of enzymes like peptidases, proteases, and lipases in solid tumors is often greater compared to their concentrations in normal tissues. Based on these over expressed enzymes, in the past few years, a wide variety of micelles have been developed as the enzyme-responsive systems for selective and efficient targeted drug delivery (Dai et al., 2019)

Thermo-responsive Micelles

Elevation of temperature or hyperthermia in specific tissues can either occur due to certain diseases such as tumor, inflammation, or infection. Temperature/thermo-responsive drug delivery system is among the most investigated stimuli-responsive strategies for cancer therapy. The thermo-sensitive micelles comprise of polymer with thermo-responsive block which undergo a quick change in their physical properties in response to the change in temperature to destabilize the micelles which result in release of drug. The most frequently used thermo-responsive polymer for the preparation of thermo-sensitive micelles is poly(*N*-isopropylacrylamide) (Farjadian et al., 2020).

EMULSIONS

Emulsions are meta-stable colloidal system which comprises of droplets of one liquid dispersed within another immiscible liquid. Emulsion technology has been used extensively in the pharmaceutical industry for drug delivery. These emulsions are either oil-in-water or water-in-oil type (Figure 2). In general, there are three main types of emulsion systems as nanoemulsions, microemulsions and macroemulsions (Figure 3) (Kale and Deore, 2017).

Nanoemulsions

Nanoemulsions are commonly referred to as ultrafine emulsions and are usually blue-white to semi-opaque in nature. Droplet sizes in nanoemulsions are usually in the size range 100–400 nm in diameter and are formulated by high-pressure processes such as homogenization. The uptake of drugs delivered in nanoemulsions is through receptor mediated endocytosis. Kinetically stable and metastable nanoemulsions are finding an increased number of applications in location of tumours by magnetic targeting and

using oil-in-water nanoemulsions and derivatized ferromagnetic fluids or phase contrast fluorinated colloids for ultrasound imaging (Barkat et al., 2020).

Microemulsions

Microemulsions are transparent, thermodynamically stable mixtures of oil and water stabilized by emulsifiers. Contrary to their terminology, microemulsions consist of the smallest droplet sizes found in emulsion systems and droplet sizes range from 10 to 100 nm in diameter. Due to very small droplet sizes results in improved bioavailability of drug (Ohadi et al., 2020).

Figure 2. Schematic representation of water in oil (left) and oil in water (right) emulsions

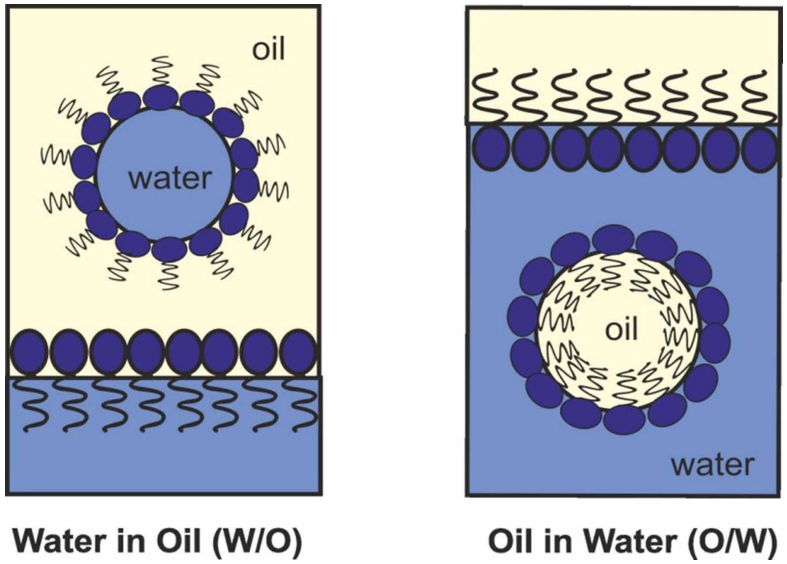
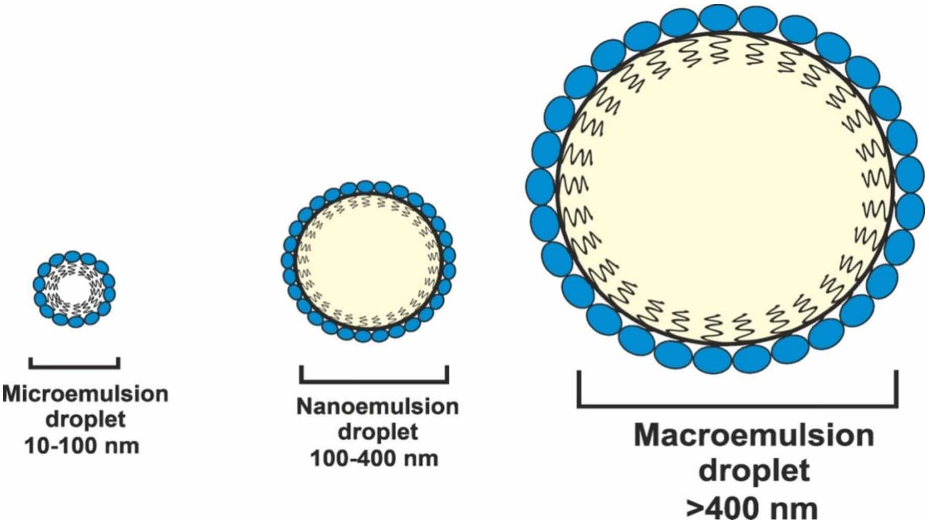


Figure 3. Types of emulsions on basis of size and diameter of droplet



Macroemulsions

Macroemulsions are often referred to as coarse or opaque emulsions due to their relatively large droplet sizes which results in a turbid solution. In general, macroemulsions consist of droplets larger than 400 nm in diameter. Due to their larger droplet sizes, macroemulsions are kinetically and thermodynamically unstable (Callender et al., 2017).

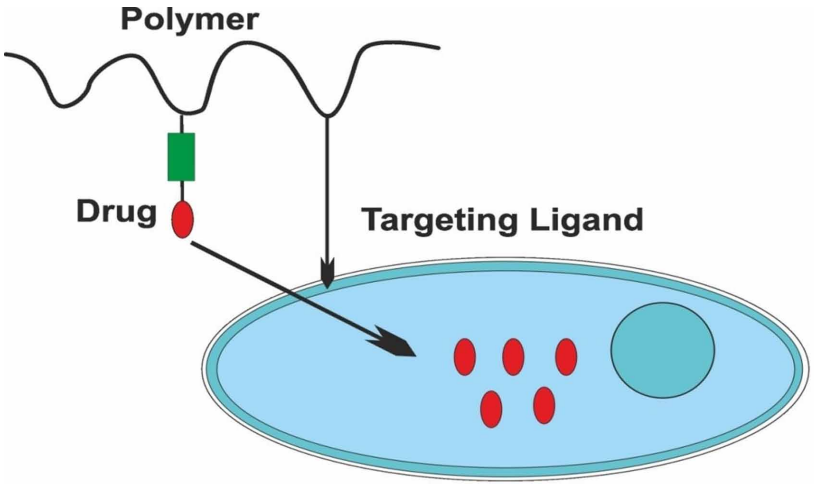
POLYMER

Polymers represent a promising class of drug delivery system that can activate delivery in response to specific stimuli (Figure 4). Polymers and their formulations in different forms, such as hydrogels, micelles, and polymerases which can play important role in cancer diagnosis and treatment. Polymeric formulations are popular in many areas due to properties such as their eco-friendly nature, unique design capacity, easy production, and cost-effectiveness. In composites, interactions between the polymer matrix and the nanofiller are critical in determining the properties of the hybrid structure (Calori et al., 2020).

Biopolymers

Biopolymer is any polymer that can be degraded by enzymes or through decomposition from the actions of microorganisms. Their nano-formulations of biopolymers are gaining attention for use as antimicrobials, drug carriers, and sensors and in disease diagnosis, tissue engineering, wound healing and cancer therapy. Insoluble biopolymers such as collagen, elastin, chitosan, keratin, and silk can be converted into soluble derivatives through chemical and enzymatic hydrolysis and can be used for drug delivery for treatment of cancer. Polysaccharides which have low immunogenicity can be used for diagnosis, bioactive therapy, controlled drug delivery, gene therapy, theranostics, cell encapsulation, tissue engineering, and medical devices (Harting et al., 2019).

Figure 4. Representation of polymer drug conjugate targeting cell



Synthetic Polymers

Synthetic polymers can be an interesting alternative for the preparation of nanocarriers in the place of traditionally used biodegradable polymers for treatment of cancer. Synthetic polymers such as polylactic acid, polyethylene glycol, polyvinyl pirrolidone-co-vinyl acetate copolymer, polyacrylic acid and polyvinyl alcohol-co-albumin copolymer for the delivery of the drugs are used (Feldman, 2019).

Polymer Micelles

Polymer micelles are an important group of drug carriers widely used in chemotherapy therapy. Polymeric micelles consisting of hydrophobic polyethyleneimine and cis 1,2-cyclohexandycarboxylic anhydride were used to deliver drugs such as Candesartan and Paclitaxel in the chemotherapy treatment of cervical cancer. Study conducted by Wu et al., 2016 suggested that cross linked mPEGylated starch with 3,3′-dithiodipropionic acid micelles hold great potential as ideal drug delivery carriers for cancer treatment. Another recent study by Zhang et al., (2018) have reported poly[L-co-*N*,*N*′-bis(acryloyl) cystamine-co-dodecylamine] micelles could be potentially used for effective drug delivery.

Polymer Hydrogels

Hydrogels have specific characteristics that make them applicable in some areas of medicine, such as tissue repair, sensors, drug carriers, and many others. These hydrogels are widely used in the pharmaceutical and medical fields, such as in biosensors, materials for contact lenses materials, synthetic skin, and lining for hearts. A recent study conducted by Patra et al., (2018) suggested that hydrogel based on glycogen and 2-hydroxy ethyl methacrylate using ethylene glycol dimethacrylate crosslinker was used to carry 5FU drug effectively killed MG-63 cancer cells. Another study carried out by Fathi et al., (2015) reported that anticancer properties of Mitoxantrone drug were enhanced when loaded with Fe_3O_4 nanoparticles-based hydrogels.

Polymersomes

Polymersomes are vesicle membranes made of amphiphilic synthetic block co-polymers. Polymersomes have attracted much attention as versatile drug carriers due to their tunable membrane properties and ability to encapsulate or integrate a broad range of drugs. Examples of polymersome block copolymers are poly(acrylic acid-co-distearate acrylate), the poly(trimethylene carbonate)-co-poly(L-glutamic acid) copolymer, polybutadiene-b- poly(ethylene oxide), poly(ethyl ethylene)-b- poly(ethylene oxide) and polystyrene-poly(ethylene oxide) (Meerovich et al., 2019).

PEPTIDES

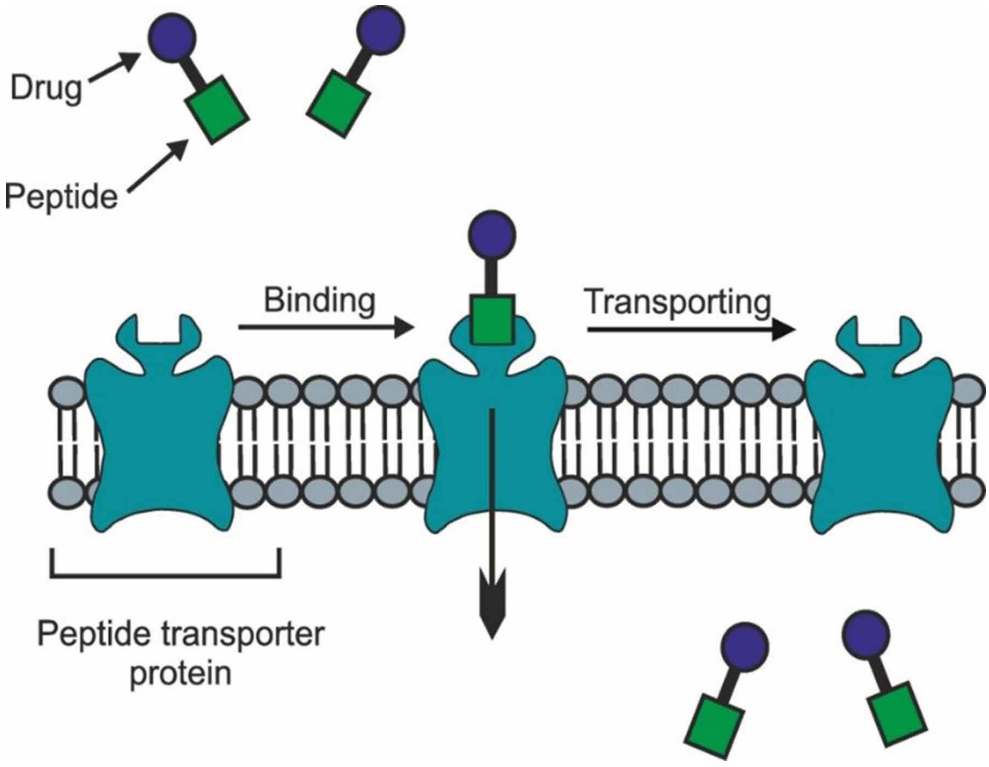
Due to rapid growth in pharmaceutical industry industrial capacity of producing a large amount of potential therapeutic peptides and proteins has enhanced. Endogenous peptides and proteins play an important role in the regulation and integration of life processes and act with high specificity and potency. Peptide–drug conjugates are an emerging class of pro-drugs, manufactured through the covalent attachment of a

specific peptide sequence to a drug. The incorporation of peptides enhances functionality of drug, as the amino acid sequence can control the physicochemical properties of the conjugate and active targeting due to presence particular receptor on the tumor cell surface. A common issue among traditional small molecule anticancer drugs is their poor solubility in aqueous solutions. This problem can be overcome by conjugating drug with water soluble peptide. The two most commonly used water-soluble peptide that can enhance drug delivery are cell surface targeting and cell-penetrating peptides (Lee et al., 2019).

Cell Surface Targeted Peptide–Drug Conjugates

First point of interaction between any drug or delivery formulation and a cell is at the outer cell membrane (Figure 5). Abnormally expressed receptors present on cancerous cells provide a focal point for targeted delivery. Integrins are transmembrane receptors that facilitate cell-extracellular matrix adhesion. In the past two decades, various kinds of integrin targeted peptide–drug conjugates have been designed (Arosio et al., 2017). A study conducted by Arap et al., 1998 found that coupling the free carboxylic groups of RGD4C to the 3′-amino position of doxorubicin gave a conjugate that exhibits an effective inhibition of *in vivo* tumor growth and metastasis inhibition as compared to free doxorubicin.

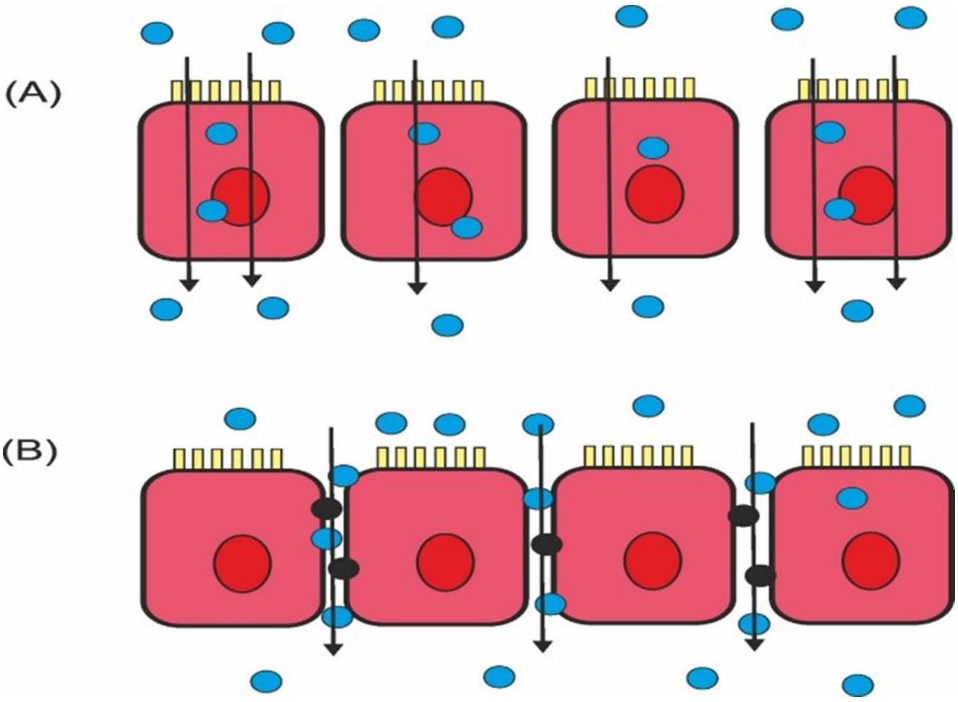
Figure 5. Schematic representation of peptide–drug conjugates targeting cell surface



Cell Penetrating Peptide–Drug Conjugates

A novel approach of cell-penetrating peptides, to overcome issue cell membrane impermeability and to deliver a large variety of drugs and macromolecules into cells has been recently emerged. (Figure 6). Guanidinium-rich drug delivery conjugate, a typically highly water-soluble conjugate is found to readily pass through the non-polar membrane of a cell and for some across tissue barriers. Multi-drug resistance in tumor cells is one of the main reasons for the failure of many cancer chemotherapeutic agents. Guanidinium-rich drug delivery can greatly assist in overcoming the issue of drug resistance in tumor cells (Al-azzawi and Masheta, 2019).

Figure 6. The absorption of therapeutic proteins/peptides by transcellular (A) and paracellular pathways (B)



CONCLUSION

The lipid, polymers and peptide are significant candidates for the improvement of drug delivery systems. These drug delivery formulations have potential to overcome the barriers that impede effective delivery of drugs to cancer cells. Encouragingly, many of these approaches were more effective than the conventional methods treatment of variety of cancer and tumors. To ensure these biomolecules-based drug delivery methods achieve the clinical success that is widely expected.

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Chapter 9
Apoptotic Pathway: A Propitious Therapeutic Target for Cancer Treatment

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ABSTRACT

Cancer is a major killer disease caused by uncontrolled growth and invasion of cells. Apoptosis is the cell's natural mechanism of death, which maintains tissue homeostasis. Any mutation that disturbs the apoptotic pathway leads to deregulated proliferation, resistance, and evasion of apoptosis. This evasion is one of the hallmarks of malignant developments. Apoptosis takes place via two distinct pathways i.e. the intrinsic and the extrinsic pathways. These pathways use cleaved caspases to execute apoptosis which in turn cleave many downstream proteins to kill the cells. They can also be inhibited through various means that include up-regulation of anti-apoptotic and down-regulation of pro-apoptotic factors. The authors here aim to impart a comprehensive understanding of the biochemical characteristics of these pathways that render scientists target these pathways and assess apoptosis restoring abilities of the novel drugs and natural products for cancer treatment.

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INTRODUCTION

To carry out essential biological functions, the structural and functional organization of all the cells in our body and the macromolecules (DNA, RNA, proteins etc.) within should be perfectly balanced and carefully regulated. Typically, a cell grows and divides to increase its number according to the requirement of the body and keeps the individual healthy. Any alteration in the expression or functions of the factors responsible for cell cycle progression and normal cell division, can either lead to abnormally high or poor growth and division of cells. The abnormal proliferation can lead to highly abnormal cell growth turning them cancerous. A cancerous cell performs abnormal functions and colonizes territories which are reserved for normal cells. From these aberrantly growing cells, a tumour- the neoplasm (reliantly growing mass of abnormal cells) may arise. It is estimated that 20% of males and 17% of females get cancer at some point in their lifetime and about 13% of males and 9% of female die from it (Jemal et al., 2011). Roughly 55% of cancer death occurs in less developed regions of the world that are the countries with a low or medium level of the Human Development Index (Bray et al., 2012). According to recent reports, cancer is a major killer disease of humans (Priestly et al., 2019). It is expected that approximately 1.8 million new cancer cases with about 0.6 million death will occur in 2020 in the United States itself (Siegal et al., 2020). In short, cancer is one of the most aggressive kind of diseases and proper treatment of which would be a boon to mankind. Unfortunately, cancer is mostly associated with poor treatment efficacy which makes the researchers constantly search for the novel and effective treatment strategies to target cancer cells efficiently and specifically.

As discussed above, the development and progression of cancer are caused by the alteration of various cellular pathways. In 2011, Hanahan and Weinberg proposed eight hallmarks of cancer and the two enabling characteristics of it (Figure 1) that still continue to provide a solid foundation for understanding the biology of cancer. Understanding of these hallmarks may help in the development of the unique and efficient treatment modalities against cancer by targeting one or more of these pathways. One such pathway is the apoptotic pathway that is well established to serve as a blockade to cancer progression (Adam and Corry, 2007). There are various pro-apoptotic and anti-apoptotic proteins that promote and down-regulate apoptosis respectively. A Cancer cell manages to suppress pro-apoptotic proteins and up-regulate anti-apoptotic proteins, thereby resisting cell death via apoptosis, which in turn favours cell proliferation. In this chapter, apoptosis and all its modulators and players are being discussed in detail.

APOPTOSIS AND OTHER TYPES OF CELL DEATHS

The word "apoptosis" comes from the Greek words "απο "(apo) and "πτωσιζ "(ptosis) that means "dropping off" in context to the falling of leaves from trees during autumn. It is a type of programmed cell death. In a biological perspective, it is used to describe cell death upon receiving some stimuli. It was first described by Kerr, Wyllie, and Currie in 1972.

It is a part of normal developmental and ageing phenomena that maintains the homeostasis of cell populations in tissues. It also occurs as a defence mechanism in the immune system or when cells are attacked by a certain disease or harmful agent (Norbury and Hickson, 2001).

Figure 1. Hallmarks of cancer as suggested by Hanahan and Weinberg (2011)

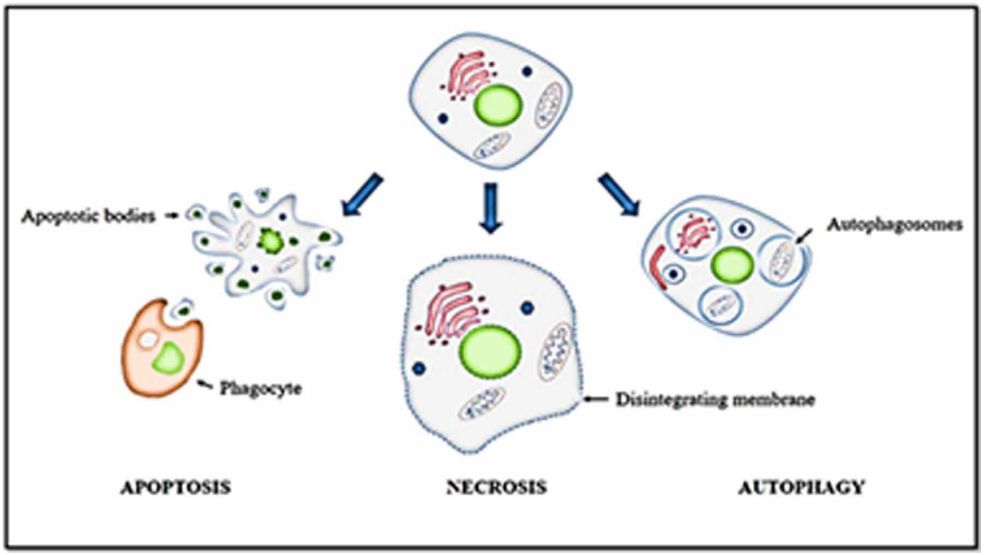
Being a highly selective process, it is also physiologically and pathologically important. Irradiation, various drug, hormones etc. could induce apoptosis in some cells. Whereas in some cells apoptosis is induced in response to certain ligand binding and protein crosslinking. It should be noted that all cells don't die in response to the same stimulus.

Beside Apoptosis, there are other forms of cell death these are Necrosis and Autophagy. Necrosis is characterized by the loss of plasma membrane integrity and may be programmed (induced in response to some stimuli) or non-programmed (in response to injury). On the other hand, autophagy is a survival mechanism which involves a catabolic process where cytosol and specific cell organelles are engulfed and then degraded by a membranous structure called autophagosomes (Green and Lambi, 2015). Morphological and Molecular features of apoptosis are discussed in subsequent sections. The difference between all these different types of cell deaths are mentioned in Table 1 and illustrated in Figure 2, so that they are not confused with each other.

Table 1. Characteristic differences between apoptosis, necrosis and autophagy

	Apoptosis	Necrosis	Autophagy
Stimuli	Oxidative stress, drug, death ligand binding etc.	Injury (non-programmed) or chemical exposure, radiation etc. (in programmed)	Hypoxia, starvation, deprivation of growth factor, drug treatment
Morphological characteristics	Pyknosis, nuclear membrane blebbing, formation of apoptotic bodies, No loss of membrane integrity, No degradation of organelles.	Loss of membrane integrity, swelling of cell organelles, Karyolysis	Vacuolisation, degradation of organelles and proteins
Biochemical changes	Caspase Cleavage, DNA degradation	Random DNA degradation, release of cellular proteins, acidosis	LC3I lipidation to LC3II, p62/ SQSTM1 degradation, increased lysosomal activity
Clearance	Phagocytosis of apoptotic bodies by macrophages.	Cells may be ingested by macrophages (if death is programmed), significant inflammation (if death is non-programmed).	Cell is eaten itself and content recycle for the survival of the tissue

Figure 2. Difference between apoptosis, necrosis and autophagy



MORPHOLOGICAL CHARACTERISTICS OF APOPTOSIS

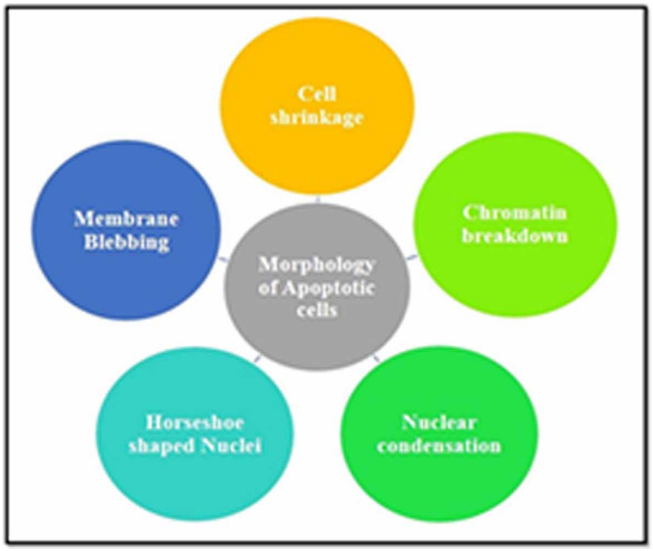
A cell undergoing apoptosis shows many morphological changes that are relatively constant among all cell types and species (Figure 3) (Hacker. 2000; Saraste and Pulkki, 2000). These are enlisted below: -

1. One of the characteristic features of it is cellular fragmentation that occurs several hours after the initiation of apoptosis.
2. Nucleus also undergoes chromatin condensation and nuclear fragmentation, along with rounding up of the cell, reduction in cell volume known as *pyknosis* and pseudopods retraction (Kroemer et al., 2005).
3. Chromatin condensation begins at the nuclear membrane periphery, forming a crescent or horseshoe-like structure.
4. Chromatin breaks up inside the cell with the plasma membrane and nuclear membrane still intact (*karyorrhexis*) (Majno and Joris, 1995).
5. Later stages of apoptosis show membrane blebbing, modification of cell organelles and a loss of membrane integrity (Kroemer et al., 2005).
6. Apoptotic cells are usually engulfed by phagocytic cells like macrophages even before apoptotic bodies occur, that is why at times apoptosis goes unnoticed.

BIOCHEMICAL ALTERATIONS DURING APOPTOSIS

All the morphological changes that we observe in apoptosis are the result of the biochemical changes that a cell undergoes in response to various stimuli. Typically, there are three types of biochemical changes that occur in apoptosis, these are:

Figure 3. Morphological characteristics of an apoptotic cells



Changes in Membrane and Recognition by Phagocytic Cells

At the beginning of apoptosis, phosphatidylserine (PS) is "flipped out" from the inner layers to the outer layers of the cell membrane. This is to aid the recognition of apoptotic cells by macrophages for phagocytosis so as to prevent the release of pro-inflammatory cellular products (Hengartner, 2001).

Breakdown of DNA and Protein

Recognition of apoptotic cells is followed by a breakdown of DNA into 50 to 300 kilobase of pieces (Vaux and Silke, 2003). Later on, inter-nucleosomal cleavage of DNA into in multiples of 180 to 200 base pairs occurs by the action of endonucleases. Although this feature is characteristic of apoptosis, yet it is not specific, it can be seen in necrotic cells too (McCarthy and Evan, 1997).

Activation of Caspases

Caspases belongs to the cysteine protease family of enzymes and have a property of cleaving after aspartic acid residues (Kumar et al., 2014). Caspases when activated cleave crucial cellular proteins and break down the nuclear scaffold and cytoskeleton. They can also activate DNAses that help in further degradation of DNA (Lavrik, 2005).

The caspases may belong to either of two categories:

1. Related to caspase 1 (like caspase-1, -4, -5, -13, and -14). These are usually involved in the processing of cytokines during inflammatory processes and
2. Related to apoptosis (like caspase-2, -3. -6, -7, -8, -9 and -10). These caspases can be further classified into:
   a. *Initiator caspases* (like caspase-2, -8, -9 and -10).

These are mainly responsible for the initiation of the apoptosis and

b. *Effector caspases* (like caspase-3, -6 and -7).

These are responsible for the hydrolysis of cellular components in apoptosis (Fink and Cookson, 2005). They play an important role in the execution of apoptosis.

MECHANISM OF APOPTOSIS

Apoptosis is initiated by chronological activation of the caspase family of enzymes, broadly through two different but congregating pathways, viz, intrinsic and extrinsic pathways (Adams, 2003: Shi, 2006) (Figure 4).

The Intrinsic Pathway

It is also known as 'stress' or 'mitochondrial' pathway and it is prominently controlled by the family of Bcl-2 protein. It is a two-step process. Firstly, various stimuli cause an increase in the permeability of mitochondria, which in turn help in the release of apoptotic factors from the outer membrane. These factors disturb the electrochemical gradient of the inner mitochondrial membrane. All these events are sensed by mitochondrial permeability transition complex which is a multi-protein complex that sits at the junction of inner and outer mitochondrial membranes, forming permeability transition pore (PTP) that cause mitochondrial dysfunction (Zamzami and Kroemer, 2001). Secondly, this dysfunction causes disturbance of plasma membrane integrity (necrosis) and/or the activation of specific apoptotic proteases (caspases) by leakage of cytochrome c (apoptosis-inducing factor) through PTP into the cytosol, this finally activates apoptosis (Kluck et al., 1997).

To ultimately execute apoptosis, the released cytochrome c needs assembling of a multiprotein caspase activating complex known **as** 'apoptosome'. Cytochrome c binds to WD-40 domain of the key apoptotic protease activating factor 1 (Apaf-1), which in the absence of cytochrome c, exists in its auto-inhibited monomeric form. Apaf-1 then binds to dATP/ATP and then to pro-caspase 9 via its caspase recruitment domain (CARD) and executes apoptosis by activating pro-caspase 9 to caspase 9 (Zou et al., 1997; Baig et al., 2016).

This intrinsic pathway is initiated in response to various internal stresses stimuli, like severe DNA-damage, activation of oncogenes, an overload of Ca2+, deprivation of growth factors, oxidants, hypoxia and microtubule-targeted drugs (Hassan et al., 2014). The mitochondrial dysfunctional consequences that lead to cell death can be exploited in cancer therapeutic strategies via induction of apoptosis in cancer cells. (Kroemer et al., 1998; Kroemer and Reed, 2000).

There are other players in the intrinsic pathway that lead to cell death. Besides, cytochrome c, a second mitochondria-derived activator of caspase (SMAC) and Omi can also be released through transition pore. Omi inhibits XIAP (X-linked inhibitor of apoptosis protein) which is an inhibitor of caspases (Lopez and Tait, 2015). SMAC, on the other hand, inhibit IAP (inhibitor of apoptosis proteins), so that apoptosis shall continue as the apoptosome is formed (Zaman et al., 2014).

This intrinsic pathway as mentioned above is tightly controlled by Bcl-2 family members. The Bcl-2 family of proteins has an important role to play as the gatekeeper of the apoptotic cascade. It is com-

prised of related proteins with pro-apoptotic and anti-apoptotic members that interact with each another especially through the intrinsic pathway as they lie upstream of irreversible cellular damage and mostly act at the mitochondria level (Gross, 1999).

The first identified protein of this family was Bcl-2, it is encoded by the *Bcl-2* gene, deriving its name from B-cell lymphoma 2, and having t (14; 18) chromosomal translocation (Tsujimoto et al., 1984). Bcl-2 family has Bcl-2 homology (BH) motifs that are short sequences of amino acids. Each member of the Bcl-2 family has at least one BH motif members. These homology motifs have a role to play in the function of the protein (Strasser et al., 2011). All the members of this Bcl-2 family are located on the outer mitochondrial membrane. They are dimers which are responsible for membrane permeability either in the form of an ion channel or through the creation of pores (Minn et al., 1997).

The Bcl-2 family of protein is classified into 3 groups based on its function:

1. Anti-apoptotic proteins such as Bcl-2, Bcl-xl, Mcl-1, Bcl-w, A1/Bfl-1 etc.
2. Pro-apoptotic *effectors*, e.g. Bax, Bak, and Bok/Mtd and
3. Pro-apoptotic *activators*. e.g. Bid, Bim, Puma, Noxa, Bad, Bmf, Hrk, and Bik. According to the preclinical studies, the *activators* contain only a single BH3 motif and they are the mediators in the cellular response to stresses such as DNA damage (Certo et al., 2006).

Effectors are found to be closely associated with the mitochondrial membrane. These pro-apoptotic effectors disrupt the mitochondrial membrane thereby forming a pore and releasing cytochrome c that forms 'apoptosome' which constitutes caspase-9, Apaf-1 and cytochrome c. Apoptosome activates other effector caspases and executes apoptosis. The effectors do so in response to the BH3-only *activators* (Garcia-Sáez, 2012; Wei et al., 2001).

Anti-apoptotic Bcl-2 family members directly inhibit the apoptosis promoting effects of both the effectors and activators (Llambi et al., 2011).

It has been observed that Bcl-2 binds and sequesters BH3-only activators, thus, preventing their interaction with the pore-forming effectors. Similarly, it can also affect the effectors to prevent mitochondrial pore formation (Figure 4). A dynamic balance is required between the anti-apoptotic members, such as Bcl-2, and the pro-apoptotic members that help in determining if the cell would go for apoptosis or not (Strasser et al., 2011; Garcia-Sáez, 2012). Disruption in the balance of anti-apoptotic and pro-apoptotic members of this family cause dysregulation in apoptosis in the affected cells either by overexpression of one or more anti-apoptotic proteins or an under-expression of one or more pro-apoptotic proteins or a combination of both.

The Extrinsic Pathway

It is also known as the death receptor-mediated apoptosis pathway. It is engaged when certain death receptor ligands, like FAS ligand or TNF, tie up their death receptors with the cell membrane, this activates caspases-8 via FADD (Fas Activated Death Domain) and TRADD (TNF-R1 Associated Death Domain).

Activation of caspases here is brought about by the formation of death receptor (DR) signaling, initiated by DRs at the cellular surface (Lavrik, 2009; Schleich and Lavrik, 2013, Lavrik, 2010). These DRs are expressed on the plasma membrane and are a part of tumour necrosis factor (TNF) receptor gene superfamily are characterized by the presence of a death domain (DD) that is crucial for apoptotic

signaling (Schleich, 2013). Six members of the Death Receptor family have been recognized so far (Lavrik, 2010; Schleich and Lavrik, 2013):

1. TNF-R1,
2. CD95 (APO1/FAS),
3. DR3,
4. TRAIL-R1,
5. TRAIL-R2 and
6. DR6

The role of DR3 or DR6 has not been defined clearly (Walczak and Krammer, 2000). However, TL1A (TNF-like ligand 1A or TNFSF15) - a relatively new cytokine is observed to be a specific ligand for Death Receptor 3 (DR3). Binding of TL1A to DR3 causes activation of NF-κB and trigger apoptosis in cells (except primary T-cells) with both endogenous and overexpressed DR3 (Migone et al., 2002). Corresponding ligands of the TNF superfamily include death receptor ligands like CD95 ligand (CD95L), TNFα, lymphotoxin-α (the latter two binds to TNF-R1), TRAIL and TWEAK, a ligand for DR3 (Walczak and Krammer, 2000).

The CD95 receptor is involved in the regulation of apoptosis in different cell types, especially in the cells of the immune system (Krammer, 2000). It is a type I transmembrane receptor, expressed on activated lymphocytes and many tumour cells (Krammer, 2000). CD95 ligand (CD95L) is made by immunologically active T cells and regulate the immune system by inducing autocrine or paracrine death in lymphocytes or other target cells (Krammer, 2000). Cancer cells may have high CD95L expression on them that may result in immune escape of tumors (Krammer, 2000). Constitutive expression of this death receptor ligands on cancer cells may kill the attacking antitumor T cells through induction of apoptosis via CD95/CD95L interaction. However, this model of tumour counterattack is not fully established (Igney and Krammer, 2002).

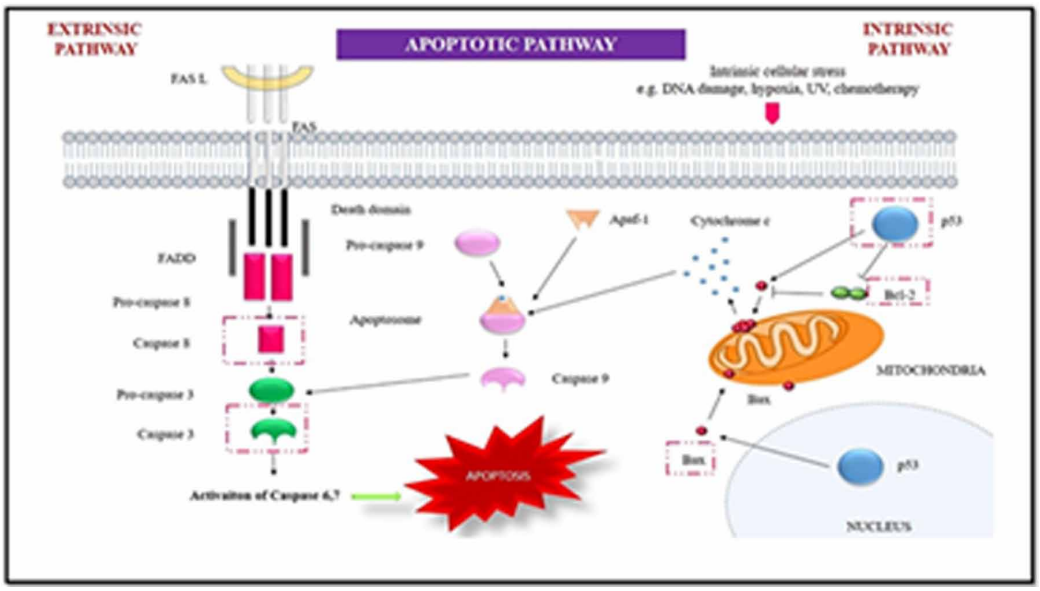
TNF-related apoptosis-inducing ligand (TRAIL) /Apo-2L is constitutively expressed in a wide range of tissues (LeBlanc and Ashkenazi, 2003). It has two agonistic TRAIL receptors, TRAIL-R1 and TRAIL-R2, contain a conserved cytoplasmic death domain motif, which makes them engaged in the cell's apoptosis upon ligand binding. TRAIL-R3 to R5 are the antagonistic decoy receptors, which bind TRAIL, but do not transmit a death signal (LeBlanc and Ashkenazi, 2003).

TRAIL receptors (R1 and R2) are propitious targets for cancer therapy (Ravi et al., 2004; Kelley and Ashkenazi, 2004; Ashkenazi, 2002; Johnstone et al., 2008). Triggering of DRs (their trimerisation and clustering) by the death ligands results in the formation of a 'death-inducing signaling complex' (DISC) (Kischkel et al., 1995, Lavrik et al., 2009). The DISC has oligomerized receptors, the Fas-associated death domain (DD containing adaptor molecule), procaspase-8 (FLICE), procaspase-10 and the cellular FLICE inhibitory proteins (c-FLIP). Formation of DISC activates procaspase-8/10 and subsequently starts a pro-apoptotic cascade of caspases (Schleich and Lavrik, 2013).

The oligomerisation of pro-caspase 8 upon DISC formation, activates it by self-cleavage and form caspase 8. Caspase 8 further activates down-stream Caspases like caspase 3.

These two apoptotic pathways congregate at the effector caspases which are caspase-3, -6 and -7 (Figure 4). Formation of tBid by caspase-8 in the extrinsic pathway could engage the intrinsic pathway and enhance the apoptotic activity. Figure 4 represents both extrinsic and intrinsic pathway diagrammatically.

Figure 4. Apoptotic pathway representing intrinsic pathway on the right and extrinsic pathway on the left along with their apoptotic modulators



The highlighted members are the ones that have been studied frequently for their implication in carcinogenesis.

IMPLICATION OF APOPTOSIS IN CARCINOGENESIS

Apoptosis has the potential to eliminate incipient cancer cells and prevent tumour progression. Hence, reduced apoptosis as discussed earlier is one of the hallmarks of cancer. There are multiple mechanisms by which a cancer cell may evade apoptosis, these are discussed below:

Disruption of the Balance of Pro-apoptotic and Anti-apoptotic Proteins

As previously mentioned, there are proteins that may exert pro- or anti-apoptotic effects in the cell. The ratio of pro-apoptotic proteins to anti-apoptotic proteins plays an important role in the regulation of cell death and progression of cancer.

Similar to oncogene addiction, tumour cells may also become dependent on Bcl-2 in order to survive (Certo et al., 2006). Stress signals like DNA damage may prompt the malignant cells to express pro-apoptotic *activators* but some cancer cells overexpress Bcl-2, which can hinder the pro-apoptotic response (Letai, 2008). As a result, the abundant pro-apoptotic *activators are* sequestered by Bcl-2. Such a cancer cell is thought to be "primed" for apoptosis because they have sufficient amounts of the pro-apoptotic *activators*, if these could be displaced from Bcl-2, the cell would undergo apoptosis. Those cancers that depend on Bcl-2 for their survival are much sensitive to Bcl-2 modulation (Deng et al., 2007). The primed state concept provides strong support for conducting anticancer research related to the targeting and inhibition of Bcl-2 to kill the cancer cells by activating pro-apoptotic proteins.

The wide variety of cancer types were observed to have associated with atypical expression of Bcl-2 is compatible with its capacity to act as an apoptotic regulator (Table 2).

In less than 5% of patients having chronic lymphocytic leukaemia a detectable Bcl-2 gene rearrangement is observed, whereas, the vast majority of patients had over-expressed Bcl-2 (Hanada et al., 1993). In the majority of acute myeloid leukaemia cases and almost all of the acute lymphocytic leukaemia, Bcl-2 is found to have over-expressed frequently (Wei et al., 2001; Gala et al., 1994).

In follicular lymphoma, Bcl-2 overexpression is caused by a t (14;18) chromosomal translocation in tumour cells (Chen-Levy et al., 1989; Tsujimoto et al., 1984). In more than 40% of diffuse large B-cell lymphoma, many patients reported had higher levels of Bcl-2 expression (Hermine et al., 1996).

In non-hematologic tumours (Solid tumorus) also, Bcl-2 may have a significant role. Inappropriate expression of it has been found in different solid tumours like prostate, breast, and small cell and non-small cell lung cancers (Karnak and Xu, 2010; Hellemans et al., 1995; Jiang et al., 1995; Anagnostou et al., 2010). In more than 90% of small cell lung cancer cases, high Bcl-2 expression has been reported (Jiang et al., 1995). Apart from these, cancers of Ovary, bladder, colorectal, some head and neck cancers and neuroblastoma all have been observed to have significant levels of Bcl-2 expression. (Henriksen et al., 1995; Lamers et al., 2012; Swellam et al., 2004; Zhao et al., 2005).

Table 2. Some studies on role of Bcl-2 in cancer

Type of malignancy	Findings related to Bcl-2 expression	References
Chronic lymphocytic Leukaemia	High expression in 95% of cases as compared to normal peripheral blood lymphocytes	(Hanada et al., 1993)
Acute myeloid Leukaemia	High expression along with poor response to chemotherapy	(Campos et al., 1993)
Acute lymphocytic Leukaemia	High levels are found in nearly all patients.	(Gala et al., 1994)
Follicular lymphoma	Chromosomal translocation most cases of follicular centre B-cell lymphomas causing over-expression of it.	(Tsujimoto et al., 1984)
Diffuse large B-cell lymphoma	Chromosomal translocations affecting Bcl-2 occur in approximately 20% of cases	(Huang et al., 2002)
Solid tumours	Inappropriate expression were found in prostate, breast, and small cell and non-small cell lung cancers	(Karnak and Xu, 2010; Hellemans et al., 1995; Jiang et al., 1995; Anagnostou et al., 2010)

It was observed that most of the cancer therapies exert their effects via apoptosis, in which Bcl-2 has a significant role to play (Plati et al., 2011; Herman et al., 2010, 2011). Bcl-2 also helps in the potentiation of chemotoxic agents (Tse et al., 2008; Tahir et al., 2007). It was revealed in preclinical studies that overexpression of anti-apoptotic Bcl-2 family members, renders cancer cells resistant to cancer treatment. (Strasser et al., 2011; Reed, 2008; Stolz et al., 2008). It was probably for the similar reason, it was observed that overexpression of Bcl-2 in prostate cancer, neuroblastomas, breast cancer inhibited apoptosis (Raffo et al., 1995; Fulda et al., 2002). It was found that a mutation in Bax made colorectal cells resistant to drugs by hampering apoptosis (Miquel et al., 2005). Similarly, in the case of Chronic Lymphocytic Leukaemia (CLL), overexpression of anti-apoptotic Bcl-2 and lesser expression of Bax

inhibited apoptosis *in vivo*. In 1997, Pepper et al. reported that in CLL, when these cells were cultured *in vitro*, drug-induced apoptosis was found to be inversely related to Bcl-2/Bax ratios.

Beside Bcl-2, other pro-survival or anti-apoptotic proteins like BCL-xl, BCL-w, Mcl-1, A1, NR-13, BHRF1, LMW5-HL, ORF16, KS-BCL-2 and E1b-19K have also been observed to be over-expressed in cancer cells (Pfeffer and Singh, 2018).

The p53 protein, also known as Tumour Protein 53 (or TP 53), is a tumour suppressor protein encoded by the TP53 gene (Levine et al., 1991). Initially, it was thought to be slightly-oncogenic but later on, it was found that this was due to a p53 mutation, or i.e., a "gain of oncogenic function" (Bai and Zhu, 2006). p53 is known to play a role in the induction of apoptosis, cell cycle regulation, development, differentiation, gene amplification, DNA recombination, chromosomal segregation, cellular senescence etc. (Oren and Rotter, 1999) and that is why it is also known as the "guardian of the genome" (Lane, 1992). It is estimated that more than 50% of human cancers are caused by defects in the p53 tumour suppressor gene (Bai and Zhu, 2006).

The tumour suppressor protein p53 has a critical role in the regulation of the Bcl-2 family of proteins; however, the exact mechanisms have not yet been completely elucidated (Schuler and Green, 2001). It is observed that expression of Puma and Noxa -the proapoptotic activators is induced by p53 protein and thus p53 has an indirect role in the induction of apoptosis. Moreover, the expression of both Bcl-2 and Bax is regulated by the p53 tumour suppressor gene (Miyashita, 1994). p53 can activate DNA repair proteins when DNA has sustained damage, can hold the cell cycle at the G1/S regulation point on DNA damage recognition, and can initiate apoptosis if the DNA damage proves to be irreparable (Pietenpol and Stewart, 2002).

Thus, if the p53 gene is damaged, then tumour suppression is severely reduced. It is observed by researchers that some target genes of p53 involved in apoptosis and regulation of cell cycle are abnormally expressed in melanoma cells, causing abnormal activity of p53 and contributing to the cancerous growth in these cells (Avery-Kiejda et al., 2011). Many studied have confirmed the abnormalities in p53 are associated with impaired apoptosis in cancer cells/tissues. N-terminal deletion of p53 in mice has shown to reduce survival, more profound tumour spectrum, reduced apoptosis and an intense pro-inflammatory phenotype (Slatter et al., 2011). However, wild type p53 restoration resulted in apoptosis induction in cancer cells. (Vikhanskaya et al., 2007).

Reducing Caspase Function

Decreased levels of caspases or impairment in their function affect apoptosis and promote carcinogenesis. In a study, down-regulation of caspase-9 was observed in patients with stage II colorectal cancer (Shen et al., 2010). on the other hand, in breast, ovarian, and cervical tumours, caspase 3 mRNA levels were observed to be either undetectable (breast and cervical) or decreased (ovarian). It was also reported that caspase-3-deficient breast cancer (MCF-7) cells undergo apoptosis in response to the anticancer drug by restoring the expression of caspase-3 (Devarajan et al., 2002). In some instances, more than one caspase can be down-regulated, contributing to tumour cell growth and development. Fong et al., (2006) observed a co-down-regulation of caspase-8 and -10 may contribute to the pathogenesis of choriocarcinoma.

Impairing Signalling by Death Receptors

Extrinsic pathway (Death receptor pathway) of apoptosis can be invaded by cancer cells by various mechanisms. These may include suppression of the receptor or impairment in their function and/or reduced level in the death signals, causing impaired signalling. Nonetheless, one of the mechanisms of drug resistance is the down-regulation of receptor surface expression. Reduced expression of these membrane death receptors and aberrant expression of the decoy receptors also play a role in the evasion of the death signalling pathways in many cancers (Fulda, 2010). Researchers also observed that the loss of Fas and the dysregulation of FasL, DR4, DR5, and TRAIL in the cervical intraepithelial neoplasia (CIN)-cervical cancer sequence play a role in the progression of cervical cancer (Reesink-Peters et al., 2005).

Blebbishield Formation

A new mechanism of apoptosis evasion in cancer stem cells is discovered recently which is the formation of blebbishields. Blebbishields are the spherical structures formed by the fusion of apoptotic blebs (Jinesh et al., 2013). Blebbishield activation often linked to evasion of the immune system and apoptosis, induction of tumourigenesis, enhanced glycolysis, generation of chromosomal instability, drug resistance and metastasis (Jinesh and Kamat, 2016a; Jinesh et al., 2013; Jinesh, 2017, Jinesh et al., 2016). This is a kind of emergency program that is activated in order to save apoptotic cancer stem cells from apoptotic death (Jinesh and Kamat, 2016). Visual signs of apoptosis are present in the cell, but the mechanism is halted and the cell survives (Jinesh et al., 2013). Endocytosis and endocytosis-driven serpentine filopodia formation are observed in blebbishields to prevent the apoptotic response. Typically, apoptosis may end in secondary necrosis due to a lack of ATP but in blebbishields, this secondary necrosis is prevented through activation of glycolysis (Jinesh et al., 2016).

It is observed that Caspases, Bad activation and K-ras signalling play an important role in the formation of blebbishields. K-ras signalling regulates the phosphorylation of Bad and activated Bad regulates glycolysis. beside Bad, Bax and Bak also enhance glycolysis and provide sufficient ATP to blebbishields to avoid secondary necrosis. On the other hand, Bax p18 fragment that is responsible for the formation of mitochondrial transition pore is found at low levels during blebbishield formation. Thus, in blebbishield formation, the mitochondria remain intact and ensure adequate production of ATP to prevent secondary necrosis (Jinesh and Kamat, 2016a; Jinesh et al., 2013; Jinesh, 2017, Jinesh et al., 2016).

This survival of cancer stem cells by blebbishield formation can be stopped through therapies targeting apoptosis. Inhibitors of Caspase inhibitors, Smac mimetics and internal ribosome entry site (IRES) translation inhibitors are the potential candidates for such therapies. IRES translation controls the anti-apoptotic proteins like cIAP-2 and XIAP. Thus, inhibition of IRES translation would prevent initiation of the blebbishield formation. N-Myc is an IRES-translational target that has been targeted to prevent blebbishield formation (Jinesh, 2017; Jinesh and Kamat, 2016b).

TARGETING APOPTOSIS IN CANCER TREATMENT

Every defect in apoptotic pathway serves like a double-edged sword that may also be exploited as a potential target for cancer treatment. Treatment strategies that can restore the apoptotic signalling pathways to normal can eliminate the cancer cells, which rely on these abnormalities to stay alive.

Many plants derived compounds when used/tested for cancer therapy have shown to exert their effect by targeting the apoptotic pathway. Resveratrol-a phytoalexin from grapes is reported to initiate CD95 signalling–dependent apoptosis in HL60 cells and T47D (breast carcinoma cells) by enhancing CD95L expression (Clément et al., 1998). 7-hydroxystaurosporine (UCN-01), which is an alkaloid has been observed to have apoptotic effects in ovarian cancer cells (Taraphdar et al., 2001). Graviola fruit has been reported to induce apoptosis by inhibiting Bcl-2 proteins and simultaneously increasing proapoptotic (Ko et al., 2011). This property of Graviola makes it a promising therapeutic against cancer (Ioannis et al., 2015). Other natural compound includes black cohosh of *Actaea racemosa* by activating caspase (Grant and Ramaswamy, 2012), Juglone from *Juglans mandshurica by* increasing *caspase 9 cleavage* (Lu et al., 2013) and genistein (A phytosterol from flavonoid family) from soybeans by arresting cell cycle, induce apoptosis in cancer cells (Schnekenburger et al., 2014). Quercetin, a naturally occurring flavonoid is also reported to have an apoptotic effect by inducing cytochrome c release and caspase 9 activations. (Schnekenburger et al., 2014). Epigallocatechin-3-gallate from Green tea also exhibit an apoptotic effect on cancer cells (Levitsky and Dembitsky, 2015). Aloe-emodin, found in *Rheum palmatum* initiate apoptosis in cancer cells by activating caspase function through cytochrome c release (Levitsky and Dembitsky, 2015). Curcumin, a polyphenolic compound present in turmeric, has been extensively studied for its anticancer properties and have been approved by both the Food and Drug Administration and the World Health Organization (Kanai, 2014). A formulation of curcumin into nanoparticles, called curcumin-ND (curcumin nanodisks), has been reported to show enhanced biological effect by inducing apoptosis via reactive oxygen species generation and activation of the caspase-3 pathway, as well as cell cycle arrest at the G1-S phase (Singh et al., 2011). Curcumin interacts with both apoptotic pathways. It can interfere at many different points in the signalling cascade. Bcl-2 and XIAP are inhibited by curcumin which leads to increased expression of Bax and Bak. Curcumin also increases the ability for mitochondria to undergo mitochondrial membrane permeability leading to increased release of cytochrome c which causes caspase activation and the apoptotic response (Ravindran et al., 2009).

Besides these natural products, some of the therapeutic drugs that are approved by FDA (Food and Drug Administration) exert their effect by targeting the apoptotic pathway. They include vinca alkaloid of Vinblastine and Vincristine interfere with microtubules function and terminate cell cycle and result in cell death through the induction of apoptosis (Islam and Iskander, 2004). Vanetoclax that has been recently approved by the FDA, induce apoptosis in Chronic Lymphocytic Leukaemia (CLL) and Acute Myeloid Leukaemia (AML) by inhibiting Bcl-2 protein. It binds to this protein and displaces pro-apoptotic heterodimers e.g. Bim (Li et al., 2019).

Thus, the efficacy of all the above-mentioned plant-derived compound and drugs against cancer cells by influencing apoptosis, provide sufficient evidence that indeed apoptosis is a promising target for anticancer therapies and would be of great importance in assessing and designing future therapeutic in cancer.

CONCLUSION

Apoptosis is a form of cell death and one of the hallmarks cancer. Many alterations, defects and mutation in the both extrinsic and intrinsic pathway of apoptosis in cancer cells make them escape the apoptosis mediated cell death and lead to malignant developments. Thus, targeting the apoptotic pathway promises a fascinating approach for designing treatment modalities for a disease like cancer. Any therapy that

could restore the normal apoptotic pathway in cancer cells would provide a more universal cancer therapy and would open the avenues for researchers to assess the role of novel drugs and natural compounds for their apoptosis-inducing abilities.

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Chapter 10

Strategies to Suppress Tumor Angiogenesis and Metastasis, Overcome Multi-Drug Resistance in Cancer, Target Telomerase and Apoptosis Pathways

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ABSTRACT

Cancer has been a worldwide topic in the medical field for a very long time. As angiogenesis is essential for tumor growth and metastasis, controlling tumor-associated angiogenesis is a promising tactic in limiting cancer progression. In cancer patients, multidrug resistance (MDR) is most widely used phenomenon by which cancer acquired resistance to chemotherapy. This resistance to chemotherapy occurs due to the formation of insulated tumor microenvironment which remains a major hurdle in the

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cure of various types of cancer. The mechanisms that cause malignant growth of cells include cell cycle control, signal transduction pathways, apoptosis, telomere stability, and interaction with the extracellular matrix. This chapter focuses on current strategies to suppress tumor angiogenesis for cancer therapy, various mechanisms involved in the development of MDR in cancer cells, which in turn will help us to identify possible strategies to overcome these MDR mechanisms and a variety of procedures that involves targeting apoptotic and telomerase pathways to suppress tumor progression.

STRATEGIES TO SUPPRESS TUMOUR ANGIOGENESIS AND METASTASIS

Introduction

Angiogenesis speaks to a sign of malignancy. A few proteoglycans associate with cell surface receptors and manage angiogenesis inside the tumour microenvironment (TME) (Chakraborty et al., 2020). Cancer growth metastasis comprises of different, complex, communicating and associated steps (Isaiah J. Fidler, 1990). Inability to finish any one of these steps may keep the tumour cell from developing a metastasis. It has already been established that the process of angiogenesis is basic to the development of different types of tumours (Folkman, 1986). The enlistment of angiogenesis is intervened by positive and negative administrative molecules secreted by both tumour and host cells (I. J. Fidler & Ellis, 1994; Liotta et al., 1991). Key to the science of tumour angiogenesis is the commitment of the host environment scale condition (Takahashi et al., 1996).

Angiogenesis and Metastasis

Angiogenesis is a fundamental natural procedure including the growing and development of fresh blood vessels from the prior vascular primordium (Folkman, 1995) and assumes a key role not just in the guideline of different physiological exercises (e.g., early-stage improvement, wound recuperating, female regenerative cycle, and so forth.) but also in tumour development and metastasis (Folkman, 2001). Tumours that develop past a size of 2 mm^3 are reliant on oxygen and supplements provided by encompassing newly formed blood vessels (Eskens, 2004).

Agrin Repertoire Involved in Angiogenesis

Agrin crosstalk between cancer cells and endothelial cells (ECs) is underlined by the perception that high levels of Agrin associates with poor prognosis (Chakraborty et al., 2017). Like Hepatocellular carcinoma (HCC) cells, Agrin is communicated and secreted in a large scale but at lower levels than in malignant growth cells (Njah et al., 2019). Significantly, the angiogenic job of Agrin depends on its receptor complex comprising of Integrin β1, Lrp4, and MuSK, which are likewise communicated in these ECs. Like HCC cells, focal adhesion kinase (FAK) is likewise basic as a component of downstream effectors for Agrin-interceded angiogenesis in ECs. Agrin and its receptors likewise intercede a solid bond of invading ECs to tumour cells. Fragment of Agrinmay promoted an angiogenic phenotype in several ECs and an ex vivo rodent metatarsal sprouting assay. This fragment of Agrin may also effectively recruited more blood vessels in cell-free subcutaneous Matrigel plugs, suggesting a promising avenue for Agrin as an in vivo mediator of angiogenesis.

VEGF Molecules Involved in Angiogenesis

The different pro-angiogenic and anti-angiogenic particles coordinate various steps in vessel arrangement, alongside their capacities. Vascular Endothelial Growth Factor (VEGF), as of now is considered the most basic proangiogenic factor VEGF increases vascular penetrability, advances movement and multiplication of ECs. EC endurance factor can assemble endothelial progenitor cells (EPC) populaces from the bone marrow, and is known to upregulate leukocyte attachment on ECs (Dvorak, 2002; Ferrara et al., 2003; Melder et al., 1996). During tumour movement, or with treatment, the quantity of particular angiogenic particles created by tumours can increase (Casanovas et al., 2005; Yoshiji et al., 1997). Thus, after VEGF flagging is hindered, a tumour may depend on other, elective angiogenic factors (e.g., basic fibroblast growth factor [bFGF], stromal cell-derived factor 1α [SDF1α], placental growth factor [PlGF], or interleukin-8 [IL-8]) (Jain et al., 2009). Other positive regulators of angiogenesis may include the angiopoietins which are involved in stabilizing micro blood vessels and controlling vascular permeability; different proteases engaged in dissolving and remodelling matrix and releasing growth factors; and organ-specific angiogenic stimulators (i.e., endocrine gland VEGF)(Jain, 2003; LeCouter& Ferrara, 2003; Yancopoulos et al., 2000). Angiogenesis inhibitors incorporate endogenous receptors of different proangiogenic ligands (e.g., sVEGFR1/sFLT1) and particles that downregulate the proangiogenic ligands (e.g., interferons) or that interferes with the arrival of the triggers or official with their receptors (e.g., platelet factor 4). Thrombospondins are among the first and best portrayed endogenous inhibitors that meddle with the development, grip, movement, and endurance of ECs (Bouck et al., 1996). Other endogenous inhibitors incorporate parts of different plasma or framework proteins (e.g., angiostatin, a section of plasminogen; endostatin, a piece of collagen XVIII; tumstatin, a piece of collagen IV) (Maeshima et al., 2002; O'Reilly et al., 1994, 1997). Neither the systems of activity of the grid determined inhibitors nor their physiologic job is well understood. The age of proangiogenic and antiangiogenic particles can be activated by metabolic pressure (e.g., low pO_2, low pH, or hypoglycaemia), mechanical pressure (e.g., shear pressure, strong pressure), insusceptible or fiery cells that have invaded the tissue, and hereditary changes (e.g., actuation of oncogenes or erasure of silencer qualities that control the creation of angiogenesis regulators) (Bouck et al., 1996; Brown et al., 2001; Fukumura et al., 2001; Garkavtsev et al., 2004). These molecules can radiate from malignant growth cells, ECs, stromal cells, blood, and extracellular network (Brown et al., 2001). Because the typical host cells among organs are different, therefore, the fundamental systems of angiogenesis may rely upon the particular host-tumour connections working inside a given tissue (Blouw et al., 2003; Tsuzuki et al., 2001). Furthermore, because the tumour microenvironment is probably going to change during tumour development, relapse, and backslide, profiles of proangiogenic and antiangiogenic particles are probably going to change with time and space (Izumi et al., 2002; Jain, 2005). The test at present is to build up and bring together theoretical structure to portray the transient and spatial profiles of this inexorably different cluster of angiogenesis controllers with the point of creating successful restorative strategies (Izumi et al., 2002; Jain et al., 2009).

Possible Therapy and Strategies to Prevent Angiogenesis and Metastasis

Anti-angiogenic treatment involves tumour cells starving by targeting especially endothelial cells and blocking angiogenesis (Imai & Takaoka, 2006). Anti-angiogenic compounds are small-molecule, which have advantages over monoclonal antibodies. For example, small molecules agents are less expensive and more affordable and convenient to administer (Imai & Takaoka, 2006). Nonetheless, the advancement of

medication obstruction and constrained viability limit their broad clinical application (Gacche & Meshram, 2014). Thus, there is a requirement for novel kinds of small particle with hostile to angiogenic action.

Diterpenoids are a class of bioactive regular compounds that are found in numerous therapeutic herbs and have incredible potential in the treatment of illnesses including irregular angiogenesis malignancy. Oridonin, an antibiotic medication diterpenoid isolated from Rabdosiarubenscens, was appeared to prompt apoptosis and restrains the relocation and attack of exceptionally metastatic human breast malignancy cells (Wang et al., 2013). Oridonin suppressed tumour development and metastasis by blocking tumour angiogenesis through the downregulation of VEGF-initiated Jagged/Notch signalling (Wang et al., 2013). Another cause of a characteristic enemy of angiogenic compound is triptolide, a diterpenoid epoxide isolated from Tripterygium wilfordii Hook F. Triptolide prevent angiogenesis and spread of human anaplastic thyroid carcinoma cells by blocking nuclear factor-κB flagging (Zhu et al., 2009) and instigated apoptosis while smothering the development and angiogenesis of human pancreatic disease cells through negative guideline of cyclooxygenase 2 and VEGF articulation (Cheung et al., 2001). These reports show the capability of diterpenoids as hostile to anti-cancer agents.

Andrographolide (Andro; Scheme 1) is a bioactive labdane diterpenoid. It was recently detailed that the Andro substance of dried entire *Andrographalis Paniculata* (Burn. f.) plant is as high as 4%, proposing that Andro is the most copious diterpenoid in this therapeutic plant (Cheung et al., 2001). Andro is known to have anticancer (Peng et al., 2018; Zhou et al., 2008) against angiogenic (Blanchard et al., 2018), mitigating, antidiabetic (Yu et al., 2015) and neuroprotective (Yang et al., 2019) activities. Andro and A. paniculate restrain tumour angiogenesis by stifling angiogenic atoms, e.g., VEGF and nitric oxide and improve the declaration of hostile to angiogenic factors (i.e., interleukin 2 and TIMP metallopeptidase inhibitor 1) (Sheeja et al., 2007). Also, Andro can block tumour angiogenesis by preventing VEGF-A-prompted enactment of VEGFR2 and downstream mitogen-activated protein kinase (MAPK) signalling (Kajal et al., 2019). It is interesting to know the anticancer and anti-angiogenic impacts of AGS-30 in vitro and in vivo and the hidden systems of activity. Besides, the current investigation likewise showed that adjustment of the C14 position in the synthetic structure of Andro fundamentally improved its anticancer and hostile to angiogenic impacts. These discoveries give a basis for the future improvement of Andro as novel angiogenesis inhibitors for the treatment of malignant growth. Key to the science of tumour angiogenesis is the commitment of the host environment scale condition (Takahashi et al., 1996).

OVERCOME MULTI-DRUG RESISTANCE

Introduction

Global Cancer Report issued by the World Health Organization, reported over 10 million new cases of cancer each year and over 6 million annual deaths (Stewart & Kleihues, 2003). The disappointment of the treatment of malignant growth patients frequently happens because of inborn or gained resistance of the tumour to chemotherapeutic agents. The resistance happens not exclusively to a solitary cytotoxic medication used, but happens as a cross-resistance to an entire scope of medications with various structures and cell targets. This phenomenon is called multiple drug resistance (MDR). Once MDR shows up, utilizing high portions of medications to beat resistance is not possible, poisonous impacts show up and resistances are additionally invigorated. MDR seriously constrains the adequacy of chemotherapy in an assortment of basic malignancies and is answerable for the general poor viability of disease che-

motherapy (I. Akan et al., 2004; Ilhan Akan et al., 2005; Ambudkar et al., 1999; Choi, 2005; Liscovitch & Lavie, 2002; Thomas & Coley, 2003). Targeting TME with anti-cancer chemotherapy synergistically could provide enormous success in the prevention of the development and cure of various types of cancers. To solve this mystery, the present chapter aimed to understand various mechanisms involved in the development of MDR which in turn will help us to identify possible strategies and/or potent targets to mitigate these MDR mechanisms.

Tumor Microenvironment and MDR

Traditionally, most of the anti-cancer drug development has been focused on targeting the tumour cell cycle. However, recently, there has been a shift in research focussing on efforts to target cancer cells directly to the attractive alternate or synergistic targeting of components within the tumour microenvironment (TME). In cancer patients, multidrug resistance is the most widely exploited phenomenon by which cancer acquires resistance to chemotherapy. This resistance to chemotherapy possibly may occur due to the formation of insulated TME which remains a major hurdle in the cure of various types of cancer. Several cellular and noncellular mechanisms are involved in developing TME around cancer cells toward chemotherapy. Targeting TME with anti-cancer chemotherapy synergistically could provide enormous success in the prevention of the development and cure of various types of cancers (Albini & Sporn, 2007; Quail & Joyce, 2013). MDR has been associated to poor prognosis and reduced survival in gastric cancer, gliomas, ovarian cancer, sarcomas, breast cancer, pancreatic cancer, and haematological malignancies, including childhood acute lymphoblastic leukaemia and acute myeloid leukaemia (Dahiya & Deng, 1998; Declèves et al., 2006; Hiddemann et al., 1999; Jamroziak & Robak, 2004; Kitange et al., 2001; Leighton & Goldstein, 1995; Swerts et al., 2006; Valera et al., 2004; Van den Heuvel-Eibrink et al., 2000; Zhang & Fan, 2007). To understand the physiological mechanisms of MDR it is necessary to have an appreciation for the TME and for the selection pressures that contribute to tumour progression. Characteristics of TME include hypoxia and changes in the regulation and/or expression of oncogenes, tumour suppressors, and apoptotic factors (Nelson et al., 2004). Relative to normal cells hypoxic cancer cells have a substantially reduced intracellular pH level (Harris, 2002; Kizaka-Kondoh et al., 2003). This acidic cellular environment is associated with the activation of a subset of proteases that may lead to metastasis (Harris, 2002). Additionally, hypoxic cells mainly depend on energy on anaerobic metabolism, obtaining ATP from glucose to lactic acid conversion pathway instead of oxidative metabolism (Guppy, 2002).

Mechanism of Multidrug Resistance

A few speculations are clarifying the development of medication resistance, including changed medication transport over the plasma membrane, hereditary reactions, improved DNA repair, alteration of target molecules, access to target cells, metabolic effects, and growth factors. A portion of the mechanisms utilized by cancer cells to oppose cytotoxic drugs are likewise seen in normal cells as a major aspect of a barrier system against ecological cancer-causing agents.

Treatment resistance occurs due to “pumps” which are present in tumour-cell membranes that effectively pump out chemotherapeutic medications from the cell, and maintain a strategic distance from medication or the drug. The pumps that give chemoresistance in disease cells are P-glycoprotein, the

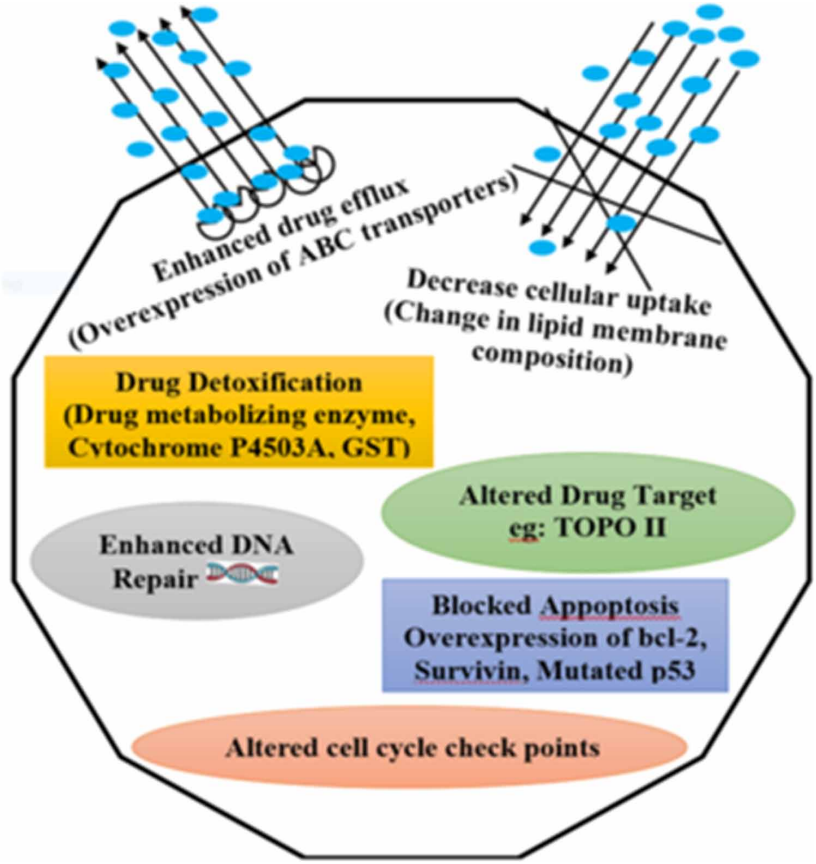
alleged multidrug resistance-associated protein (MRP). Due to their capacity and significance, they turned into the objectives of a few anticancer medications.

Recognizing the mechanisms prompting intrinsic or acquired multidrug resistance (MDR) is significant in growing increasingly compelling treatments (Ilhan Akan et al., 2005). The medication resistance in cancer cells frequently results from raised articulation of specific proteins, for example, cell-film transporters, which can bring about an expanded efflux of the cytotoxic medications from the disease cells, therefore bringing down their intracellular focuses (Ambudkar et al., 1999; Gottesman et al., 2002; Thomas & Coley, 2003). The mechanism of resistance is called typical or classical MDR when overexpression of the membrane efflux siphons is associated with MDR (Choi, 2005). The traditional MDR is expected for the most part to expanded efflux siphons in the cell membrane of cells siphoning anticancer medications out of cells (I. Akan et al., 2004; Ilhan Akan et al., 2005; Choi, 2005).

ATP- binding cassette (ABC) transporters are a group of transporter proteins that add to drug obstruction employing adenosine-tri-phosphate (ATP)- subordinate medication efflux pumps (Leonard et al., 2003). To date, over 100 ABC transporters from prokaryotes to people and 48 human ABC genes have been recognized that offer arrangement and auxiliary homology (Choi, 2005; Dean et al., 2001; Leonard et al., 2003; Thomas & Coley, 2003). The elements of 16 genes have been resolved and 14 genes are connected with a few sicknesses present in people (Choi, 2005; Dean et al., 2001; Efferth, 2001). Even though the safe proteins have a place with the ABC superfamily, they are very unique as for gene, locus, amino corrosive arrangement, structure and substrate (Choi, 2005). ABC proteins are available in totally known living species, with a generally preserved structure that contains a mix of moderated ABC and transmembrane domains (TMDs). In warm-blooded animals, dynamic ABC proteins comprise of at any rate four of such spaces, two TMDs and two ABCs. These domains might be available inside one polypeptide chain (full transporters), or inside two separate proteins (half-transporters). ABC transporters need the dimerization of the above explicit half-transporters (Sarkadi et al., 2004). High sequence homology in the ATP-restricting spaces known as nucleotide-restricting folds permits recognizable proof and arrangement of individuals from the ABC transporter family (Leonard et al., 2003). These transporters utilize the energy released from the hydrolysis of ATP to drive the vehicle of different atoms over the cell layer (Dean et al., 2001; Thomas & Coley, 2003). They are associated with the vehicle of numerous substances, including the release of poisons from the liver, kidneys and gastrointestinal tract. It has been progressively perceived that transporter-intervened forms altogether adjust sedate retention, dispersion, metabolism and secretion (Sarkadi et al., 2004). Notwithstanding their physiologic articulation in ordinary tissues, many of them are over-communicated, in human tumours (Thomas & Coley, 2003). There are seven subfamilies delegated ABC transporters (ABC-A through ABC-G) that are communicated in both ordinary and malignant cells (Leonard et al., 2003). Fig. 1 illustrates the various factors that contribute to MDR.

The overexpression of membranes ATP binding cassette (ABC) transporters comprises one of the fundamental mechanisms of MDR (Schinkel & Jonker, 2003). The physiological capacities and restriction of ABC transporters in human tissues influence the general adsorption, distribution, metabolism, elimination and toxicity of any medication class (Wu et al., 2011). ABC drug transporters, including P-glycoprotein (Pgp; ABCB1), MRP1 (ABCC1) and ABCG2 (BCRP; MXR) truly influence disease chemotherapy. ABC transporters contain a couple of ATP-restricting areas, otherwise called nucleotide restricting folds and 2 transmembrane domains containing 6 layers spreading over α-helices. The particles pump substrates in a single direction, commonly out of the cytoplasm. For hydrophobic compounds, this development is regularly from the internal leaf of the bilayer to the external layer or an acceptor molecule

Figure 1. Diagrammatic representation of different contributing factors of multidrug resistance



(Dean et al., 2001). ABC transporters use energy derived from hydrolysis of ATP to effectively ship anticancer medications across biological membranes, forestalling drug aggregation and prevent them to reach the target inside a cancer cell.

Pgp (ABCB1)

Pgp is the principal molecule from the ATP- binding cassette (ABC) transporter, which goes about as a physiological barrier and releases toxins and xenobiotics from the cells (Sharom, 2011). Pgp is fundamentally found in epithelial cells covering the colon, small intestine system, pancreatic ductules, bile ductules, kidney proximal tubules, and adrenal gland (Beaulieu et al., 1997). Pgp in humans has 2 isoforms. The Class I isoform (MDR1/ABCB1) is a drug transporter, though the Class II isoform (MDR2/3/ABCB4) trades phosphatidylcholine into the bile (Amin, 2013). Pgp can bind anticancer medications (Martins et al., 2010).

MRP1 (ABCC1)

MRP1 (ABCC1) has a 5-domain structure with a third NH2-proximal membrane spreading over space with 5 transmembrane fragments and an extracytosolic NH2 end (D. R. Hipfner et al., 1997). MRP1 is a functioning ATP-subordinate transporter of cysteinyl leukotriene LTC4; 17b-estradiol 17-(b-D-glucuronide), Exo and endo glutathione (GSH) conjugates of the mycotoxin aflatoxin B1 (David R Hipfner et al., 1999).

ABCG2 (BCRP; MXR)

ABCG2 (BCRP; MXR) is found in a group of stem cells and human tissues, including the placenta, liver, kidney, and digestive system, protecting them from exogenous and endogenous poisons. Low-oxygen conditions instigate ABCG2 expression in tissues and ABCG2 protects cells and tissues from the protoporphyrin congregation under hypoxic conditions by connecting with heme and porphyrins (Krishnamurthy & Schuetz, 2006).

MDR Modulators

Various therapeutic agents, also called MDR modulators have been explored in various clinical investigations to target and/or prevent multidrug resistance in cancer. These MDR modulators (chemosensitizers or MDR inhibitors) can be classified into the first, second and third-generation MDR modulators (Ferry et al., 1996; Saraswathy & Gong, 2013).

First-generation MDR drugs had other pharmacological exercises and were not explicitly created for limiting MDR. Their fondness was low for ABC transporters and required the utilization of high portions, bringing about unsuitable high toxicity which restricted their application (Ferry et al., 1996; Krishna & Mayer, 2000; Thomas & Coley, 2003). Clinical preliminaries with original (first-generation) MDR drugs fizzled for different reasons, frequently because of reactions (Ferry et al., 1996; Krishna & Mayer, 2000; Liscovitch & Lavie, 2002; Theis et al., 2000; Thomas & Coley, 2003). A large number of the original chemosensitizers were themselves substrates for ABC transporters and contended with the cytotoxic medications for efflux by the MDR siphons. Hence, high serum centralizations of the chemosensitizers were expected to create adequate intracellular fixations (Ambudkar et al., 1999). These confinements provoked the advancement of new chemosensitizers that are progressively intense, less toxic and specific for the Pgp and other ABC transporters (Krishna & Mayer, 2000; Thomas & Coley, 2003).

Novel point of view to strife with multi-drug resistance mechanisms second-generation chemosensitizers were intended to diminish the reactions of the original medications. Second- generation MDR modulators have a superior pharmacologic profile than the original mixes, still, they hold a few attributes that limit their clinical convenience. Co-organization of an MDR modulator for the most part hoist plasma groupings of an anticancer medication by meddling its flexibility or hindering its digestion and release, accordingly prompting inadequate harmfulness that requires chemotherapy portion decreases in clinical preliminaries down to pharmacologically ineffectual levels (Liscovitch & Lavie, 2002; Thomas & Coley, 2003). The inclination of second-age MDR drugs towards ABC transporters was too low to even think about producing huge inhibition of MDR *in vivo* at tolerable dosages (Ferry et al., 1996).

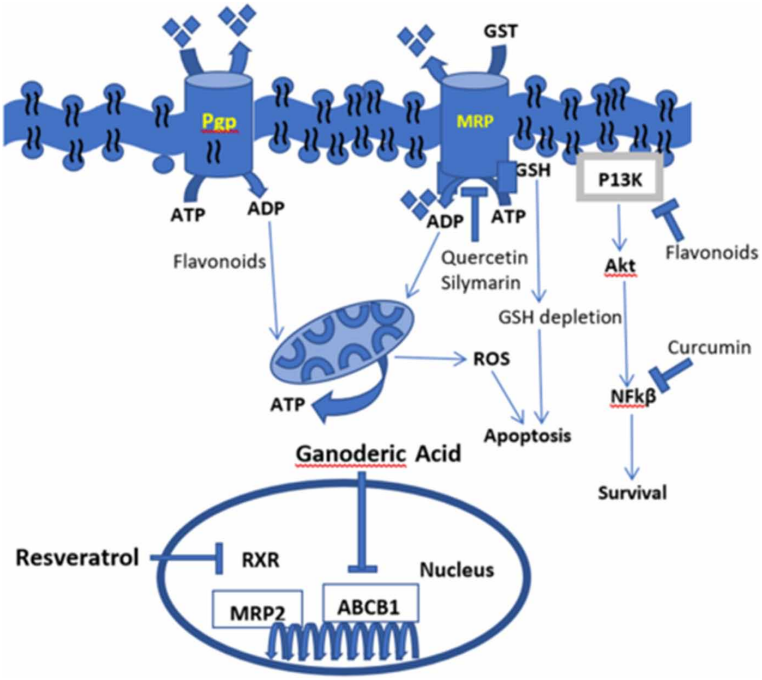
Third-generation molecules have been created to defeat the impediments of the second era MDR modulators (Krishna and Mayer, 2000; Thomas and Coley, 2003). They are not used by cytochrome

P450 3A4 and they don't modify the plasma pharmacokinetics of anticancer medications. Third-age operators explicitly and powerfully hinder Pgp and don't repress other ABC transporters (Thomas & Coley, 2003). None of the third-age agents tried so far have caused clinically applicable changes in the pharmacokinetics of the co-regulated anticancer medications. Due to their explicitness for Pgp transporters and the absence of communication with cytochrome P450 3A4, third-age Pgp inhibitors offer significant enhancements in chemotherapy without a requirement for chemotherapy portion decreases (Thomas & Coley, 2003).

Natural Product Modulators

Recently, various scientists started to screen normal ligands as MDR modulators for putative low harmfulness chemosensitizers (Molnár et al., 2010). Organically dynamic segments acquired from plants and parasites (flavonoids, stilbenoids, coumarins, carotenoids, diterpenes, and curcumin subordinates) have been utilized as MDR modulators after cleaning and molecular portrayal as a result of their putative low poisonousness. A few examinations have shown that herbal mixtures could act synergistically with anticancer agents and converse MDR in diseased cells (Fig. 2).

Figure 2. The mechanism of affecting multidrug resistance by natural products



Anticancer Drugs Capable of Overcoming MDR

Anticancer medications that are not substrates of ABC transporters may offer improved therapeutic results for patients who experience the multidrug resistance impact. Anthracycline-adjusted medications

including annamycin and doxorubicin-peptide are among this classification of anticancer medications. Lareotaxel, a semisynthetic taxoid subsidiary, is another model. Since it is a poor substrate for Pgp related medication resistance, it can act viably against taxane protected malignant growth models. The component of antiproliferative activity is like that of different taxanes. To be specific, Lareotaxel causes tubulin assembly and represses microtubule elements. The epothilones are a class of antineoplastic specialists created from myxobacterium, which prompt the microtubule packaging arrangement of multipolar shafts, prompting mitotic capture. Mainly, four epothilones—specifically, patupilone, BMS-310705, KOS-852 and ZK-EPO—are explored for their anti-cancer properties in various clinical examinations. Furthermore, ixabepilone affirmed for treating breast cancer by the Food and Drug Administration (FDA) in 2007 (https://www.cancer.gov/cancertopics/druginfo/fdaixabepilone). These epothilones, unlike anthracyclines and taxanes, have low weakness to regular resistance systems including Pgp efflux and adjustments in β-tubulin articulation. Vinflunine, a fluorinated vinca alkaloid, is additionally successful against taxane safe malignant growth models. DNA intercalating operator, trabectidin (ET-743), which can down-control Pgp/MDR1, has additionally been very much contemplated. Eribulin mesylate (E7389), a derivative of halichondrin B with successful antimicrotubular action, has additionally been demonstrated to be compelling in treating taxane safe malignant growth. As of late, eribulin and ixabepilone have been affirmed by the US Food and Drug Administration as cytotoxic operators (Saraswathy& Gong, 2013).

Novel Perspective to Conflict With Multi-drug Resistance Mechanisms

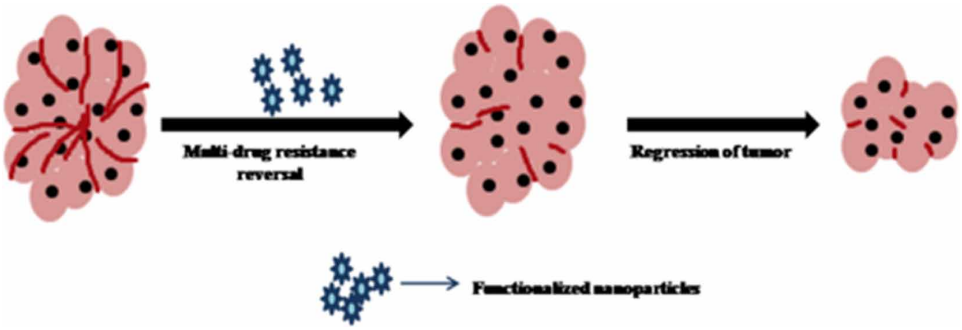
The troubles experienced with MDR inhibitors have driven a few elective ways to deal with MDR therapy. These methodologies can be isolated into two groups. One group of studies comprises trials intended to inhibit MDR components in a novel manner and the other group centers around trials to go around MDR mechanisms (Liscovitch & Lavie, 2002). There are a few ways to deal with restraining components engaged with guideline of MDR transporters. MDR protein gene articulation in tumour cells is initiated upon treatment with cytotoxic medications, while this quality expression is hindered by a few pharmacological inhibitors that influence the flagging pathways. It was exhibited that taxol stimulated MDR1 and cytochrome p450 3A4 (CYP3A4) expression using its immediate cooperation with steroid and xenobiotic receptor (SXR) which in turn prompted expanded drug-resistance (Liscovitch & Lavie, 2002). Thus, antagonists of the nuclear steroid and xenobiotic receptor might be used related to anticancer medications to adapt to the acceptance of MDR1 and CYP3A4 (Liscovitch & Lavie, 2002). Ongoing advances in antisense oligonucleotide innovations propose another option and a more explicit approach to adapt to MDR than the utilization of customary MDR inhibitors (Bouffard et al., 1996). Downregulation of ABC transporter proteins and chemicals associated with cancer cell growth resistance utilizing antisense oligonucleotides may give a productive way to defeat MDR. An ideal MDR reversal agent must have broad-spectrum ABC-transporter inhibitory activities, good pharmacokinetics, no trans-stimulation effects and minimal or no sign of toxicity (Bugde et al., 2017). In advance, nano carrier-based drug delivery systems containing both the cytotoxic drug and reversing agent may represent an effective approach to reverse MDR with minimal drug toxicity (Fig. 3).

TARGET TELOMERASE AND APOPTOSIS PATHWAYS

Introduction

In the normal human cells, the chromosomal end comprises (TTAGGG)n repeated sequence, known as telomeres (De Vitis et al., 2018). The sequence repeats for about 3000 to 20,000 times by the telosome, also shelterin complex, consisting of TRF1 (Telomere Repeat binding Factor 1), TRF2 (Telomeric repeat-binding factor 2), TPP1 (Tripeptidyl Peptidase 1), TIN2 (TRF1- and TRF2-Interacting Nuclear Protein 2), POT1 (protection of telomeres protein 1) and RAP1 (repressor/activator protein 1) (Fan et al., 2019). Telomeres are very crucial to maintain the stability of the genomic sequence by disabling the function of DNA damage response (DDR). During each cell cycle, about 200 nucleotides are lost from the telomeres, making it shorter in length and ultimately lead to senescence or apoptosis. This process occurs in all cells except, germinal cells, stem cells and cancer cells. In these cell types, Telomere Maintenance Mechanisms (TMMs), namely alternative lengthening of telomere (ALT) and telomerase-mediated telomere maintenance, are initialized to shun telomere shortening function (De Vitis et al., 2018).

Figure 3. The nanoparticles-based therapy to reverse multidrug resistance in cancer cells



In cancer cells, ue to telomerase-mediated telomere maintenance, the unstoppable division of cells occurs as the telomere length is conserved by the telomerase reactivation. Telomerase includes different subparts, such as human telomerase RNA and human telomerase reverse transcriptase (hTERT). It is observed that the high level of hTERT is present in approximately 90% of types of cancer. Therefore, if the telomerase activity is suppressed and telomere length is reduced, there is a chance to form an anti-cancer mechanism (Huang et al., 2019).

Programmed cell death (PCD), also known as apoptosis, is a unique pathway towards the death of a cell in all the multicellular organisms. The process maintains balance such that the surrounding healthy cells are not being affected during the removal of the toxic or unwanted cells. Apoptosis involves condensation of the nucleus followed by its fragmentation, shrinking of the cell, bleb formation in membrane and ultimately loses its hold of the neighbouring cells or extracellular matrix, via intrinsic and extrinsic pathway. Failure in any part of the process results in several human diseases, for instance, cancer. As a result, the apoptosis pathway comprising caspases, anti-apoptotic Bcl-2 (B-cell lymphoma 2) family members, p53, inhibitor of apoptosis (IAP) proteins, extrinsic pathway, FLICE-inhibitory protein (FLIP) is targeted to induce apoptosis and suppress cancer (Goldar et al., 2015).

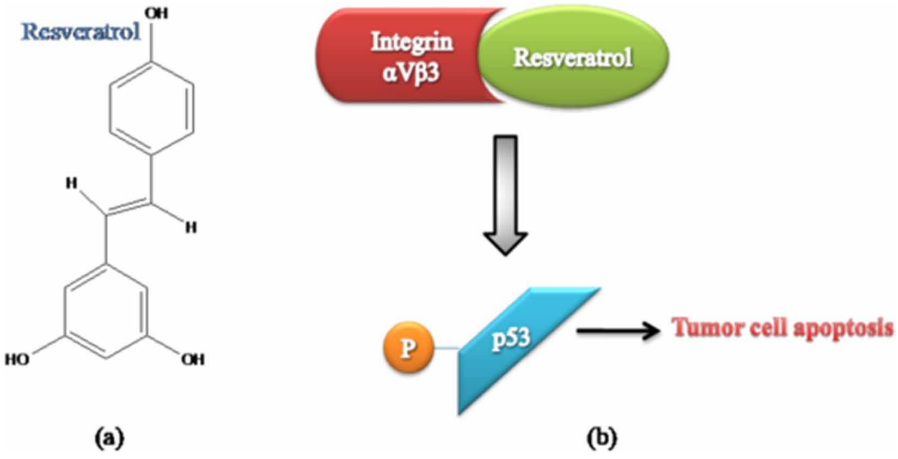
Target Telomerase Pathways in Cancer

Targeting STAT3, NF-κB and Akt Signalling Pathways

Signal transducer and activator of transcription 3 (STAT3) and Nuclear factor κB (NF-κB) are the transcription factors present in the cancer stem cells that are specifically activated. The function of STAT3 is to transmit signals from different growth factors and cytokines to the nucleus from the cell membrane. When a variety of growth factors and cytokines gets activated, these phosphorylate Tyrosine 705 in STAT3 forming pSTAT3 ensuing the transcriptional activation of its target genes. The management of the survival of cancer cells is mainly done by NF-κB. It also regulates the resistance of drugs, while providing an immune response to inflammation. Therefore, targeting these signalling factors in cancer stem cells could be a potential target in cancer therapy (Chung et al., 2019).

A natural compound, Resveratrol, obtained from grapes, berries and nuts, has been found to have importance in the medical field. It is formed in the stress condition, fungal infection, or injury. It has several properties such as anti-inflammatory, antioxidant, anti-aging, lowering the threat of cardiovascular disease and induces insulin production. Additionally, it also possesses anti-cancer properties. It was stated that integrin αVβ3 in breast cancer cell line MCF7 had a receptor site for resveratrol that causes apoptosis by p53 phosphorylation (Fig. 4). Also, in glioma cells, resveratrol transduces pro-apoptotic signals by plasma membrane integrin αVβ3 utilizing extracellular-regulated kinases 1 and 2. In addition to this, resveratrol can increase the effectiveness of chemotherapy and/or radiotherapy in cancer cells.

Figure 4. (a) Structure of Resveratrol. (b) Apoptosis induction via p53 phosphorylation due to binding of Resveratrol to integrin αVβ3



5-Fluorouracil (5-FU), a drug containing enzymatic action of thymidylate synthase in DNA replication, is being used to treat different solid tumours. It induces apoptosis by stopping the G_1/S cell cycle. But the adjuvant chemotherapy accompanied by 5-FU, leucovorin and oxaliplatin (FOLFOX) that is given to the patients at stage II and stage III starts resistance towards drug and develops harmful side-effects such as liver damage and can relapse later. Keeping this in mind, a study has been done that ex-

perimented by combining the above two drugs, that is, resveratrol with 5-FU and reported that it hinders the STAT3 as well as Akt (protein kinase B or PKB) signalling pathway. It stops the phosphorylation of STAT3 and subsequently, its binding to human telomerase reverse transcriptase (hTERT) promoter (Chung et al., 2018).

Nitrogen-containing curcumin derivative has been also tested and accounted to have the same properties as the above combination of resveratrol and 5-FU contain, and suppresses the STAT3 binding to hTERT at the promoter site. Additionally, it halts the cell cycle at the G1 phase and promotes apoptosis (Chung et al., 2019).

PinX1

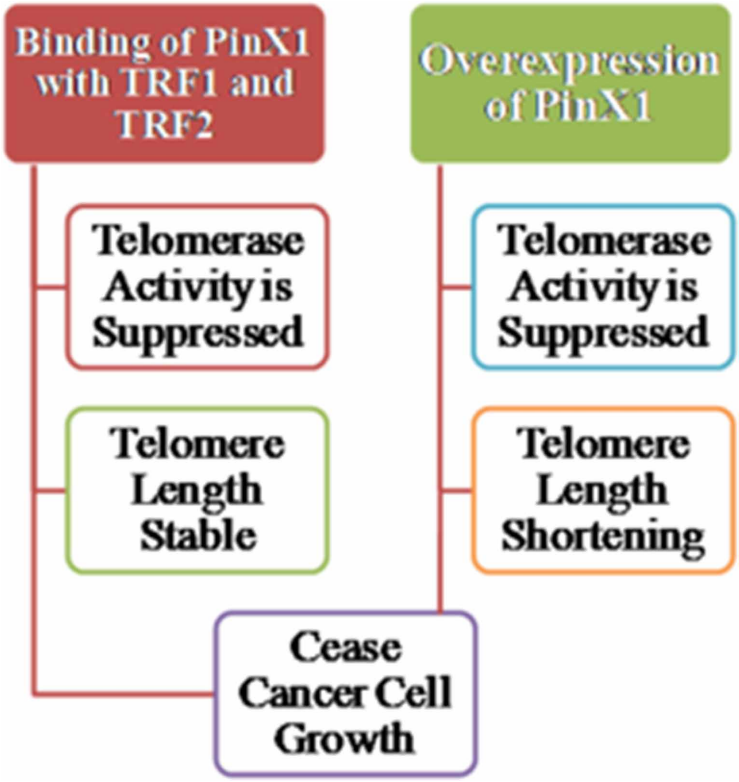
Pin2/ telomeric repeat factor (TRF) 1 –interacting telomerase inhibitor 1 (PinX1) gene is present at human chromosome 8p23 (Li et al., 2016). Its expression product is found in the nucleolus at the two sites, nearby telomerase subunits and with telomeres. It is known to have an important role in telomeres length maintenance as well as instability of the chromosome. It is interesting to know that it can bind with hTERT as well as the telomere-binding protein hTRF1 (Banik & Counter, 2004). PinX1 when binds with TRF1 or with TRF2, it can maintain the integrity of telomere by suppressing the activity of hTERT (Fig. 5), but the exact function of PinX1 is not yet discovered (Shen et al., 2018). Some studies have shown that it can be the cause of the initiation of tumour and cancer progression. However, the overexpression of PinX1 has a suppressing effect on telomerase functioning that leads to telomere shortening (Banik& Counter, 2004) (Fig. 5). In cancer cells, the Telomerase Inhibitory Domain (TID) fragment at C-terminal in PinX1 stops the expression of telomerase (Li et al., 2016). These tumour-specific biological markers (Shen et al., 2018), such as TID fragment (Li et al., 2016), are not expressed in normal cells, but only in cancer cells to suppress the telomerase activity and stops the increase in the growth of cancer cells and metastasis (Shen et al., 2018).

c-Myc, a proto-oncogene, that binds at the promoter site of hTERT, is the main cause of hTERT upregulation. But, c-Myc is in the competition with Max dimerization protein 1 (Mad 1) for its binding to the ubiquitous binding partner Max, which results in the suppression of hTERT activation. Keeping this in mind, it has been shown that in the gastric cancer cells, the expression of telomerase by the PinX1 gene can be suppressed through the Mad1/c-Myc pathway. Another study shows that the transcription of PinX1 gene can be stopped when the p53 level decreases by (E6 protein of human papillomavirus type 16) HPV16 E6 that boosts the telomerase function. Hence, it was assumed that by increasing the activity of PinX1 gene by Mad1/cMyc/p53 as well as by TRFs, the telomerase activity can be suppressed and stop the progression of cancer stem cells. From the above assumption, a research showed that upregulation of PinX1 in CD133+ in cancer stem cells has resulted in suppression of hTERT and prevention of growth of cancer cells ultimately leading to apoptosis, at the same time, decreasing c-Myc mRNA level and increasing the Mad1, TRF1 and p53 mRNA levels. Alternatively, by masking PinX1, it showed the opposite effect leading to tumour progression (Shen et al., 2018).

Mechanistic Target of Rapamycin (mTOR) Pathway

The mechanistic target of rapamycin (mTOR), an evolutionarily conserved prototypic pathway is a signalling pathway of serine/threonine kinases which is involved in various functions such as cell cycle regulation and angiogenesis, mediate cell growth, maintenance and production, generating ribosome,

Figure 5. Control in cancer cell growth via PinX1



protein formation and autophagy using various growth factors and nutrients. The dysfunction of a part of mTOR, phosphatidylinositol kinase-related protein kinase (PIKK) family as well as other proteins occurs in cancer that results in drug resistance to different chemotherapeutics. mTOR, in humans, is available as two individual macromolecular complexes, i.e., mTOR complex 1 (mTORC1) plus mTOR complex 2 (mTORC2). The phosphorylation of Akt by mTORC2 forms a means by which mTORC1 and mTORC2 can be regulated by each other (Gopalakrishnan et al., 2018).

Telomerase versus Alternative Lengthening of Telomeres (ALT)

Apart from telomerase, Alternative lengthening of Telomeres (ALT) can also be the cause of cancer. In cancer cells, where there is no or low activity of telomerase is identified, it is shown that ALT can maintain the telomere length. In other cases, when the telomerase-targeting drugs are given, the cancer cells trigger the ALT mechanism (De Vitis et al., 2018). Hence, ALT targeting drugs can also be considered for cancer treatment.

Target Apoptosis Pathway in Cancer

Extrinsic Pathway

The TNF-family receptor (TNFR), Fas ligand (FasL) is vital for cytotoxic T cells-mediated kill, moreover, TRAIL (TNF-related apoptosis-inducing ligand or Apo2 ligand) is important for natural killer cell-mediated inhibition in cancer. However, some cancer cells can escape the immune response created by T cells by resisting FasL. The resistance to FasL can be due to various reasons, such as suppression of the Fas receptor, secretion of a soluble form of Fas receptor at high level, production of non-functional Fas receptor. This seizes the Fas ligand or its response directly on the cancer cell surface. Although, some cancer cells can respond by a Fas ligand-mediated "counter-attack" resulted in the reduction of apoptosis of tumour infiltrated by lymphocytes. Also, some cancer cell lines have shown the resistance to TRAIL, verifying that malignant tumours can evolve by forming important cell receptors in vivo and survive the immune response. Therefore, anticancer therapy that can reinstate the ability of the extrinsic pathway could be profitable (Hassan et al., 2014).

Fas/Apo1 (apoptosis antigen 1) plus Killer/DR5 (death receptor 5) are the two pro-apoptotic elements of TNFR superfamily that in the presence of several anticarcinogenic drugs act as they are p53-dependent. The upregulation of p53 can elevate Fas production as well as transportation of Fas via Golgi to the cell surface. Additionally, it has been studied that in several types of cancer, different microRNAs are required in the extrinsic pathway, like miR-196b, miR-20a, miR-21 and miR-590 can modulate Fas/FasL system (Pistritto et al., 2016).

It is reported that ABT-737, a new drug that inhibits Bcl-2, is acknowledged as an apoptosis inducer in glioblastoma cells by detaching Bax pro-apoptotic protein from Bcl-2. The drug is also known to make cancer cells sensitive towards TRAIL and another chemotherapeutics (Mohammad et al., 2015).

Intrinsic Pathway

Bcl-2 family proteins, the main regulators of the intrinsic pathway that can upregulate or downregulate the mitochondrial membrane permeability to secrete cytochrome c (Cyt-c) and other proteins involved in apoptosis. Hence, a slight change in this pathway can lead to apoptosis inhibition. Therefore, it is the central pathway for apoptosis stimulation. In most cancer types, anti-apoptotic proteins Bcl-2 or Bcl-xl overexpression causes resistance to chemotherapeutics agents as well as to myriad apoptotic stimuli (Hassan et al., 2014).

In a study, Mortenson and colleagues showed that when Bcl-2 is overexpressed in cancer, it enhances AKT and IKK function plus NF-κB transcription (Mortenson et al., 2007). Kumar and colleagues observed that Bcl-2 and interleukin-8 are responsible for the proliferation and invasion of tumour cells, respectively (Kumar et al., 2008). A recent study reported by Tucker and colleagues shows that the upregulation of Bcl-2 results in cyclin D1a expression maintenance, which can take place via p38 mitogen-activated protein kinase (MAPK)-mediated signalling pathways in human lymphoma cell lines (Tucker et al., 2008). Besides that, in prostate cancer cell lines, Bcl-2 suppression modulates carbonic anhydrase IX (CAIX), phosphorylated AKT (pAKT) and vascular endothelial growth factor (VEGF) expression. From the above studies, it can be established that Bcl-2 family proteins can initiate resistance to anticancer drugs and it is important to find new chemotherapeutics targeting these proteins. Thus, various inhibitors of these proteins are already developed and some are still under pre-clinical and clinical trials. Among these

tests, only one drug, namely Gossypol (AT-101), a cotton plant-derived multi-targeting polyphenol, can reach the clinical trials in glioblastoma multiforme (GBM) patients. The drug binds to the BH3 pocket in Bcl-2 family proteins plus other target proteins.

Recently, Rosenbluh et al. (Rosenbluh et al., 2012) proved that suppressing Bcl-xl inhibits proliferation in β-catenin-active cancer cell lines, showing that Bcl-xl is a significant target of navitoclax in catenin cadherin-associated protein β 1 (CTNNB1) mutant cancers. Inhibition of Proteasome was likewise adequate to overcome the apoptotic defence by Bcl-2 or Bcr Abl oncoprotein. It causes the upregulation of Bax protein within mitochondria leading to an increase in the Bax/Bcl-2 ratio, which is related to the release of cytochrome c and induction of apoptosis. It has been demonstrated that during TNF-α- instigated apoptosis, Bcl-2 protein is destroyed by ubiquitin/proteasome-dependent pathway which also leads to increased Bax/Bcl-2 ratio. Thus, degrading selectively the Bcl-2 family proteins by proteasomes can change the pro- to anti-apoptotic proteins ratio that could ultimately contribute to the apoptosis and thereby overcome resistance. It is shown that NF-κB upregulates Bcl-2 resulting in human melanoma tumorigenesis.

Authoritative NF-κB initiation in melanoma cells is related to increased survival and multiplication of cells. Prior Wang and associates have effectively exhibited that focused inhibition of Bcl-2 can stifle PDAC (Pancreatic ductal adenocarcinoma) development in vitro and in vivo (Wang et al. 2009). Recently, Abulwerdi et al., (Abulwerdi et al., 2014) oppressed Mcl-1 as a therapeutic objective utilizing small molecules in PDAC. Both these investigations altogether demonstrate that targeted inhibition of BH3 family proteins could be a practical therapeutic strategy against PDAC. The function of Bcl-2 proteins in resistance of apoptosis from retinoic acid treatment has been seriously explored. It has been demonstrated that the capacity of retinoid-instigated cells to experience apoptosis relies upon the expression level and the useful interactions between Bcl-2 and Bax. Featuring its importance, autophagy and Beclin 1, an autophagic protein, were demonstrated to be upregulated for all-trans-retinoic acid (ATRA)-initiated neutrophil/granulocyte segregation of an (acute promyelocytic leukaemia) APL-derived cells. This autophagy induction is related to the downregulation of Bcl-2 and suppression of mTOR function. Further, different investigations have indicated that an imitative BH3 domain, JY-1-106, that antagonizes antiapoptotic Bcl-2 members, i.e., Bcell lymphoma-extra-large (Bcl-xL) and myeloid cell leukemia-1 (MCL-1) on its own or with retinoids diminished viability of HL-60 APL cells. This combination had the best effect on the viability of cells by inducing apoptosis. These examinations showed that double BCL-xL/MCL-1 inhibitors and retinoids could work in cooperation in APL. Several authors noticed apoptosis induction in primary B-cells utilizing curcumin with other compounds, for example, rapamycin and epigallocatechin-3-gallate (EGCG). In the previous case, the impact was related to diminished Bcl-2 expression and a high level of pro-apoptotic factor, Bax (Mohammad et al., 2015).

Most of the small molecule inhibitors of Bcl-2 portrayed to date is Bad-like BH3 imitative. Ongoing investigations demonstrated that HA14-1 which binds BH3 can improve the sensitivity to both chemotherapy and radiotherapy in human glioblastoma cells (Mohammad et al., 2015).

FLIP

FLIP is a significant apoptosis regulatory protein highly expressed in haematological and solid cancers, in which it's overexpression, is regularly associated with a bad prognosis. FLIP expressed as short (FLIP(S)) and long (FLIP(L) splice forms, by binding to FADD results in its regulation of cell death functions. Fas-associated protein with death domain (FADD) is a basic connector protein in the extrinsic pathway

which connects FLIP to the apical caspase (caspase-8), in various complexes regulating cell death. FLIP additionally has a key function (along with caspase-8) in controlling another type of cell death named 'necroptosis'/programmed necrosis, also in other key cell procedures that affect cell survival, involving autophagy. Also, FLIP effects initiation of the intrinsic apoptotic pathway mediated by mitochondria by managing caspase-8-mediated activity of the pro-apoptotic Bcl-2-member Bid. It has been shown that FLIP can repress death receptor-mediated apoptosis, nonetheless cell death can be prompted by various clinically significant chemotherapeutics and ionizing radiation. Recently, the main functions for FLIP to promote the survival of immunosuppressive tumour-inducing immune cells have been found. In this manner, FLIP is of particular interest as a therapeutic target for cancer. FLIP has been demonstrated to be overexpressed in various malignancies, comprising meningiomas, ovarian carcinoma, stomach cancer, breast cancer, prostate cancer, pancreatic cancer, non-small-cell lung cancer (NSCLC), cervical cancer colorectal cancer, urothelial cancer, nasopharyngeal carcinoma, Burkitt's lymphoma, and acute myeloid leukaemia.

The viral FLIP K13 shows antiapoptotic action and plays a significant role in the pathogenesis of gamma-herpesvirus HHV-8 associated tumours, along with multicentric Castleman's disease, Kaposi's sarcoma, and primary effusion lymphoma. Various examinations showed that FLIP as an autonomous adverse prognostic marker and indicated both FLIP(S) and FLIP(L) to be significant in patient's treatment results. One reason behind the relationship between FLIP overexpression and poor anticipation is that FLIP presents protection against therapeutics. Downregulation of FLIP mediated by siRNA [mainly FLIP(L)] significantly upgraded chemotherapy-initiated cell death. Comparable outcomes were found with docetaxel in prostate cancer models, cisplatin in NSCLC models, and lately with ionizing radiation in NSCLC. Additionally, high levels of FLIP(S) that are initiated by AKT/mTOR/S6K1 and Ral effector protein (RalBP1) have been connected to oxaliplatin and cisplatin-induced apoptosis suppression (Humphreys et al., 2018).

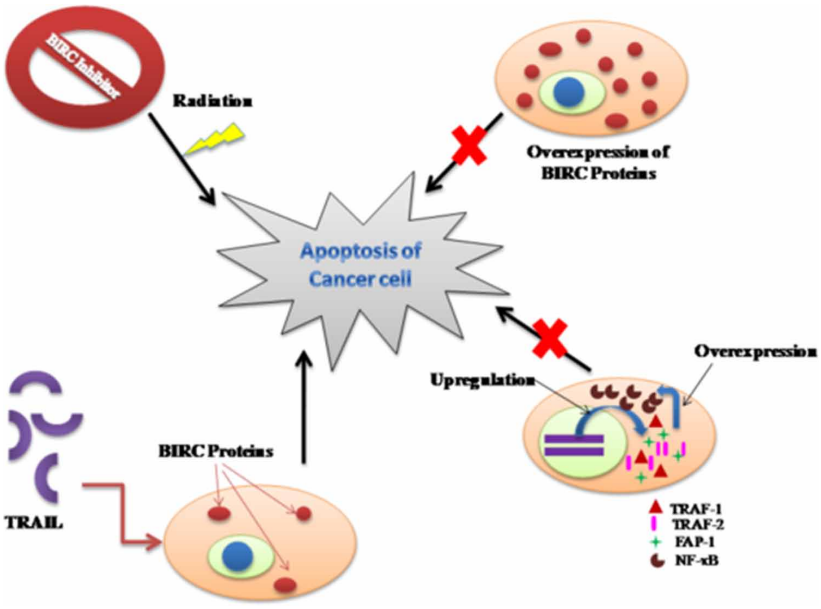
i. **Indirect Targeting:** Various known chemotherapies, for example, 5-Fluorouracil (5-FU), gemcitabine, cisplatin, etoposide, and paclitaxel showed suppression of FLIP in different tumour cell line models. The antimetabolites 5-FU and gemcitabine have shown to downregulate FLIP in colorectal and pancreatic cancer models, separately. Additionally, Cisplatin showed to lower the level of FLIP in cancer such as pancreatic cancer and glioblastoma as well as oral squamous cell carcinoma. The topoisomerase II inhibitor etoposide was additionally shown to downregulate FLIP and sensitize cells resulting in cell death due to CD95. Hence, FLIP can be downregulated by chemotherapies but the methods by which this happens are different and frequently poor as well as cell type specific. Several reports have detailed a decrease in FLIP mRNA because of treatment with inhibitors of HDAC (histone deacetylase). Entinostat surmounted FLIP(L)-mediated protection from MEK inhibitors in BRAF mutant colorectal cancer. Another comparative methodology is the utilization of CDK9 inhibitors, which appeared to downregulate FLIP and Mcl-1 expression successfully in NSCLC cell lines. An alternate potential methodology is to target the ubiquitin-specific proteases (USPs) that repress the production of FLIP by the ubiquitin-proteasome system (UPS), for example, USP8 (Humphreys et al., 2018).
ii. **Direct Targeting:** FLIP especially utilizes phenylalanine 114 to associate with histidine 9 in FADD. Interestingly, procaspase-8 especially utilizes tyrosine 8 to interact with phenylalanine 25 of FADD. This uncovers a potential chance to specifically target FLIP, maintaining the mobilization of procaspase-8 to the death-inducing signalling complexes. A comparative methodology was investigated

to create peptides from FLIP that had the option to specifically focus on the connection among FLIP and (autophagy-related 3) Atg3 without influencing its interaction with LC3 (microtubule-associated protein 1A/1B-light chain 3). These peptides independently bound to FLIP or Atg3 and appeared to instigate suppression of growth and autophagic cell death (Humphreys et al., 2018).

Inhibitor of Apoptosis (IAP)

Recently, research demonstrated that BIRC-4 (X-linked inhibitor of apoptosis protein, or XIAP) inhibitors along with radiation induce glioblastoma cell apoptosis and baculoviral IAP repeat-containing (BIRC) proteins can also make cells sensitive to TRAIL resulting in apoptosis (Fig. 6). Additionally, some research showed that in an intracranial glioblastoma xenograft model, the endogenous BIRC inhibitor Smac can altogether enhance the TRAIL's anti-cancer function. In later stages of metastatic melanoma, pro-apoptotic pathways are repressed by hyperactivated NF-κB via the upregulation of tumour necrosis factor receptor-associated factor-1 (TRAF-1) and tumour necrosis factor receptor-associated factor-2 (TRAF-2) and Fas-related phosphatase-1 (FAP-1) (Fig. 6). It is also accounted that TRAIL initiated apoptosis repression in prostate cancer is likewise. The TRAIL resistance development is both epigenetic and hereditary (Mohammad et al., 2015).

Figure 6. Effect on apoptosis of cancer cells due to various factors



Survivin (BIRC5), an inhibitor of apoptosis (IAP) protein family that suppresses caspases and obstruct cell death is overexpressed in many tumours and is related to a poor clinical result (Fig. 6). Survivin has reliably been recognized by molecular profiling examination to be related to high tumour grade malignancies and their recurrence. Survivin gene polymorphism is shown to be an asset in the biology of the cancer and can be utilized in cancer diagnosis and prognosis. Polymorphisms of the survivin gene have

been accounted to impact tumour aggression and cancer patients' survival. As survivin expression is different in normal and cancerous cells, also its functioning in various cell pathways as a nodal protein makes it an important target for therapeutics (Jaiswal et al., 2015).

Apart from Survivin, to date these IAPs have been identified, namely, IAP-like protein 2 (BIRC8), c-IAP1 (BIRC2), c-IAP2 (BIRC3), NAIP (BIRC1), X-linked IAP (XIAP, BIRC4), Apollon (BRUCE, BIRC6) and Livin/ML-IAP (BIRC7) (Wong, 2011).

Reduced Caspase Activity

It was frequently found that the downregulation of caspase-9 in patients with stage II colorectal malignancy is related to poor clinical results. In certain cases, the downregulation of several caspases leads to tumour cell development and growth (Wong, 2011). Fong et al discovered that both caspase-8 and - 10 are co-downregulated in cDNA array differential expression study and hypothesized that it might contribute to the choriocarcinoma pathogenesis (Fong et al., 2006).

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Chapter 11
Receptor-Based Combinatorial Nanomedicines:
A New Hope for Cancer Management

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ABSTRACT

Nanotechnology-based drug-delivery systems, as an anticancer therapy tool, have shown significant potentials for the diagnosis and treatment of cancer. Recent studies have demonstrated that cancer therapy could be efficiently achieved by combinatorial therapies, approaches using multiple drug regimens for targeting cancers. However, their usages have been limited due to shorter half-lives of chemotherapeutic agents, insignificant targetability to tumor sites and suboptimal levels of co-administered conventional drug moieties. Thus, nanotechnology-based drug-delivery systems with effective targetability have played a crucial role to overcome the limitations and challenges associated with conventional therapies and also have provided greater therapeutic efficacy. Herein, the authors have focused on various drug-incorporated combinatorial nanocarrier systems, the significance of various receptors-associated strategies, and various targeted delivery approaches for chemotherapeutic agents.

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INTRODUCTION

The National Nanotechnology Initiative has defined nanotechnology as the process of designing, synthesizing, characterizing, and using the materials and devices that have dimensions in the nano range scale (10-200 nm) (National Science and Technology Council Committee on Technology., 2005). Generally, nanomedicines are the fabrication of active pharmaceutical ingredients or materials for disease treatment and diagnostic purposes in the nanoscale range (Sanna et al., 2014). Nowadays, nanomedicine-based delivery systems are getting a lot of attention as they have been used to develop various approaches in delivering medications, particularly chemotherapeutics, with significant safety and efficacy. Formulation scientists have developed a diverse class of nanoparticles (NPs), demonstrated their various applications in drug delivery, observed their effects over the cellular intracellular uptake, biodistribution and dosing efficacy of incorporated drug regimen and also noticed their targetability at diseased sites rather affecting the healthy or normal cells (Farokhzad et al., 2009). A revolutionary change was seen in disease treatment with the application of nanoparticulate-based drug delivery systems. In recent years the Food and Drug Administration (FDA) has approved several liposomes and polymer-based nanoformulations for the clinical purpose (Sanna et al., 2014). The NPs have exhibited tremendous ability to improve the solubility of the drug, execute the process of drug loading and release to the targeted sites in a controlled manner (Chrastina et al., 2011). The NPs have exposed various unique features such as large surface to volume ratio, flexible exterior boundary, biodegradability, low cytotoxicity, and these features have enhanced their applications in establishing nanomedicines. Nanomedicine has also shown fruitful insights into the development and establishment of personalized medicine (Davis et al., 2008; Zhang et al., 2012). Novel NPs-based anticancer therapy symbolizes an innovative and potential drug delivery strategy to conquer the challenges and restrictions of conventional chemotherapeutic agents by improving drug uptake and selective intracellular accumulation in tumor tissues via passive and active targeting with minimal toxicity to normal cells (Blau et al., 2016; Shapira et al., 2011; Bar-Zeev et al., 2017).

The NPs have shown significant applications for developing drug delivery systems (DDSs) incorporated with hydrophobic drugs, improved their solubility, enhanced cellular uptake and targetability at the target sites with no or reduced cytotoxicity. Further such approaches can surmount the drug resistance in malignancy. With this concept, it makes possible to design a rational DDS to target the cancer cells and even establish personalized medicine or therapy. The nanotechnology science has exhibitednumerous potentials including initial recognition of cancer cells, active and passive targeting, improved biocompatibility, and versatility of applying imaging and therapeutic proficiencies in chemotherapy (McNeil., 2009).

Surface-engineered NPs based therapeutics offers several clinical advantages in disease treatment. Polyethylene glycol (PEG) is usually employed for modifying NPs surface, has prevented the clearance of NPs from the blood circulation and thus increased the circulation time and cellular uptake of a drug at the targeted sites (Farokhzad et al., 2009; Chow et al., 2013). The functionalized NPs surface not only enhances the drug efficiency but concurrently minimizes the dosage, and thus provides a unique approach to optimize drug pharmacokinetics (Chow et al., 2013). NPs can deliver the drug to the targeted sites through epithelial or endothelial barriers, in response to the passive and active targeting process (Chrastina et al., 2011). There are few examples of surface-engineered NPs which are designed to overcome challenges and limitations for cancer drug delivery.

The application of the NPs-based approaches has facilitated enhanced drug solubility, minimized cytotoxicity, and better drug pharmacokinetics, an example is Doxil® and Genexol-PM®. In 2006, Noble prize in physiology brought a revolution in the field of gene targeting and silencing, which evolved new

therapeutic prospects. The specific approaches for regulating gene expressions increased the curiosity of researchers to establish drug delivery systems associated with small interfering RNA (siRNA) and microRNA (miRNA) tools (Whitehead et al., 2009). Though it is very difficult to deliver nucleic acid into cells, it is unsafe because nuclease enzyme is present everywhere in blood and its negative charges obstruct cell internalization. Additionally, the process of non-specific cytoplasmic interferon response due to foreign nucleic acid moieties has found to be a major obstacle for clinical translation (Whitehead et al., 2009; Shi et al., 2011; Xu et al., 2015). Thus, for preventing these obstacles, an idyllic siRNA-based delivery system could be established which effectively encapsulates the siRNA part (negatively charged) and thus assists the intracellular uptake and release the therapeutic moieties by preventing degradation of endogenous enzymes (Xu et al., 2015).

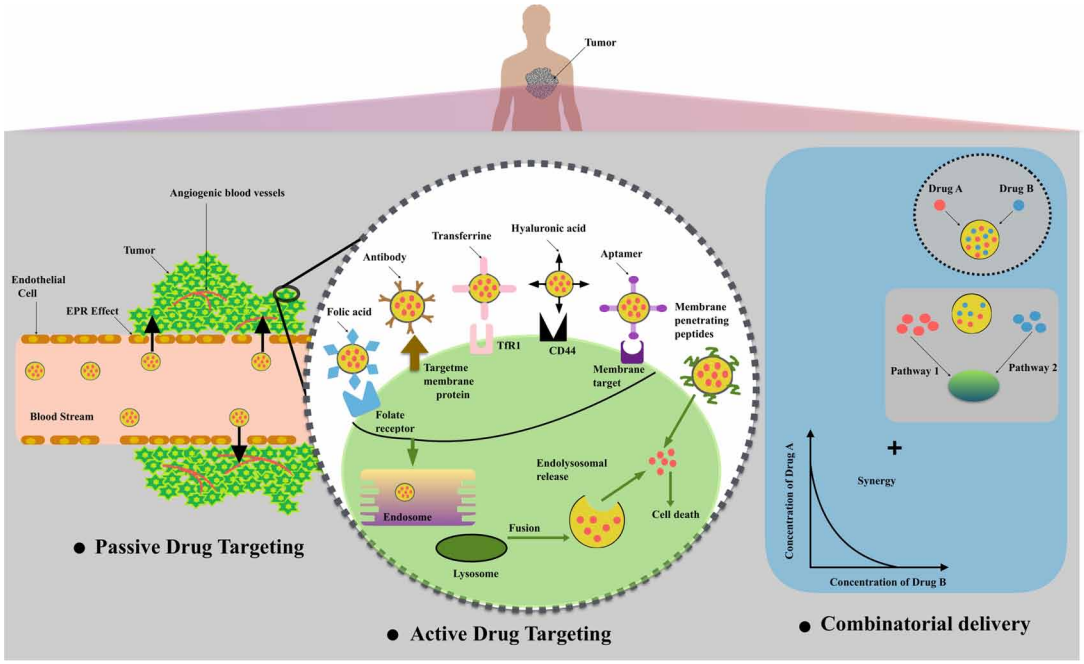
TARGETED STRATEGIES TO OVERCOME CANCER BARRIERS

Passive Targeting

The most important advantages of nanomedicines include the enhancement of bio-distribution of therapeutically active agents via passive targeting, a prominent characteristic of NPs. Furthermore, tumors can preserve more polymeric NPs, liposomes and micellar assemblies as compared to the normal cells and this has been observed due to the improvement in the permeability and retention (EPR) effect (Kamaly et al., 2012; Acharya et al., 2011; Maeda et al., 2009). Physically maximum tumors are typically dense and have a penetrable vasculature produced by the triggering of vascular endothelial growth factor (VEGF). It has been noticed that the particles having a size >2 nm can't pass through the endothelial cells and get prevented due to tight junctions in normal vasculature. Particle size in the range of 10-500 nm extravasate and gather inside the tumor vessels and this happens due to irregularities in the tight junctions and base membranes of tumors vasculature (Torchilin., 2011; Bertrand et al., 2014). Also, the lymphatic drainage system is damaged in tumors, which causes microparticles entrapment and delay in their clearance (Fang et al., 2011; Maeda et al., 2013). The particle size and permeable neovasculature of tumors lead to success for passive targeting (Figure 1). An extended blood circulation possibly by "stealth" alterations (e.g. PEGylation), causes accumulation of NPs due to enhanced permeability and retention effect (EPR effect) (Torchilin., 2011). The concept of NPs employed as a vehicle for therapeutic object exit from decades and in market nanoformulation has reached with significant success (Venditto et al., 2013). In 1995 Doxil® was permitted clinically for the treatment of AIDS-associated Kaposi's sarcoma, ovarian cancer, etc. (Barenholz., 2012). The blood circulation half-life, and concentration of doxorubicin (DOX), in a tumor, increased when it was encapsulated within the PEGylated NPs. Similarly, Genexol-PM®, a polymer-based micellar formulation comprised of poly (D, L-lactide) for programmed delivery of medicine for the therapeutic purpose (Harshita et al., 2019). siRNA-encapsulated ALN-TTR02 lipid nanoparticles were developed for targeting a preserved sequence in the 3' untranslated section of the TTR (transthyretin) gene. The electrostatic interactions involved in the encapsulation of siRNA and the structure of the nanoparticulate system comprised of a neutral lipid (PEG) and an ionizable cationic lipid which counters the negative charge of siRNA (Semple et al., 2010). This nanomedicine has been used for the treatment of disease transthyretin amyloidosis, where the condition like an accretion of TTR amyloid in exterior heart and nerves has occurred due to circumstances formed by mutant transthyretin gene (Coelho et al., 2013). Further, a successful approach has been observed, in which ALN-TTR02-

specified delivery ensued for effective and targeted delivery to the hepatic environment, however, the NPs accumulated due to uptake by the reticulo-endothelial system (RES). In another study, the TKM-PLK1 lipid NPs, structurally alike to ALN-TTR02, was encapsulated with siRNA and this system inhibited the PLK1 (protein polo-like kinase 1), which plays a significant role in the phosphorylation of Cdc25C and regulates the damage of DNA checkpoints, nucleation in microtubules, chromosomal abridgment (Shi et al., 2010). The effects of dose rise in the solid tumors measured in clinical trials (Phase I) on the advanced stage of cancer patients have a perfect safety and efficacy results, this process continues and rises in an ongoing clinical trial (Phase II) with progressive Adrenocortical Carcinoma (ACC). Further, the effect of EPR extents and it varies from tumor to tumor and even intratumoral, because of the differences in the heterogeneity and vascular penetrability within the discrete tumor (Bertrand et al., 2014; Danquah et al., 2011). Within a tumor, a high interstitial fluid pressure (IFP)occurs which causes the NPs to extravasate from the interior regions to the external regions and lead to the intensification of the tissues (Kamaly et al., 2012). The prime objective of the nanomedicine is to sort out the challenges associated with the targeted delivery system by improving the drug residual time in the blood circulation and by targeting the NPs into the targeted sites. Therefore, it is very important to focus on various approaches required for designing a formulation that synergizes passive targeting of therapeutically active moieties towards targeted cells or tissues, rather than normal or healthy cells or tissues, for superior therapeutic efficacy.

Figure 1. An illustrative representation of passive targeting, active targeting and combinatorial delivery of nanomedicine



Active Targeting

The EPR effect and PEGylation have enhanced the biodistribution of drugs and increased their accumulation in the RES-associated organs (liver and spleen) (Albanese et al., 2012). The advantage and major role of active drug targeting, seen in the cancer patients, is to minimize the harmful effects of the NPs on the normal cells and to target these NPs into the tumor cells. For targeted delivery of the drug, the NPs surfaces are polyvalent decorated with a ligand that could efficiently enable the conjugation with a biomarker particularly found in targeted tissues and trigger receptor-facilitated endocytosis (**Figure-1**) (Zhang et al., 2012). The modification of NPs could be done by the involvement of ligands, which might be an antibody-engineered molecule, protein, peptide and other small biomolecules (Harshita et al., 2020). The ligand-receptor interface could be preferentially used to target a drug regimen at a disease *in vivo*, with an improved delivery profile (Chrastina et al., 2011; Ge et al., 2013). Moreover, the formulation quantity of ligand is an important factor and it can be used to optimize the activity of targeted moieties (Kamaly et al., 2012; Valencia et al., 2011). Various preclinical studies have demonstrated good results for the NPs implemented in active targeting, however, in some cases, the therapeutic efficacy of the NPs-conjugated delivery systems was hindered (Chrastina et al., 2011; Albanese et al., 2012; Mahon et al., 2012).

TARGETING SPECIFIED RECEPTORS

Folate Receptor

In the cancer cells, folate receptors are active to interact with anticancer medicine. Keeping this concept in mind, research has been focused on functionalizing the nanoparticle's surface with folic acid (Mansoori et al., 2007; Barkat et al., 2020; Kukowska-Latallo et al., 2005). Russell-Jones and coworkers performed experiments on four murine tumor models by employing folic acid to aid in the delivery of pHPMA conjugated daunomycin. The results indicated that a lot of survivors had tumor experimented folic acid targeted daunomycin-HPMA conjugate and the survival time was noted down. It was concluded that folic acid is highly effective in enhancing the efficacy of many polymeric system bound cytotoxins (Russell-Jones et al., 2012). Also, in another study reported by Kukowska-Latalloto and groups, the folate conjugated methotrexate dendrimers in the immune-compromised asthmatic nude female mice were studied. In the performed experiment, nano-conjugate injections were given by the lateral tail vein, twice a week. By comparing the resulting efficacy of methotrexate and conjugated methotrexate, it was found that there was a tenfold increased efficacy in conjugated methotrexate and lowered toxicity, and ultimately the lengthened life span of mice (Kukowska-Latallo et al., 2005). The efficacy of nanocomposites of folate receptor targeting doxorubicin was examined on a cancer patient. The PEGylated doxorubicin folate conjugates had an average diameter of 200 nm and the in vitro studies on cancer cells revealed higher and more selective targeting of these nanomicelles to folate receptors while the in vivo experiments showed tumor suppression (Zwicke et al., 2012; Yoo et al., 2004). Overall from the experiment, it was clear that the folate receptors could be successfully targeted with the use of folic acid linked nanoparticles to help in selective drug delivery.

Transferrin Receptor

CALAA-01, synthesized from a cyclodextrin integrating cationic polymer, the outer coating of PEG and targeting ligand, human transferrin (Tf), was the first fabricated nanoparticle system to cargo siRNA. (Davis., 2009). The nanocomposites functionalized with Tf could interact with their receptors (TfR) which was overexpressed on cancerous cells after their internalized via receptor-mediated endocytosis. To melanoma patients, the intravenously administered siRNA-containing targeted NPs can localize in the tumor cells (Davis., 2009; Davis et al., 2010). A quantitative relation is found between the amounts of intracellular localized NPs and administered dose if biopsies of the tumor are performed. As well as, the level of the specific messenger RNA and the protein is seen to be lower post injecting the targeted nanoparticles. TfR, hence, appears as a potential target, specifically in upregulating cancer cells as well as in stimulating the cellular uptake by clathrin-coated pits. Many research studies have tried to target the TfR to bind the drug and promote its cell entry because of its overexpression in certain tumor cells to enhance the iron uptake. Several Tf conjugated materials have been employed to target cancer cells, like Tf linked anticancer drugs, RNases, antibodies, peptides and even toxic proteins (Kawamoto et al., 2011; Daniels et al., 2012). Kawamoto et al. discovered the usefulness of Tf-lytic mixed peptides in selectively targeting the cancer cells. The in vivo studies using MDA-MB-231 cell bearing athymic mice model, revealed that the intravenously administered peptides were detrimental to the cancerous cells and negligibly to the healthy cells. It was also demonstrated that the Tf-lytic peptides could break the plasma membranes of T47D cancerous cells within 10 mins without hampering the normal healthy body cells (Kawamoto et al., 2011). Bellocq et al., research showed that a minor modification on transferrin could impart more stability to the fabricated nanomaterials in the physiological normal body salt concentration and transfect the leukemia tissues with improved efficacy. The Tf functionalized NPs are useful for the systemic delivery of therapeutics derived from nucleic acid in treating metastasis (Bellocq et al., 2003; Dass et al., 2006).

Luteinizing Hormone-Releasing Hormone Receptor

The cancer cells of the breast, ovarian, and prostate membrane are having luteinizing hormone-releasing hormone receptor (LHRH) receptors. Luteinizing hormone-releasing hormone is hence employed as ligands for targeting these cells (Sun et al., 2008; Dharap et al., 2005). Farokhzad et al. reported a new technique for the delivery of the drug in the cytosol of cancerous cells. They have developed nanoparticle in which the drug when reached inside the cell's fluid, easily dissolved and drug were released in a controlled manner. The nanoparticles were "decorated" on the surface for the selective targeting is called aptamers, or small genetic sequences that aim to identify the biomolecules present on the exteriors of cancerous cells. The additional stability imparted from PEG molecules helps the nanoparticles in not being obliterated by macrophages (Sutradhar et al., 2014). Asialoglycoprotein (ASGP): present in hepatoma, ASGP is a receptor example that could be targeted by nanoparticles to deliver chemotherapeutic agents. Sung and co-workers prepared paclitaxel loaded hepatoma cells targeting biodegradable nanoparticles, of almost 140 nm size, from a copolymeric block of poly (-γ-**g**lutamic acid)-poly (lactide) with the technique of emulsion solvent evaporation. Through amide linkage, NPs were conjugated with galactosamine (GAL) which binds to the ASGP receptors and increases the formulation's uptake in HepG2 cells. A dual-particle tumor-targeting complex was used in the selective inhibition of hepatoma angiogenesis.

The first particle was ganciclovir linked to galactosamine which was loaded in a nanoparticle system. The second component of the system was HSV thymidine kinase gene loaded nanoparticle. Following the cell internalization of both the nanoparticles, thymidine kinase would degrade the ganciclovir which would result in cytotoxicity of cancer cells (Praetorius et al., 2007).

Antibody-Mediated Targeting

Strength and intensification of immune systems can be enhanced by using specific monoclonal antibodies (mAbs) for targeting tumors. The surface proteins of neoplastic cells are generally targeted by these antibodies. When the nanoparticles are linked to a tumor-specific antibody, selective drug/gene delivery could be accomplished (Sutradhar et al., 2013). Single hybridoma cell clones are used to synthesize mAbs. The hybridoma cell is produced by mixing myeloma cells with normal plasma cells that are stimulated by antigens. The function of the myeloma cells is to produce antibodies while the normal plasma cells can specifically bind tumor antigens. This generated mAbs could eradicate the tumor cells by various mechanisms like blocking the growth factor receptors, apoptosis, and anti-idiotype development. In addition to these, the indirect mechanism is available to kill the cancer cells by stimulating complement-mediated cytotoxicity as well as antibody-dependent cytotoxicity (Praetorius et al., 2007). A fragment or the whole antibodies in its natural form could be employed for targeting cancer cells. Hollow protein NPs were fabricated Kuroda and fellows against hepatic cancer. The hollow protein NPs could be used to encapsulate the cytotoxic agents that contain the cancer-treating gene (Sudarshanet al., 2005; Micheau et al., 1997).

Tissue-specific Receptor

An example of the tissue-specific receptor is the Prostate-specific membrane antigen (PSMA). Nanomedicine BIND-014 modified with PSMA targeting ligand is now under clinical trials. For the treatment of solid tumor, docetaxel (DTXL) encapsulated in a polymeric nanoparticle were also a modification of PSMA substrate analog inhibitor, S, S-2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid (ACUPA) that targets the prostate cancer upregulated PSMA. A good result was revealed in the Phase-I trials for patients possessing advanced tumor cells (Sanna et al., 2014). It was noticeable that the patients who did not respond to alternate therapies showed a marked tumor recession (Hrkach et al., 2012). Another prostate as well as non-small cell lung cancer targeting formulation, BIND-014 is presently undergoing Phase- II trials. The use of specific targets for drug and gene delivery has garnered a lot of research attention. For instance, the presence of a plethora of mAbs has fostered research interest in developing nanoparticles that are modified with these antibodies (Johnston et al., 2012; Steichen et al., 2012).

COMBINATION THERAPY

In 1965, the concept of combination therapy was introduced by Emil Frei and group, used in the treatment of acute leukemia (Frei et al., 1965). Combination therapy of four drugs (methotrexate, vincristine, 6-mercaptopurine, and prednisone) was used for the pediatric patients and results were found to be fruitful, even the approach reduced the tumor with prolonged remission (Frei et al., 1965; Mokhtari et al.,

2017). It has been expected that two or more therapeutically active agents could synergistically target various genes, cellular pathways, or cellular checkpoints and improve cancer therapy to eliminate the carcinogenic cells (Figure-1). As per oncologists, an ideal approach for cancer research uses a low dose of combinatorial drugs with reduced cytotoxic effects and improved efficacy (Kamaly et al., 2012). As compared to individual therapies, the survival of the patient was found to be more in the case of combination therapy. For instance, the combination of Navelbine (vinorelbine) and Platinol (cisplatin) is used for the management of non-small cell lung carcinoma. Also, the combination of Taxol, Carboplatin, and Herceptin (TCH) is used for the effective management of HER2/neu-progressive tumors (Douillard et al., 2006; Robert et al., 2006). Challenge with combination therapy includes that the different drug has dissimilar pharmacokinetic and biodistribution, and different metabolism rate inside the body. So, the ratio of the drug (two or more) must be optimized to overcome the challenges, otherwise, the main objective of the combination therapy would fail (Valencia et al., 2013). Liposomal NP CPX-351 was used to develop the combination therapy of anticancer drug cytarabine (CTB) and daunorubicin (DNB), in a ratio of 5:1, for effective management of acute myeloid leukemia. Clinically it has used but the efficiency was inadequate, showed poor solubility and incompatible pharmacokinetic profile and thus required co-administration with the toxic solvents (Ma et al., 2013). Moreover, CPX-351 during the clinical trial (Phase I & II) improved the complete existence in the first-degeneration patients and prevails in phase III of a clinical trial (Feldman et al., 2011). CPX-1, the combination of drugs (irinotecan/floxuridine) and paclitaxel/tanespimycinis under pre-clinical and clinical exploration (Ma et al., 2013; Batist et al., 2009; Katragadda et al., 2013).

Restrictive combinations (RC) is a new approach for cancer therapy, it is an emerging but yet not experimented on human patients. A planed dosing and administration of the drug to skip the normal cells and targeted the cancer cells with a cytotoxic effect. Combination regimens recognize the minor difference between normal cells and carcinogenic cells, in normal cells P53 is absent (Blagosklonny., 2008). For instance, a p53-tempting agent, like a lower dose of DOX, caused cell cycle arrest at the G1/G2 phase in the non-targeted cells (Blagosklonny., 2008; Frei et al., 1965).

NANOMEDICINES APPROVED FOR CLINICAL PRACTICE

Currently, several nanomedicines based anticancer agents are in the clinical trial and has not been accepted by the FDA or other governing bodies for commercialization. **Table-1** enlists the nanomedicines based pharmaceutical formulations that are currently being available in the market while, **Table-2** highlighted the ongoing clinical trials on nanomedicine based formulations, respectively. FDA accepted several nanomedicines such as Doxil, Abraxane, Thermo Dox, Rexin-G based pharmaceutical formulation for marketing. These gain more popularity because of its unique feature, superior biocompatibility and lower or no cytotoxicity. In recent time, FDA also considered some metal-based nanoformulation like Aurimune (based upon colloidal gold) and AuroLase (based upon gold-coated silica NPs), are in different phases of preclinical/clinical trials (Chang., 2007; Stern et al., 2008; Wang et al., 2013).

Table 1. Glimpse of Nanomedicine based pharmaceutical formulations approved by FDA

Generic name	Drug delivery carrier system	Active pharmaceutical ingredients	Therapeutic Use	Company
Liposomal doxorubicin (Doxil)	PEGylated liposome	Doxorubicin	HIV-related Kaposi sarcoma, ovarian cancer, and multiple myeloma	Ortho Biotech (acquired by JNJ)
Liposomal daunorubicin (DaunoXome)	Liposome	Daunorubicin	HIV-related Kaposi sarcoma	Galen
Liposomal vincristine (Marqibo)	Liposome	Vincristine sulfate	Acute lymphoblastic leukaemia	Onco TCS
Liposomal irinotecan (Onivyde or MM-398)	Pegylated liposome	Irinotecan	Post-gemcitabine metastatic pancreatic cancer	Merrimack
Nab-paclitaxel (Abraxane)	Albumin NP	Paclitaxel	Breast, lung and pancreatic	Celgene
Oncaspar®	PEG-L-asparaginase	Asparagine	Acute lymphoblastic leukemia	Enzon pharmaceuticals

Table 2. Glimpse of Nanomedicine based pharmaceutical formulations under clinical development

Nanoparticle	Drug delivery carrier system	Targeting ligand	Therapeutic indication	Developing Organization	Clinical status	Govt Identifier
CALAA-01	TfR-targeting Polymeric NP	Transferrin	Solid tumors	Calando Pharmaceuticals	Phase I	NCT00689065
CRLX101	Cyclodextrin NPs/ Camptothecin	Passive	Non-small cell lung cancer/rectal cancer/ renal cell carcinoma	Cerulean Pharma	Phase II	NCT01380769 NCT00333502 NCT02010567
ALN-TTR02	Patisiran	Passive	Transthyretin amyloidosis	Alnylam Pharmaceuticals	Phase III Phase I Phase II	NCT01960348 NCT01559077 NCT01617967
CPX-351	Liposomal cytarabine and daunorubicin	Passive	Acute myeloid leukemia	Celator Pharmaceutical	Phase III	NCT00822094
MBP-426	Oxaliplatin	Transferrin	Gastroesophageal adenocarcinoma	MebiopharmCo.,Ltd.	Phase II	NCT00964080
SGT53-01	Transferrin targeted liposome with p53 gene	Antibody fragment	Solid tumors	SynerGeneTherapeutics	Phase I	NCT00470613
TKM-PLK1/ TKM-080301	Small interfering RNA	Passive	Hepatocellular Carcinoma, Neuroendocrine Tumors, Adrenocortical Carcinoma, Solid tumors	Arbutus Biopharma Corporation	Phase I/II	NCT01262235
BIND-014	PLGA/PLA NPs/ Docetaxel	Small molecule	Metastatic Cancer, Prostate cancer, Solid tumors	BIND Therapeutics	Phase I	NCT01300533
Atu027	Liposomal small interfering RNA (siRNA)	Protein kinase N3	Carcinoma, Pancreatic Ductal, Solid tumors	Silence Therapeutics GmbH	Phase I/II	NCT01808638

continues on following page

Table 2. Continued

Nanoparticle	Drug delivery carrier system	Targeting ligand	Therapeutic indication	Developing Organization	Clinical status	Govt Identifier
Cyclosert	Cyclodextrin nanoparticles (Cyclodextrin NP/ SiRNA)	small molecule	Solid tumors	Insert Therapeutics (now Calando Pharmaceuticals)	Phase I	
S-CKD602	PEGylated liposomal CKD602 (topoisomerase inhibitor)			Alza Corporation	Phase I/II	NCT00177281
CPX-1	Liposomal irinotecan		Colorectal cancer	Celator Pharmaceuticals	Phase II	
LE-SN38	Liposomal SN38		Colorectal cancer	Neopharm	Phase II	
INGN-401	Liposomal/FUS1		Lung cancer	Introgen	Phase I	
NC-6004	Polymeric nanoparticle (PEG-polyaspartate) formulation of cisplatin		Pancreatic cancer	Orient Europharma Co., Ltd.	Phase III	NCT02043288
NK-105	Polymeric nanoparticle (PEG-polyaspartate) formulation of paclitaxel		Various cancers	Nippon Kayaku Co. Ltd.	Phase II	
NK-911	Polymeric nanoparticle (PEG-polyaspartate) formulation of doxorubicin		Various cancers	Nippon Kayaku Co. Ltd.	Phase I	
NK-012	Polymeric micelle of SN-38		Various cancers	Nippon Kayaku Co. Ltd.	Phase II	
SP1049C	Glycoprotein of doxorubicin		Various cancers	SupratekPharma Inc.	Phase II	
SPI-077	PEGylated liposomal cisplatin		Head/neck and lung cancer	Alza Corporation	Phase II	
ALN-VSP	Lipid nanoparticle formulation of siRNA		Liver cancer	Alnylam Pharmaceuticals	Phase I	
OSI-7904L	Liposomal thymidylate synthase inhibitor		Various cancers	OSI Pharmaceuticals	Phase II	
OSI-211	Liposomal lurtotecan		Various cancers	OSI Pharmaceuticals	Phase II	
Combidex	Iron oxide		Tumor imaging	Advanced Magnetics	Phase III	
Aurimune	Colloidal gold/TNF		Solid tumors	CytImmune Sciences	Phase II	
ADI-PEG20	PEG-arginine deiminase		Hepatocellular carcinoma	Polaris	Phase I	
PEG-IFNα2b	PEG-Intron		Melanoma, multiple myeloid, and renal-cell carcinoma	Merck	Phase I/II	
PEG-IFNα2a	PEG-asys		Melanoma, chromic myeloid leukemia, and renal-cell carcinoma	Genentech	Phase I/II	
PEG-PGA and DON	PEG-glutaminase combined with glutamine antimetabolite 6-diazo-5-oxo-l-norleucine (DON)		Various cancers	EvaluatePharma	Phase I/II	
SGT53-01	Transferrin targeted liposome with p53 gene	Anti-transferrin receptor single-chain Ab fragment (TfRscFv)	Solid tumors		Phase I	

NANOMEDICINES OF FUTURE

CytImmune, a nano therapy platform, performed research on nanomedicines for the development of the anticancer drug. Globally, it has been popularized for nanomedicine establishments. Started with a diagnostic company, now it covers the clinical development, focuses on the detection, progress, and commercialization of targeted approaches for tumor and cancer therapy. In their findings, they have completed the phase I clinical trial for CYT-6091, employed colloidal gold NPs for delivering drugs straight into the cancer tumors. Tumor's blood vessels are leaky so the chances of NPs to come out are maximum, so to prevent this, researchers need to design the nanoparticulate systems in such a way that it could stay there and maximum therapy would be attained (Jain et al., 2012). NPs are attached with a tumor-killing agent, the tumor necrosis factor-alpha (TNF- α) and Thiol-functionalized PEG (PEG-THIOL). The PEG-THIOL protects the TNF-α and freely circulates in the bloodstream. Furthermore, the NPs safely carry the drug to cancer cells through the bloodstream and allow them to target specific sites (Pillai., 2014; Sebastian., 2017).

CONCLUSION AND FUTURE PERSPECTIVES

Combinatorial DDSs or nanomedicines have played a significant role as an emerging and influential approach; however, it is still in its preliminary stages and its accurate perspective needs to revealed yet. The conventional approaches are found too time-consuming, unidimensional, labor exhaustive and economically incompetent. On the other hand, combinatorial approaches have offered incredible potential as a multipurpose and customizable system where the features of the delivery systems could be personalized according to the distribution of small molecules, nucleic acids, protein or peptide-based therapeutics in chemotherapy. Also, NPs have presented important applications for developing combinatorial DDSs with improved solubility, enhanced cellular uptake, and targetability at the target sites. The receptors-based targeting approaches have also gained lots of importance for establishing apt DDSs as a potent tool in chemotherapy. The selection of a modest yet sophisticated synthetic approach has allowed negligible post-synthetic development of products, thus making the approach amenable to high-throughput screening for rapid output. Apart from this, the US-FDA has implemented strict guidelines that need to execute before any DDS passages from laboratory to clinical translations, with safety as a vital parameter. Hence, substantial work is required for establishing this potential area of research, however, the preliminary success has indicated a promising future. Also, in the future, *in vivo-in silico* simulation studies would help for optimizing materials and processes for establishing DDSs with no or minimal toxicity and high therapeutic efficacy towards the management of cancer.

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Chapter 12
Targeted Drug Delivery in Cancer Treatment

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ABSTRACT

In a conventional oral or intravascular drug delivery approach, therapeutic factors are distributed throughout the body and only a limited part of the drug reaches to tumor site. Packaging of cytotoxic agents in drug delivery systems like nanoparticles could enhance its delivery to specific targets in the tumors and could be potential candidate for therapeutics advancement. Targeted drug delivery holds the potential to overcome the present therapeutics of cancer by selective delivery of an arbitrary amount of drug at the tumor site. Loading of cytotoxic agents in drug delivery systems could enhance its delivery to specific targets based on strategy to reach the tumor site. This chapter explores the detailed of innovative methods of drug delivery, challenges of targeted drug delivery, and their implications.

INTRODUCTION

Cancer is the second most leading cause of death in the world, estimated 9.6 million deaths in 2018 (World Health Organization). The treatment of cancer is based on surgery, radiotherapy, hormone therapy, and mainly chemotherapy. The radiation therapy induces DNA damage and kills cells within the localized tumor microenvironment. (Baudino et al. 2015). Chemotherapy is widely used treatment for cancer patients, produce systemic toxicity to not only growing and dividing cancer cells but also to proliferating normal cells. Success rate of present therapeutics remains low due to limited accessibility

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of therapeutic drugs at tumor site, non-target killing of normal cells, undesirable side effects, intolerable toxicity, development of multi-drug resistance and the dynamic heterogeneity of the growing tumors (Vasir and Labhasetwar, 2005; Bahrami et al., 2017).

Targeted drug delivery holds potential to overcome the demerits of present therapeutics of cancer by accumulating the arbitrary amount of drug at particular tumor site translating the Paul Ehrlich's "magic bullet" concept in cancer therapy (Strebhardt and Ullrich, 2008). The magic bullet strategy of drug delivery in cancer seeks attraction in the preferential killing of cancer cells without any toxicity to normal healthy cells. This could be achieved by coupling cytotoxic agents with targeting ligands or entrapment of the drug into ligand-directed delivery systems to enhance its accumulation at designated target (Bahrami et al., 2017; Senapati et al., 2018). Furthermore, direct conjugation of drugs to the targeting ligand could directly impact the ligand and receptor interaction and potentially able to alter the characteristics of the drug. A receptor-directed drug delivery system is an emerging strategy in development of cancer therapy (Yu et al., 2010). The therapeutic efficacy of present drugs could be improved through this approach by specifically killing of the cancer cells by direct toxic action of drug or indirectly due to bystander effect of the therapy. This chapter explores the detailed of innovative methods of drug delivery, challenges of targeted drug delivery and their implications.

STRATEGY OF DRUG DELIVERY

The loading of cytotoxic agents in drug delivery systems increase its penetrance to cellular barriers, prevent the leakage to normal healthy cells, enhance controlled drug release and reduce side effect. Moreover, delivery of drugs through carriers overcome the multidrug resistance (MDR) caused by P-glycoprotein, drug efflux transporters frequently overexpressed in tumor cells (Piddock 2006; Yu et al., 2010). The delivery of drug carriers to specific targets is based on different strategy to reach the tumor site: Passive targeting or active targeting.

Passive Targeting

Passive targeting of drugs carriers could be done due to its specific physiological conditions of tumors including hypoxia, abnormal vasculature, temperature, pH, and surface charge of tumor cells. The increased penetrance of drug carriers in tumors is favored by enhanced permeability and retention (EPR) effect, which is firstly reported in 1986 (Matsumura and Maeda, 1986). The unchecked growth of tumor requires continuous oxygen and nutrient supply that is fueled by continued angiogenesis resulting poor vascularization (Hanahan and Weinberg, 2000). The chaotic and poorly formed new blood vessels often got leaky and allow increased mass transport of macromolecules, such as drug carriers, into the tumor (Maeda et al., 2000). This is coupled with impaired lymphatic drainage that enhanced the accumulation and retention of drug carriers in tumor cells (Leu et al., 2000).

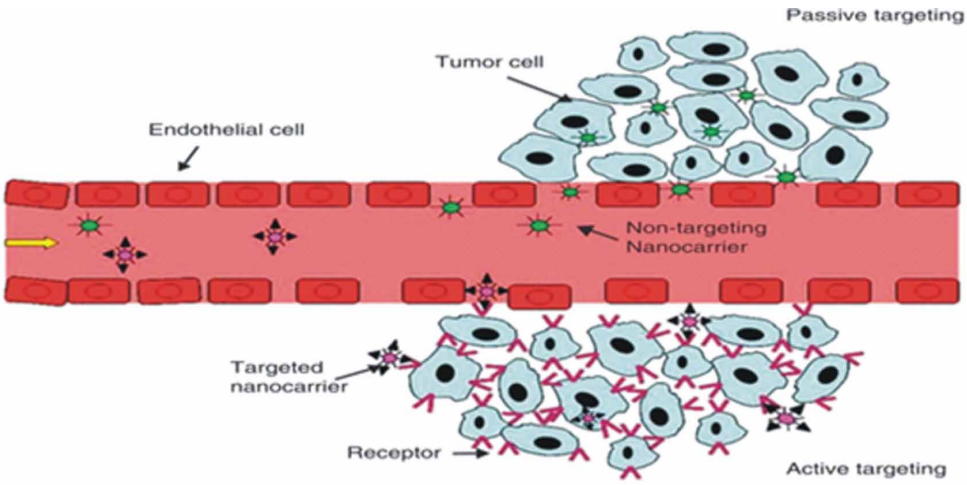
Although passive targeting is interesting approach, however, it suffers from the serious limitations such as limited drug diffusion into tumor cells, the random nature of targeting, and the lack of EPR effect in some tumors.

Active Targeting

This drawback could be combat by conjugation of particular tumor specific ligands with drug carriers that targets to specific receptor/ligand binding proteins and purports delivery to a specific target on tumor cells referred as 'active targeting'. (Leonor Pinzon-Daza et al; 2013)

There are several targeting moieties such as monoclonal antibodies and their variable fragments, peptides, vitamins, carbohydrates and aptamers specifically bind to receptors over expressed in tumors. Among the various targeting ligands, folate, transferrin, epidermal growth factor receptors (EGFRs), and glycoproteins are the most investigated for active targeting (Dixit et al., 2015; Vinothini et al., 2019). The receptors act as specific biomarkers, often, overexpressed on tumor cells but less or no expression on normal cells; produce high specificity binding to ligands of drug loaded carriers. The cytotoxic activity of drugs is promoted by ligand-receptor biding of drug carriers followed by internalization in tumor cells through receptor mediated endocytosis. (Bahrami et al., 2017) (Figure 1).

Figure 1. A schematic diagram representing the accumulation of nanocarriers in tumor sites by passive or active targeting



Both targeted nanocarriers and non-targeted nanocarriers reach tumors selectively through the leaky vasculature in the tumors. Upon arrival at tumor sites, targeted nanocarriers can bind to the target tumor cells or enter the cells via receptor mediated endocytosis. (Courtesy: Yu et al., 2010).

Combining passive and active targeting in a single platform could further improve the efficiency of therapeutic index of carrier loaded drugs. Drugs can be encapsulated in a vesicle, entrapped in a matrix, or solubilized within a hydrophilic or a hydrophobic component of nanoparticles (Bonacucina et al. 2009, Mishra et al. 2010). In addition to tumor cell targeting, the site-specific active targeting strategy also includes vascular targeting and nuclear targeting.

Vascular Targeting

The vascular endothelium exhibits many targets including the endothelial cells, and specific stromal components; specific for cancer therapy. In addition to tumor targeting, vascular targeting is evolving as a promising alternative in destruction and killing of tumor cells by shutting down the supply of oxygen and nutrients by targeting endothelial cells of the tumor vessels (Decuzzi et al., 2010).

Nuclear Targeting

The anticancer drugs are toxins that target nuclear DNA to cause its damage or topoisomerase inhibition to induce cell death (apoptosis). However, Cancer cells limit the drug entry to their nuclei via the cell-membrane associated multidrug resistance and various intracellular drug resistance mechanisms.

CHARACTERISTICS OF DRUG CARRIERS

Drug Carriers

Drug encapsulation in a carrier offers several advantages as compared to bare drugs such as protection from degradation, increased circulation half-life of drugs in body and enhance their accumulation in tumors site which is in part related to the physiological properties of nanoparticles, deregulated tumor vasculature and enhanced permeability and retention (EPR) effects. Certain characteristics including type of material, size, surface charge, molecular weight, and hydrophobic or hydrophilic feature influence the targeted delivery of drug loaded carriers at particular tumor site. (Lee et al., 2016; Alavi and Hamidi, 2019). Nano-sized drug carriers (10 ~ 400 nm) seeks potential attention as they are capable to carrying large amount of drugs, designed to have prolonged circulation time (especially when surface PEGylated), and facilitating selective tumor accumulation through the EPR effect (Reddy et al., 2010).

Properties of Good Drug Carrier

These drug carriers should be stable during the route of delivery. It should have ability to maximize the accumulation of cytotoxic drug to tumor site through enhanced permeability and retention (EPR) effect. Drug carrier should be able to solubilize and carry hydrophobic drugs and remained stable during transportation and reached to tumor site in an intact form. A carrier should also provide a stable barrier to bar entry of drug to normal healthy cells. The barrier protect drug from defense mechanism of body, increase its circulation time and facilitate its transportation to tumor site. The carrier stability prevents leakage of drug; reduce side effect and increase its therapeutic efficacy. The carrier should have ability to increase its circulation time by avoiding renal clearance and escaping the reticuloendothelial system (RES) of the body (Yang and Yu, 2016). The leaky vessels (400 nm) promote accumulation of macromolecules including drug carriers through EPR in tumors; (Tee et al., 2019). Particles smaller than 10 nm are rapidly cleared from kidneys (about 70,000 Da) (Bahrami et al., 2018) whereas large-sized particles are preferentially eliminated from blood circulation through reticulo-endothelial system. There are several reports emphasizing particle size in the range of 100-200 nm that is favored for effective accumulation and retention in the tumor matrix through EPR effect (yang et al., 2016; Liu et al., 2013). The integrity

of vasculature in normal healthy cells prevents permeation of drug loaded carriers and increases its circulation and accumulation at tumor site; moreover, reduce the side effects of drugs. Surface charge of drug carriers is also an important parameter. Neutral and zwitterionic nanoparticles exhibit longer circulation time than positively and negatively charged nanoparticles due to its less susceptibility to RES clearance as compared to later (yang et al., 2016). In addition, RESclearance of nanoparticles that was promoted by opsonisation could also be reduced by surface coating of polyethylene glycol (PEG) and modifications of nanocarriers, resulting in prolonged circulation time (Tee et al., 2019).

Drug Carriers and Tumor Microenvironment

Drug carrier should have additional strategy to target the cancer microenvironment. Carriers should be sensitive to physiology of the tumor micro environment. The tumor microenvironment constitutes unique organizational structure and metabolic characteristics, such as a tumor cells extracellular matrix (ECM), high redox homeostasis, low pH, and specific enzyme metabolism. The active targeting strategy is based on the conjugation of drug loaded carrier with tumor sensitive linkers/ligands that inhibit drug release outside the tumor microenvironment. Cancer cells are often more acidic (pH of 4.0–6.5) than normal tissues due to the high accumulation of lactic acid through anaerobic glycolytic pathway. Moreover, it creates hypoxic conditions due to angiogenesis and continued vasculature formation. Tumor hypoxia or low oxygen concentration serves as good potential target for anticancer treatment (e.g. reduced pH, elevated reactive oxygen species, glutathione levels, or metabolic activation by overexpressed enzymes) (Chen et al., 2017). The high concentration of metalloproteinases in the tumor has inspired the development of drug carriers with targeting moieties cleavable by matrix metalloproteinase-2 (Zhou, 2019). The drug carrier could be developed in manner to trigger its activity and release in response to acidic pH of tumor (Qiu et al., 2017).

Structure of Drug Carrier

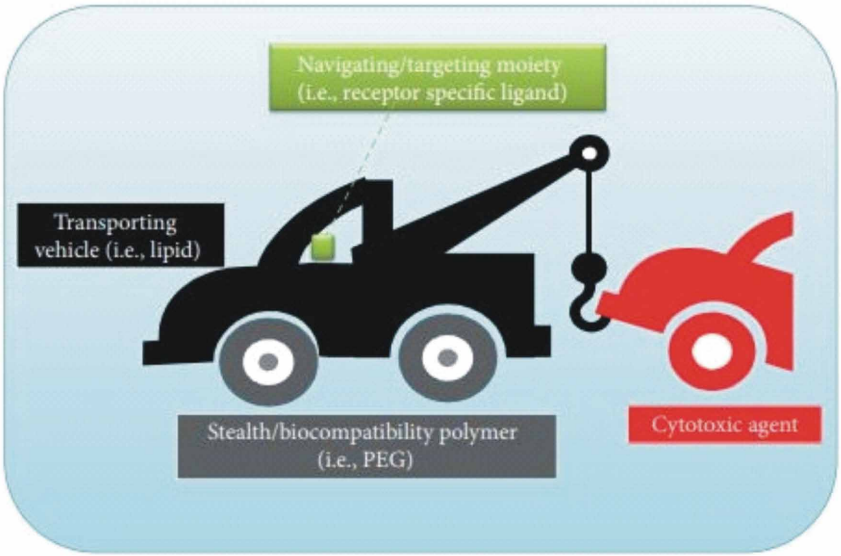
The drug loaded carriers/vehicles should consist of a multidimensional architecture: transporting vehicle (lipid), the cytotoxic agent (drug), the "programmable" navigating/targeting agent (receptor specific ligand) that selectively navigates through appropriate delivery routes to tumor cells avoiding toxicity to healthy proliferating cells and the "stealth" nanocarriers (biocompatibility polymers, i.e., PEG) that enhance the short plasma half-life of the drug-loaded transporting vehicle. The structure of drug carrier includes ABCD paradigm A: active pharmaceutical drug, B: lipids, C: a stealth/biocompatibility polymer layer (like PEG), D: targeting layer-receptor specific ligand, Figure 2 and 3 (Tzakos et al., 2013).

NANOPARTICLE BASED DRUG DELIVERY SYSTEM

Nanoparticles (NPs) are nanosized (1–100 nm) materials holds potential attention for specific tumor targeting, diagnosis (imaging) and therapy. The scaffold structure of nanoparticle provides unique biological characteristics like small size (diameter within 1–100 nm) and large surface area to volume ratio allow efficient binding, absorption and transportation of therapeutic agents, such as drugs, gene or imaging agents. (Farokhzhad and Langer, 2009; Petros and De Simone, 2010).There are several types of nanoparticle based drug-delivery systems including liposomes, inorganic polymers, metal based nanoparticles,

dendrimers, micelles, mesoporous silica, quantum dots, magnetic nanoparticles, layered double hydroxides and carbon nanotubes have been reported for successful targeted cancer treatment(Table 1) Figure 4). Till so far, several nanoparticle-based therapeutics are clinically approved and many are in various stages of clinical or preclinical development. First generation FDA-approved nano-chemotherapeutics; liposome formulation of doxorubicin (DOX) (Doxil® or Caelex®), daunorubicin (DaunoXome®) and albumin-bound paclitaxel (PTX) (Abraxane®) (Fernandes et al., 2018) (Table 2).

Figure 2. Architecture representing navigated drug delivery nanoparticle
(Courtesy: Tzakos et al., 2013).



1. Liposomes

Liposomes are spherical lipid emulsions with bilayers are able to carrier hydrophilic therapeutic factors within the vesicles and hydrophobic agents in lipid bilayer. Conventional liposome is highly biocompatible but showed low stability and rapid clearance from circulation by the reticuloendothelial system (RES) unless they are surface coated by hydrophilic polymers such as polyethylene glycol (PEG) that eventually increased circulation time (Yang and Yu, 2016)

PEGylated liposomes are more effective in passive targeting of cancer cells but can easily modified by coupling ligands that promote active targeting and internalization of liposome-drug conjugates into specific target cells (Hu et al., 2010). Moreover, PEG coated liposomes showed high degree of nuclear transfection. Liposomal antisense oligonucleotides (ASO) have been reported be effective to overcome resistance of multidrug resistant tumors (Pakunlu, et al., 2006). There are few FDA approved liposomal based chemotherapeutics are available in the market DaunoXome®, Myocet®, VincaXome®, DepoCyt®, Doxil®, Caelyx® (Misra et al., 2010; Bahrami et al., 2017) and many are under clinical development. Paclitaxel encapsulated A7RC peptide modified liposomes served as promising antimitotic chemotherapeutic drug for promoting antitumor and antiangiogenic therapies. (senapati et al., 2018).

Figure 3. Navigated drug delivery: the drug delivery nanocarrieris equipped with a "programmable navigation system" that allows the transportation of the "Anticancer" cytotoxic agent in the targeted location. In the absence of the drug delivery vehicle that is tagged with navigating delivery routes, toxicity is triggered on healthy proliferating cells against the anticancer agent, and ineffective therapeutic drug concentration reaches the tumor site.
(Courtesy: Tzakos et al., 2013).

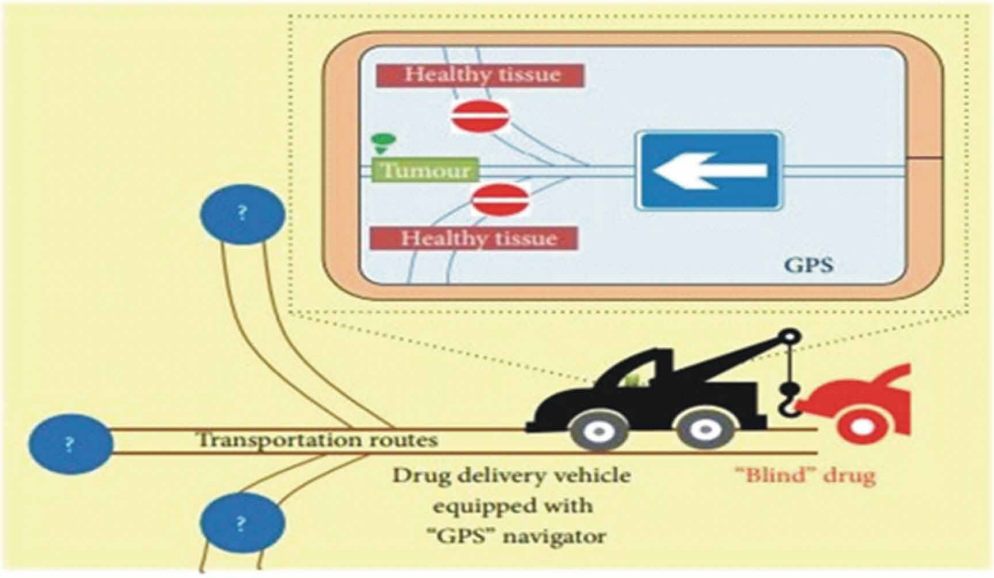


Figure 4. Different types of nanocarriers used as controlled delivery vehicles for cancer treatment
(Courtesy: Senapati et al., 2018).

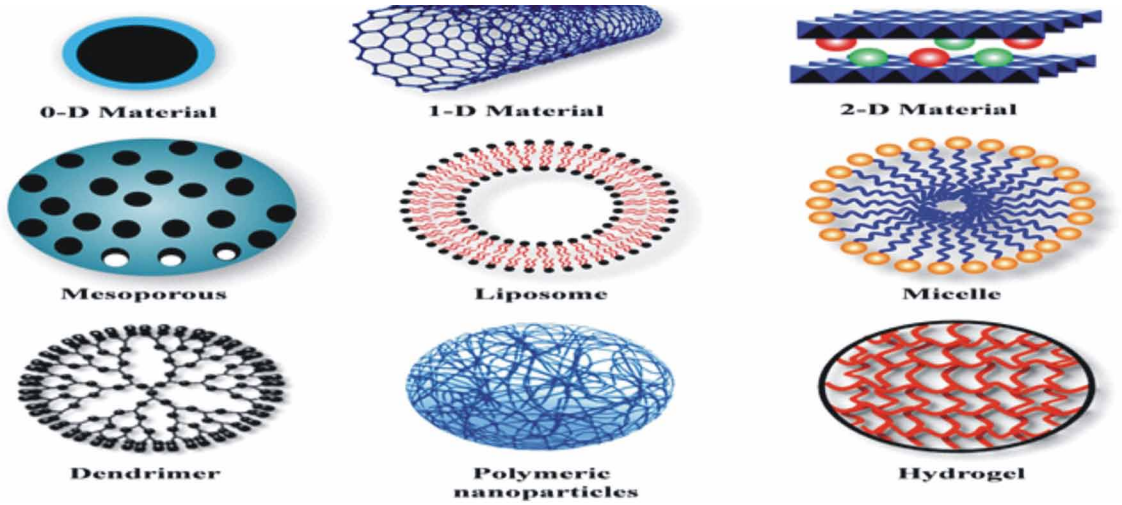


Table 1. Various drug delivery carriers used in cancer therapy (Modified from Senapati et al., 2018)

Material	Description of Carrier	Commercial name	Target	Ref
Carbon nanotubes	Anti-P-glycoprotein antibody functionalized CNT-doxorubicin		Human leukemia cells (K562)	Li et al., 2010
Layered double hydroxide (LDH)	Co-delivery of 5-fluorouracil and siRNAs Raloxifene intercalated into the interlayer gallery of LDH host		Tested on three different cancer cell lines Solid tumor	Li et al., 2014 Senapati et al., 2016
Iron oxide nanoparticles	Phospholipid-PEG coated super-paramagnetic iron oxide nanoparticles	NanoTherm	Solid cancer	Maier-Hauff, K. et al, 2011
Mesoporous silica nanoparticles (MSN)	Azobenzene-modified mesoporous silica for NIR-triggered anticancer drug delivery Endosomal pH-sensitive MSN for doxorubicin delivery		Solid tumor Solid tumor	Liu et al., 2013 Huang, et al., 2013
Polymeric nanoparticles	Cyclodextrin-PEG nanoparticles covalently conjugated with camptothecin PEG-PLGA nanoparticle formulation of docetaxel	CRLX101 BIND-014	Lung and ovarian cancer Various solid malignancies	Weis et al., 2013 Matsumura et al., 2009
Liposomes	Liposomal doxorubicin Liposomal cytarabine Liposomal daunorubicin	Doxil Myocet Daunoxome	Karposi sarcoma, ovarian cancer, multiple myeloma Intrathecal lymphomatous meningitis Karposi's sarcoma	Von Hoff etal., 2016 Barenholz et al., 2012 Mross, K. et al, 2004
Micelle	Polymericmethoxy-PEG-poly(D,L-lactide) micelle formulation of paclitaxel PEG-b-poly (α,β-aspartic acid) nanoparticle formulation of paclitaxel	Genexol-PM NK105	Breast cancer; lung cancer; ovarian cancer Gastric cancer; breast cancer	Lao et al., 2013 Cabral et al., 2014
Protein nanoparticles Human serum albumin-bound paclitaxel nanoparticles	Human serum albumin-bound paclitaxel nanoparticles Folate-conjugated bovine serum albumin- bound paclitaxel nanoparticles	Abraxane	Metastatic breast cancer Human prostate cancer cells (PC3)	Montero et al., 2011
Dendrimers	Carboxylated PAMAM dendrimers covalently conjugated with cisplatin Complexation of doxorubicin with cationic poly-L-lysine dendrimer SPL7013 conjugated with G4 lysine-based dendrimer	Vivagel	Lung cancer cells (NCI-H460) Solid tumor	Nguyen et al., 2013 Menjonge et al., 2010

2. Inorganic Nanoparticles

Gold and iron nanoparticles are some common inorganics that have metallic characteristics that provide unique advantages in therapies. Iron particles due to is ability of visualization by Magnetic Resonance imaging (MRI) have been widely used for imaging purposes in various tumors (Peng et al., 2008; Bahrami et al., 2017). Iron oxide NPs in a range of 1–100 nm are used for therapeutic goals via producing hyperthermia through conduction of external magnetic field into tumor site [(Yu et al., 2008)33]. These nanoparticles are biocompatible and biodegradable; frequently degraded into iron that absorbed

by hemoglobin in body (Sun et al., 2010)34]. Several iron oxide-based NPs are available in market for therapeutic or imaging applications which such as Ferridex I.V.®, Ferumoxytol®, and Combidex® (Kievit and Zhang, 2011). Gold nanoparticles due to its high atomic number used as imaging vectors and tumor-selective photothermal therapy (Blasiak et al., 2013). The surface of gold nanoparticles can be easily modified by amine and thiol groups for tumor specific targeting. Moreover, gold NPs show surface plasmon resonance (Park et al., 2008). Gold nanoparticles-based drugs are under clinical trials and associated with hopeful outcome (Libuti et al., 2009).

Table 2. Nanoparticle based Drug approved by FDA (Courtesy: Anselmo and Mitragotr, 2019)

Particle/Drug	Approved application	Cancer	FDA approval (Year)
VYXEOS CPX-351	Liposomal formulation of cytarabine: daunorubicin	Acute myeloid leukemia	2017
DoxilCaelyx (Janssen)	Liposomal doxorubicin (PEGylated)	Ovarian cancer; HIV-associated Kaposi's sarcoma; Multiple myeloma	1995
DaunoXome (Galen)	Liposomal daunorubicin (non-PEGylated)	HIV-associated Kaposi's sarcoma	1996
Myocet (Teva UK)	Liposomal doxorubicin (non-PEGylated)	Treatment of metastatic breast cancer	2000(EMA)
Abraxane (Celgene)	Albumin-particle bound paclitaxel	Advanced non-small cell lung cancer, Metastatic breast cancer, Metastatic pancreatic cancer	2005
Marqibo (Spectrum)	Liposomal vincristine (non-PEGylated)	Philadelphia chromosome-negative acute lymphoblastic leukemia	2012
MEPACT(Millennium)	Liposomal mifamurtide (non-PEGylated)	Treatment for osteosarcoma	2009 (EMA)
OnivydeMM-398 (Merrimack)	Liposomal irinotecan (PEGylated)	Metastatic pancreatic cancer	2015

3. Polymer Based Nanoparticle

A wide range of materials can be used for the formulation of polymeric nanoparticles including synthetic polymers, e.g., poly (lactic acid) (PLA), poly(ε-caprolactone) (PCL), poly(lactic-co-glycolic acid) (PLGA), N-(2-hydroxypropyl)-methacrylamide copolymer (HPMA) and poly(styrene-maleic anhydride) copolymer and natural polymers, such as gelatin, dextran, guar gum, chitosan, and collagen (Senapati et al., 2018). Drugs can easily be encapsulated either through dispersion in the polymer matrix or conjugation/attachment to polymer molecules for their controlled delivery. Particle sizes of polymers can be controlled in the range of 10 – 100 nm, which facilitates tumor tissue penetration and cellular uptake. (Yang and Yu, 2016). Among others, polymeric materials attain great interest in targeted drug delivery as they are biocompatible and biodegradable. The conjugation of doxorubicin with dextran and subsequently encapsulated in a hydrogel has been reported to improve its therapeutic efficacy in the treatment of solid tumors (Mitra et al., 2001)67. Nanoparticles derived from and polyglutamic acid (PLGA), are an excellent choice for controlled release of drug, as it hydrolyzed into glycolic acid and lactic acid, safe for administration and approved by FDA (Vahed et al., 2018)In vitro anticancer activity of tamoxifen was higher when embedded in PLGA nanoparticles, as compared to pure drug (Pandey et al., 2016).

68 Taxol-loaded PLGA nanoparticles has showed effective chemotherapeutic activity and near-infrared photothermal destruction of cancer cells in vitro and in vivo 69 (senapati et al., 2018).Various PLGA based chemotherapeutic formulations are currently available and FDA-approved for several types of cancer treatments including PLGA microspheres (Lupron Depotand Trelstar), PLGA-based gels (Eligard) and implants (Zoladex®) (Rezvantalab et al., 2018).

4. Dendrimers

Dendrimers are composed of the repeatedly highly branched polymeric star-like molecules with a 3D scaffolding or nanocontainers that can conjugate, complex or encapsulate the therapeutic drugs or imaging moieties, Dendrimers constitutes central core, the branches, and an exterior surface with various surface functional groups (Astruc et al., 2010) [26]. Dendrimers allow encapsulation and solubilization of drugs and its active targeting via tunable surface properties including addition of different functional groups such as COOH, COONa, NH2, or OH in structure of dendrimers (Gorain et al., 2019). Dendrimers are characterized through the addition of monomers (G) to main core; assembled through two main strategies including divergent (outward from the core) and convergent (inward towards the core) (Tomalia et al., 1998) [27]. Dendrimers are the smallest nanocarriers generated in the range of 1.9 nm for G1 and 4.4 nm for G4,display several advantages and can transport large amounts of drug into specific areas and at the same time and can be used for monitoring the progress of the treatment and diagnostic (imaging) (Turrin and Caminade, 2011; Carvalho et al., 2020). Vivagel® is the first dendrimer-based compound approved by the FDA (Menjonge et al., 2010).

5. Albumin Based Nanoparticles

Nanoparticles based on albumin possess several specific advantages including biocompatibility, biodegradable properties, easy preparation, high drug binding capacity along with high half plasma circulating life and ease of covalent modification with targeting ligands. The albumin based nanocarriers can be accumulated in tumor tissue through both passive and active targeting mechanisms (Yu et al., 2010). The functional groups (amino and carboxylic groups) on albumin nanoparticles surfaces facilitate binding of targeting ligands (Elzoghby et al., 2012). Doxorubicin loaded in human serum albumin-based nanoparticles showed better in vitro antitumor efficacy than the pure drug in neuroblastoma cell lines (UKF-NB3 and IMR 32) (Dreis et al., 2007). The paclitaxel-coupled with bovine serum albumin based nanoparticles has also exhibited potential therapeutic effect in human prostate cancer cell line (PC3) (Zhao et al., 2010).Abraxane (paclitaxel-albumin nanoparticle), is the first FDA-approved commercial product accumulate in solid tumors and effectively used in the treatment of metastatic breast cancer (Elzhogby et al., 2016), however, another chemotherapeutics Nab-docetaxel is under clinical trials. (senapati et al., 2018).

6. Hydrogels

Hydrogels are three-dimensional (3D) polymeric and hydrophilic network shaving excellent biocompatibility, biodegradabile properties, lower toxicity (Xu et al., 2014; Rossi et al., 2014). Hydrogel can encapsulate biomacromolecules, including proteins, DNA, both hydrophilic or hydrophobic drugs and produce controlled drug delivery at tumor sites in response to specific environmental stimuli e.g., heat,

pH, light, and ultrasound (Sun et al., 2019)The acidic pH of tumor (pH < 5.5) favor drug release as compared to the normal cell conditions pH ~ 7.0. Cellular uptake of doxorubicin (DOX) released from hydrogels nanoparticles has effectively shown in A549 and HepG2 cells, exhibiting hydrogels to be promising vehicle for anticancer drug delivery. Biodegradable Nanohybrid hydrogel beads made up of carboxymethyl cellulose/ graphene oxide (CMC/GO) physically crosslinked with FeCl3.6H2O has shown controlled release of an anticancer drug (DOX) (Rasoulzadeh and Namazi, 2017).

7. Mesoporous Silica Nanoparticles (MSNs)

Mesoporous silica nanoparticles (MSNs)highlighted as promising candidates for tumor targeted drug delivery due to its highs table nature that make it amenable to various chemical modifications and increase its binding efficiency to almost all types of functional groups, such as metal/metal oxide, targeting ligands, polymers and fluorescent agents. It exerts several advantages as compared to other nanoparticles including large specific surface area and pore volume, tunable pore (2 to 50 nm) and particle size (10 nm to micron range), good compatibility to drugs protect it from degeneration or denaturation. Moreover, it serves as a universal transmembrane carrier for impermeable hydrophobic drugs and also used for intracellular drugs delivery and imaging application. Furthermore, active targeting could be achieved by the functionalizing of MSNs with targeting ligands, such as folate (FA), antibodies, peptides, EGF41 and magnetic nanoparticles. In the targeting mechanism, particle size and surface modification of MSNs critically influence particle cellular uptake, pharmacokinetics, and biodistribution profiles. The shielding of PEG mask the of MSN surface exposed silanol groups, prevent non-specific protein binding, form hydrophilic layer around nanoparticle, minimize opsonisation and enhanced drug delivery efficiency. (Yang et al., 2016; Senapati et al., 2018) methotrexate (MTX) functionalized MSNs drug delivery systems due to folic acid served as both a targeting ligand and a cytotoxic agent (Rosenholm et al.2010). Conjugation of hyaluronic acid (HA) on MSNs are potential in targeting drugs to different cancer cell and proven to be efficient for cancer therapy (Yu et al., 2013; Haung et al., 2018). Aptamer MSNs bioconjugates also showed improved cell targeted drug delivery system and controlled drug release in different cancer cell lines (Li et al., 2012; Yang et al., 2019).

8. Quantum Dots (QDs)

Quantum dots (QDs) is as zero dimensional (0- D) fluorescent nanoparticles (1–10 nm), is emerging promising candidate for both targeted and traceable drug delivery systems, real-time monitoring of intracellular processes and display of molecular imaging because of its unique physicochemical properties, such as highly tunable photoluminescence, resistant to photobleaching, uniform size, large surface-to-volume ratio, multicolor fluorescence imaging and detection (Probst et al., 2013; Yang et al., 2016). A multifunctional platform of synergistic therapy composed of graphene quantum dots (GQDs) as caps and local photothermal generators and magnetic mesoporous silica nanoparticles (MMSN) as drug carriers is reported to improve controlled drug release (Yao et al., 2017). It was reported that shielding of PEG increased accumulation of quantum dots (QDs) up to for two years in the body and offered various advantages in tumor-targeted drug delivery (Elzoghby et al., 2016).

9. Carbon Nanotubes (CNTs)

Carbon nanotubes (CNTs) are synthetic one-dimensional (1D) carbon based tubular nanomaterials, used in diagnosis and treatment of cancer. CNTs are being investigated due its high potential to cross biological barriers and effective transportation of drugs or gene molecules into the cytoplasm of targeted cells and tissues without producing a toxic effect (Madani et al., 2011). The entrapment of chemotherapeutic drug molecules can be performed by its conjugation to functional groups on the CNT surface or polymer coatings of CNTs. CNTs can also act as potential antigen-presenting carriers for antitumor immunotherapy as it can improve weakly immunogenic tumor specific peptides/antigens to trigger a humoral immune response within the tumor. Doxorubicin-Loaded and Folic Acid-Conjugated Carbon Nanotubes Poly (*N*-vinyl pyrrole) showed synergistic effect through targeted chemo–photothermal effect owing to its outstanding efficiency in cancer treatment (Wang et al., 2017).

10. Graphene-based Nanomaterials

Graphene-based nanomaterials, particularly grapheme oxide (GO) and reduced GO (rGO), have arisen as promising candidates for photothermal therapy (PTT)-based cancer treatment due to their multifunctional physicochemical and optical properties including extremely large surface area, strong photothermal effect, tunable active groups, guided imaging and good biocompatibity. (Chaen et al., 2016; Liu et al., 2018). It is advanced drug delivery system act either as modifiable carriers or active agents; increase the specificity of the delivered drug molecules like doxorubicin in prostate cancer (SreeHarsha et al., 2019).The composite of graphene-based nanomaterials with magnetic nanoparticles or gold nanoparticles offers key advantage in the modern biomedicine due to enhanced controlled drug delivery (Alegret et al., 2017; Jafariziad et al., 2017).

11. Layered Double Hydroxides (LDHs)

Layered double hydroxides (LDHs), is two-dimensional (2D) structure also known as anionic nanoclays or hydrotalcite like compounds, have recently got attraction for potential delivery carriers due to excellent biocompatibility, high drug loading efficacy, anion exchange ability, high protection to loaded drugs, pH-sensitive drug or DNA release, easy and cost effective preparation, potential cell membrane penetration, controlled drug delivery, good endosomal escape, biodegradation in the cellular pH (between 4 and 6), moreover, the drug release rate can be tuned by changing the interlayer anion. Anionic drugs and genetic materials like DNA, peptides, proteins, etc. can easily be intercalated in the interlayer thereby conferring protection from enzymatic degradation during circulation in biological fluids (Rives et al., 2014; Senapati et al., 2016). The intercalation of an anticancer drug, raloxifene hydrochloride (RH), into a magnesium aluminum double layered with different interlayer exchangeable anions ($NO3^{-}$, $CO3^{2-}$, and $PO4^{3-}$) has been reported to exhibit controlled release of drug at tumor site (Senapati et al., 2016).

12. Hybrid Protein-inorganic Nanoparticle

Hybrid protein-inorganic nanocarriers offer an advantage for both targeted drug delivery and cancer diagnostics by combining the merits of inorganic material like iron oxide, gadolinium, gold, silica, calcium phosphate NPs, carbon nanotubes, and quantum dots with naturally-occurring protein. Combining

characteristics of protein nanocarriers like prolonged systemic circulation, high drug accumulation at tumor tissues and of Inorganic NPs like high biocompatibility, inert nature and stability; highly increased efficacy of tumor-targeted delivery and longtime accumulation in the body (Elzhoghby et al., 2016; Freag and Elzoghby, 2018). The PEG modification of theragnostic lysozyme proteins protein–inorganic hybrid quantum dots nanoparticles loaded with anti-cancer drugs *i.e.*, paclitaxel (PTX) showed high active targeted tumor accumulation as well as effective tumor inhibition (Xie et al., 2019). The modification Protein-drug conjugate with PEG coated-AuNPs increase its bioavailability and cytotoxicity in target cancer cells. (Kalimuthu et al., 2018)

Hyaluronic acid based nanocarriers can be used for the delivery of various drugs such as nucleic acid, proteins and drugs. It has several advantages such as, increase bioavailability, reduced side effects, controlled drugs release and long-lasting effect in the body. It is also a good candidate to replace PEG, being investigated for new protein or peptide drug carrier. HA and polycation conjugates greatly improve the circulation half-life and bind to specific receptors overexpressed in tumor cells (Huang and Huang, 2018)

The multifunctional targeting drug delivery systems is promising approach for most effective and prevailing drug targeting and increase in therapeutic index of drug. Conjugation to different kinds of nanoparticles quantum dots, single-walled carbon nanotubes and nanographene oxide,(Liu et al., 2007; Cen et al., 2013) to vascular targeting ligand including vascular endothelial growth factor (VEGF) or arginine−glycine−aspartic acid (RGD) peptides could be generally applied to most or all types of tumors enhanced its accumulation in tumor vessels and increased therapeutic efficiency of drugs (Li et al., 2016).

The efficacy of nuclear targeted drugs doxorubicin could be increased by development of nuclear-targeted delivery systems through surface conjugation of different nanoparticles to nuclear localization signal like TAT, cell penetration peptide (CPP) including silver nanoparticles including quantum dots, magnetic nanoparticles and gold nanoparticles. (Ali et al., 2017; Maity and Stepensky, 2017; Hua et al., 2019).

TARGETING LIGANDS FOR NANO CARRIERS

Nanoparticles are usually decorated with different kinds of ligand or target moieties that bind to tumor specific receptors or macromolecules and facilitate release of drugs in a controlled manner. PEG is usually used as a linker to reduce steric interference between nanocarriers and receptors; maximizing its targeting efficiency. Broad range of ligands including vitamin (folate), peptides, proteins (antibody or antibody fragments), glycoprotein (transferrin), nucleic acid (aptamer) are currently being explored for the development of targeted nanocarriers in cancer.

Folate Receptor (FR)

Folic acid (folate or vitamin B9) is an essential requisite for the synthesis of purines and pyrimidine taken up by folate receptors (FRs) in all living cells in a non-destructive, recycling endosomal manner (Murthy, 2007). The high expression of folate receptor (FR) has been reported in different types of tumors such as ovarian, colorectal and breast cancer etc. Makes it potential candidate for targeting of folate ligated nanocarriers like liposomes, polymers with (Vinothini et al., 2019; Joshi et al., 2019).

Transferrin Receptors

The human transferrin receptor1 (TfR1) is over articulated in fast dividing cancer cells as compared to its negligible expression in normal cells, thus, is a lucrative target for drug delivery in cancer treatment (Yuan et al., 2015; Joshi et al., 2019). TfR-mediated delivery can be directed by conjugation of transferrin (Tf), transferrin peptide, antiTfR antibody, antibody fragments or ferritin into different types of nanoparticles. (Dixit et al., 2015 Li et al., 2019; Yang et al., 2019).

Integrin

Integrins are proteins that attach the cell cytoskeleton to the extracellular matrix; it's over expression in different cancer cells increase disease progression through angiogenesis and metastasis (Ramage et al., 2010).There are 24 different integrins composed of heterodimers made up of α and β chains (Rathinam and Alahari, 2010); several of them provide hallmark for targeting nanoparticles in different types of cancer. Integrins αvβ3, αvβ5, and α5β1 are reported to be involved in angiogenesis of cancer whereas, and integrins αvβ6 and α6β4 are overexpressed in tumor cells (Rathinam and Alahari, 2010; Desgrosellier and Cheresh, 2010). The tripeptide Arg-Gly-Asp (RGD) containing peptides e.g. cRGDfX, cRGDeV, and cRGDyV is usually used as targeting moieties for different types of integrins, overexpressed in tumors (Wang et al., 2014; Wu et al., 2017).

Glycosylation Mediated Targeting

Targeting of various nanocarriers ligated with glycan structures could be achieved due to aberrant glycosylation in tumors and the tumor-associated microenvironment (Dalziel, et al. 2014 Bahrami et al., 2017). The glycosylated nanocarriers can be used for targeted delivery of small-molecule drugs, genes, RNAs and vaccines. In addition to lectins, other glycans including mannose, fucose, galactose is used as for glycosylation mediated drug targeting. (Gupta et al., 2009; Cai et al., 2018).

Epidermal Growth Factor Receptor

The epidermal growth factor receptor (EGFR) is a transmembrane receptor regulates several cell processes, including cell growth, proliferation, migration, survival and tissue invasion. It has been demonstrated that Deregulation of EGFR is reported in the majority of human epithelial tumors and serves as appealing drug target in cancer treatments. Different peptides are engineered to target drug loaded nanoparticles to tumor site (Fan et al., 2016; Soudy et al., 2017).

Aptamers

Aptamers are single-stranded DNA or RNA (20-100 nucleotides) folded to form unique three-dimensional (3D) structures can bind, hold and target nucleic acids or proteins in a highly specific manner. It directly or in conjugation to drug nanocarriers specifically bind to receptors on the cell membrane or release drug based on tumor specific physiological conditions (Vahed et al., 2018). Aptamer-conjugated to paclitaxel entrapped graphene oxide nanocarrier, released drug *in vitro* in a pH-responsive manner (Hussien et al., 2018).

APPLICATION OF NANOPARTICLES IN CANCER THERAPY

Gene Therapy

Gene therapy is emerging tool to specific targeting of oligonucleotides that regulate abnormal genetic expressions in tumor cells. The Nanoparticles protect encapsulated gene molecules, nucleotides and other small interfering RNA (siRNA), microRNA (miRNA)that enhance gene transfection, improve cellular uptake and increase silencing efficiency of aberrated gene expression in tumor cells (Lee et al., 2016)

Prodrug Cancer Therapy

Prodrugs are the inactive or less active derivatives of drug molecules which metabolized and regenerate into active drug at a specific site or at specific physiological conditions and causing toxicity to tumors in a selective manner without harming healthy tissue (Padma et al., 2015). Prodrugs hold several advantages over conventional therapeutics including increased solubility, stability and bioavailability, enhanced permeability, prolonged half-lives and reduced side effects. The combined approach of nanoparticles and prodrugs attained attraction due to enhance storage stability, controlled prodrug release and tumor-targeted delivery in cancer treatment (Fang and suwayeh, 2012).A peptide linker is incorporated either into the prodrug structure or prodrug nano carrier as a 'trigger' ligand allowing the diffusion and cleavage of the prodrug into specific physiological conditions of tumor microenvironment including pH, hypoxia, glutathione level etc. (Ling et al., 2018). There are different strategies of prodrug therapy such as gene-directed enzyme prodrug therapy, virus-directed enzyme prodrug therapy (VDEPT), and antibody-directed enzyme prodrug therapy (Padma et al., 2015; Souza, 2019).

Targeted Cancer Therapy

Targeted therapy refers to a new generation of cancer drugs designed to act on specific molecular changes/ molecular targets including genes, proteins, or the tissue environment that have a critical role in tumor growth or progression. The identification of appropriate targets is based on a detailed understanding of the molecular changes underlying cancer (Sawyers, 2004). There are three main types of targeted cancer therapies; 1) small molecule inhibitors, 2) monoclonal antibodies and 3) immunotoxins (Baudino, 2015).

Targeted Immune Therapy

The main aim of immunotherapy is to enable immune system of patient's to specifically target and destroy cancer cells. Several methods are involved in cancer immunotherapy including and monoclonal antibodies cytokines, cancer vaccines.

Antibodies are served as both targeting agent and as therapeutic agent and proven effective due to its high specificity against the wide range of tumor associated antigens. Herceptin® (Trastuzumab) is an unconjugated monoclonal antibody (mab) targeted against tyrosine kinase, Her-2, over expressed in breast cancer cells. Avastin® (bevacizumab) is mab targeted against tumors vascular endothelial growth factor (VEGF) involved in angiogenesis in; given in combination with standard chemotherapy in colorectal cancer (Kabbinavar et al., 2003). Antibodies can also be attached to drugs directly or to drug carriers that increase its efficacy called as immunoconjugates have been widely used in the treatment of cancer.

Gemtuzumab (Mylotarg®) is composed of CD-33 specific monoclonal antibody conjugated with cytotoxic derivative of calicheamicin given effect results in the treatment of acute myeloid leukemia (Baron and Wang, 2018). Whole antibody or its fragments including scFv, Fab, nanobody, bispecific antibody, bifunctional antibody, diabody and minibody are functionalized on surface on nanocarriers for targeted drug delivery that bind to specific epitopes of tumor-specific antigens and tumor-specific antigens (Fay and Scott, 2011; Alibakhshi et al., 2017). Increase in targeting efficiency has been reported on conjugation of Anti-EGFR antibodies derived fragment and nanoparticles directed to epidermal growth factor receptor (EGFR), which is over expressed in lung adenocarcinomas (Aston et al., 2018).

IL-6 are ubiquitously tumor deregulated cytokines proven to be potential target candidate for IL-6-conjugated toxins, CNTO 328 antibody (Siltuximab), BE-8 and mAbs against IL-6 and IL-6R in different types of human cancer including multiple myeloma ovarian cancer and prostate cancer(Guo et al., 2012; Yao et al, 2014).

Cancer vaccine can be developed by aim to induce strong, specific T cell responses. This could be achieved by targeting different antigens like receptor kinases, Toll-like receptors, and C-type lectin receptors expressed on cell surface of dendritic cells that efficiently stimulate T cell responses. It showed promising targets for vaccine design against cancer. IFN-gamma plays crucial role in both functional maturation of dendritic cell and dendritic cell activation of helper T cell in the presence of Tol like receptor (TLR) ligation. IFN-gamma together with Toll-like receptor agonists are novel targets to enhance antigen specific T cell responses and can be used for development of vaccines and drug targets in cancer (Tzakos et al., 2013). Combining the antibody-directed enzyme prodrug therapy immunotherapy, and a nanotechnological approach (or a nano-enabled approach) could provide synergic immune response to combat cancer (Souza, 2019).

Dual-drug Therapy

Dual-drug delivery system is reported an outstanding candidate for the treatment of cancer by combining two chemotherapeutic agents, offer advantages over drug-resistance by cancerous tumors and therefore increase its efficacy to eradicate tumor. Self-assembled nanogels fabricated with poly (ethylene glycol) methyl ether (mPEG) and chitosan are used for construction of combination drugs, paclitaxel and 5-fluorouracil (Tran et al., 2018).

CHALLENGES OF TARGETED DRUG DELIVERY

Targeted drug delivery has greatly increased the bioavaibility of chemotherapeutic agents and offers advantage over convention chemotherapy, however, there are still many challenges need to address for their successful clinical implications. Although, nanocarriers offer many advantages as drug carrier systems; their lack of biodegradation, poor stability in the circulation, lack of controlled drug loading capacity, inadequate tissue distribution raise concerns over there successful long-term administration. The heterogeneity and physiological condition of tumor microenvironment and its clinical implication challenge the current paradigm of targeted drug delivery. More investigations are required to understand the barriers offered by tumor microenvironment in accumulation of drug nanoparticles. Many tumors offer insensitivity to drug targeting due to lack of enhanced permeability and retention effect. The tendency of cancer cells to evolve their behavior over time and its abundant heterogeneity of tumors microenvi-

ronment should also be considered in designing methods of drug delivery that is often correlated with resistance to chemotherapy. The poor vascularization and angiogenesis results in hypoxic condition that create obstacle for diffusion of nanoparticle into oxygen devoid regions. High Interstitial fluid pressure in tumors and its extracellular matrix also prevents the accumulation of nanoparticles through enhanced permeability and retention effect that is highly influenced by characteristics feature of nanoparticle. Most of the drug nanoparticles tested successful in small laboratory models fails in clinical trial. The translation of laboratory studies on human is most challenging job due to differential metabolism and physiology associated with cancer development and its sensitivity to chemotherapy. More clinical data are needed to completely understand the merits and demerits of these nanocarriers.

CONCLUSION

Targeted drug delivery systems ensure drug localization at specific tumor site without harming healthy cells. This holds a promising approach for cancer treatment as compared to conventional chemotherapeutics in future. Understanding the interaction of drug carrier with tumor microenvironment is critically important for drug delivery; some aspect has been focused in chapter. Several nanoparticles-based drugs delivery system has been discussed that are being evolved in recent years for effective cancer therapeutics. The application of these nanoparticles-based drugs in different types of cancer therapy (as discussed in chapter) could provide a new horizon for advanced cancer therapeutics.

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Chapter 13
Advancements in Cancer Therapeutics:
Targeted Drug Delivery in Cancer Treatment

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ABSTRACT

Targeted drug delivery in cancer treatment is a very convenient method for increasing the effectiveness of drugs and reducing their toxic side effects. Nano drug delivery systems have unique physical, chemical, mechanical, and optical properties. Nanoparticles, which have large surface areas and functional groups for the binding of therapeutic agents, benefit the drug distribution with nanoparticle formulations and can provide new features. They also enable personal oncology for diagnosis and treatment, which is appropriate for the personal molecular profile structures of cancer patients. The tumor-targeted active substances are attached to nanoparticles and the active substance loaded nanoparticles are targeted to the tumor area; these nanoparticles can be used with a high tendency to bind and specificity, to target tumor antigens or vessels. This chapter, besides traditional chemotherapy and radiotherapy methods in the field of cancer treatment, is aimed to give information about targeted drug delivery systems for cancer cell targeting without damaging normal tissues.

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INTRODUCTION

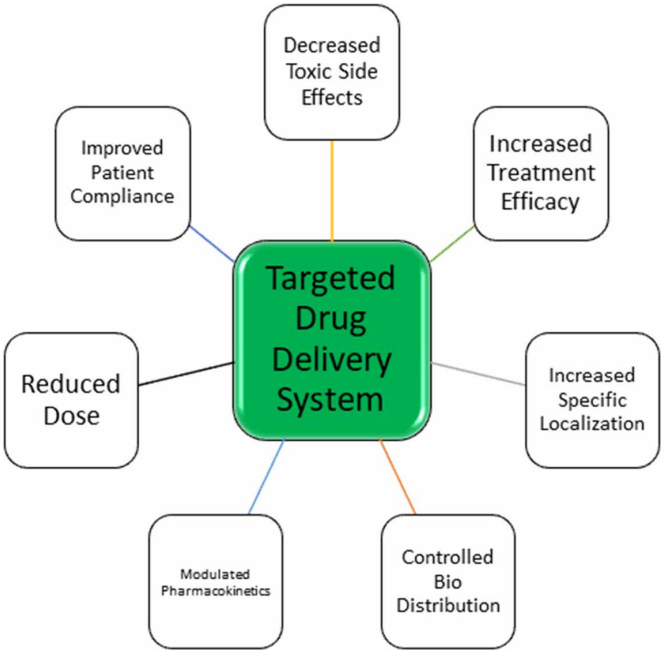
Cancer, one of the deadliest diseases worldwide, is one of the serious health problems of the 21st century that can start and spread anywhere in the body with uncontrolled cell proliferation resulting from a significant decrease in apoptosis in cells. Cancer cells need to get the oxygen, glucose and amino acids necessary for survival (i.e. to divide and multiply), so they successfully compete with normal body cells. Tumors can grow to about 2 mm^3 without forming blood vessels. A significant proportion (more than 85%) of hundreds of cancer types is solid. Considering the location and behavior of tumor cells, it is divided into five main classes. These are carcinoma, sarcoma, lymphoma, leukemia and CNS cancers (Ferlay et al., 2017). This disease can show therapeutic resistance and leads to occur clinical diversity with complexity at the genetic and phenotypic levels (Zhao & Rodriguez, 2013). Various treatment methods are applied for cancer treatment. These are treatment methods such as surgical removal, chemotherapy, radiotherapy and hormone treatment (Jabir et al., 2012). It can be given good result to remove the tumor tissue formed in a certain and limited region by surgical intervention. However, it is not a suitable method for cancer types such as leukemia or cancers spread over more than one region. Chemotherapy, one of the most widely used methods, is the method in which controlled anticancer drugs are administered to the diseased person in order to prevent the spread of cancer cells that proliferate uncontrollably. There are many types of chemo used in this method. The treatment to be applied varies according to the type of cancer. However, in this method, medications and drug doses can cause serious side effects, and blood, mouth, digestive system and hair follicles cells can be damaged. The method called radiotherapy or radiation therapy is a treatment method applied to the tumor area with X-rays. Cancer cells can be prevented from division and proliferation and destroyed with high doses of radiation. This method, which is suitable for use in almost all types of cancer, is mostly used in cancer types such as skin cancer, head and neck cancers, brain tumor, breast, prostate, gynecological cancers. There are various side effects in this method. A large area is used to send a sufficient dose of beam to the cancerous area. This causes damage to healthy tissues. In this method in which radiation therapy is applied, the different radiation resistance of each organ applied can create different side effects. It is one of the treatment methods that can be used in cancer types that occur in hormone producing regions such as ovarian and prostate cancer. This method has various side effects due to hormone treatment (Hassanpour & Dehghani, 2017).

As it is understood from here, the absence of targeting or limited targeting in cancer treatment causes many side effects. The most difficult part for the therapeutics used in cancer treatment is to distinguish between cancerous and normal body cells. Therefore, the main purpose is to enable the drug to recognize cancer cells. Interaction with normal cells is inevitable as targeting to cancer cells cannot be achieved in chemotherapy methods (Mousa & Bharali, 2011). The insufficiency of drugs used in cancer treatment for drug delivery also bring about the development of new treatment methods and techniques. Drug delivery targeting can be classified as active, passive and physically in itself, and can target organs, tissues, organelles and cells (Yu et al., 2016). Targeted drug delivery systems are promising tools for the fewest side effects of diseases and high therapeutic effects. As an alternative to traditional methods, it is aimed to minimize negative side effects for effective drug transport in studies of cancer treatment systems with the nanomaterials having a size between 1-100 nm (Liyanage et al., 2019). The most important differences that distinguish nanomaterials from other materials are the increased surface area and quantum effects. Properties such as electrical properties, in vivo behavior, reactivity are related to properties that distinguish nanomaterials from other materials (BASHYAL, 2018).

TARGETED DRUG DELIVERY

The biological effects of an anticancer drug on the patient are related to the pharmacological properties of the drug and these effects occur as a result of drug and receptor interactions. The effectiveness of drug-target interactions is associated with delivering the drug at the appropriate concentration and rate for low side effects and high therapeutic effect. Chemotherapy treatment using one or more anticancer drugs in cancer treatment can also cause death of healthy cells as well as cancer cells. In traditional drug delivery methods, it can cause irregular pharmacokinetics, due to drugs such as tablets and capsules taken by mouth being affected by the metabolic pathways of the body. This situation can lead to toxic side effects with the application of more doses. In short, the inability to target drugs to cancer cells in traditional methods can adversely affect treatment (Yu et al., 2016). In the methods developed and being developed to prevent such negative situations, it is aimed to reduce toxic side effects and to be least affected healthy tissues with the help of nanoparticles created using biodegradable and biocompatible polymers (Sinha, Kim, Nie, & Shin, 2006).

Figure 1. Advantages of targeted drug delivery system



The transport of molecules used for treatment or marking can be provided with systems called nanocarriers created using nano materials. Different types of nanostructures are responsible for carrying the determined molecules to the diseased area where they are targeted without interacting with healthy areas. Targeted drug delivery systems have significant advantages such as targeting, localizing, protecting, and increasing the interaction of a diseased tissue with a drug molecule. The methods developed before the targeted drug delivery systems have disadvantages such as the very low success rate in reaching the diseased area and the more side effects of the drugs, despite the high doses of the drug. Considering

these disadvantages in the development of new treatment methods, it is necessary to examine in detail the specific features of the drug, its side effects, the route taken to deliver the drug, the target region and the type of disease (Saltzman & Torchilin, 2008; Trafton, 2009). For targeted drug delivery systems to be successful, there are four important requirements: retention, avoiding, targeting and releasing. For the drug to show the desired effect, the drug must be correctly loaded on the drug carrier vehicle. In addition, it is an important requirement for these delivery carriers to escape from the secretions of the body, to remain in the circulation of the body for a long time, and to reach the targeted area and release the drug (Mishra, Pant, Porwal, Jaiswal, & Aquib, 2016).Targeted drug delivery or, in other words, smart drug delivery (Muller & Keck, 2004), is a drug delivery system used in certain parts of the patient's body for the drug to be found more than other parts. This form of delivery delivers the drug to the specified specific area of the body. The drug delivery system concentrates drug density in specific body areas such as targeted tissue or organ and reduces it in other parts of the body. This increases the effectiveness of the drug while minimizing the side effects. Different targeting methods are used to apply the targeting process to the body area such as the tissue and organ determined (Mishra et al., 2016).

The shape, size and surface properties of nano-carriers used in targeted therapeutic applications are also of great importance. Surface charge, along with charge density and polarity, is an important factor affecting the transmission and cellular uptake of nanoparticle. It has been shown in studies that charged particles show more cytotoxic effects than unloaded ones (Maiti, 2012; Rahul, 2015). It is also known that positively charged among the charged nanoparticles are more cytotoxic than negatively charged. Charge density is a factor affecting the cellular uptake of the nanoparticle. Cellular uptake involves the electrostatic interaction of the membrane and positively charged nanoparticle with each other, which support the adhesion of the nanoparticle onto the cell surface. Surface chemistry of nanoparticles is also an important factor for long circulation time in the body (Rahul, 2015). Circulation time, which is one of the important features of targeted delivery systems, is affected by the size and density of the nanoparticles used.

Targeting process is carried out according to the targeted region. Active targeting, which is one of the targeting methods, is affected by ligand density, nanoparticle structure, and surface and ligand load. The nanoparticles used in the processes are targeted using important methods such as physical and chemical factors such as heat, pH, proteases and magnetic field, or cell-specific binding and targeting. Specific nanocarrier systems can release drugs at certain pH values, while the pH specification allows drugs to be distributed directly in a tumor area. Tumor cells, which are more acidic than normal cells, have a pH of about 6.8. By using this feature, the drug can be released in acidic tumor environments by using nanocarriers that release drugs at certain pH values (W.-Y. Qian et al., 2012). Highly acidic environments provide the drug to be released by causing the nanocarriers to disintegrate.

Low pH environments ensure that a large proportion of drugs are released simultaneously, so drugs with anti-cancer properties begin to kill tumors quickly. This rapid process affects the tumor becoming drug resistant and the time it takes to mutate. In addition to the pH effect, the temperature also has an effect on the delivery of drugs by some nanocarriers. Tumor temperature is warmer than other parts of the body and is known to be around 40 ° C (Rezaei, Nabid, Niknejad, & Entezami, 2012).

Strategies of Drug Targeting

Targeting for a specific area of the body is of great importance for increasing the therapeutic effect and reducing the toxic effect that may occur. Targeting process is generally carried out with different strategic methods.

Active Targeting

Active targeting gives information about drug targeting interactions by considering ligand and receptor interactions. In order to interact between a ligand and receptor, the distance between them must be less than 0.5 mm (Bae & Park, 2011; Mishra et al., 2016). Binding of drug carrier nanoparticle systems used in active targeting to ligands supports the uptake of drug molecules with the help of receptor targeted ligand molecules (Abd El-Karim, El-Zahar, & Anwar, 2015). The most important factors affecting active targeting are the density and charge of the ligands used in anticancer studies, the size and shape of the nanoparticles having the carrier-role and the surface charge. It has been aimed to use ligand-receptor interaction or antibody-antigen recognition pathways to target the nanoparticles used in studies with the active targeting method to the cancerous region (Malam, Loizidou, & Seifalian, 2009; Praetorius & Mandal, 2007; Sutradhar & Amin, 2014). Cancer cells being less than relatively healthy cells may cause nanoparticles to miss the targeted cell. Various and many ligands can be used to eliminate such problems (Yu et al., 2016). The active targeting method can be used to direct nanoparticle systems to cell surface receptors and antigens. Active targeting is divided into three groups: receptor targeting, antibody-mediated targeting and anti-angiogenesis.

Receptor Targeting

EGFR (Epidermal growth factor receptor), Folate receptor, Tf (Transferrin) receptor and asialoglycoprotein receptors are important in receptor targeting.

EGFR (Epidermal growth factor receptor): The EGF family is known as receptor tyrosine kinase (RTK) class 1. EGF (epidermal growth factor) is an important protein that stimulates cell growth and differentiation by binding to its receptor, EGFR (Carpenter & Cohen, 1990). EGFR is a transmembrane protein that acts as a receptor for the members of the EGF family of extracellular protein ligands (Herbst, 2004). Overexpression of EGFR known as upregulation and amplification as a result of mutations, is associated with different types of cancer, such as lung adenocarcinoma, anal cancers, glioblastoma and head and neck epithelial tumors. EGFR mutations cause uncontrolled cell proliferation. EGFR and its associated mutations and amplifications can be seen in epithelial cancers. For these reasons, it is aimed to develop various therapeutic approaches intended for EGFR. Gefitinib (Paez et al., 2004), erlotinib, afatinib, brigatinib and iconitib (Liang et al., 2014) for lung cancer, and cetuximab for colon cancer are anticancer therapeutics intended for EGFR. Many therapeutic studies developed for EGFR are examples of monoclonal antibody inhibitors such as cetuximab and panitumumab. Monoclonal antibodies block the activation of tyrosine kinase by blocking the extracellular ligand binding site. In another method, when molecular kinase inhibitors such as gefitinib, erlotinib, brigatinib and lapatinib are used, EGFR cannot activate itself, which is necessary for the binding of downstream adaptor proteins. In connection with this, tumor proliferation and migration are reduced. It has been reported that studies to target EGFR on mice in 2014 were promising (Stuckey, Hingtgen, Karakas, Rich, & Shah, 2015).

Folate receptor: The task of folate receptors is to bind folate and reduced folic acid derivatives and assist in the introduction of tetrahydrofolate into the cell (Wibowo et al., 2013). The folate receptor is over-expressed in many tumors. Especially folate receptor-alpha is over-expressed in 40% of human cancers. Folate receptor-Beta is expressed in active macrophages and on the surface of hematopoietic originated malignant cells (BASHYAL, 2018; Low & Kularatne, 2009). Folate receptors are specifically expressed on the surface of different types of tumor cells such as breast, kidney, gastric and pancreatic (Yi, 2016). Targeting the folate receptor has been shown to have a supportive effect on drug intake (Costantino et al., 2009).

Tf (Transferrin) receptor: The transferrin receptor is a transferrin carrier protein that plays an important role in the uptake of iron into intracellular and is regulated in response to intracellular iron concentration (Z. M. Qian, Li, Sun, & Ho, 2002). The transferrin receptor can be activated quickly, so it provides more intake and leads to rapid cell proliferation. Its vital role in cancer cell pathology has led to the prominence of this receptor in cancer treatment (K. Cho, Wang, & Nie, 2008; Daniels, Delgado, Helguera, & Penichet, 2006; Pastorino et al., 2006). Transferrin receptor, which can be conjugated with different materials for targeting in cancer studies, come into prominence with positive results in studies. For example, Kawamoto et al working on the TfR-lytic hybrid peptide, reported that this conjugated structure caused the death of cancer cells to a large extent (Daniels et al., 2012; Kawamoto, Horibe, Kohno, & Kawakami, 2011).

Asialoglycoprotein receptors: Asialoglycoprotein receptors are lectins that have the carbohydrate binding function found in the plasma membrane of liver cells (Reithmeier, 1996). Lectins are important structures that play a role in recognition at the cellular and molecular level (Brudner et al., 2013; Rutishauser & Sachs, 1975). Asialoglycoprotein receptors undertake the task of keeping target glycoproteins out of circulation. The asialoglycoprotein receptor has been reported to have high expression in hepatomas used in cancer targeting (BASHYAL, 2018), human carcinoma cell line and liver cancer (Das, Kudale, Dandekar, & Devarajan, 2019; Roggenbuck, Mytilinaiou, Lapin, Reinhold, & Conrad, 2012). Lectins can be incorporated into nanoparticles and used as targeting fragments directed towards cell surface carbohydrates (Minko, 2004).

Vascular endothelial growth factor (VEGF) receptor: Angiogenesis is a physiological event in which new vessels form from existing blood vessels. Angiogenesis, a physiologically natural phenomenon such as growth, development and wound healing, is also a pathological event in tumors, inflammatory diseases and degenerative macular eye diseases (Aydin, 2008; BASHYAL, 2018; Konukoğlu & Turhan, 2005). Growth factors and their receptors play important roles in angiogenesis. Angiogenesis begins with hypoxia and inflammatory stimulation, with an increase in activator factors and a decrease in inhibitory factors (Aydin, 2008; Kılıç, Yıldırım, Şahin, & Pamir, 2005; Konukoğlu & Turhan, 2005). One of these activator and inhibitory factors is VEGF and VEGF inhibitor. More than half of malignant tumors express VEGF at high concentrations. Active targeting of the tumor vasculature is possible by targeting VEGF receptors with nanoparticles (BASHYAL, 2018). Bevacizumab (Avastin) is an anti-VEGF mAb that inhibits the growth factor of new blood vessels and is the first angiogenesis inhibitor approved in 2004 for use in the treatment of colorectal cancer (Ferrara, 2005; Peer et al., 2007).

Antibody mediated targeting

Specific monoclonal antibodies (mAb) are used in anticancer studies. Monoclonal antibodies have different uses, such as radioisotope conjugates, chemotherapy-monoclonal antibody conjugate, toxin-

linked conjugates (Dillman, 1984). The intended use of monoclonal antibodies is to increase the immune response and antitumor capacity of the immune system. The target of monoclonal antibodies is abnormally expressed proteins in neoplastic cells. In drug delivery studies, conjugation of nanoparticles with antibodies has been developed against the tumor antigen. When specific antibodies are targeted by the drug, they bind to specific cancer cells (BASHYAL, 2018). In studies conducted by Wartlick et al in 2004, trastuzumab (Herceptin®), a specific antibody to the HER2 receptor, was conjugated to the nanoparticle surface, and specific targeting studies were performed for cells that overexpress HER2. Binding of the conjugated antibody-nanoparticle system to the surface of the cells that overexpress HER2 is related to time and dose. In the study, it has been shown that nanoparticles are effectively internalized by cells that overexpress HER2 by receptor-mediated endocytosis (Wartlick et al., 2004).

Table 1. Various targets with nanoparticles used in cancer studies

Nanocarrier System	Targets	Tumor Cells	References
Liposomes	Tf receptor/Tf	C6 glioma	(Ying et al., 2010)
Magnetic nanoparticles	VEGF/anti-VEGF mAb	Human Liver Cancer	(Cheng et al., 2008)
Liposomes	EGRF receptor/anti-EGRF Mab	MDA-MB-468,U87 glioma	(Mamot et al., 2005)
Polymeric NPs	HER2	Breast Cancer	(Alexis et al., 2008)
Liposomes	HER2	Breast Cancer	(Kirpotin et al., 2006)
Polymeric NPs	Folate receptor	Ovarian Cancer	(Werner et al., 2011)
Micelles liposomes	Asialoglycoprotein receptor	HepG2 B16 melanoma	(Y.-C. Wang, Liu, Sun, Xiong, & Wang, 2008)

Antiangiogenesis

The formation of new vessels from existing blood vessels is called angiogenesis. If the molecular mechanism of angiogenesis can be resolved, new treatment approaches for tumors can be developed and successful results can be achieved. Tumoral angiogenesis is not like physiological angiogenesis (Kılıç et al., 2005; ÖZUYSAL, 2001). Physiological angiogenesis is stable and self-limiting, but tumoral angiogenesis is neither stable nor limited. Angiogenesis is a complex phenomenon in which growth factors, cytokines and their receptors play a role. New studies on cancers have focused on targeted therapy, targeting growth factors and receptors, signal conduction pathways, angiogenesis, and the extracellular matrix (Hicklin & Ellis, 2005). The most important mediator in angiogenesis is VEGF (Aydin, 2008). Therefore, the most important target in the treatment of angiogenesis are VEGF and VEGF receptors. Active targeting of the tumor vasculature is carried out using nanoparticles by targeting the VEGF receptors (VEGFRs), integrin receptors, and other angiogenic factors. Integrins, which interact with the extracellular matrix, are transmembrane receptors and play important role in angiogenesis process. The enhance of amount of integrins increases the survival, growth, and invasion of both tumor and endothelial cells (Desgrosellier & Cheresh, 2010; Sutradhar & Amin, 2014).

Passive Targeting

In the passive targeting method, tumor vascular systems with anatomical and functional differences are utilized to ensure that drugs reach and accumulate in the tumor area with appropriate nano carriers (Au, Jang, & Wientjes, 2002; Vasir & Labhasetwar, 2005). The leaky endothelium of the tumor vascular system makes the accumulation process faster. This condition is called increased permeability. The deficiency in the lymphatic system, known as the drainage system, is associated with the accumulation of macromolecules in tumor cells, this is called the retention effect. Both increased permeability and retention effect are commonly known as the EPR effect (Hossen et al., 2019). In studies for cancer treatment, the use of EPR effect-mediated nanocarriers is an important development. The EPR effect (Fang, Sawa, & Maeda, 2004; Iyer, Khaled, Fang, & Maeda, 2006; Maeda, 2001; Maeda, Bharate, & Daruwalla, 2009), first reported by Maeda et al, allows extravasation of macromolecules greater than 40 kDa from the tumor vessel that allows the accumulation of macromolecules to the interstitial space. Despite that tight junctions in normal endothelial cells do not allow this extravasation. Tumor-targeted drug delivery in anti-cancer studies is promising for the future, as the extravasation permit originating from the EPR effect is located in the tumor area (Kumari, Ghosh, & Biswas, 2016). Idealization of the EPR effect is related to the fact that the active substance and the nano carrier carrying it are not removed by the body's immune system and can provide long-term circulation in the body. These properties, which are associated with the idealization of the EPR effect, are affected by the size, shape and surface charge of the nanoparticle (Yu et al., 2016). It is known that the concentration values of the anticancer active substances carried to the tumor region by the binding of active substances with anticancer properties to a polymer structure or another molecular carrier increase in this region and the concentration of the polymer-drug conjugates can reach 10-100 times higher than in the free drug (Sinha et al., 2006). Nano-carrier system with a size in the range of 10-100 nm is important and appropriate to have properties such as no charge or anionic charge and protection from the effect of RES (the reticuloendothelial system) (SAYINER & ÇOMOĞLU, 2016). While negatively charged NPs have the ability to circulate in the blood for a longer period, positively charged NPs are more easily absorbed by cancer cells with a negative surface charge. In cancer studies conducted with passive targeting, it was reported by O'Neal et al. that 130 nm diameter nanoshells could pass through the vessel walls and accumulate in tumor tissue (O'Neal, Hirsch, Halas, Payne, & West, 2004). The direct drug delivery technique in passive targeting allows the drug to target tumor tissue directly, avoiding circulation. Experiments were carried out by giving local drug with intratumoral applications in the passive targeting technique. In a study by Nomura et al. in 1998, mitomycin was applied to tumor tissue and the concentration of the drug was increased in the tumor area and it was reported that toxicity decreased (Nomura et al., 1998).There are some obstacles in the use of passive targeting in cancer studies. Despite the EPR effect, the vast majority of passively targeted NPs cannot reach the targeted tumor area by intravenous application (Yu et al., 2016). Due to the association of passive targeting with the tumor vascular system and angiogenesis, the extravasation of nano carriers varies in relation to tumor type and region in the body. High pressure in solid tumor tissues prevents the homogeneous distribution of drugs accumulating in the tumor (Bae, 2009; Heldin, Rubin, Pietras, & Östman, 2004).

Physical Targeting

One of the strategic methods used to target drug release is physical targeting through external stimulation. Physical targeting can be done using ultrasound and magnetic field. Targeting the ultrasound waves to the cancerous region can be used to ensure the uptake and release of anticancer substances carried by polymeric micelles in the cell. Although the targeting mechanism has not been resolved precisely, it is believed that the micelles excited by ultrasound are extravasated into the tumor area and the release of the anticancer substance transported by micelles occurs only in the ultrasound-irradiated cancerous area (NY Rapoport, Christensen, Fain, Barrows, & Gao, 2004; Natalya Rapoport, Marin, Luo, Prestwich, & Muniruzzaman, 2002). This targeting technique has been used in in vitro studies for drugs of the anthracycline class, a class of chemotherapy agents, and for the delivery of anthracycline drugs to MDR ovarian A2780 carcinoma cells (Husseini, Christensen, Rapoport, & Pitt, 2002; Natalya Rapoport, 2004; Vasir & Labhasetwar, 2005).Another physical targeting technique is to target an anticancer substance by applying an external magnetic field by coating it with a nanomaterial sensitive to magnetic field and injecting it into the vein. Magnetic liposomes and magnetic ferrofluids can be shown as examples of magnetic field sensitive nanomaterials (Häfeli, 2004). In the Phase 1 clinical studies on patients with advanced sarcoma, magnetic targeting of drug-epirubicin was performed, but the results were not satisfactory because most of the carriers had low magnetic field sensitivity. It was emphasized that targeting systems should be more independent from the patient and the disease for the trial studies to show more positive results (Lübbe, Alexiou, & Bergemann, 2001; Vasir & Labhasetwar, 2005). In studies sensitive to magnetic field, the studies have shown that the geometry of the magnet used, and the distance between the tumor tissue and the magnet are important in drug delivery (Price, Mahmoud, Al-Ghamdi, & Bronstein, 2018; Shamsi et al., 2018; X. Wang et al., 2018).

The limitations of Active, Passive and Physical Targeting

Drug Targeting systems have disadvantage besides advantages. Active or passive targeted nanoparticle systems face great difficulties in releasing drugs in neoplastic cells. Because lysosomal enzymes can quickly destroy nanoparticles and drugs inside the cells (Sutradhar & Amin, 2014). During passive targeting drugs have some of limitations. Extravasation of nanocarriers varies with tumor types and anatomical sites because the passive targeting is attached to the degree of tumor vascularization and angiogenesis. Additionally, successful uptake and homogenous distribution of drugs in the tumor is difficult because of high interstitial fluid pressure of solid tumors. Interstitial fluid pressure composing a significant barrier prevents the penetration of nanocarriers inside the tissues.The high protein content in the interstitial space causes the development of colloidal pressure, and the development of this condition causes blocking the ingress of a macromolecule from the bloodstream (Bae, 2009; Heldin et al., 2004; Kumari et al., 2016). In physical targeting, if the energy of ultrasound radiation on plasma membranes of cells is low, this increase the intracellular uptake of drug, while the energy of ultrasound radiation higher than the cavitation threshold can seriously harm the cell membranes (Vasir & Labhasetwar, 2005).

USAGE OF NANOTECHNOLOGY IN CANCER TREATMENT

Conventional drug forms are used frequently and repeatedly. There may be undesirable situations when the dose used to concentrate the active substance released into the system drops below the sufficient amount or goes above the toxic level. Such adverse conditions can be avoided by using drug delivery systems that reduce the dose of the active substance, extend the dosing interval, minimize side and toxic effects, and deliver the active substance to the target area. By using nanotechnological techniques in drug delivery systems, the active substance concentration in the blood can be kept constant at the desired therapeutic level for a longer period, and elimination of the active substance in the body can be minimized. Thus, the most effective use of the active substance takes place and the benefit from the drug can be increased. Unfortunately, it is not possible to achieve this with the oral use of drugs, which are among the most common forms of administration. During the preparation of drug delivery systems, the selection of polymers is very important, which allows us to control the active substance output. By using such systems made of biocompatible polymers, it is ensured that these delivery systems break down in the body over time and that the decomposed products do not have toxic effects. Many advantages are provided by using nano carriers in drug carrier systems. It is especially preferred in the release of cancer drugs because it reduces the toxic effect of drugs and prevents multiple drug resistance.

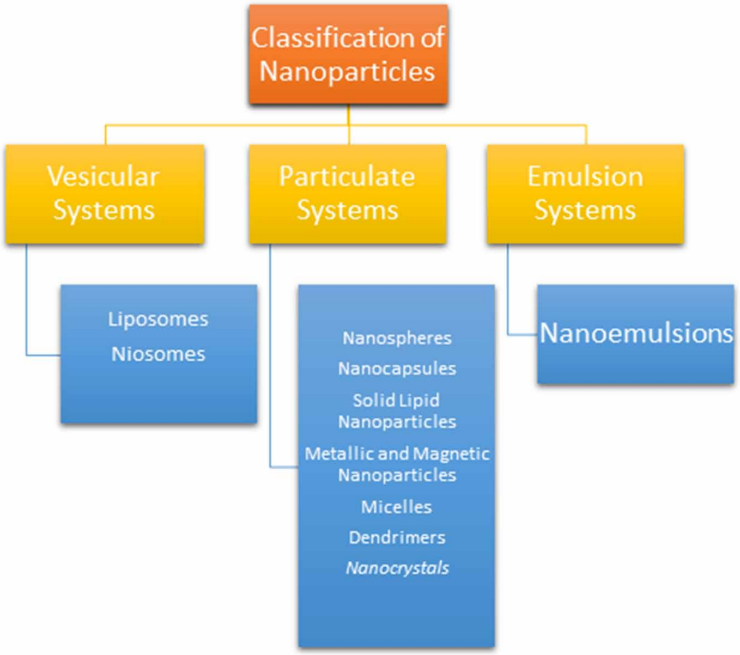
Table 2. Nanodrugs approved by clinical studies

Name	Type	Active drug	Type of cancer	References
Zinostatinstimalamer	Polymer protein conjugate	Styrene maleic anhydride neocarzinostatin (SMANCS)	Renal cancer	(Tran, DeGiovanni, Piel, & Rai, 2017)
Doxil/caelyx	Liposome (PEGylated)	Doxorubicin	HIV-associated Kaposi's sarcoma, ovarian cancer, metastatic breast cancer, multiple myeloma	(Cainelli & Vallone, 2009; Tran, DeGiovanni, Piel, & Rai, 2017)
DaunoXome	Liposome (non-PEGylated)	Daunorubicin	HIV-associated Kaposi's sarcoma	(Chen et al., 2010)
Lipo-Dox	Liposome	Doxorubicin	Kaposi's sarcoma, breast and ovarian cancer	(Conner, Bawa, Nicholas, & Weinstein, 2014)
DepoCyt	Liposome	Cytosine arabinoside (cytarabine)	Neoplastic meningitis	(Conner et al., 2014)
Abraxane	Nanoparticle albumin bound	Paclitaxel	Advanced non-small-cell lung cancer, metastatic pancreatic cancer, metastatic breast cancer	(Miele, Spinelli, Miele, Tomao, & Tomao, 2009)
Oncaspar	PEG protein conjugate	l-Asparaginase	Leukemia	(Conner et al., 2014)
Genexol-PM	PEG-PLA polymeric micelle	Paclitaxel	Breast cancer, Lung cancer, Ovarian cancer	(Kim et al., 2004)
MEPACT	Liposome (non-PEGylated)	Mifamurtide	Osteosarcoma	
NanoTherm	Iron oxide nanoparticle		Thermal ablation glioblastoma	(Ledet & Mandal, 2012)
Marqibo	Liposome (non-PEGylated)	Vincristine	Philadelphia chromosome negative acute lymphoblastic leukemia	(Silverman & Deitcher, 2013)
MM-398 (Onivyde)	Liposome (PEGylated)	Irinotecan	Metastatic pancreatic cancer (2nd line)	(Bayever et al., 2017)

Nanotechnology Based Delivery Systems

Targeted drug delivery systems allow drugs to be delivered to the target more effectively and relatively practically to current drugs. Most of the drugs in use do not show their effectiveness effectively in the hydrophobic areas in reaching the target cells. In addition, the inability of the drugs to show their effects within the specified time and their effect on the whole body, except for the target tissue, is one of the undesired side effects. Another undesirable situation is that the active substance cannot overcome the barriers in the body and cannot reach the target area. Thus, nanotechnology produces solutions with several methods in solving these similar problems that arise in the use of active substances.Thanks to the development of nano carriers, drugs are delivered to the target tissue by overcoming various anatomical and biological barriers such as blood-brain barrier, bronchioles in the respiratory system and tight connections in the skin. As a result of the advantageous situation brought about by its dimensions, nano delivery systems, which have a better distribution in narrow areas in the body, especially facilitate the dissolution of low solubility drugs. Nano carrier systems, which have brought new properties to pharmaceutical agents, also reduce drug toxicity and enable more efficient drug distribution. In drug delivery systems, the fragmentation and loss of active substances used is also minimized. By connecting hydrophilic molecules such as PEG to carrier systems, the drug is allowed to continue its effect in circulation for a longer period of time. More than one active substance can be loaded into nano-carrier systems as well as multiple targeting molecules. The classification of nanoparticles was given in Figure 2.

Figure 2. The Classification of Nanoparticles



Polymer-Drug Conjugates

There are many superior aspects of polymer-based nanoparticles such as low systemic toxicity and low cytotoxicity, free of organic solvent residues, large scale production possible, targeted active substance in tissues, and controlled active substance release. The second feature is that the surface area / volume ratio is very high compared to microparticles, making them more preferred in both in vitro and in vivo studies. (SAYINER & ÇOMOĞLU, 2016; Tüylek, 2019)

Nanoparticulate Drug Delivery Systems

Nanoparticles which are matrix systems that are prepared using natural or synthetic polymers, whose sizes vary in the range of 10 - 1000 nm, called nanospheres or nanocapsules according to the method of preparation, in which the active substances are dissolved, trapped and / or adsorbed or attached to the surface. There are two main advantages of nanoparticles. The first is that it has small particle sizes, thus enabling the active substance to accumulate in the target area. The second is the use of biodegradable materials in the preparation of small particles, which ensures long-term release of controlled active substances thanks to biodegradable materials. Besides these; nanoparticles ensure the stability of drugs / proteins or peptides. Active substance loading capacities are also high and can be easily sterilized. Thus, the release and bioavailability of the drug given in the form of nanoparticles increases in oral administration.

Vesicular Systems

Liposomes

Liposomes are spherical shaped closed vesicles containing an aqueous phase between the layers and in the middle inner part consisting of one or more lipid double layers. Liposomes are biocompatible, nonimmunological, reversible vesicular systems with phospholipid double layer structure, sizes vary in nanometers and several micrometers. Liposomes, which play an important role in the formulation of drugs, attract great attention due to their versatility and strengthening the therapeutic effect. Thanks to liposomes, various problems such as poor solubility, poor bioavailability, short half-life, and potent side effects of drugs can be largely overcome. Since liposomes have the ability to increase vascular permeability in tumor tissues, they are especially used in the treatment of cancer treatment (Amreddy et al., 2018; Pandey, Rani, & Agarwal, 2016; H. R. Pawar, Bhosale, & Derle, 2012).

Niosomes

Niosomes are created by including non-ionic surfactant and cholesterol as excipients. Niosomes are more capable of penetrating than previous emulsion preparations. Its two-layer structure is structurally similar to liposomes. Both hydrophilic and lipophilic drugs have the ability to retain in an aqueous layer or a vesicular membrane made of lipid material (Ag Seleci, Seleci, Walter, Stahl, & Scheper, 2016; Moghassemi & Hadjizadeh, 2014; Torchilin, 2006).

Table 3. Liposome anti-cancer drugs

Name	Trade name	Route of Administration	Company	Indication
Liposomal daunorubicin	DaunoXome	Parenteral	Gilead Sciences	HIV-related Kaposi's sarcoma
Liposomal doxorubicin	Myocet	Parenteral	Zeneus	Combination therapy with cyclophosphamide in metastatic breast cancer
Liposome-PEG doxorubicin	Doxil/Caelyx	Parenteral	Ortho Biotech, Schering-Plough	HIV-related Kaposi's sarcoma, metastatic breast cancer, metastatic ovarian cancer

Table 4. The applications of Some Niosomes

Targeted tissue	Loaded therapeutic agent	Targeting molecule	Ref.
Brain	Doxorubicin	N-Palmitoyl glucosamine	(Marco Bragagni, Mennini, Ghelardini, & Mura, 2012)
	Dynorphin-B	N-Palmitoyl glucosamine	(M Bragagni et al., 2014)
	Vasoactive intestinal peptide	N-Palmitoyl glucosamine	(Dufes, Gaillard, et al., 2004)
Breast cancer	Doxorubicin	Transferrin	(Tavano, Muzzalupo, et al., 2013)
Chronic myelogenous leukemia	Doxorubicin	Magnetite	(Tavano, Vivacqua, et al., 2013)
Epidermoid carcinoma	Hydroxycamptothecin	Transferrin	(Hong, Zhu, Jiang, Tang, & Pei, 2009)
	Doxorubicin	N-Palmitoyl glucosamine	(Dufes, Muller, et al., 2004)
Melanoma	Doxorubicin	N-Palmitoyl glucosamine	(S. Pawar & Vavia, 2016)

Particulate Systems

Nanospheres

Nanospheres are drug delivery systems consisting of a polymeric matrix. In matrix system nanospheres, drugs are homogeneously and uniformly dispersed. The drug may be dispersed in the polymeric sphere or adsorbed to the surface. They can be prepared with a natural polysaccharide structure and using synthetic polymers. Drug release is realized by diffusion between the polymeric structure, erosion of the polymeric structure or the combination of both mechanisms (R. Singh & Lillard Jr, 2009). Drug-loaded nanoparticles, which selectively target tumor cells, offer promising solutions as they prevent damage to healthy cells. Nanoparticles can be designed to contain a wide variety of chemotherapeutic or diagnostic agents by creating flexibility (Yurgel, Collares, & Seixas, 2013).

Nanocapsules

Nanocapsules are vesicular systems. The drug is placed in a cavity and bounded by the polymer membrane. They are drug carrier systems that consist of a sheath made of polymer biomaterial and an oily

inner core. Due to its ability to pass easily through the veins, they are easy to get into the systemic circulation. When the surface area increases, the bioavailability increases with drug solubility. The drug can be easily targeted to the region where it will be applied. Due to its good stability and long shelf life, it is possible to prepare new drugs.

Solid Lipid Nanoparticles

They are solid nanoparticles with a particle size of 50-1000 nm, formed by the use of solid lipids in oil / water emulsions instead of oil. In addition, they are carriers with high physical stability that can be controlled and sustained release. SLN is an attractive colloidal drug delivery system due to the successful incorporation of active compounds and their benefits. The lipid matrix of SLN has drug loading and deterioration protection features. The evacuation of drugs in the target tumor tissues can also be controlled depending on the surface coating of the SLN and its constituent lipids. SLN is also used in both preoperative and intraoperative brain tumor detection (Mathur et al., 2010).

Metallic Nanoparticles

Metallic nanoparticle formulations are preferred for optical or heat-based therapeutic methods as well as their intense surface functionalization potentials. Metallic nanoparticles are particularly advantageous in cancer immunotherapy applications because of their ability to control size, shape, load and surface modifications.

Table 5. The metallic nanoparticles and examples of their cancer immunotherapy applications

MNP	Approach	Mechanism	Outcome	Ref.
Aluminum oxide	Adjuvant	Enhances anti-cancer effects of tumor cell vaccines	Observed smaller tumor sizes and more CTLs when co-administered with a tumor cell vaccine	(Sun et al., 2010)(Evans, Bugga, Asthana, & Drezek, 2018)
Cobalt oxide	Antigen delivery	Induce macrophage activation	Increased antigen-specific CTLs *in vivo*	(Chattopadhyay et al., 2016)
Cuprous oxide	Alter tumor microenvironment	Alter expression of drosophila transcription factor	Induced myeloid infiltration and systemic immunity	(Kheirolomoom et al., 2015)
Gold	Antigen/adjuvant delivery; Photothermal therapy	Increased CTL responses; tumor ablation released tumor antigens	Reduced tumor growth *in vivo*; prevented tumor growth *in vivo*	(Dreaden, Mackey, Huang, Kang, & El-Sayed, 2011)
Iron oxide	M1 macrophage polarization; Protein delivery; Photothermal therapy	Increased pro-inflammatory macrophage proliferation; IONP-HSP chaperoned antigens to APCs; thermal tumor ablation	Inhibited tumor growth; IONP-HSP70 led to tumor-specific CTL responses; ablation led to protective immunity	(Shevtsov et al., 2015; Toraya-Brown et al., 2016; Zanganeh et al., 2016)
Silver	Reduce tumor-promoting cytokines	Decreased IL-1β signaling in tumor microenvironment	Inhibited fibrosarcoma tumor growth *in vivo*	(Chakraborty et al., 2016)
Titanium dioxide	Immune stimulation induced by ultrasound	ROS generation increased pro-inflammatory cytokines and interleukins in the tumor	Suppressed tumor growth *in vivo*	(You et al., 2016)
Zinc oxide	Antigen delivery (pulsed DCs)	Improved antigen-specific CTL responses	Delayed tumor growth *in vivo*	(N.-H. Cho et al., 2011)

Magnetic Nanoparticles

Magnetic nanoparticles are widely used to reduce or eliminate the side effects of traditional cancer diagnosis and treatment due to their unique physical properties, magnetic susceptibility, biocompatibility and stability. Magnetic nanomaterials which have superior properties such as size, morphology, surface chemistry, biodegradability, and specific targeting, and thus, nanoparticles are also most preferred as contrast agents for hyperthermia, drug release and Magnetic Resonance Imaging (MRI) for cancer diagnosis and treatment. Tumor tissues can retain more MNP than healthy tissues due to the high permeability and retention effect caused by damaged and infiltrated vessels. In addition, polymer-coated MNPs can be adopted by the Reticuloendothelial System (RES) organs, such as liver, spleen, lymph nodes, and bone marrow, by macrophages. Therefore, it is also widely used as targeting mechanisms to create adequate imaging contrast between tumor and healthy tissues. Thanks to its targeting mechanisms, MNPs can distinguish tumor tissues from normal tissues. It has developed an innovative cancer therapeutic approach for the sequential release of two cytotoxic agents in breast cancer cells (Kumar et al., 2015). In another study of the application of magnetic nanoparticles, 5-fluorouracil, polylactic-co-glycolic acid (PLGA) was loaded into magnetic nanocapsules, and the results revealed that the synthesized nanoparticles show excellent anti-tumor activity against colon cancer (Shakeri-Zadeh, Khoee, Shiran, Sharifi, & Khoei, 2015). In a study conducted in 2014, it was shown that PEGed iron oxide nanoparticles containing doxorubicin (DOX) were synthesized and could be used as a potential drug carrier (ERDOĞAN, 2018; Hałupka-Bryl et al., 2014).

Micelles

It is defined as a spherical particle that has been made more durable with hydrophilic polymer chains and morphologically hydrophobic blocks in core. Micelles, which are used as drug delivery systems, increase the bioavailability as they dissolve low solubility agents. Micelles, which have nano size, allow the active substances to accumulate in areas with weak vascularity. Micelles prepared with the PEG-poly (D, L-lactic acid) copolymer (<50 nm) of paclitaxel for use in the treatment of breast cancer were approved in 2007 in Korea under the name Genexol-PM (Samyang-Korea) (Kim et al., 2004). Clinical studies of polymeric micelles containing cisplatin and doxorubicin are also ongoing. Based on increased permeability and retention (EPR) in cancer tissue and low pH microenvironment in cancer tissue, nanoscale pH sensitive polymeric micelles are considered as sensitive treatments that can release diagnostic and therapeutic agents to the cancerous area at the same time (G. H. Gao, Li, & Lee, 2013). Polymeric micelles are ideal for drug delivery due to their size. Ultrasound applications in the field of chemotherapy are also available in the literature, using a micelle-sized carrier that secures the chemotherapeutic agent and prevents its interaction with the rest of the body, but can then release the agent at the desired place and time (Husseini & Pitt, 2008).

Dendrimers

They are large spherical molecule structures consisting of symmetrical branching units that repeat each other. It's intertwined structures, end groups that can be reactive, and the ability to add various molecules between its branches are among the most distinctive characteristics. Branching units allow dendrimers to grow repeatedly. Thanks to their branched functional groups, excellent encapsulation properties and

great controllability, they are used in drug handling applications. Dendrimers are an ideal drug delivery to explicitly study the effects of polymer size, charge, composition and architecture on biologically relevant properties such as lipid bilayer interactions, cytotoxicity, internalization, blood plasma retention time, biodistribution and tumor uptake. In recent years, there have been significant advances in the use of anti-neoplastic and contrast agents in therapeutic and diagnostic use of dendrimers for cancer treatment, including advances in neutron capture therapy, photodynamic therapy and photothermal therapy applications (Wolinsky & Grinstaff, 2008).

Nanocrystals

They are nano-sized crystals or nanoparticles of crystal character. Pharmaceutical nanocrystals are defined as a crystalline drug active substance that has been reduced to nanoscale by various methods. Nanosuspension formulations created by suspending nanocrystals or nanocrystals are being developed to increase the bioavailability of low-resolution drugs. Difference from other systems: Almost all of it consists of active substance, does not contain carrier, it is made durable by adding stabilizer, they have a crystal structure. Drug nanocrystals with impressive physicochemical properties are preferred drug delivery systems for effective cancer treatment due to their high drug loading efficiency, excellent structural stability, continuous dissolution and long circulation times (Miao, Yang, Feng, Lin, & Huang, 2018). In recent years, many hydrophobic or lipophilic drugs such as paclitaxel (PTX), camptothecin (CPT), timectacin, busulfan, cyclosporine A, 2-devinyl-2- (1-hexloxyethyl) pyrophosphophobic (HPPH), have been used in cancer treatment with nanocrystal forms.

Nanoemulsions

Nanoemulsions are colloidal particle systems ranging in size from 10 to 1,000 nm, acting as carriers of drug molecules. These carriers are solid spheres and their surfaces show negatively charged, amorphous and lipophilic properties. Nanoemulsions are effective drug delivery systems for delivering lipophilic cytotoxic antineoplastic agents to targeted areas due to their superior properties such as optical clarity, biocompatibility, non-immunogenic, biodegradable, drug encapsulation, sustained and controlled release, nanometric size, large surface area, ease of preparation, and thermodynamic stability (Sahu, Das, Mishra, Kashaw, & Kashaw, 2017).

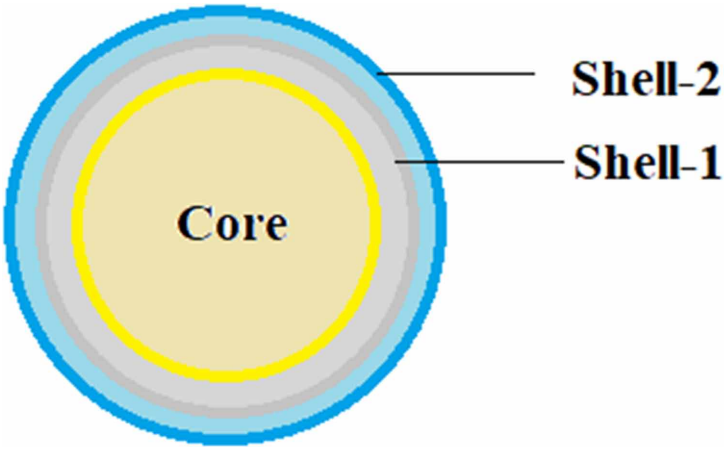
Cancer Imaging with Nanoparticles

Using nanoparticles as contrast agents has been shown to have a remarkable potential for cancer imaging. The studies that are still ongoing today are a fragment of important developments in the diagnosis and treatment of cancer in the future. Contrast agents are important tools to help visualize features that are difficult to detect (Smith, Kuncic, Ostrikov, & Kumar, 2012).

Optical Imaging with Quantum Dots

Nanocrystals of a semiconducting materials with diameters in the range of 2-10 nanometers such as silicon, cadmium selenide, cadmium sulfide or indium arsenide, are called quantum dots. QDs are either produce of one or several materials based on the core and shell construction principle, as well

Figure 3. The structure of a free core-shell quantum dot



as several coating layers are also possible to construct (Murray, Kagan, & Bawendi, 2000). Due to the fluorescence properties, quantum dots which having small size, adjustable emissions and photostability, are a better alternative to organic dyes for biological imaging and sensing (Bruchez, Moronne, Gin, Weiss, & Alivisatos, 1998).

The electronic and optical properties of quantum dots and their adjustment based on core shell structures make QDs very important for many applications (Murray et al., 2000). QDs have been in a wide range of optoelectronic devices and have been used in many in vivo and in vitro imaging, labelling and sensing techniques. Inorganic-organic composite nanomaterials have shown extreme efficiency in cancer diagnosis in vivo. However, to achieve their fully potential, QDs, which do not contain toxic heavy metals, and very efficient light emitters must be used (Efros, 2019). Systemic injection of multifunctional QD probes were used to achieve sensitive and multicolor fluorescence imaging of cancer cells in the Gao's study on living animals (X. Gao, 2007; X. Gao, Cui, Levenson, Chung, & Nie, 2004). With the advancement of synthesis and modification of QDs, the impact of QDs on the investigation of tumor metastasis will become ever more significant in the future.

MRI (Magnetic Resonance Imaging)

Magnetic Resonance Imaging (MRI) is a medical imaging technique used to obtain the image of anatomy and physiological processes of the body in the field of radiology. In this technique, which is an application of nuclear magnetic resonance, imaging is performed using strong magnetic fields and radio waves. This imaging technique is applied with the help of hydrogen atoms, which are abundant in water and oil in humans and all other living things. Nuclear magnetic resonance signals from hydrogen nuclei provide information about the anatomy and physiological process. In cancer studies, contrast agents that improve image contrast can be used in the diagnosis and treatment of tumors. Many probes have been developed to examine cell functions without damaging living organisms which are in the molecular size. Nanoparticles, especially iron oxide, gold and gadolinium NPs, have been used as contrast enhancing agents in magnetic resonance imaging (MRI) and have been shown to show significant effects in diagnostic medicine (Asl, 2017). Gold nanoparticles are non-toxic, biocompatible and inert

(Bhattacharya & Mukherjee, 2008) are the most suitable inorganic structures for gene therapy and drug release applications (Templeton, Wuelfing, & Murray, 2000). TNF-α was released using these particles and increased tumor breakdown (Alexis, Pridgen, Langer, & Farokhzad, 2010). Gold nanoparticles are also used in photothermal therapies (Bikram, Gobin, Whitmire, & West, 2007). The excellent physical, chemical and optical properties of gold nanoparticles are strongcandidates for their use to design new biosensors and imaging system.

For MRI, studies are emphasized with super magnetic nanoparticles that increase contrast. These nanoparticles consist of an iron oxide core coated with polyethylene glycol or dextran. Supermagnetic iron oxides (SPIO) that increase MRI contrast, Lumirem ®, Endorem ® (Kanematsu et al., 2006) and ultra-small paramagnetic iron oxide (USPIO) Sinerem ® (Keller et al., 2004) are the ones used in the market. Commercially available SPIOs and USPIOs can be used to screen the gastrointestinal tract, liver and spleen tissues, and the blood pool (Abd El-Karim et al., 2015). Cross-linked iron oxides known as CLIO, developed for active targeting, which is one of the targeting methods, has been developed. These structures can be conjugated to monoclonal antibodies or receptor-seeking agents (Delaloye, 2000).

Nuclear Medicine Imaging and Therapy

Increasing advances in nanotechnology is also important for imaging studies in therapeutic nuclear medicine. Liposomes, one of the nano-carrier materials, can be used to transport radioactive compounds due to the fact that radioactive compounds can be easily attached to liposomes. Since most of the liposomes are captured by the reticuloendothelial system (RES), the transmission of agents to RES is easy. Long-circulating liposomes were obtained by passing liposomes through a number of modifications so that substances could be delivered to different parts of the body other than RES (Abd El-Karim et al., 2015; Medina, Zhu, & Kairemo, 2004).

Ultrasonography

Pulses of ultrasound waves sent to a patient's body and interact with complex tissues. Echoes producing due to reflection or scattering are received and after the digital processing a gray scale image of the body or tissue cross-section are formed. The contrast in ultrasonic pictures is due to variations in the tissue's acoustic impedance being imaged (Mallidi et al., 2009). It is known that US exposure causes some cell damage for both normal and cancer types of cells, while the effect is somewhat greater for cancer cells. However, when NPs are added to the system, the resulting cell damage is much larger for cancer cells, than for the normal cells (O. K. Kosheleva, Lai, Chen, Hsiao, & Chen, 2015). Nanoparticle presence significantly increases the efficacy of ultrasonic treatment of solid tumors (O. Kosheleva et al., 2017). Nanoparticles in general is easier to be trapped in tumor due to the high porosity. Nanoparticles especially with metallic or magnetic nanoparticles, the contrast between normal tissues compared to nanoparticle is much higher than normal tissue to tumor. High resolution ultrasound imaging should have no disadvantages. Many approaches have been made to add nanoparticles to improve ultrasound imaging (Ng et al., 2019).

Challenges and Future Opportunities of Targeted Drug Delivery and Nanomedicine for Cancer Treatment

New drug delivery systems show promise with controlled drug release and safer and more efficient treatment studies in the treatment of many diseases, especially cancer treatment (Senapati, Mahanta, Kumar, & Maiti, 2018). Nanotechnology, which ensures the control of substances at the atomic and molecular level, has brought innovations to the drug distribution and continues to bring. Nanoparticles used in drug development studies showed positive developments in drug targeting, delivery and release processes. The ability of nanoparticles to combine diagnosis and treatment has provided a very important place in nano-medicine studies. The most important objectives of this field are to improve stabilization in the biological environment, to mediate biodistribution, to load, target, transport and release drugs. Nanoparticular drug delivery systems are important structures that have great potential with their application in different disease treatments (Gupta, Yadav, Kesharwani, Mishra, & Singh, 2010). Drug distribution studies using nanoparticles are on the way to become the future hope of cancer studies. Nanoparticles, which have multiple functions such as widely available, easily functionalized and biocompatible, can detect and target cancer cells and eliminate them. For many years, researchers have been working on developing new formulations to present new anticancer agents and existing agents. Therefore, studies in the field of nanotechnology will continue to move forward with more rational designs, taking into account efficiency and safety (Abd El-Karim et al., 2015). Cancer treatment studies are carried out by making use of cancerous and normal cell differences in specific targeting. Passive targeting based on the EPR effect, active targeting based on ligand-receptor interaction, and physical targeting at the clinical trial stage are strategies used in cancer studies (Yu et al., 2016). It is more possible to obtain information about cancer-specific symptoms by improvements in proteomics and genomics. Carrier design and targeting methods are related to the type, location and development process of cancer. Cancer cell and normal cell differences can be utilized with the help of conjugation of biodegradable and biocompatible nanoparticles with tumor-specific inhibitors (Sinha et al., 2006). Besides the important advantages and positive effects of nanoparticle studies, it is known to have many difficulties. Perhaps the most important one of the important difficulties in nanoparticle studies is the cytotoxicity of degradation products (Yu et al., 2016). While targeting drugs to certain tissues is quite complicated, the spread of tumor cells will lead to further growth of targeting problems. Molecular targets are associated with the metastasis region of cancer cells. Skeletal metastasis is a common complication seen in breast and prostate cancers where solid tumor structures are common and it is difficult to treat (Coleman, 2001). Although new drug delivery systems seem generally advantageous, they also have disadvantages. The high costs of nano medicine application have important disadvantages such as the cytotoxic effects of the nanoparticles used. Some nano materials (such as nano-shell, nano-tube, nano-pores) are difficult to apply to the body of some patients such as babies and elderlies. All drugs cannot be applied through nanoparticles, carriers or devices because they cannot be incorporated into the polymer matrix or broken down. The prodrugs are new chemical entities and need a lot of evaluation before being used as carriers (N. Singh & Khanna, 2012).

Although it is known that the deficiency of drug delivery systems still exists, a drug delivery approach is needed that can overcome anatomical and physiological barriers and provide drugs especially in the bone metastatic region (Bagi, 2005). In the event of such developments, the side effects of the drugs are reduced, and the therapeutic drug concentration is increased in the region where the disease exists (Vasir & Labhasetwar, 2005). In the process of distributing drugs using nano-carrier systems to the cells,

insufficient nanocarriers and / or the possibility of delayed drug release may lead to drug resistance. Besides nano-carrier organic polymers, toxic effects of materials such as gold, silver and nanotubes are important problems in clinical applications. Information about the distribution and location of the targeted nanoparticles may be incomplete after oral or intravenous applications. Lack of real-time in vivo studies of nanoparticles in many studies leads to a lack of information on biodistribution and therapeutic effects. Such disadvantages must be overcome before nano-carrier studies to be developed for the detection of cancer cells and the study of treatment efficacy (Kumari et al., 2016).

CONCLUSION

In this book section, information about targeted drug therapy used in cancer treatment and the use of nanotechnological systems is given. Even if all these described diagnostic and treatment methods are not successful in clinical applications, it promises great hope for the future and is believed to be a special and important treatment option for new treatment options in the near future. Targeted administration of drugs can make existing methods more effective in the diagnosis and treatment of cancer and many other diseases. It is known that there are new drugs developed and approved in recent years and some of them are under clinical trials (Abd El-Karim et al., 2015). The targeting methods and delivery systems described in this book section show the great potential for solving important clinical problems (Torchilin, 2000). It is hoped that scientists and healthcare professionals can work together using very small ones to do big jobs and cure many diseases (Sinha et al., 2006).

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Chapter 14
Surfactant-Based Anhydrous Nano Carrier System for Poorly Aqueous Soluble Anti-Cancer Drugs

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ABSTRACT

Around 40% of new chemical entities and drugs are lipophilic or poor aqueous soluble in nature. Among them many anti-cancer drugs are also consist lipophilic properties. Available poorly water soluble anti-cancer drugs are paclitaxel, etoposide, and docetaxel. To get better stability of those anti-cancer drug via encapsulation and searching suitable carrier system for the controlled release, design and development requires of anhydrous nano carrier system. However, to deliver and entrapment of these kind of anti-cancer drugs are very essential with avoidance of water free preparation to get suitable controlled release application and achieve targeting site. The primary objective of proposed chapter is to develop and design novel stable anhydrous or non-aqueous nano emulsion carrier system and provide suitable carrier system for poorly aqueous soluble anti-cancer drugs. Another important aim is to design and develop better stabilizing agent by combining different type of surfactant, co-surfactant, and co-solvent.

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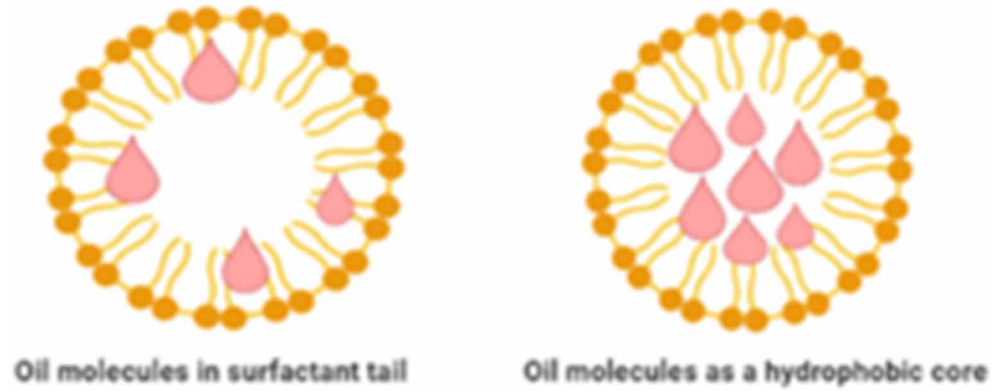
INTRODUCTION

The traditional drug delivery system is very useful and popular with a variety of advantages among physicians, surgeons and patients. But there are some drawbacks of the traditional drug delivery system which can overcome by adapting controlled drug delivery system(Patra, 2018).Controlled carrier provides reduced dosing frequency for patients with controlled release. In nutshell-controlled drug delivery carrier systems are patient compliance. There are many advantages and some disadvantages of controlled drug delivery system(Tiwari, 2017). In the past few decades, growth of controlled drug delivery system has been very rapid. Which has mainly worked on the dispersion system as it can popularly useful as oral, topical and parenteral. The surfactant based controlled dispersion system consists of microemulsion, nanoemulsions, multiple emulsions, submicron emulsions, and anhydrous nanoemulsion, of which anhydrous nanoemulsion is different from other controlled dispersions as it does not use water phase(Jaisawal, 2015).Anhydrous nanoemulsions are rationally nano carrier system to deliver a poorly aqueous soluble drug in controlled manner and system stabilized with various mixture and combination of surfactant (Verma, 2011). The special feature of Anhydrous Nanoemulsion is that it easily ingests water-insoluble drug due to absence of water phase in system. Other controlled delivery systems are unable to do for poorly aqueous soluble drug. There are many types of controlled drug carriers available at present and research is going on in a positive direction, looking at the additional potential in the future beyond that, then why the need to develop surfactant based nano carrier system (Verma, 2017). Answer would be around 40 percentage new chemical entities and drugs are lipophilic or poor aqueous solublein nature. Among them many anti-cancer drugs are also consist lipophilic properties. Available poorly water-soluble anti-cancer drugs are paclitaxel, etoposide and docetaxel (Narvekar, 2014). To get better stability and protection of those anti-cancer drug via encapsulation and searching suitable carrier system for the controlled release, design and development requires of anhydrous nano carrier system(Din, 2017). However to deliver and entrapment of these kind of anti-cancer drugs are very essential with avoidance of water phase preparation to get suitable controlled release application and achieve targeting site. Primary objective of chapter is to develop and design novel stable surfactant based nano carrier system i.e. anhydrous or non-aqueous nanoemulsion and provide suitable carrier system for poorly aqueous soluble anti-cancer drugs (Olusanya, 2018). Other important aim is to design and develop better stabilizing agent by combining different type of surfactant, co-surfactant and co-solvent for anhydrous nanoemulsion.

Various advantages of surfactant based nano carrier system i.e. Anhydrous Nanoemulsion makes it different from others and more useful (Kour, 2016). They are:

1. Minimize drug degradation and loss via encapsulation and entrapment
2. Prevent harmful side-effects by maintain therapeutic index zone
3. Increases drug bioavailability and the fraction of the drug accumulated in the required zone.
4. Controlled and sustained release of drug
5. Solubilization of poorly aqueous soluble anti-cancer drugs in to water free dispersion system
6. Minimum size and larger surface area
7. Stability over an extended period of time
8. Avoidance of phase separation, minimize toxic effect and adverse reaction.

Figure 1. represents Incorporation of surfactants in oil phase to stabilize Anhydrous Nano Emulsion dispersion



Principle and Mechanism of Anhydrous Nanoemulsions

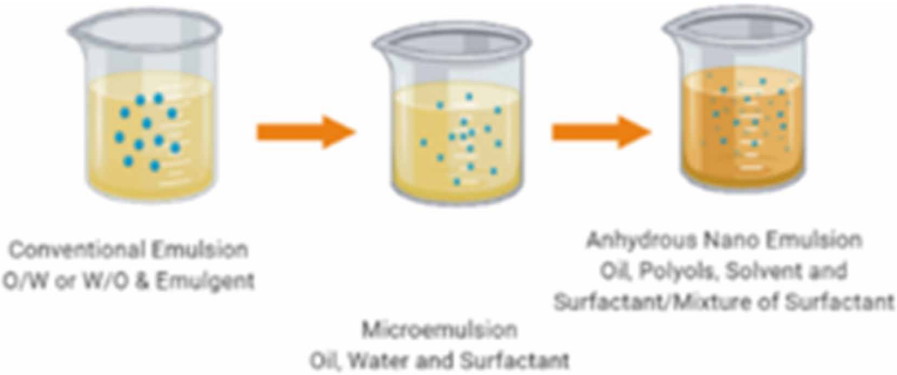
To avoid and overcome various problem associated with conventional and modern dispersion system a mandatory need to develop and design water free nano dispersion system. However, surfactant based anhydrous nano carrier for poorly aqueous soluble anticancer drug plays vital role to deliver drug in controlled manner. Anhydrous nano carrier system can replace water phase with oil, suitable organic phase or polyols and stabilized with surfactant, co-surfactant and different combination of compatible surfactant mixture. These anhydrous nano carrier system termed as anhydrous nanoemulsions (Jaitely, 2004).

Some rationale plans of action can be considered when searching for suitable stable anhydrous nano carrier systems i.e. anhydrous nanoemulsions (Torchilin, 2001).

1. To design and develop surfactants and co-surfactant with two incompatible blocks, each of which is selectively soluble in either of the immiscible liquid of anhydrous system (Verma, 2012).
2. To search for a compatible oil immiscible polar solvent or polyols that can substantially replace water using surfactant.

A solvent or liquid capable of replacing water in nano emulsion should have an appreciable polarity to make it immiscible with oils and to make it a good solvent for the solvophilic part of the surfactant molecules. Hydrogen bonding in the polar liquid is expected to play a role in solvating ionic and non ionic surfactants. The principle strategies for anhydrous nanoemulsion is to develop stable formulation for extended period of time by finding suitable surfactants whose to structural part was selectively soluble in either of the immiscible phase. The significant factor for the stabilization of the surfactant in internal phase, lowering of interfacial tension. The concept of anhydrous nanoemulsion is unique. It provides water free nano carrier system for poorly aqueous soluble anticancer drugs to controlled release in a physiological system and also applicable to reach infected targeted site.

Figure 2. shows composition and difference among conventional emulsion, Microemulsions and Anhydrous Nanoemulsions



Fundamentals of Anhydrous Nanoemulsion

Three major components require to formulate and prepare anhydrous nanoemulsion i.e. oil, organic solvent or polyols and surfactant. The selection of solvents for formulating anhydrous nanoemulsion is a very important. The basic concept of the selection of the polar solvent and polyols and predicting and identifying their respective miscibility. Suitable characteristics of a surfactant is required. The choice and selection of the two phases in anhydrous dispersion system depends mainly on the polarity of the solvents (Ha, 2000). It is difficult to assess combination of molecular properties can be used to predict and identify with any certainty a stable system formed with a given surfactant and co-surfactant, nevertheless, hydrogen bonding appears to influence in determining the stability. However, formamide is closest to water in terms of hydrogen bonding and dielectric constant and was chosen as the external phase. Designing of suitable surfactant is essential to get stable anhydrous nanoemulsion such as diblock copolymers of polystyrene and polyisoprene were able to stabilize DMF and hexane emulsions for almost one day. Searching of suitable oil-immiscible polar solvent that can replace water produces stable anhydrous nanoemulsion. Initial strategies show, the drawback of necessitating the specific design of a new surfactant for each combination of system. The emulsifying effects of several ionic and non-ionic surfactants on the anhydrous system of glycerin and olive oil have been reported, the anionic agents, dioctyl sodium sulfosuccinate, diamyl sodium sulfosuccinate and the calcium stearate failed to produce stable emulsion under the condition of this studies, but some anionic agents were effective in producing emulsification of glycerin and olive oil(Hamill, 1966).Sodium lauryl ether sulphate, and sodium lauryl sulphate produced stable, somewhat opaque preparation. The amines, 3 – propandiol, trisamminomethane, ethanolamine, triethanolamine and ammonia gas at very low concentration, formed saponification product, which results in clear, stable nanoemulsion. The cationic agents benzalkonium chloride likewise failed to stabilize the system. but also, cationic agents, cetyl pyridinium chloride and the stearyl dimethyl benzyl ammonium chloride produce stable nanoemulsion of this system. Several non-ionic agents, sorbitan monolaurate (S-20), sorbitanmonopalmitate (S-40), S-60, sorbitan monooleate (S80), sorbitansesquioleate (S-83), were tested for their capacity to emulsify the anhydrous system of glycerin and oil. Authors have used anhydrous nanoemulsions in the preparation of Nanoparticle or as templates in the formation of silicate microstructures, usually without providing details of formulation issues

developed stable concentrated oil based formamide and sulfoxide emulsions using commonly available non-ionic surfactants. Several other polar solvents are turned out not to produce stable anhydrous nano-emulsion with these surfactants. It is not clear that exactly which combination of surfactant properties measures the emulsifying effect, but hydrogen bonding clearly plays major role than polarity. Another important method ostwald ripening was exhibit to be a major factor in the stability of these anhydrous nanoemulsion, which is considerably faster than in aqueous systems because of the higher solubility of oils in nonaqueous polar solvents (Jaitley, 2004).Ostwald ripening could be completely arrested by dissolving a compound about 1% in the oil with an extremely low solubility in the continuous phase.

Interfacial Properties of Anhydrous Nanoemulsions

Anhydrous nanoemulsion contains two liquid phases. In a large amount of liquid, the molecules are completely surrounded by other molecules. Attraction forces are identical in each and every direction but at the interface, forces towards an inward attraction. Therefore, small drops to assume a spherical shape and soap films to contract (Che Marzuki, 2019). The propensity of an interface to contract in order to minimize the interfacial area. It shows a state of tension between oil and solvent or polyols hence surface tension is the tension along the surface of a liquid in air. It is the force acting at right angles to a line of unit length in the surface. Figure 3 represents the interface of a pure liquid in air and Figure 4a pure liquid/liquid (Morales, 2003). In anhydrous nanoemulsion with the formation of interface work is done and there is an associated free energy change because the molecules of oil and organic solvent at the interface have an excess of free energy (Shafiq-un-Nabi, 2007). The per unit area of excess free energy are the same as those for interfacial tension but are only numerically equal for pure liquids in equilibrium with two solvents or liquids make contact at a plane interface. Normally, the value of interfacial tension of two different liquids is less than the maximum discrete surface tension of one of the solvents or liquids because the reciprocal attraction is abated by all the molecules elaborated. Authors consider it adequate to explain both interfacial tension and interfacial free energy as the work required to enhance the area of an interface isothermally and reversibly by unit amount (Sonneville-Aubrun, 2004).

Figure 3. The interface of a pure liquid in air

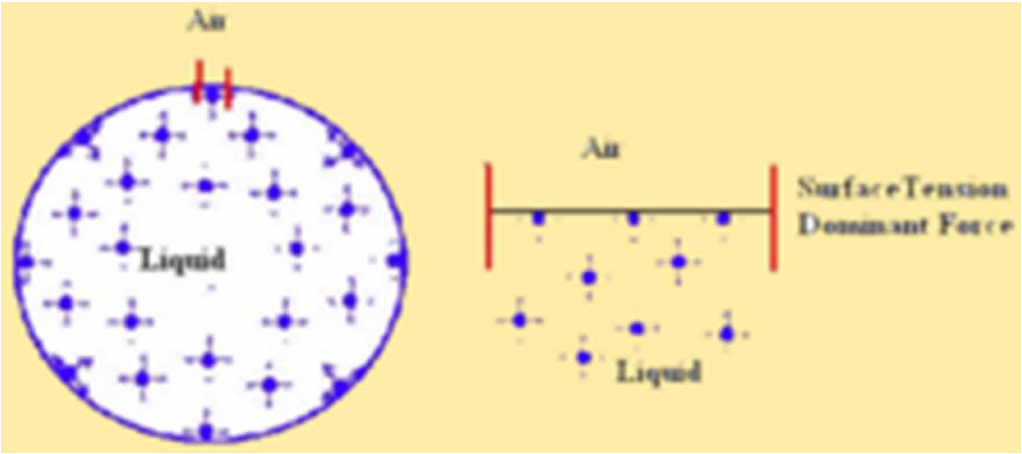
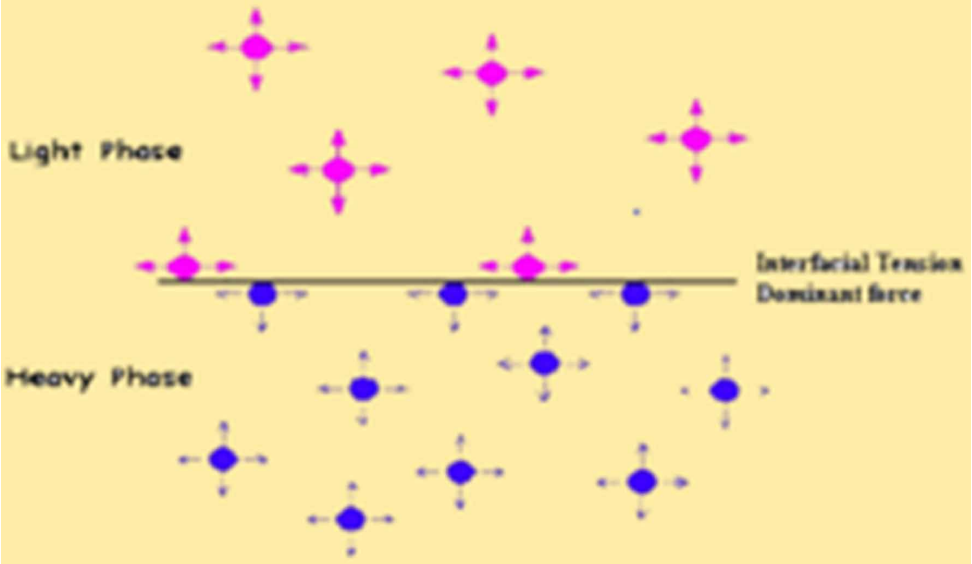


Figure 4. A pure liquid/liquid



Free Energy Considerations

The most important and fundamental property of any interface is that it possesses a positive free energy. Essentially, this means that the molecule at the interface are in a higher energy state than if they were located in the bulk phase. The greater the preferences of the molecule of interest for the bulk, as compared with the interface, the higher the interfacial energy (Fryd, 2012). Although Interfacial free energies cannot be measured directly, interfacial values give reasonably good approximations. As a rule, accurate interfacial and surface tension measurements are very difficult to obtain, since impurities in the systems and instruments wetting problems introduce large errors (Qian, 2010). Since most of the surfactant systems used in industry are either mixture of surfactants, or contain significant amount of impurities, surface or interfacial values associated with these systems have little meaning. Most often these measurements are carried out without purpose, for there is usually no apparent relation between surface or interfacial tension and the formulation parameter of concern. Whenever there is a really need to determine surface or interfacial tension, the Wilhelm plate apparatus is the instrument of choice for rapid and accurate determinations. No correction factors are necessary, and wetting problems are usually absent. If any event, surface or interfacial tension should simply be looked on as a mathematical approximation of surface or interfacial free energy. The principal problem for formulations is natures continuous attempt to reduce this positive interfacial free energy value to zero by various means. One approach is simply to reduce the amount of interface; that is a twofold reduction of the amount of interface results in a similar reduction in the interfacial free energy of the product. For example, when emulsion droplets collide they can either bounce away or coalesce into larger droplets, ultimately leading to the destruction of the emulsion. The later event will result in a reduction of interfacial free energy and, unless barriers are placed in the way, will occur with each collision (Nguyen, 1978).Thus in the absence of an emulsifying agent, oil and water will separate almost instantaneously. Most often compounders are not changing the ultimate thermodynamic fate of the product by altering the formulation, but merely

changing the thermodynamic path which in practical terms, means increasing the self-life of product. Another important method that nature uses to reduce interfacial free energy is to vary the composition of the interface to make it rich in surface active material, and poor in highly polar compounds (e. g. water; a surface active agent or surfactant contains at least one prominent polar group and one prominent non polar group). This mechanism is advantageously used by introducing materials into the formulation that concentrate at the oil-water droplet interface and present barriers to droplet coalescence. The principal mechanism by which emulsifiers stabilized emulsions is not a reduction of interfacial free energy of the system, but involves the introduction of a mechanical barrier to delay the ultimate destruction of the system. Although the concentration of surface active emulsifier is greater at the oil water interface than in either of the bulk phases, most of the emulsifier molecules are in the water phase or in oil phase, and not at the emulsion droplet interface. A reduction of the interfacial free energy probably does help somewhat in the ease of preparing the emulsion, but it is not a major factor for long term stability. Finally, proper orientation of the molecules at the interface (Polar groups directed towards the water phase and non polar groups directed toward the oil phase) further reduces interfacial energy. It is extremely important for the formulator to keep in mind that, throughout the processing of the formulation (Whether by simple mixing with a stirring rod or the use of high-energy shear equipment), the emulsifier molecules are continuously partitioning between the bulk phases and the interface, and are continuously changing their orientation at the interface. Moreover, when a combination of emulsifier is used, the ratio of the hydrophilic and hydrophobic emulsifier at the interface continuously changes during the preparation of the emulsion (Galenko, 2007). Since equilibrium is never established, the final configuration is very much a function of processing the emulsion in such a manner that small changes in processing and storage variables do not result in large changes in the properties of the emulsion, are extremely important considerations.

Rheology of Anhydrous Nanoemulsion

Rheology deals with the flow of liquid and deformation of solids in anhydrous nanoemulsion. It is extremely important parameter for formulation and application of dispersion system. Anhydrous nanoemulsionis a viscous dispersion of oil, organic solvent or polyols and surfactant (Amovilli, 1997). However, it is widely administered through topical, oral and parenteral route. Surfactant concentration plays vital role to maintain and determine flow behavior of formulation. Rheological behavior of anhydrous nanoemulsion system influence stability and spreadibilty of formulation.

Surfactant and mixture of surfactants are major component of anhydrous nanoemulsion and it may cause system to flocculate (Beveridge, 1989). The flocculation of dispersion nanoemulsion globules by high concentration of surfactant is believed to be due to depletion force. The depletion force in anhydrous nanoemulsions is an attractive force that arises between colloidal particles that are dispersed in to continuous phase. Dispersed particle in an anhydrous nanoemulsion containing free polymer approach each other to within a distance that is smaller than the diameter of the polymer molecule (Tatar, 2017). This phenomenon results in an attractive force between the particles due to lowering of osmotic pressure in the region between the particles, and consequently flocculation of particles occurs. In the case of nanoemulsions, it is the exclusion of surfactant micelles between approaching nanoemulsion droplets that results in flocculation. Suitable rheological behavior of any colloidal dispersion determined applicability of formulation (Hamed, 2016).

Component of Anhydrous Nanoemulsion

Anhydrous naoemulsions are water free dispersion system where poorly aqueous soluble anticancer drugs are incorporate to achieve controlled release (Komaiko, 2016). The major components of anhydrous nanoemulsions are:

1. Oil
2. Organic solvents or Polyols
3. Surfactant/Co-surfactant
4. Drug
5. Additives and miscellaneous

Oil

(A) Anhydrous nanoemulsion contains organic oils such as natural oils derived from animal, vegetable, or mineral sources, are suitable. Common organic oils also safe and used for preparation of anhydrous nanoemulsion. Examples are, cacao butter (Theobroma oil), carrot seed oil, castor oil, citrus seed oil, coconut oil, corn oil, cottonseed oil, cucumber oil, egg oil, jojoba oil, lanolin oil, linseed oil, mineral oil, mink oil, olive oil, palm kernel oil, peach kernel oil, peanut oil, rapeseed oil, safflower oil, sesame oil, shark liver oil, soybean oil, sunflower seed oil, sweet almond oil, tallow (beef) oil, tallow (mutton) oil, turtle oil, vegetable oil, whale oil, and wheat germ oil(Jadhav, 2015).

(B) Medium Chain Triglycerides, Fatty Ester (Ethyl palmitate)

(C) Nonvolatile polydimethylsiloxanes having a viscosity generally in the range of around five to about thousand centistokes, and fragrances of myrrh and musk.

Organic Solvents or Polyols

When the nanoemulsions are intended for Pharmaceutical and personal care application, then the organic solvent or polar solvent should be one recognized as universally acceptable in preparation of anhydrous nanoemulsion dispersion system of poorly aqueous soluble anticancer drugs (Hejazifar, 2016).In a preferred embodiment, polar liquid that exhibits a dipole moment of from 0.9 to 4.5should be selected. Some acceptable anhydrous polar organic liquids, can be used, for example, in a preliminary preferred incarnation polar hydroxylic solvents. They are one or more of glycols, alcohols, polymeric glycols, polyhydric alcohols and mixtures thereof. Preferably, the polar solvent contains monohydroxy alcohol, such as ethanol, propyl alcohol and iso-propyl alcohol, a diolsandtriols, such as propylene glycol, dipropylene glycol, tripropylene glycol, butyleneglycol, iso-butylene glycol, 2- and methyl-3-propane diol, a polyhydric alcohol, such as glycerin erythritol and sorbitol, or a polymeric glycol, such as polyethylene glycol, polypropylene glycol mono alkyl ethers and polyoxyalkylene copolymers(Sabale, 2012). In a highly preferred embodiment, the polar liquid is selected from ethanol, propyl alcohol, isopropyl alcohol, propylene glycol, dipropylene glycol, tripropylene glycol, butyleneglycol, iso-butylene glycol, 2-methyl-3-propane diol, glycerin, erythritol sorbitol, polyethylene glycol, polypropylene glycol mono alkyl ethers.

Surfactant

Surfactant act as a stabilizing agent in anhydrous nanoemulsion dispersion system. Rationale of stable anhydrous nanoemulsion is that it should be surfactant based. Surfactant is principle component of anhydrous nanoemulsion. Stability of anhydrous nanoemulsion depend upon type and concentration of added surfactant in to formulation. Various type of surfactant like anionic, cationic, nonionic, ampholytic and Zwitter ionic used to prepare stable anhydrous nanoemulsion dispersion system. Various surfactants were screened and identify by using visual assessment for their ability to form anhydrous dispersion systems with medium-chain and long-chain triglycerides. The productive systems were formed by surfactant and co-surfactant with mostly unsaturated acyl chains, silicone-containing emulsifying agents, emulsifying agents derived from sorbitan compounds and emulsifying agents derived from fatty alcohols and polymeric emulsifiers. Amongst these the most efficient were oleates with HLB values of approximately 11. Polysorbate and Sorbiton monostearates are very popular and useful for nanoemulsion dispersion (Klang, 2010).Apart from above popular surfactant some versatile surfactant listed below:

Table 1. Anionic Surfactant Used to Prepare Stable Anhydrous Nanoemulsion

1-Octanesulfonic acid sodium salt	**1-Octanesulfonic acid sodium salt**
Chenodeoxycholicacid	Cholic acid from oxorsheep bile
Dehydro-cholic acid	Glycocholicacidhydrate
Glycodeoxycholicacidmonohydrate	Sodium dodecyl sulfate
Sodium lycolcholatehydrate	Sodium cholatehydrate
Sodium taurolithocholate	Lithium dodecyl sulfate
Lithium 3,5-diiodosalicylate	Taurocholicacid sodium salt hydrate

Table 2. Cationic Surfactant Used to Prepare Stable Anhydrous Nanoemulsion

Amproliumhydrochloride	1-Octanesulfonic acid sodium salt
Benzalkonium chloride	Cyclohexylmethyl β-D-maltoside
Benzethoniumhydroxide	Hexaethyleneglycolmonodecylether
Benzyl dimethyl hexadecyl ammonium chloride	Methoxypolyethyleneglycol
Dimethyldioctadecylammoniumbromid	N-Decanoyl-N-methyl glucamine
Hexadecyl pyridinium bromide	Pentaethyleneglycol mono dodecyl ether
Hexadecyl pyridinium chloride monohydrate	N-Nonanoyl-N-methyl glucamine

Preformulation Study

A preformulation study is a preliminary study prior to making any final permanent formulation. In the preformulation study, apart from the active drug, all the ingredients are used to make the final dosage form. Drug substances contains active property and mobility. It determines the whole formulation and vitality of nano dispersion system. In present study solubility of drug is core issue during formulation

Table 3. Nonionic Surfactant Used to Prepare Stable Anhydrous Nanoemulsion

Ethyleneglycol Mono decylether	Diethyleneglycolmonohexylether
Ethyleneglycol mono dodecyl ether	Heptaethyleneglycol mono decyl ether
Ethyleneglycol mono hexadecyl ether	Decyl-β-D-1-thiogluco pyranoside
n-Dodecyl β-D-glucopyranoside	N-Octanoyl-N-methylglucamine
Poly(ethyleneglycol)diglycidylether	Sucrosemono decanoate
Polyoxyethylene10 tridecylether	Tween (Polysorbate)20,21,40
Polyoxyethylene80 stearate	Span20,40,60, 65,80

and preparation of system (Fofaria, 2016). Authors have taken poorly aqueous soluble anticancer drug i.e. paclitaxel, etoposide and docetaxel to incorporate in to anhydrous nano system. The objective is to enhance stability and solubilization of drug in to water free dispersion system for patient's compliance. To make safe and effective dosage form. It would be possible to vary the formulation parameters according to the preformulation characteristics of the drug and other formulation excipients. An insight in to such parameters allows formulation to select the various additives and formulation conditions for the successful and effective drug delivery to the biological system(Ma, 2013).Therefore, a preformulation study of the selected model drugs paclitaxel, etoposide and docetaxel was carried out which included test for identifications (infrared spectra and ultra violet maxima), solubility analysis, partition coefficient and drug compatibility with the formulation additives. Since anhydrous nanoemulsions are dispersion system of oil, polar solvent and surfactant therefore essential to perform primary rheological behavior of added ingredients.

Preformulation studies of anhydrous nanodispersion requires to secure the design and development of a stable, safe and therapeutically effective dosage form. It is a stage of development during which the physical pharmacist characterizes the physiochemical properties of the drug substances and its interaction with various formulation components. The preformulation is the phase of development that decides about the formulation conditions related to process variables and selection of compatible ingredient (Debnath, 2011).

Emulsifying Effect of Various Surfactants on Anhydrous Nanoemulsion

The present chapter focused on to develop stable anhydrous nanoemulsion by using different oil, polar solvents/polyols and surfactant combination, exploring also the possibility of using such systems as nanoemulsion vehicles for controlled drug release. To achieve stability of anhydrous nanoemulsion, designing and searching a suitable oil-immiscible polar solvent and polyols that can substantiallyreplace water using existing surfactants (Heiati, 1998). Anhydrous dispersion system for hydrophobic drug requires surfactant, co-surfactant and various combination of surfactant to get complete emulsification effect. Emulsifying effect experiment is a well-organized trial and error procedure carried out with the aim ofverifying or establishing the validity of a hypothesis. Experiments provide insightinto cause-and-effect by demonstrating what outcome occurs when a particular factor is practically performed. In present investigation attempt is aimed to achieveemulsifying effect of several surfactants for stable anhydrous nanoemulsion system(Kabalnov, 1992).

Example: Emulsifying effect of various surfactants on the stability of glycerin- olive oil anhydrous system. Detailed study and observation given in table no. The system employed in experiment consisted of equal weights of glycerin and olive oil with a variable concentration of surfactants. The glycerin and olive oil used were I.P. grade. Every reasonable precaution was taken to avoid undue exposure of these reagents to the atmosphere. The glycerin was heated to 180^0C and sealed to insure the absence of water. Therequiredquantitiesofpolymericsurfactantwereweighedandaddeddirectlytoaweighedquantityofglycerin. This mixture was then sonicated in bath sonicator (JP-040S, Japan) for 20minutes.The oil was added drop wise with continuous Sonication (JP-040S, Japan), to form the anhydrous system. After addition was complete, the prepared anhydrous nanoemulsion was allowed to stand. Appearance and period of anhydrous nanoemulsion stability was noted. The emulsions were than examined for emulsifying effect of various surfactants on the stability of anhydrous nanoemulsion system (Riess, 2004). Given in Table 4.

Table 4. Emulsifying effect of various surfactants on the stability of glycerin- olive oil anhydrous system

SN.	Agent	%	Method	Emulsion/Appearance
1.	Polysorbate20 (Tween 20)	1% 0.5 3% 5%	Emulsification Emulsification Emulsification Emulsification	Clear/Separate Clear/Separate Clear Clear
2.	Polysorbate60 (Tween 60)	1% 0.5 3% 5%	Emulsification Separation Separation Emulsification	Opaque --------- Separate/Clear Clear
3.	Polysorbate40 Tween 40	1% 0.5 3% 5%	Emulsification Separation Separation Separation	Opaque ---------- ---------- ----------
4.	Polysorbate80 Tween 80	1% 0.5 3% 5%	Emulsification Part. Separation Emulsification Emulsification	Clear Opaque Opaque Opaque
5.	Ethanolamine 2-Aminoethanol	1% 0.5 3% 5%	Part. Emulsification Separation Separation Separation	---------- Semi solid Opaque Clear
6.	Sorbitan monostearate (Span 80)	1% 0.5 3% 5%	Separation Separation Emulsification Emulsification	Opaque Opaque Opaque Opaque
7.	Sodium Lauryl SO_4	1% 0.5 3% 5%	Part. Emulsification Separation Part. Separation Emulsification	Opaque Opaque Clear Clear
8.	Benzalkonium Chloride	1% 0.5 3% 5%	Separation Separation Separation Separation	---------- ---------- ---------- ----------

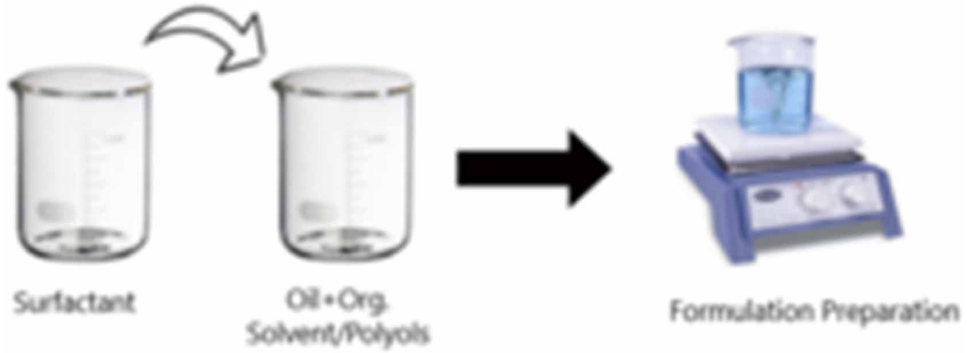
Method of Preparation

1. Sonication Method
2. High Pressure Homogenizer

Sonication Method

This method is useful for preparation on small batches of anhydrous nanoemulsions. Sonication method worked on principle of ultrasonic wave in which droplets or globule size of anhydrous nanoemulsions minimized. It reduces the droplet size of traditional emulsion. Probe and bath sonicators are used to prepare anhydrous nanoemulsions. Probe sonicator used for bulk level preparation and bath sonicator used for small scale or laboratory level preparation. Fine and uniform droplets of dispersion found in this method. Temperature maintained at room temperature during preparation. After complete formation of dispersion, samples are preliminary observed under high resolution microscope for size and shape determination further it goes through electron microscopy. Measured amount of oil and polar solvent or polyols are taken in suitable glass container. The ingredients of the formulations were listed in (Table 6.4 to 6.9) Drug was first pre-dissolve in measured volume of polar solvent, and all ingredient including oil and surfactants, co- surfactant incorporated into polar solvent-drug mixture and all ingredients were sonicated using a probe or bath sonicator (JP-040S, Japan). Meanwhile concentration of surfactant and co-surfactant should be checked to stabilize formulation. Subsequently, increase the sonication of formulation for 5 mnt. to complete the emulsification.

Figure 5. Method of preparation



High Pressure Homogenizer

In high pressure homogenizer method, high pressure is applied over the combination having oil phase, polar solvent or polyols phase, and surfactant or cosurfactant with the help of homogenizer. Due to high energy involved during preparation, it also called high energy homogenizer method. It is used for the preparation of anhydrous nanoemulsion of less than 20% oil phase. To one side from being choosy more problems associated with this experimental method include poor yield and ingredient decline due to generation of too much heat.

Large unruly forces are provided by the use of high energy homogenizers which produce globules of smaller size. The globule size depends on the type of equipment, conditions during production, such as temperature and time, as well as the characteristics and composition of sample. This method consumes large amount of energy with high pressure; therefore, they are much costly. The major advantage of high pressure homogenizer method is that they allow good control of globules or droplet size with stability carrier system (Jasmina, 2017).

Preparation of Paclitaxel Containing Anhydrous Nanoemulsion Formulation (Glycerin-Olive Oil-Surfactant-Drug)

Paclitaxel was first pre-dissolve in glycerin, and all ingredient including olive oil and polysorbate 20 (Tween20) or polysorbate 80 (Tween80) incorporated into glycerin-paclitaxel mixture. Oil phase added slowly with continuous sonication and prepared dispersion were sonicate using a probe or bath sonicator for 15 mnt. Subsequently, increase the sonication of formulation for 5 mnt to complete the emulsification. To test the stability of anhydrous nanoemulsion, prepared formulation were centrifuge at 5000 rpm for 20 mnt to check their stability. Composition given in Table 5.

Table 5. Anhydrous nanoemulsion formulation of two different system

S. No.	Formulation	Ingredients			
System I		paclitaxel	Glycerin	Olive oil	polysorbate20
1.	Anh. Naoemulsion 1	3%	40%	35%	22%
2.	Anh. Naoemulsion 2	3%	45%	32%	20%
3.	Anh. Naoemulsion 3	3%	38%	45%	14%
System II		Indomethacin	Glycerin	Olive oil	polysorbate80
1.	Anh. Naoemulsion 1	3%	42%	50%	5%
2.	Anh. Naoemulsion 2	3%	45%	49%	3%
3.	Anh. Naoemulsion 3	3%	45%	50%	2%

Preparation of Docetaxel Containing Anhydrous Nanoemulsion Formulation (PEG-Castor Oil-Surfactant-Drug)

Docetaxel was first pre-dissolve in glycerin, and all ingredient including olive oil and sorbiton mono-stearate 20 (Span 20) or polysorbate 60 (Tween 60) incorporated into PEG-paclitaxel mixture. Oil phase added slowly with continuous sonication and prepared dispersion were sonicate using a probe or bath sonicator for 15 mnt. Subsequently, increase the sonication of formulation for 5 mnt to complete the emulsification. To test the stability of anhydrous nanoemulsion, prepared formulation was centrifuge at 5000 rpm for 20 mnt to check their stability. Composition given in Table 6.

Table 6. Anhydrous nanoemulsion formulation of two different system

S. No.	Formulation	Ingredients			
System I		docetaxel	PEG	Castor oil	Sorbitan monostearate 20
1.	Anh. Naoemulsion 1	3%	32%	40%	25%
2.	Anh. Naoemulsion 2	3%	38%	29%	30%
3.	Anh. Naoemulsion 3	3%	40%	20%	37%
System II		docetaxel	(PEG)	Castor oil	Polysorbate 60
1.	Anh. Naoemulsion 1	3%	42%	50%	5%
2.	Anh. Naoemulsion 2	3%	45%	49%	3%
3.	Anh. Naoemulsion 3	3%	45%	50%	2%

Evaluation Parameters of Anhydrous Nanoemulsion

Anhydrous Nanoemulsionsareevaluate for safe and efficient dosage form in terms of many physico-chemical parameters like size and shape, viscosity, zeta potential, drug release, percentage entrapment and stability etc. (Baspinar, 2010).

MORPHOLOGICAL CHARACTERISTICS

The surface morphology of anticancer drug loaded anhydrous nanoemulsion was determined by using high resolution and electron microscopy. Transmission Electron Microscopy used for proper size distribution and specific shape determination.

Vesicle size and size distribution of optimized formulation was determined by photon correlation spectroscopy by using Delsa Nano C Zeta Sizer (Beckman coulter Delsa™ Nano C, USA). For the measurement of globule size.

Zeta Potential for Surface Charge

Surface charge on droplets determines the physical stability of anhydrous nanoemulsion. Surface charge on droplets is quantified as zeta potential value which is measured via electrophoretic mobility of particles in an electrical field. Malvern Zetasizer ZS (Malvern Instruments, Worcestershire, UK) by laser Doppler electrophoresis is a popular and potential instrument used to determine surface charge (Honary, 2013). The core objective to perform zeta potential study is to provide information on the repulsive forces between globules in the dispersion system. It helps to avoid coagulation or aggregation of thousands of smaller globules or droplets in system.

Viscosity Measurement

Anhydrous nanoemulsions are viscous dispersion of nano range molecules therefore viscosity is important parameter to characterize dispersion system. Viscosity influence formulation and application of

anhydrous nanoemulsion. Variety of equipment and instruments are employed for measuring viscosity such as Brookfield viscometer, Ostwald viscometer, falling ball viscometer, Stormer viscometer, and Ferranti-Shirley viscometer. Among all Brookfield is the preferred one for measuring the viscosity of anhydrous nanoemulsion dispersion(Pal, 2016). Viscosity of the anhydrous nanoemulsion dispersion was measured as such without dilution using rotational viscometer RheolabQC (Anton PaarGmbh, USA).

Measurement of Encapsulation Efficiency

To know the amount of drug entrapped in the nanoemulsion formulation, weighed amount of formulation is dispersed in organic polar solvent by ultrasonication and the drug is extracted into suitable buffer. Drug content is estimated by analyzing the extract spectrophotometrically at λmax of drug after making suitable dilutions against suitable blank. The entrapment efficiency (EE) and loading efficiency (LE) of the drug can be calculated by using the following Eqns.

drug EE = drug content in the product obtained (mg)/total amount of drug added (mg)×100 and drug LE = drug content in the product obtained (mg)/total product weight (mg)×100. Drug content could also be determined using reverse phase high-performance liquid chromatography (HPLC) techniques. Singh et al. employed this technique for finding primaquine concentration and reported 95% encapsulation efficiency of formulated nanoemulsion (Gurpreet, 2018).

Determination of Drug Content

Drug containing anhydrous nanoemulsions were dissolved in 100 ml of 0.1 NaOH and ethanol in volumetric flask. The drug was allowed to dissolve in the solvent and centrifuged it up to 5000 rpm for 15 mnt. 1 ml centrifuged sample was taken in 50 ml of volumetric flask and diluted up to mark with 0.1 NaOH and ethanol, and analyzed spectrophotometrically at suitable nm. (Kumar, 2014). The concentration of drug was obtained by using standard calibration curve of the drugs. Drug content studies were carried out in triplicate for each formulation.

In-vitro Drug Release Study

The in-vitro drug release studies of drug from various anhydrous nanoemulsion formulation were determined to evaluate the effect of the formulation variables. The drug release studies were performed using franz diffusion cells fitted with 0.45 μ cellulose acetate membrane (Sartorius)at37±0.1°Cusing a thermostatic water pump. (Cyberbath, CB 2000, USA) The effective diffusion area was 2.54 cm^2 (18 mm orifice diameter), and the receptor compartment was filled with 13.5 ml of phosphate buffer pH 7.4. The receptor fluid was constantly stirred by externally driven Teflon coated star headmagnetic bars. Accurately weighed drug was placed in the donor compartment. Samples (0.5ml) were withdrawn from the receptor fluid at predetermined time interval for up to 9 hrs after the application. An equal volume of the fresh phosphate buffer was immediately replenished after each sampling to adjust the sink condition. Samples were filtered, diluted and assayed at each interval for drug content released at λmax of different nm using double beam UV-Spectrophotometer (Shimadzu 1800, USA). The amount of drug present in the samples was calculated with the help of appropriate calibration curve constructed from reference standards. (Drais, 2015). The drug release experiments were performed in triplicate for each

batch (n=3) in order to minimize the variation error. Drug released at specified time periods was plotted as percent drug release vs time (mnt.) curve.

Stability Study- Effect of Temperature in Growth of Droplet Size

The prepared anhydrous nanoemulsions were carefully studied for any positive signs of separation. Two of the agents (surfactants) were selected to stabilize the each anhydrous nanoemulsions systems for the extended time-temperature study of droplet size growth. The surfactant concentrations employed were determined as the lowest concentration producing stability for at least maximum days at room temperature.

In extended time-temperature study of droplet size growth for system Glycerin-Olive oil with Polysorbate 20 and polysorbate 80 surfactant; six solutions of the surfactants were prepared. The agents used were polysorbate 20 and polysorbate 80. The concentration of polysorbate 20 employed were 2, 3 and 5 ml, while 10, 15 and 20 ml of polysorbate 80 were employed. All anhydrous nanoemulsions for given concentration were prepared at the same time. This allowed for a more meaningful observation of droplet size change (Ali, 2013).

The temperatures involved were 0^0C, 25^0C, 35^0C and 45^0C three seats of anhydrous nanoemulsions were prepared to represent each temperature and surfactant concentration to be used in the study. The three seats were carefully compared for the first seven days of the study period after which one set of anhydrous system was used for the rest of the study. The temperatures were maintained with in $\pm$ 20C for 120 days. Above procedure used to perform stability study with time-temperature effect on growth of droplets.

Application of Anhydrous Nanoemulsion

Developed anhydrous nanoemulsions are clear, transparent, thermodynamically stable dispersion of polar solvent or polyols in oil, stabilized by various surfactant, co-surfactant and mixture of surfactants. It showed great potential in the field of pharmaceuticals like.

- Water free liquid preparation of a number of drugs. Versatile vehicle for poorly aqueous soluble drugs. It has capability to easily incorporate hydrophobic drug within anhydrous nanoemulsion system and provide best carrier system for poorly aqueous soluble anticancer drugs.
- Due to its highly dispersibility property and ease of penetration into various layers of skin the developed preparation are suitable for topical applications.
- Anhydrous nanoemulsions are also suitable vehicle for lipophilic drugs.
- Best suited for topical and pediatric dosage form.
- Greater efficacy with minimum side effect.
- The components of formulations are economical and manufacturing equipments are easily accessible.
- Clear, transparent, thermodynamically stable dispersion of polar solvent or polyols in oil, stabilized by surfactant and mixture of surfactants provides stable preparation over an extend period of time.
- Avoidance of phase separation.
- During the investigation, it was found that all the ingredients were compatible with each other and showed versatile reservoir vehicle.

- Anhydrous nanoemulsions showed novel system of delivery for poorly aqueous soluble drugs with controlled release pattern.
- All the prepared formulations were explored sustained release profile.
- All the characterization parameters of anhydrous nanoemulsion systems were similar to other carrier of novel and controlled systems.
- Anhydrous nanoemulsions are vital for delivery of cosmetic substances. Due to smaller size of system it has potential application in cosmeceuticals.
- Anhydrous nanoemulsions are best suited for vaccine and ocular delivery.

CONCLUSION

Anhydrous Nanoemulsions offer many advantages. Anhydrous Nanoemulsions specially designed and developed for delivery poor aqueous soluble drugs and it is best suited for it. It plays versatile vehicle for number of drugs and phytoconstituents. Anhydrous Nanoemulsions are applicable for almost all routes of delivery therefore used as a promising drug delivery for different fields including ocular, vaccine, cosmetics and biotechnology. Anhydrous Nanoemulsions developed to overcome various problem and disadvantages associated with traditional emulsion and water containing novel microemulsions. Its water free preparation provides better stability for water unstable drugs.

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Chapter 15
Precision Medicine in Cancer

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ABSTRACT

Cancer is the one of the deadliest diseases and takes the lives of millions of people every year across the world. Due to disease heterogeneity and multi-factorial reasons, traditional treatment such as radiation therapy, immunotherapy, or chemotherapy are effective only among a small population of the patients. Tumors can have different fundamental genetic causes and protein expressions that differ from one patient to another. This variability among individual lends itself to the field of precision and personalized medicine. Following the completion of human genome sequencing, significant progress has been observed in the characterization of human epigenome, proteome, and metabolome. Pharmacogenetics and pharmacogenomics use this sequence to study the genetic causes of individual variations in drug response and the simultaneous impact of change in genome that decide the patient's response to drug respectively. On summation, identify the subpopulation of patient and provide them tailored therapy

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thus increasing the effectiveness of treatment. All these evolved the field of precision or personalized medicine that plays a crucial role in cancer prevention, prognosis, diagnosis, and therapeutics. These tailored therapies are characterized by increased efficiency and reduced toxicity. Not all cancers have genetic variability; some are also influenced by polymorphism of gene encoding enzymes that play an important role in pharmacokinetics of drug. The discoveries of cancer predisposition genes allow diagnosis of a patient at risk of cancer development and let them make the decision on précised individual risk modification characteristic. The use of CYP2D6 genotyping for breast cancer, mutation in KRAS in colorectal cancer, genomic variation in EGFR in small lung cancer, melanoma are some of the examples of importance of cancer predisposition genes. In recent times, distinct molecular subtypes of cancers have been identified with requirement of different treatment for each subtype. Precision medicine shifts the trend from reaction to prevention and forestalls disease progression.

INTRODUCTION

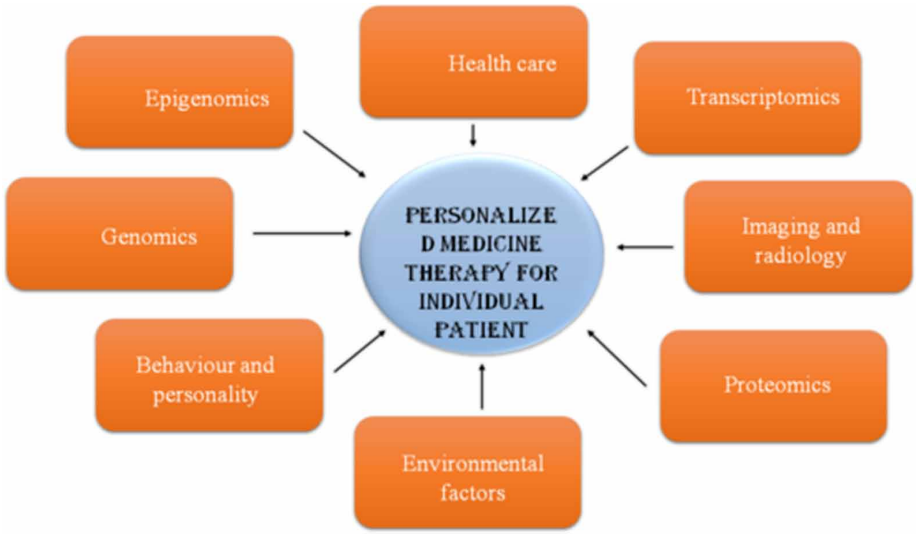
Cancer is a multi-factorial disease that originates in any organ or tissue in the body when abnormal cells grow uncontrollably and migrate to adjoining parts of the body. The latter process is called metastasis and is the major cause of death. According to WHO, cancer is the second leading cause of death accounting about 9.6 million deaths in 2018. Most common cancer in women are breast, colorectal, cervical, thyroid and lung cancer whereas, common cancer in men are lung, colorectal, stomach, prostate and liver cancer (WHO, 2018). A tumor may be benign or malignant. Benign tumor or neoplasm remains confined to the original location, whereas malignant tumors have the property of invasion to the nearby tissue and metastasize to other body parts. Only malignant tumor is properly referred to as cancer (Cooper et al., 2000). These are classified according to the type of cell they arise from. Most of the cancer falls into six major categories: carcinoma, sarcoma, myeloma, leukemia, lymphoma and mixed types. Carcinomas include 90% of cancers are of epithelial origin. Sarcomas refer to tumors originated from connective tissue and supportive tissue such as muscle, bone, cartilage and fibrous tissue. Myeloma and leukemia refer to cancer in the blood cells originated from the bone marrow. Lymphoma develops in the glands and nodes of the lymphatic system and mixed types include tumors from different categories (Kindt et al., 2007). Development of cancer is a multistage process: tumor initiation, promotion, and progression. Some of the carcinogenic agents that cause cancer include radiation, chemical, hormone and viruses. Radiation and chemical carcinogens cause DNA damage and induce mutations in the cells (Koeffler et al., 1991). These carcinogens are called initiating agents. Some of the examples of initiating agents are UV radiation, tobacco smoke and particulate matters such as asbestos (Barnes et al., 2018). Hormones particularly estrogens, are important tumor promoters, with proliferation of cells of uterine endometrium leading to endometrial cancer and mammary gland causing breast cancer. Out of total human cancer cases, 20% constitute virus oncogenesis (Rodriguez et al., 2019). Seven viruses are associated with human cancer and considered to be oncogenic viruses. These include Hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus (HPV), Human herpes virus 8 (HHV8), Human T-lymphotropic virus type-1 (HTLV-1), Epstein Barr virus (EBV) and Merkel cell polyomavirus (MCPyV). The molecular mechanism of viral oncogenesis include induction of chronic inflammation, disruption of host organism genetic and epigenetic homeostasis, intrusion in DNA repair mechanism causing genetic instability and cell cycle dysregulation by insertion of viral genome into the host genome. The viral oncoproteins upon

expression modulate the cellular signaling pathways and alter the gene expression (Luo et al., 2015). Treatment for this disease depends upon the diagnosis and the current grade or stage of cancer. The commonly used cancer treatments constitute chemotherapy, radiation therapy, surgery, immunotherapy, hormone therapy, stem cell transplant therapy and targeted & precision medicine (Abbas et al., 2018). The most common treatments used include surgery, radiation and chemotherapy. All these therapy have their respective side effects. Precision medicine is a kind of medication where cancer patient are given right drug at right dosage with minimal toxicity on the basis of their genetic makeup (Verma et al., 2012). Precision medicine considers the pharmacogenetics of individual patient i.e. the variability in drug response due to variation in the DNA sequence and the pharmacogenomics which spotlights on the identification of molecular determinants at the genome, transcriptome, and proteome levels, typically via a change in drug pharmacokinetics or via alteration in the pharmacodynamics. (Mini & Nobili et al., 2009). This chapter discuss about the art of precision medicine in the field of oncology.

WHAT IS PRECISION OR PERSONALIZED MEDICINE?

The center for disease defined personalized medicine as an approach for protecting and treating a disease that works on the basis of a person's genome, environmental condition, and behaviors. The national research council explained that precision medicine can be explained as tailoring of medical treatment according to the individual characteristics of every patient. All these definitions direct towards a 'person-centered approach' (Maier et al.,2019). Thus precision translates to a person-centered approach. Precision medicine classifies individuals into subpopulations based on their difference in susceptibility to disease, diagnosis or prognosis of the disease, their response to specific treatments and minimizes the adverse effects due to drugs (Fig. 1).

Figure 1. Factors contributing to Personalized Medicine



HISTORY

Ancient Persian sages like Avicenna were aware of the concept of personalized medicine and used different patterns to recognize patterns in an individual to select medications to be administered (Moeini et al., 2017). This variability in drug response came to spotlight in early 1950s which led to the new discipline with consideration of genetics, genomics, pharmacology and biochemistry known as pharmacogenetics and pharmacogenomics. Upon commercialization, this concept was tagged as personalized medicine (Vogenberg et al., 2010). With the completion of Human Genome Project (HGP) by 2003, mapping various genes in disease lead to increased pace in designing effective medicine while causing least adverse effects of medications for different subset of people (Yu et al., 2017, McGonigle et al., 2016).

THE NEED FOR PRECISION MEDICINE IN CANCER TREATMENT

The development of chemotherapy for cancer treatment was lauded but it was not functional for all patients and for types of cancer, also the relapse of the cancer was another drawback of chemotherapy and these drawbacks on part of traditional cancer treatment strategies lead to the evolution of personalized medicine in cancer treatment (Turnbull et al., 2015). Cancers are characterized by the accumulation of genetic variability in the genes and the alteration of the molecular pathways they control. Knowing the genetic variation among the genes involved in metabolizing drugs may help in understanding the toxicity of given drugs and the an individual's specific response to them (Pinto et al., 2011). Through recent research, we come to know that no two individuals have the same genetic makeup of cancer. So, based upon the "omics" data from human genome sequencing the precision medicine may be able to develop a unique treatment for each subtype of cancer (Doisneau-Sixou et al., 2014). Thus, the integration of PM into cancer research helps fight against cancer.

STRATEGY

The modern day approach for the development of personalized medicine (Figure 2) includes, collecting data for the variability in gene makeup, metabolism and environmental stress for different subsets of patients and cancer types, followed by integration of this data with the ongoing research for identification of most suitable target for the drugs to be developed.

DATA COLLECTION FOR PM

With the completion of human genome project (HGP), knowing the genetic variations in a patient helps in determining, who is at risk of developing a cancer? The study about these variations is generalized with the term "Omics". Omics include genomics, transcriptomics, proteomics and metabolomics (Lowe et al., 2017).

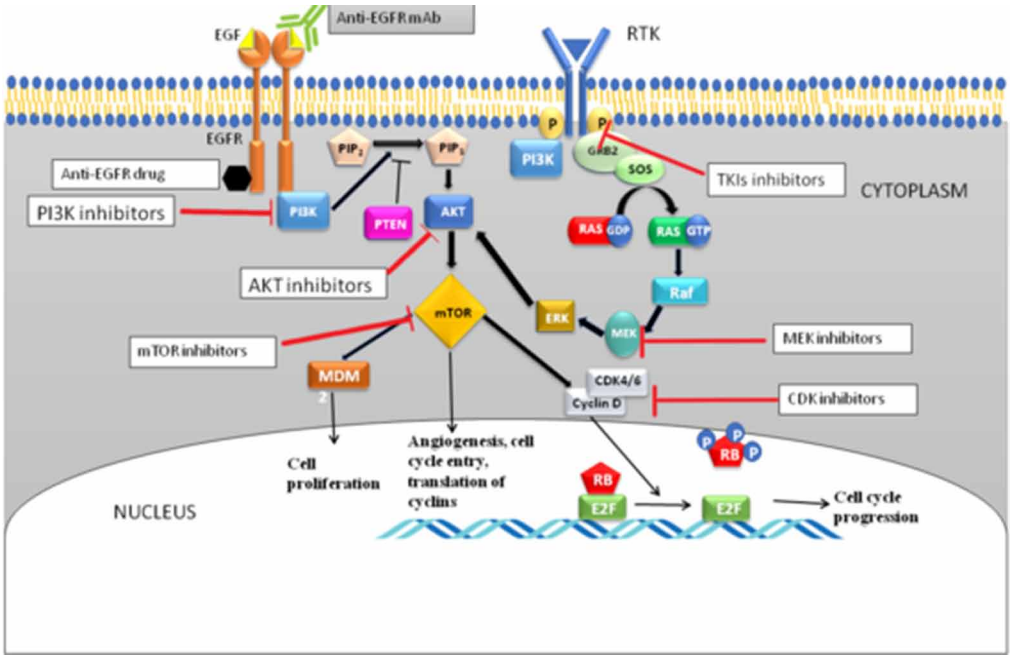
Genomics can be defined as the characterization of whole human genome sequence. With the advancement of technology, the cost and time required for mapping whole genome of an individual has become easy. By referencing the personal genome and personal medical records, more proactive therapy could

be designed (Tremblay et al., 2013). Though genomic data is important, there is a need to fill in the gap between the genotypic effect and the phenotypic event to understand the drug action in physiological. Transcriptomics deals with the total mRNA quantity within the sample and it is done through the high-throughput techniques including microarray and RNA sequencing (RNA-Seq) methods (Parente et al., 2005). Proteomics refers to the measuring concentration of proteins, protein structure, cellular localization, protein-protein interactions and post-transcriptional modifications at a particular time in a cell. Data on post transcriptional modifications (PTM) and the expressions of proteins in tissue are important for early disease diagnosis, prognosis and to analyze the disease progression (Pandey et al., 2000).

Metabolomics refers to the analysis of metabolite, a small intermediate product in a reaction. Metabolites were analyzing as they reflect the effect of genetic and environmental influences (Clish et al., 2015). Metabolomics, along with other omics is a promising technique for PM.

Epigenomics deals with the characterization of epigenetic modifications (DNA methylation and histone modifications) as they influence and regulate the gene expression without any change in respective DNA sequence. Techniques used in analysis of DNA methylation include methylation-specific PCR, sequencing bead array, bisulfide conversion etc., and analysis of histone modifications is done by chromatin immunoprecipitation (ChIP) followed up by Quantitative PCR (Kronfol et al., 2017). The obtained data defines the location and regulation of genes and their promoters, enhancers and repressor, noncoding transcripts. This detailed information is made into epigenomic map (Bernstein et al., 2010).

Figure 2. A flowchart of strategies in personalized medicine



PREPARATION OF PERSONALIZED MEDICINE

Once data have been collected and stored, they need to be analyzed in order to identify biomarkers, aberrant pathways in disease, mutations and treatment outcome. The tools allow integration of multi omics data for disease subtyping, prediction of diagnostic biomarkers and study on disease biology. The approaches used by these tool use similarity, networking, fusion, Bayesian, correlation based and multivariate methods. The portals/tools used to interpret and visualize omics data include GENEASE, firebrowse, linked omics, UCSC xena, cBioPortal, 3Omics etc. (Subramanian et al., 2020, Vasaikar et al., 2018).

Majority of omics tests are used to validate and predict the biomarkers which has not yet become successful for the use of test in clinical trials (as bioassay). Institute of medicine in adherence with U.S. National cancer institute established guidelines, for the approval of omics test to be practiced in an institute they should receive a FDA approval (Micheel et al., 2012). Upon obtaining the required clearance and following successful omics trial the treatment of the individual can be tailored using the approaches like targeted drug delivery, stem cell transplantation, CRISPR-CAS-9 based gene editing, monoclonal antibody treatment etc.

BIOMARKER TESTING

To get gate entry of PM, there should be accessibility of tumor molecular profiling and centers that perform high-quality tests (Table 1). Biomarkers, a measurement of macromolecules such as proteins, DNA, RNA and used as an indicator of normal or pathological processes as well as therapeutic indicators. Biomarker testing was performed for the diagnosis of disease and distinguishing subtypes among them thus facilitating personalized treatment (Sung et al., 2016).

Table 1. Utility of Biomarkers

Biomarker use	Clinical objective
Screening	Early detection of disease in a group of asymptomatic patients
Diagnosis/differential diagnosis	A specific establishment of stage and presence of disease
Classification	With the identified biomarkers patients are classified in disease subtypes
Prediction/treatment	Predict the response to the therapy or drug
Therapy related risk management	Measurement of adverse effect due to the given therapy
Therapy monitoring	Monitoring of intended action of drug in parallel with adverse effects caused.
Posttreatment monitoring	To check the relapse of disease or improvement of complications if any.

Technologies used for the biomarker testing on routine basis were Sanger sequencing, ARMS, RFLP and Next Generation Sequencing as mentioned in companion diagnostics. Apart from their advantages, they post specific challenges (Sung et al., 2016).

Types of biomarker tests include: single-analyte tests, suite of multiple-single analyte tests, multiple analyte panel and complete Next-generation sequencing (NGS) of entire genome.

Single analyte: ECRG4 can be used as a single biomarker for the differential diagnosis between urocystitis and bladder tumors (Rose et al., 2020).

Suite of multiple, single analyte tests: a group of single analytes test considered to opt multi targeted therapy. Consideration of estrogen receptor/progesterone receptor along with HER2 amplification for targeted therapy is one example.

Multiple analyte panel: testing multiple biomarkers in single in vitro testing. Five biomarkers, CA125, IL (interleukin)-8, IL-6, CRP (C- Reactive Protein), SAA (Serum Amyloid A) were assessed using in vitro diagnostic multivariate index assays which show positivity of ovarian cancer and also difference between benign and malignant tumor (Autelitano et al., 2012).

Immune biomarker testing: Biomarker testing for immune checkpoint inhibitor such as PD-L1/ CTLA4 using immunohistochemistry with approved antibodies directed against the immune checkpoint inhibitors (Fred et al., 2018).

NGS: It is a high-throughput technique that led us to sequence multiple target genes including genome sequencing, transcriptome sequencing and epigenetic profile (Fred et al., 2018). Hang Au et al., reported 77 mutations in 24 genes of 37 patients (total of 50 patients) in myeloid neoplasm (Au et al., 2016).

Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), both are shed by the tumors and categorized as noninvasive cancer diagnostics. Due to rarity of CTC (frequency of one cell per 10^6-10^7 leucocytes) in the blood, implementing them as biomarkers is biggest challenge. However, CTC detected may help in enhancing omics information from cancer sample through NGS, EPISPOT immunoassay, cytometry, RNA-sequencing and migration assays. CTCs used in preparation of functional assay which help assessing the effectiveness of drug. The result of study conducted by the Iwatsuki et al., showed that primary HER-2 negative gastric cancer, CTCs showed HER2 gene amplification on cancer progression. This suggest that CTC sequencing required for the targeted therapy (Lee et al., 2019).

Whereas ctDNA unlike CTCs, can easily detected in tumor samples and does not require specialized isolation technique. ctDNA are released into the bloodstream due to apoptosis or anoikis of tumor cells. Information related to mutation and methylation status can be assessed through PCR (digital PCR, methylation PCR) and tagged-amplicon deep sequencing. One of the examples, US FDA approved two drugs erlotinib and osimertinib against EGFR mutation for non-small-cell-lung cancer, where EGFR mutation was detected using ctDNA (Chang et al., 2017).

One major challenge is time taken to get the result. For rightly treatment, testing on appropriate time is essential. Delay in testing may results in initiation of first line treatment for patients with advanced stage in tumor. This may pose severe side effects, unnecessary treatment or even cost the life of patient. In addition, small tissue size, poor sample quality, and economic and access limitation to biomarker testing seems other challenges. The difficulties in the invasive techniques led scientist to more easily available circulating biomarkers (Sung et al., 2016).

We are already being in the era of targeted medicines. Molecular testing / genomic profiling couple patients with their best targeted therapies. These arenas evolve with respect to molecular diagnostics and therapeutics applications. Minimization of existing painful invasive techniques remain the next milestone to be achieved in biomarker testing thus guiding us towards best treatment options (VanderLaan et al., 2018).

COMPANION DIAGNOSTICS (CDX)

With the great interest in the field of PM, the central concept of CDx has also became an interest among lab professionals and clinicians. As per the definition of US FDA, companion diagnostic is an in-vitro diagnostic technique which give information of safety and effectiveness of therapy against the particular disease. In simplest form it can be described as outcome predictor and therapy monitor. The very first predictive assay was performed for the drug Trastuzumab for Herceptin positive in breast cancer. The main aim of CDx assay is to predict whether a patient is profited from the drug or not. Some of the technologies used are RT-qPCR, DNA microarray, immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH). In addition to the outcome prediction, CDx were used for monitoring patent's response to treatment. Due to increased apoptosis in cancer cell, DNA and RNA were released into the blood stream. Estimation of these free DNA and RNA in the blood is termed as liquid biopsy (as mentioned in before). But, most of the present CDx assay is based on 'monotherapy', which on long run may develop resistance and relapse. To overcome this there should be a shift from one biomarker: one drug approach to multigene approach that integrates many biomarkers and drugs (Jan et al., 2015). Does imaging can be used for companion diagnostic? Imaging have the ability to measure the target expression that change from site to site which cannot be accessed by the biopsy from one place. It is of noninvasive nature and let us repeat. In addition, they also avoid difficulties in biopsy. For example, imaging to determine the Estrogen Receptor and Progesterone Receptor in breast cancer patient. Currently PET ER imaging with estradiol analogs, ER imaging with positron emitting radiopharmaceutical (Mankoff et al., 2016). Researchers need to study more on this. Recent approach, that use multi modal technology is NanoString technology. It is based on the identification of targeted mRNA molecules digitally with a sequence specific tagged color probes, succeeded by high-resolution imaging technique. It is advantageous due to its simplicity, user friendly, high sensitivity and reproducibility. It detects multigene in single go which saves reagent and cost. Nanostring technique application includes analysis of gene expression in cancer chemotherapy, disease prognosis and detecting epigenetic changes in cancer progression. Limitation lie in normalization of gene expression level and probe designing (Eastel et al., 2019).

APPLICATION OF PERSONALIZED MEDICINE IN CANCER

Ovarian Cancer

Ovarian cancer is an umbrella terminology referring to malignancies that arise from an ovary. It is a world second most occurring cancer and most dangerous gynecologic malignancy. Morphologically they are classified as – non-epithelial ovarian cancer (NEOC) and epithelial ovarian cancer (EOC) (Reid et al., 2017). With the advancement of genomics techniques genetic changes were observed in protein such as BRCA1/2, PIK3CA mutations, loss of function of p53, p21, p27 and pathways including PI3K/AKT/mammalian target of rapamycin (mTOR) pathway, vascular endothelial growth factor (VEGF)/hypoxia inducible factor 1 alpha (HIF-1α) pathway, interleukin-6 (IL-6)/STAT3 pathway, Rb pathway, p16, KRAS pathway, wnt pathway and MDR pathway (Li et al., 2017). From above data, the regimen of personalized medicine was developed which included angiogenesis inhibitors, PARP inhibitors, EGFR tyrosine kinases inhibitors, folate receptor α inhibitors, immunotherapy – checkpoint inhibitors, immune modulators, adoptive T-cell transfer, vaccines and other palliative treatment against malignant ascites

formed due to tumor spread (Cortez et al., 2018). Recent studies reported that ovarian cancer is often associated with malignant ascites formation and suggested that therapies against VEGF and EpCAM lead to slower accumulation of ascites e.g., catumaxomab (against EpCAM) (Pejovic et al., 2013, Romero & Bast et al., 2012). A comprehensive list of PMs for ovarian cancers is given in table 2. Immunotherapy is another approach which uses the body's own immune system to remove the tumor cells. Immunotherapy includes checkpoint inhibitors (pembrolizumab – anti-PD-1 antibody), vaccine (p53-MVA – for OFPC patients) and adoptive T cell transfer (NY-ESO-1 antigen-reactive TCR) (Koury et al., 2018)

Table 2. Personalized medicine in ovarian cancer and their targets

CATEGORY	DRUG NAME	TARGET	DESCRIPTION	REFERENCES
PARP inhibitors	Olaparib, Rucaparib	PARP1,2,3	Used in women with platinum sensitivity and tumor with BRCA ness	Chen & Du et al., 2018, Staropoli et al., 2018, Mittica et al., 2018, Dal Molin et al., 2018, Gonzalex-Martin et al., 2019, Coleman et al., 2019.
	Veliparib, Talazoparib, Niraparib	PARP1,2		
PI3K inhibitors	LY294002	PI3K p110α	Irreversible binding at c-20 position of furan ring of PI3K p110α	Mazzoletti et al., 2010, Mabuchi et al., 2015
	ZSTK474	PI3K	Oral ATP-competitive inhibitor	Mazzoletti et al., 2010, Mabuchi et al., 2015
AKT inhibitors	Perifosine	AKT	Lipid based phosphatidyl inositol drug, arrest signal transduction thus preventing membrane localization of AKT	Mazzoletti et al., 2010, Mabuchi et al., 2015
	GSK690693	AKT	Inhibit AKT in ATP-competitive manner	Mazzoletti et al., 2010, Mabuchi et al., 2015
	MK2206	AKT	Oral inhibitor, allosteric inhibitor of PH domain preventing membrane localization	Mazzoletti et al., 2010, Mabuchi et al., 2015
mTOR inhibitors	Temsirolimus	mTOR	Standard therapy for ovarian carcinoma carboplatin	Mazzoletti et al., 2010, Mabuchi et al., 2015
	Everolimus	mTOR	Increase the platinum sensitivity of human ovarian carcinoma	Mazzoletti et al., 2010, Mabuchi et al., 2015
VEGF inhibitors	Bevacizumab	VEGFA	mAb that inhibit the ligand and receptor interaction	Monk et al., 2016, Khalique et al., 2017
	Aflibercept	VEGFA,C, placental growth factor	Dominant negative chelation of target molecules	Monk et al., 2016, Khalique et al., 2017
VEGF receptors	Cediranib, Sunitinib	VEGFR1-3	Inhibit vessel growth and sprouting	Monk et al., 2016, Orbegoso et al., 2017
	Nintedanib	VEGFR, FGFR, PDGFR	Oral tyrosine kinase inhibitors	Monk et al., 2016, Khalique et al., 2017
	pazopanib	VEGFR, PDGFR,c-kit	Inhibition of angiogenesis and tumor proliferation	Monk et al., 2016, McLachlan et al., 2015
Folate receptor α inhibitor	Farletuzumab	folate receptor- α	inhibiting the interaction between the FRα and lyn kinase leading to apoptosis of cells	Monk et al., 2016
EGFR inhibitor	Gefitinib, Erlotinib	EGFR	Competitively inhibits interaction of ATP at the ATP site on EGFR	Ohta et al., 2012, Glaysher et al., 2013
MEK inhibitor	Selumetinib, Trametinib, Binimetinib	MEK	Treatment of low-grade serous cancer	Fernandez et al., 2019

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Table 2. Continued

CATEGORY	DRUG NAME	TARGET	DESCRIPTION	REFERENCES
Immunotherapy	Catumaxomab	EpCAM	Uses EpCAM and CD3 binding domain to stimulate immune response	Mantia-Smaldone et al., 2012
Anti-CTLA4	Ipilimumab	CTLA4	Monotherapy platinum sensitive ovarian cancer	Mantia-Smaldone et al., 2012
Anti-PD1	Pembrolizumab	PD1	Platinum resistant ovarian cancer	Mantia-Smaldone et al., 2012
DNA vaccine	PANVAC	----	Cancer vaccine therapy that contain transgenes for carcinoembryonic antigen and T cell stimulatory molecules	Mantia-Smaldone et al., 2012

Prostate Cancer

Prostate cancer (PCa) is the second most frequently diagnosed non-cutaneous malignancy in males worldwide after lung cancer and the fifth leading cause of death (Ku et al., 2019, Rawla et al., 2019). Most of PCa are considered sporadic, which mainly occurs through somatic mutations and are not hereditary while only few are familial and passed on to next generation (Alvarez-Cubero et al., 2017). PCa is hormone dependent tumor which is often characterized by alteration in androgen receptors (AR) and its pathway which lead to cellular growth (Mullane et al., 2016). The castration-resistant prostate cancer (CRPC) is the most lethal state where the tumor has formed resistance to androgen deprivation therapy. This resistance may be due to intrinsic (TP53 mutations) factors or due to stress after treatment (AR amplification). A study of 150 metastatic castration-resistant prostate cancer (mCRPC) patients by the international Stand Up To Cancer-Prostate Cancer Foundation (SU2C-PCF) Dream Team reported some common mutations: majority showed AR mutation or amplification, TP53 and PTEN deletion while less frequently RB1 loss, BRCA1/2 mutation and CDK12 mutation, along with PI3K, WNT, cell cycle regulation and DNA repair mechanism pathways alteration in some caes (Ku et al., 2019).

Currently diverse classes of anti-androgens are available. These anti-androgens (i.e. flutamide, bicalutamide and nilutamide) inhibit the continuous activation of AR receptor by competing with AR ligand and blocking the androgen-androgen receptor interaction. These drugs increased the overall survival rate (Dole & Holdsworth et al., 1997). Bicalutamide is effective against the stage 3 or 4 prostate cancer (Scher et al., 1997). Recently developed drugs such as Enzalutamide, Apalutamide, and Darolutamide been effective by inhibiting androgen binding as well as acting at DNA level (inhibiting nuclear translocation). Niclosamide, EPI-001 and Niphatenones disrupt the AR –DNA binding (McCrea et al., 2018).

The most preferred androgen are testosterone and dihydrotesterone, formed from precursor 17α-hydroxypregnenolone and dehydroepiandrosterone (Table 3). The key enzyme involved is cytochrome P450 17A1 (CYP17A1) which catalyzes conversion of pregnenolone to 17α-hydroxypregnenolone and 17α-hydroxypregnenolone to dehydroepiandrosterone by its hydroxylase and lyase activity respectively. Abiraterone, blocks reactions which are catalyzed by CYP17A1 and Seviteronel specifically arrest its lyase activity (McCrea et al., 2018).

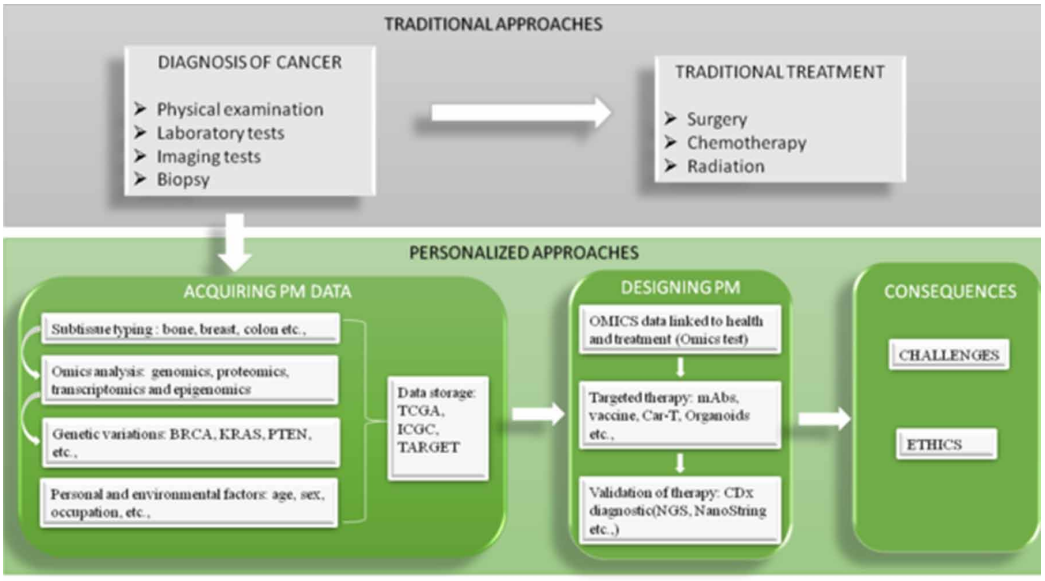
Another widely found mutation in CRPC is found in phosphoinositide 3-kinase (*PI3K*). These mutations include loss of PTEN, amplification and activating mutation in PIK3CA/B and AKT1 respectively. Mutations in PIK3CB and PTEN cause over expression of PIK3CB beyond PIK3CA. This mechanism

emphasizes the necessity of selective PI3K inhibitors. There exists the evidence of cross-talk between PI3K and HR pathway/AR signaling respond to PARP inhibitors (Figure 3) and ARPI therapy respectively (Ku et al., 2019, Mullane et al., 2016).

Some groups of prostate cancer patients harbor hypermutation and microsatellite instability in mismatch repair genes (MLH1, PMS2, MSH2 and MSH6) and loss of function of cyclin-dependent kinase-12 (CDK12). This subset is associated with over expression of PD-L1 and immune infiltration (Rosty et al., 2014). So this subset is responsive to immune checkpoint inhibitor, such as pembrolizumab, an anti-PD-1 antibody (Daud et al., 2016). Ipilimumab and nivolumab are under phase II clinical trial for CDK12 mutation.

Unphosphorylated RB1 keeps the cell from entering the cell cycle and maintains in senescence. In cell cycle G1-S phase transition is due to CDK4-CDK6 phosphorylation of RB (tumor suppressor). Phosphorylated RB1 drives the cell to Sphase. Disruption of RB1 gene results in cell proliferation. Palbociclib, an inhibitor of CDK4 and CDK6, bring about cell division arrest which is under phase II clinical trial (Ku et al., 2019, Mullane et al., 2016).

Figure 3. PARP enzymes play a pivotal role in repair of DNA damage. PARP bind mainly to SSB (single strand break) and recruit other proteins to repair the damage



SSB if not repaired result in DSB (Double strand break). thus, PARP inhibitors cause DNA damage in a cell. There are five DNA repair mechanism base-excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), Homologous recombination, and nonhomologous end joining (NHEJ). HR contains variety of enzymes. One among them is BRCA1/2. BRCA1/2 get mutated in some solid tumors (breast and ovarian cancer). PARP inhibitors are sensitive in cells with dysfunction of BRCA genes. In SSB, PARP inhibitors inhibit it activity by binding to it whereas in DSB they trap PARP on damaged DNA and this is known as DNA-PARP inhibitor trapping.

Table 3. Personalized medicine in prostate cancer

CATEGORY	DRUG NAME	TARGET	DESCRIPTION	REFERENCES
Androgen receptor inhibitor	Enzalutamide, Darolutamide, Aplalutamide	Androgen Receptor	Competitively inhibits androgen binding to AR, nuclear translocation of AR, DNA binding	Ku et al., 2019, Nevedomskaya et al., 2018, Kurihara et al., 2019, Wang et al., 2017
	Bicultamide, Flutamide, Nilutamide	Androgen Receptor	Inhibit the binding of androgen interaction with androgen receptor	Ku et al., 2019, Nevedomskaya et al., 2018
	Niclosamide	Androgen Receptor	AR V7 splice variant inhibition through proteasome dependent degradation of AR.	Ku et al., 2019, Nevedomskaya et al., 2018, Sobhani et al., 2018
Androgen antagonist	Abiraterone, orteronol	Cytochrome P450	blockage of reactions catalyzed by CYP17A1	De Bono et al., 2011, Stein et al., 2014
	VT-464	Cytochrome P450	Bifunctional inhibition of 17-αhydroxylases &17,20 lyase activity	De Bono et al., 2011, Stein et al., 2014
AKT inhibitor	Capirasertib	AKT	E17K mutated prostate cancer	Nevedomskaya et al., 2018, De Bono et al., 2011
	Ipatasertib	AKT	Selective ATP-competitive small molecule	Nevedomskaya et al., 2018, De Bono et al., 2011
PD-1 inhibitor	Pembrolizumab	PD-1	Used in Enzalutamide- resistant CRPC	De Velasco et al., 2018, Comiskey et al., 2018
Anti-CTLAA4	Ipilimumab	CTLA-4	Therapy for chemotherapy naïve CRPC	De Velasco et al., 2018, Comiskey et al., 2018
	Nivolumab	CTLA-4	CRPC	De Velasco et al., 2018, Comiskey et al., 2018
DNA vaccine	Sipuleucel-T	PAP	mCRPC dendritic cell-based vaccine	Reimers et al., 2019
	PROSTVAC	PSA	Incorporated gene for PSA and various cell co stimulation molecules	Reimers et al., 2019
PARP inhibitors	Olaparib	PARP enzymes	Act by trapping PARP enzymes at the side of DNA damage, used for germline mutation of BRCA1/2	Geethakumari et al., 2017, Kamel et al., 2018
	Niraparib	PARP enzymes	Used in Advanced castrate resistant prostate tumor	Geethakumari et al., 2017, Kamel et al., 2018, Virtanen et al., 2019
	Veliparib	PARP enzymes	Still underdevelopment	Kamel et al., 2018, Virtanen et al., 2019

Lung Cancer

Among all lung cancers, non-small cell lung cancer (NSCLC) constitute 85% of lung cancers which include lung adenocarcinoma (~45%), squamous cell carcinoma (~25%), and large cell carcinoma (10%) and remaining 20% are classified as small cell lung carcinoma (SCLC) (Politi et al., 2015, Liu et al., 2017). Important biomarkers of lung cancer include KRAS, HER2, MET, ROS1, RET, PI3K catalytic subunit-a (P/K3CA), PTEN and tumor immune and microenvironment—PD, PDL1 and vascular endothelial growth

factor A (VEGFA). Other biomarkers include FOXC2, TGF-β, ERCC1 and ribonucleoside-diphosphate reductase (RRM) (Vargas et al., 2016).

EGFR signaling plays a crucial role in epithelial tissue growth and maintenance and is found as an oncogenic driver in many lung cancer patients (Table 4). Drug targeting EGFR over-expression are tyrosine kinase inhibitors (TKIs). Gefitinib and Erlotinib are first generation of TKIs which have shown increase in overall survival rate among EGFR positive patients (Xu et al., 2015). Apart from EGFR, ALK is another common gene rearrangement caused due to inversion or translocations of chromosome 2. The irregular activation of fusion genes, ALK and echinoderm microtubule associated proteins like-4 (EML-4) cause growth of lung cancer and are often equally exclusive with other oncogenesis drivers, EGFR, ROS1 and KRAS. It's a mutation observed in 4-7% of NSCLCs (Hirsch et al., 2017, Jiang et al., 2018). It has been found that ALK inhibitors can counter the lung cancer. Crizotinib is the leading drug of class ALK-inhibitor that is designed to target ALK fusion genes, ROS1 and MET. These act as a competitive inhibitor binding to ATP binding domain thus inhibiting the carcinogenic kinase activity and preventing ALK fusion protein formation (Jiang et al., 2018, Sahu et al., 2013). Patients receiving Crizotinib showed rapid and resilient response in phase 1 and phase 2 trials. Soon studies showed that relapse occurs within 1-2 years along with resistance due to the 1151Tris, S1206Y, C1156Y, L1152R and G1202R (Friboulet et al., 2014). This lead to the formulation of second generation drugs which include Ceritinib and Alectinib (Beardslee et al., 2018).

Table 4. Personalized medicine in lung cancer

CATEGORY	DRUG NAME	TARGET	DESCRIPTION	REFERENCES
EGFR TKIs	Gefitinib,Erlotinib	EGFR	First generation	Hirsch et al., 2017, Wang et al., 2017
	Afatinib, Ganetespib, Neratinib	EGFR	Second generation	Hirsch et al., 2017, Wang et al., 2017, Kurihara et al., 2019
	Osimertinib, Rociletinib	EGFR	Third generation (target T790M EGFR mutants)	Hirsch et al., 2017, Wang et al., 2017, Murtuza et al., 2019
	EAI045	EGFR	Fourth generation, first allosteric inhibitors that designed against the T790M & C797S EGFR mutants	Hirsch et al., 2017, Wang et al., 2017
ALK TKIs	Crizotinib	ALK	First generation, inhibit the phosphorylation of ALK thus inhibiting ALK fusion protein that cause cell survival	Friboulet et al., 2014, Thai & Solomon et al., 2018, Sgambato et al., 2018, Gadgeel et al., 2018
	Ceritinib	ALK	Second generation. 20 times more potent than the Crizotinib	Thai & Solomon et al., 2018, Sgambato et al., 2018, Gadgeel et al., 2018
	Alectinib	ALK	Second generation and overcome the resistance caused by Ceritinib	Friboulet et al., 2014, Thai & Solomon et al., 2018, Sgambato et al., 2018
	Lorlatinib	ALK	Third generation can cause blood brain barrier	Sgambato et al., 2018, Gadgeel et al., 2018, Solomon et al., 2018

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Table 4. Continued

CATEGORY	DRUG NAME	TARGET	DESCRIPTION	REFERENCES
ROS1	Crizotinib	ROS1	Longer response thanin ALK rearrangement	Sehgal et al., 2018, Lin & Saw et al., 2017, Shaw et al., 2014
	ceritinib	ROS1	Active in Crizotinib naïve ROS1 rearranged NSCLC	Sehgal et al., 2018, Lin & Saw et al., 2017, Shaw et al., 2014
	Entrectinib	ROS1	Activity against ROS1 mutation like L2020M,G2032R	Sehgal et al., 2018, Lin & Saw et al., 2017, Shaw et al., 2014
RET	Cabozantinib, vandetanib, lenvatinib, sorafenib	RET kinase	Bind the ATP binding domain in active conformation	Falchook et al., 2016, Bronte et al., 2019
	Alectinib, Sunitinib, Nintedanib, Regorafenib, Ponatinib	RET kinase	Bind the ATP binding domain in inactive domain	Falchook et al., 2016, Bronte et al., 2019
MET inhibitors	Altiratinib, golvatinib	MET receptor	Multikinase MET inhibitors, bind intracellular tyrosine domain	Drilon et al., 2017, Pasquini & Giaccone et al., 2018
	Capmatinib, Tepotinib	MET receptor	Selective inhibitor, ATP competitive in nature	Drilon et al., 2017, Pasquini & Giaccone et al., 2018
	Tivantinib	MET receptor	Selective inhibitor, ATP non-competitive in	Drilon et al., 2017, Pasquini & Giaccone et al., 2018
	Onartuzumab, Emibetuzumab	MET receptor	Anti-MET mAbs, bind to extracellular domain of MET receptor	Drilon et al., 2017, Pasquini & Giaccone et al., 2018
Anti-HER2	Dacomitinib	HER2	Used in combination with chemotherapy	Hirsch et al., 2017
Anti-CTLA4	Ipilimumab	CTLA4	Human IgG1. Mostly suggested in combination with chemotherapy	Fellner et al., 2012
Anti-PD1	Nivolumab, Pembrolizumab	PD-1	Human IgG4 monoclonal antibody in SCLC	Calles et al., 2019, Villanuvera & Bazhenova et al., 2018
	Atezolizumab	PD-1	IgG1 human monoclonal antibody that disrupt the interaction between PD-1&B7-1 receptor	Calles et al., 2019

Viral Oncogenesis

Except for gastric cancer caused by Helicobacter pylori and other parasitic infection associated cancer most of the cancer is caused by virus. Some of the virus that cause cancer are human papillomavirus (HPV), hepatitis C virus (HCV), hepatitis B virus (HBV), gamma herpesvirus, Epstein Barr Virus (EBV), Kaposi's sarcoma- associated herpesvirus (KSHV)), & human T cell lymphotropic virus type-1 (HTLV1). Merkel cell polyomavirus and human cytomegalovirus were identified in Merkel cell carcinoma and glioblastoma multiforme respectively, though their role is yet to be elucidated (Smith & Khanna et al., 2017). Here we are discussing the personalized medicine in HPV and EBV briefly.

HPV causes cervical cancer and it was found that increased mRNA levels of APOBEC3A and APOBEC3B are reported in both low- and high-grade cervical lesions. This up-regulation is due to oncoproteins (E6/E7) in HPV16. APOBEC3 protein generates clusters of marked mutations in host genome by causing C to T substitution. In addition they are also involved in editing of virus genome, interrupt viral replication and make C to U substitution. APOBEC mediated mutation was found majorly in PIK3CA gene. All these lead to the idea that APOBEC mutagenesis plays a vital role in cervical cancer. Potential therapeutic benefits may be agents targeting PI3K signaling. Future studies rely on finding genetic makeup of the cancer that enables tailored therapy (Chen et al., 2017).

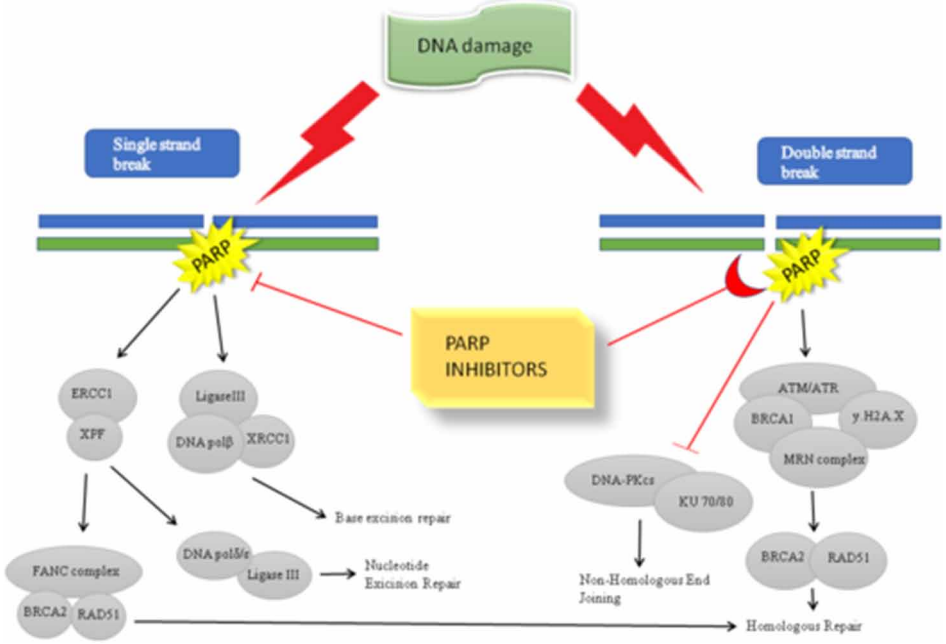
EBV is associated with different malignancies such as Hodgkin lymphoma (HL), diffuse large B-cell lymphoma (DLBCL) or NK/T cell lymphoma (NKTL) (Pei et al., 2017). EBV causes the conversion of B cells into lymphoblastoid cells (LCL). These insights lead to the development of the concept of adaptive cellular therapy (ACT). Affected B cells go into a resting phenotype and on the basis of gene expression of latent antigens (EBNA1 and LMP1 and 2) are classified as latency 0, latency I and latency II type cells (Rowe et al., 1992). The transplantation of hematopoietic stem cell (HSCT) in immune-compromised patients lead to post transplant lymphoma's (PTLD), characterized by latency III and it is a consequence of a T cell immunity failure to act against the latent antigens (Bollard et al., 2012, Ogonek et al., 2016). Thus ACT focuses on rejuvenation of immunity by generation of T cells against latent antigens in-vitro. Immunotherapy approaches in HPV associated cancers are designed against E6 and E7 antigens (Rohaan et al., 2018). In-vitro culture of tumor infiltrating lymphocytes from refractory cervical carcinoma patients and transplantation into patients showed increased number of HPV- specific T cells thus creating memory cells and controlling disease establishment (Geukes Foppen et al., 2015). Unlike EBV and HPV, hepatocellular carcinoma (HCC) caused by HBV does not have any well-developed ACT. Transgenic T cells or combined antigen receptor (CAR) against viral envelop proteins are currently under development for HCC (Smith & Khanna et al., 2017).

Breast Cancer

Breast cancer affects more than 2.1 million women annually worldwide and is the cause of 15% death of all cancer patients among females (WHO, 2018). Breast cancer is characterized by the continuous malignant cell proliferation that originates from the inner lining of breast ducts. Breast cancers are classified as luminal A, luminal B, Human Epidermal Growth Factor Receptor 2 (HER2) –positive, basal-like, and claudin-low. Breast cancers are heterogeneous in nature. Wide genetic mutations involved in the breast cancer are BRCA1, BRCA2, PALB2, TP53, CDH1, HER2, and PTEN. The signaling pathway involved in breast cancer are mitogen-Activated Protein Kinase (MAPK) pathway, PI3KK/AKT pathway (Figure 4), calcium signaling pathway, notch signaling pathway, hedgehog signaling pathway and anti-apoptotic signaling pathway. In most subtypes of cancer, the signaling pathways that lead to cancer progression were PI3K/AKT/mTOR and the RAS/RAF/MEK pathways (Jeibouei et al., 2019).

PI3K- phosphoinositol 3 kinase; PTEN- Phosphatase and tensin homologue deleted on chromosome ten; AKT- Protein kinase B; mTORC- Mammalian Target of Rapamycin Complex. RAS- Rat Sarcoma; RAF- Rapidly Accelerated Fibrosarcoma; MEK- Mitogen-activated protein Kinase; ERK-Extracellular Signal Regulated Kinase; CDK- Cyclin Dependent Kinase; RB- Retinoblastoma; PIP2-Phosphoinositol 4,5 bisphosphate; PIP3- Phosphoinositol 3,4,5 trisphosphate. EGFR- Epidermal Growth Factor Receptor; EGF- Epidermal Growth Factor; RTK-Receptor Tyrosine Kinase; TKIs- Tyrosine Kinase Inhibitors.

Figure 4. PI3K/Akt/mTOR and Ras/Raf/Mek signaling pathway and their inhibitors used in the solid tumors



There are several reports that HER2 is over-expressed in HER2 positive breast cancer (Iqbal et al., 2014). HER2 is a transmembrane, tyrosine kinase that regulates/ manages the cell division and DNA damage repair in breast cells. Upon over-expression they result in uncontrolled proliferation of breast cells (English et al., 2013). Thus HER2- targeted therapies found to be promising medicament in the HER2 positive breast cancer. Trastuzumab (Herceptin) was the first antibody which targeted HER2 by binding to the extracellular domain of HER2, inhibiting the downstream signaling that is concerned in cellular division, survival and motility (Wilson et al., 2017). A list of PM in breast cancer treatment is given in table 5.

Table 5. Personalized medicine in breast cancer

CATEGORY	DRUG NAME	TARGET	DESCRIPTION	REFERENCES
PI3K inhibitors	AZD8186	PI3K	Inhibits p110β isoforms, downregulates metabolic pathways inducing cellular stress in tumor.	Dey et al., 2017, McKenna et al., 2018
	BYL719	PI3K	In specific target p110α-isoform	Dey et al., 2017, McKenna et al., 2018
mTOR inhibitors	Everolimus, Ridaforolimus	mTORC1complex	Rapalogs, on inhibition of mTOR activity decrease the phosphorylation of 4E-BP1	Dey et al., 2017, McKenna et al., 2018, Bahrami et al., 2018
	TAK-228	mTORC1/2	Inhibits the HRG signaling pathway, leading to HER2 inhibition.	Dey et al., 2017, Bahrami et al., 2018
AKT inhibitors	MK-2206	AKT	Allosteric inhibitor of AKT. G1 phase arrest and apoptosis of tumor cells	Dey et al., 2017, Bahrami et al., 2018
	AZD5363	AKT	ATP competitive inhibitor	Dey et al., 2017, Bahrami et al., 2018

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Table 5. Continued

CATEGORY	DRUG NAME	TARGET	DESCRIPTION	REFERENCES
HDAC inhibitors	Trichostatin A, Suberoylanili-dehydroxamic Acid (SAHA)	ClassI/II HDAC	Increase the apoptosis of tumor cell by increasing Bcl-2 (proapoptotic factor)	Fang et al., 2019
CDK inhibitors	Palbociclib	CDK4/6	Oral drug, high selective against CDK4/6 and inhibit Rb phosphorylation. Activity in ER+/HER2 breast cancer	Choo et al., 2018, Murphy et al., 2019, Sachdev et al., 2019
	Abemaciclib	CDK4>CDK6	FDA approved and fit adjacent fluorine atom in ATP pocket of CDK	Choo et al., 2018, Murphy et al., 2019, Sachdev et al., 2019
	Ribociclib	CDK4/6	Small-molecule inhibitor, remarkable activity in ER+/HER2	Choo et al., 2018, Murphy et al., 2019, Sachdev et al., 2019
Aromatase inhibitors	Anastrozole, letrozole, exemestane	Aromatase enzyme	First hormonal therapy, inhibit the production of estrogen thus impair growth of hormone receptor +ve Breast Cancer cells	Buch et al., 2019, Olin et al., 2014
PARP inhibitors	Veliparib	PARP	Letha to breast cancer which is characterized by the BRCA mutation	Geene et al., 2018
HER2 inhibitors	Pertuzumab	HER2	Monoclonal antibody that inhibit the dimerisation of the HER	Sachdev et al., 2019
	Tratuzumab	HER2	Monoclonal antibody	
	TDM1-emtansine	HER2	Antibody conjugated with drug, deliver drug to HER2 overexpressed mBC	
Anit-CTLA-4	Tremelimumab	CTLA4	Used in the treatment of HER2 –ve BC in combination with anti-B7H1 antibody	Yu et al., 2017, Marra et al., 2019
	Ipilimumab	CTLA4	Combination therapy with anti-B7H3 antibody in TNBC	Yu et al., 2017, Marra et al., 2019
PD-L1 inhibitors	Atezolizumab, Avelumab, Durvalumab	PD-L1Receptor	Humanized IgG1 mABs	Katz & Alsharedi et al., 2018

Colorectal (Crc) Cancer

CRC is characterized by the formation of malignant cells and adenomatous polyps. These uncontrolled proliferative cells show property of metastasis. Genes that are frequently dysregulated in CRC are EGFR, RAS, RAF, PIK3CA, PTEN, BRAF, mismatch Repair genes and KRASs. These genes are involved in tumor formation, invasion and progression. The novel pathways include sonic hedgehog, wnt/beta catenin, TGF-β/SMAD, EGFR and notch pathway (Tiwari et al., 2018, Koveitypour et al., 2019, Ren et al., 2015).

EGFR is a growth signal receptor expressed in epithelial cells which play a pivotal role in carcinogenesis and metastasis of CRC. mAbs & tyrosine kinase inhibitor are two therapeutic approaches practiced against EGFR. mAbs act by bind inhibiting the ligand binding and preventing receptor dimerisation and conformational change by interacting with the extracellular domain of EGFR (Okada et al., 2017, Nigro et al., 2016). Cetuximab and panitumumab are two mAbs which act as effective monotherapy or combination therapy in association with chemotherapy. Okada et al. (2017) reported that mAbs bind to the extracellular domain and down-regulates EGFR by increased degradation of EGFR through internalization via endosomal-lysosomal pathway. This results in the hindering cell proliferation and stimulation of apoptotic pathway. The effect varies with RAS expression in cancer cells (Okada et al., 2017). The TKIs bind to intracellular domain of EGFR and inhibit the angiogenesis, an important process in the tumor growth (Okada et al., 2017). The PM drugs for treatment of CRCs are listed in table 6.

Table 6. Personalized medicine in colorectal cancer

CATEGORY	DRUG NAME	TARGET	DESCRIPTION	Reference
Anti-EGFR	Cetuximab, Panitumumab	EGFR	Inhibit the ligand stimulated phosphorylation of EGFR.	Sanchez-Gundin et al., 2018, Guler et al., 2019
	ZD1839(Iressa)	EGFR	Inhibition of EGFR on binding intracellular domain of it.	Daneshmand et al., 2003
Anti-angigogenic drugs	Bevacizumab	VEGF-A(ligand)	Recombinant humanized mAbs that selectively bind to VEGF ligand, inhibiting the ligand- receptor interaction.	Tiwari et al., 2018, Mody et al., 2018, Frenette et al., 2017
	Regorafenib	VEGFR-1,2,3 and TIE2	Oral multikinase inhibitor block the tyrosine kinase receptor that have role in angiogenesis	Mody et al., 2018, Frenette et al., 2017
	Ramucirumab	VEGFR-2	Recombinant human IgG1 mAbs that bind extracellular domain of VEGFR-2, inhibiting downstream signaling.	Mody et al., 2018, Oholendt & Zadlo et al., 2015
BRAF inhibitor	Vemurafenib + ceutuximab/irinotecan	BRAF	Found useful in BRAF mutant CRC have been approved by FDA as BRAF inhibitor monotherapy	Guler et al., 2019, Korphaisarn & Koptez et al., 2016, Sandhu et al., 2019
	Dabrafenib	MAPK signaling pathway		
MEK inhibitor	Trametinib, Selumetinib	MEK	MEK inhibitor treated CRC acquire resistance over it. These can be overcome by combination therapy or by administration of Akt and NF-κB inhibitor	Tsubaki et al., 2019, Spreafico et al., 2013, Yau et al., 2019
HER2	Trastuzumab	HER2	Bind to the HER2 domains and	Meric-Bernstam et al., 2019
PI3K inhibitors	BKM120 (Buparlisib)	PI3K	Inhibit PI3K which is important molecule in G1 phase arrest	Yau et al., 2019, Bahrami et al., 2018
Tyrosine kinase inhibitors	Entrectinib	Tropomyosin Receptor Kinase (TRK)	Therapy for Cancer with NTRK1/2/3, ROS1 and ALK gene rearrangements	Yau et al., 2019, Drilon et al., 2017,Kotskaya et al., 2017
Anti- PD-1	Pembrolizumab, Nivolumab	PD-1 blocker	KRAS, BRAF wild type, mismatch repair deficiency, POLE	Wrobel & Ahmed et al., 2019
Anti-CTLA4	Ipilimumab, tremelimumab	CTLA-4 blocker	mismatch repair deficiency, POLE	Wrobel & Ahmed et al., 2019

CHALLENGES AND LIMITATIONS OF PERSONALIZED MEDICINE

PM transferred the healthcare system to the next era in the field of cancer biology in the notion to save much lives. It is protective, coordinated, and proven. Still many including the healthcare system do not completely understand the benefits of PM. In short, the challenges can be classified as scientific challenges, operational issues, economic challenges, and ethical challenges (Mathur & Sutton et al., 2017).

Scientific Challenges

Most of the genomic-based trial failed to show the antitumor activity in patients. The biggest scientific challenges include:

- Identification of genomic driver events and passenger events and differentiating them.
- Failure in validation of biomarkers and their signaling pathways.
- Failure to design a drug that is highly bioactive and target specific.
- Lack of trials on combination therapies as many cancer is heterogeneous thus two or more genetic alterations (Arnedos et al., 2015).
- The most exciting and promising technology used in diagnosing tumor genotype is liquid biopsy. Liquid biopsy potential role has been proved, yet they are confronted with their own limitations. The CTC found very less in the serum and fragility, current technologies used for detection limit future applications. Even though the ctDNA is more abundant the CTC no functional assay has been discovered. Whether both the CTC and ctDNA are released in the same amount by primary or metastatic tumor, whether are profile for metastatic ability are need to be assessed. Lack of standardized preanalytical conditions, low specificity, and sensitivity make them not an established technic to be followed (Wang et al 2017).

Operational Challenges

- Despite the existence of so many technologies in accessing genomic information, processing and storing that information, remain problematic. Even though there exist many centers to process and store data, managing the big-data still remains challenging. The main problems include data storage in a different manner in different hospitals, data processing, and integration of omics data and interpretation of them, cost–effectiveness of data processing (Di Sanzo et al., 2017).
- Another challenge includes the necessity of regulated supervision of laboratory and quality assurance, the steps and protocol for acceptance of tests to be used, and funds required for genetic tests (Butts et al., 2013).

Economic Challenges

- Even though the PM seems to be beneficial, the cost of testing and therapies is the biggest obstacle. Because of financial crisis, clinical trials in developing countries remain as a question mark (Salari & Larijani et al., 2017).
- The cost of testing is not affordable by the policy agencies due to the number of tests taken to gather the genetic information of patients which are costly.

Ethical Challenges

- Participant selection for clinical trial should be based on use of pharmacogenetics information. But larger groups of patients who are financially stable are more attractive than the smaller groups in the study. This leads to inequality in healthcare sector. Moreover, the pharmaceutical companies ignore drug development for limited impact groups.

- With the available genetic information, establishing bio-bank became necessary. The ethical issues concerned with the bio-bank are sample collection, their cryopreservation, informed consent, privacy, sample sharing and confidentiality (Salari & Larijani et al., 2017).
- Other challenges include the protection of acquired private information from patients during the disease course.

Lack of knowledge of clinicians to interpret and act on pharmacogenetic information and patient's knowledge will limit the personalized medicine. Policy challenges exist concerning the association between government research and regulatory agencies.

ETHICS IN PM

Physician-patient Relationship

The Physician-patient relationship is one of the major factors in the medical field. In traditional medicine, a diagnosis of the genetic disorder includes a group of specialists such as geneticists, neurologists, oncologists etc., whereas in personalized medicine treating any disease based on the genetic makeup becomes the responsibility for even primary care physicians. The first problem is to ascertain whether the primary physician has enough knowledge about genomics and also well trained to provide personalized medicine. The physician should have good knowledge over the genome sequencing and formulating a strategy for prescribing personalized medicine. The second problem that arises is the lack of time. Personalized medicine includes genome sequencing and other tests which require time to perform. The patients are made clear about these tests and implication of them in their healthcare. Physicians have to interpret the test and design the treatment strategy. These need to be explained by physicians to patients. All these processes are time-consuming.

Privacy and Confidentiality

Here privacy means limited access to health information regarding an individual. The concept of confidentiality is a term which means information obtained within a relationship is not revealed to other individuals. The need for privacy: an individual may experience embarrassment and discrimination, a social harm may occur among an individual with mental illness or infectious diseases and fear of the compromised quality of health care. The health information was recorded in the electronic Health Records (EHR) for billing and repayment. This increases the risk of privacy of information due to computer viruses and hackers (Noonan et al., 2017). So congress passed a medical privacy law of consequence – the Health Insurance Portability and

Accountability Act (HIPAA) in 1996 which mandated electronic numerical identifiers for the patients, doctors etc., Join Commission on Accreditation of Healthcare Organizations (JCAHO) that take care of privacy fulfillment (Act et al., 1996).

Discrimination

Genetic discrimination is the very first concern of many scholars since the HGP. It may be rational/ irrational and legal /illegal. The fear of genetic discrimination in workplace, insurance, or other important activities may prevent the individual from undergoing genetic testing. There are legislative laws to overcome this discrimination in many countries. This is done by limiting the sharing of health information that is accessible only to the individual. As of 2014, in the USA, private health insurance agencies are not allowed to use an individual's information to decide whether they can provide insurance or not (Brothers & Rothstein et al., 2015, Sharrer et al., 2017).

SUMMARY

The field of PM has grown since the completion of HGP in 2003. Personalized medicine in cancer is more beneficial because cancer is heterogeneous and needs selective target for therapy. Characterization of a patient to obtain this information with the availability of modern technologies like DNA/RNA sequencing, imaging and other proteomics techniques set the spotlight on their response to intervention, and treating them accordingly is the soulful meaning of personalized medicine. At present, the research has moved ahead of sequencing to linking this obtained information to medical care and treatment strategy. There is a wide variety of platforms to store and process 'big data'. PM increased the patient response to the drug and improved survival rate. Some of the cancer treatment products include CTC, ctDNA and immune check point inhibitors. Organoids or in-vitro models of cancer improved the understanding of the gene-drug interaction. Besides, PM therapies are designed with careful guidelines and regulations. To keep PM moving forward a collaborative effort from researchers, pharmaceutical companies, clinicians, patients, and regulators is needed. The question, at present is, are we able to treat each patient based on their genomic structure? Immediate answer of many will be 'no'. Future technology improvement will change this answer to 'yes'.

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Chapter 16
Role of Stem Cells in Cancer Therapeutics

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ABSTRACT

Stem cells are pluripotent cells having capacity of self-renewal and produce various types of mature cells. Cancer stem cells are known to be responsible for drug resistance and tumor relapse, yet stem cells offer multiple avenues to treat same. Stem cells have been employed for treating of blood and immune systems damaged during chemotherapy and radiotherapy. Stem cell transplantation is emerged as critical therapy in cancer treatment, yet other potential applications of stem cells in cancer treatment are largely unexplored or underutilized. Recently, stem cells reengineered express different cytotoxic agents. It has shown to cause tumor regression and enhance the animal survival in preclinical studies. Stem cell therapy can be also employed for targeted drug delivery, gene delivery, and even used as virus to target cancer cell. In recent years, research is devoted on stem cells worldwide for new and newer application. Although the field of stem cells is nascent and raises many ethical concerns, scientific responsibilities, and future challenges, scientific community are still hopeful and filled with optimism. Currently, stem cell therapy represents the beginning of the new era in cancer treatment and giving a ray of hope to clinicians and also patients who are suffering from untreatable diseases and desperately looking for new therapies. In the present chapter, the authors mainly shed light on potential applications of stem cells to treat cancer. At the end, they also discussed the factor influencing stem cell therapies and current challenges in stem cell therapy.

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INTRODUCTION

Stem cells are defined as cells that have clonogenic and self-renewing capacities which differentiate into multiple cell lineages and also potency to produce replacement cells for a wide range of tissue and organs like heart, liver, pancreas and nervous system.(Law, Hunt, & Qu, 2019; Sachin & Singh, 2006). Stem cells are present from the early stages of human development until life ends.

The discovery of stem cells in the past became open to the new medicine era in the treatment of many diseases which led to extensive attention and research on stem cells. In 1981, Evans et al have isolated stem cells from mouse embryos(Evans & Kaufman, 1981). Further studies on mouse embryo led the discovery of isolation of stem cells from a human embryo which opens the door for use of stem cells in the treatment of diseases. Improvement in advance technology helped a lot to understand the potency and efficacy of stem cells especially the discovery of the oct3/4, sox2, klf-4 and myc which have capacity to turn any normal cell into stem cell. Table 1 shows the progression of stem cell research worldwide.

Table 1. Summary of the History of Stem Cell Research, adopted with permission from Hawsawi et al. (2018)

Year	Research Performed	References
1878	The first report of endeavors to fertilize mammalian eggs outside the body is published.	Caplan (2017)
1959	The first report on animals produced through IVF is published.	Caplan (2017)
1960	Studies of teratocarcinoma in the testes of several inbred strains of mice indicate that the teratocarcinoma originated from embryonic germ cells.	Stevens (1960)
1968	The first human egg in vitro fertilization is performed.	Hawsawi et al. (2018)
1970	Cultured stem cells are explored as models of embryonic development, although their complement of chromosomes is abnormal.	Hawsawi et al. (2018)
1978	Louise Brown, the first IVF baby, is born.	Johnson and Elder (2015)
1980	Australia's first IVF baby, Candace Reed, is born in Melbourne.	Verhoeven (2006)
1981	Evans and colleagues derive mouse cells (ESCs) from the inner cell mass of blastocysts and develop culture conditions for growing pluripotent mouse ESCs in vitro; they find that infusing the ESCs into mice induced the formation of teratomas. The first IVF baby in the United States, Elizabeth Carr, is born.	Evans and Kaufman (1981); Martin (1981)
1984-1988	Andrews and coworkers develop pluripotent cells (ECCs) from the Tera-2 human testicular teratocarcinoma cell line. Thus, the teratoma cells exposed to retinoic acid differentiate into neuron-like cells and other cell types	Andrews (1988); S. Thompson et al. (1984)
1989	Pera and coworkers isolate and characterize multipotent clones of human embryonal carcinoma cells, which yield tissues of all 3 primary germ layers.	Pera, Cooper, Mills, and Parrington (1989)
1994	Human blastocysts are established for reproductive purposes using IVF and are donated by patients for research. The inner cell mass is isolated and cultured.	Bongso, Fong, Ng, and Ratnam (1994)
1995-96	Nonhuman primate ESCs are derived and maintained in vitro; these cells were first isolated from the inner cell mass of rhesus monkeys and then from that of marmosets. The primate ESCs resemble human ECCs, indicating that it should be possible to derive and maintain human ESCs in vitro.	Hawsawi et al. (2018)
1998	Thompson and coworkers acquire and maintain human ESCs from the inner cell mass of human blastocysts that were produced through in vitro fertilization and were donated for research purposes. Gearhart and colleagues derived human embryonic germ (EG) cells from the gonadal ridge and mesenchymal tissue of fetal material originating from abortions at 5 to 9 weeks of gestation.	B. Thompson et al. (1998)
2000	Scientists in Singapore and Australia derive human ES cells from the inner cell mass of blastocysts donated by couples undergoing treatment for infertility. The ES cells proliferate for extended periods in vitro, maintain normal karyotypes, differentiate into somatic cell lineages derived from all 3 primary germ layers, and form teratomas when injected into immunodeficient mice.	Lohar (2019)
2001	Human ES cell lines are shared and new lines are derived in vitro. Many methods are aimed at generating human tissues for transplantation purposes.	Lohar (2019)

STEM CELL TYPE BASED ON ORIGIN

Stem cells are divided into three main groups according to the origin and distribution of cells such as embryonic stem cells, fetal stem cells, and adult stem cells.

Embryonic Stem (ES) Cells

As the name suggest, have only a single origin *i.e*. in the embryo and can give rise to all the cell types. Scientists have cultured these ES cells with particular growth factors like leukemia inhibitory factor (LIF) to maintain its pluripotency. In the absence of LIF these ES cells, in culture, gives rise to a range of embryological tissues including epidermis neuronal and glial cells, muscle cells and haemopoietic cells(Semrau et al., 2017).

Adult Stem Cells

These cells remain dispersed in various organs and tissues in a fully developed individual since the time of birth. Mammalian epidermal stem cells, haemopoietic stem cells, mesenchymal stem cells from adipose tissue, spermatogonial stem cells, prostate epithelial stem cells, etc. are to name a few(Clevers & Watt, 2018). Apart from this, stem cells from various organs like bone marrow, peripheral blood, umbilical cord, fetal liver are considered as progenitor stem cells of various organs (Figure 1).

Pluripotent Stem Cells

The third type of stem cell with similar properties to embryonic stem cells has emerged. Researchers have engineered these induced pluripotent stem cells (iPS) by modifying the expression of certain genes-"reprogramming" somatic cells back to a pluripotent cell(Hockemeyer & Jaenisch, 2016).

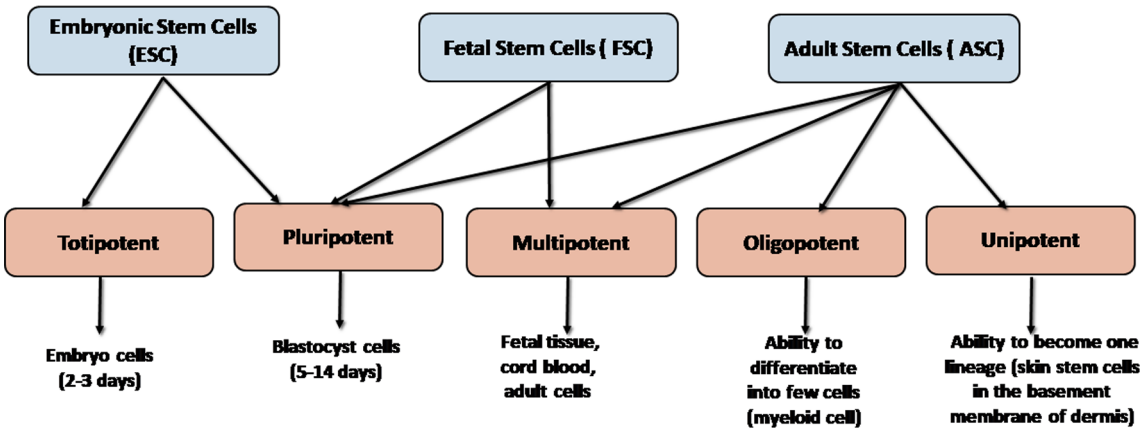
Based on differentiation potential stem cells can be divided into five types: totipotent, pluripotent, multipotent, oligopotent and unipotent. "Totipotent "can divide into embryonic and extraembryonic types of cells e.g. zygote formed at egg fertilization and first few cells that result from the division of the zygote. Whereas, "pluripotent" can produce any cell type, for example, embryonic stem cells and cells derived from endoderm, mesoderm, and ectoderm germ layers that produced in early stages of embryonic stem cell differentiation. "Multipotent" stem cells divide into any cell type i.e. mainly closely related cell family e.g. hematopoietic (adult) stem cells that can become red and white blood cells. "Oligopotent" stem cells can divide into a few cells for example, (adult) lymphoid or myeloid stem cells. Finally, "unipotent" can divide cells of their own type but have self- renewal characteristic that is required to labeled a stem cell e.g. (adult) muscle stem cell (Figure 1) (Karabekian & Sarvazyan, 2020).

Below we are also describing the different sources of stem cells according to their presence in body organs:

Human Umbilical Cord

The cord blood is one of the sources of stem cells which can be collected from the umbilical cord after the birth of a baby that consists of hematopoietic and mesenchymal stem cells(Zhu et al., 2017). The hematopoietic stem cells can form red blood cells and cells of the immune system, whereas the mes-

Figure 1. Classification of Stem Cells



enchymal stem cells involved in the generation of bone, cartilage, and other types of tissues. The cord blood can also be collected and stored in cord blood banks for future use.

Bone Marrow

It is a spongy tissue found at the center of bones such as thigh bones and hips(Birbrair & Frenette, 2016). Mesenchymal stem cells are found in bone marrow, which is the most commonly used source of mesenchymal stem cells. Subsequently, bone marrow also produces hematopoietic (RBC, WBC, and platelets) or blood stem cells that make it an attractive candidate for various purposes such as regenerative medicine and therapy of diseases.

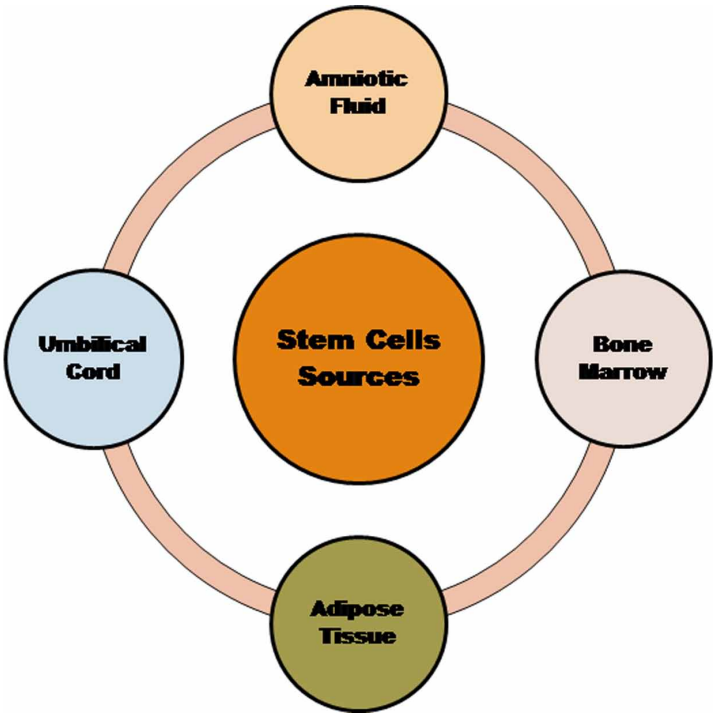
Adipose Tissue

The adipose is a loose connective tissue-derived stem cells are mesenchymal cells that have the ability for self-renewal and multipotency(Na, Ban, Lee, Im, & Kim, 2017). These cells can be differentiating into adipocytes, chondrocytes, myocytes, osteoblasts, and neurocytes, also in other cell types. These stem cells have been observed to have essential roles in reconstructive and tissue engineering fields to develop new treatments.

Amniotic Fluid

Amniotic fluid is the protective liquid that surrounds the amnion or the sac that encompasses the fetus. Stem cells from amniotic fluid can be acquired from amniotic fluid and tissue. Both the amniotic membrane and amniotic fluid are good sources of embryonic stem cells that can multiply and form any type of cell and tissue like skin, cartilage, cardiac, nerve, muscle and bone. Therefore, amniotic stem cells have important role in tissue regeneration. Although the amniotic fluid and membrane are usually discarded after birth, now a day's amniotic fluids are being cryopreserved or frozen for the therapeutic purpose(Villani, Petrosyan, De Filippo, & Da Sacco, 2018).

Figure 2. Sources of Stem Cells



These stem cells are needed for the normal physiological functions of the body. For example; stem cells are present in the skin for normal replacement of the epidermal layer which is exposed to the environmental harsh conditions. Spermatogonial cells and the follicular cells also exhibit this property for the maturation of the germ cells. But the stem cells present in the cord blood or the bone marrow are multipotent and function as the progenitor for many cell types. All these undifferentiated cells are present in the body of an individual after birth till death, although the potency decreases with age. In contrast, the cells of the heart are terminally differentiated at birth(Sudulaguntla, Gurung, Nanjwade, & Tamang, 2016).Therefore, many clinical trials have done on stem cells to evaluate it efficacy on attention towards the treatment of life-threatening diseases inrecent times which might open new directions in the medicine era. While many more professionals are also looking into the use of various types of stem cells found in the bone marrow and cord blood and mainly in mesenchymal stem cells to their uses ahead of that could be corrected by replacing cells in their own lineage. Preliminary results from clinical trials with stem cells have created mixed results that show little or minor improvements that may be accredited to extracellular factors. Most of the researchers are also facilitating the use of other variety of adult stem cells mainly neural stem cells for the treatment of diseases where the beneficial outcome might result from either in –lineage cell replacement or extracellular factors(Trounson & DeWitt, 2016).

TYPE OF STEM CELLS

Induced Pluripotent Stem Cells (iPSCs)

iPSCs are normal cell that reprogramed to behave like embryonic stem cells. It is done by inducing the expression of certain genes that define embryonic stemness(Hockemeyer & Jaenisch, 2016). Frist iPSCs were created in 2006 from normal cells in the mouse by inducing the activation of Sox2, Klf4, Oct-4, and Myc(Takahashi & Yamanaka, 2006). iPSCs are the most promising cells as any cell from the body can be converted to stem cell. It is also most clinically relevant as they do not post any immunogenicity and do not require any ethical consideration like stem cell.

Neural Stem Cells (NSCs)

NSCs are another prominent cell that is characterize by high level of Nestin, and Sox2 expression (Wang et al., 2020). NSCs can be easily grown in culture medium rich in epidermal and fibroblast growth factors. It is widely employed in anticancer therapeutics as NSCs can efficiently form clonally related progeny that can be differentiated to any type of cell.

Mesenchymal Stem Cells (MSCs)

MSCs are similar to stem cells present in the bone marrow. MSCs can be easily expanded in lab and frequently used for regeneration of cartilage, muscle, connective tissue, and tendon, etc.(Caplan, 2017). It is also used in anticancer treatment due to its wide availability inside the body.

Human Endothelial Progenitor Cells (EPCs)

EPCs are involved in the regeneration of vascular tissues. Some studies have suggested the potential utility of EPCs in cancer treatment, yet their application, suitability and safety are yet to explored in detail(Ferratge et al., 2017).

Cancer Stem Cells (CSCs)

CSCs are similar to normal pluripotent stem cells in terms of cell surface receptors and markers. CSCs are like normal pluripotent stem having the ability to give rise to all cell types but in an uncontrolled fashion, thus imparting a role in tumor development, aggressiveness and metastasis(Batlle & Clevers, 2017). Conventional therapies usually target non-stem cancer cells, while leaving CSCs to amplify and differentiate(Shimokawa et al., 2017). The aggressiveness of the tumor is inversely proportional to the differentiation level of CSCs. The frequent relapse of the tumor is believed to be due to surviving CSCs; therefore targeting CSCs may be helpful in overcoming tumor drug resistance and relapse(Nassar & Blanpain, 2016).

APPLICATION OF STEM CELLS

In past, major goal of stem cell therapy was to repair damaged cells or tissue that is unable to repair by itself(Sobhani et al., 2017). However current stem cell research is also devoted to other potential application such as targeted drug delivery or immune therapy in addition to regeneration. This gives expectation to patients who would normally not receive any treatment to treat their disease but just to mitigate the symptoms of their chronic disease. Stem cell therapies mainly involve transplanting normal or engineered stem cells into the body and facilitate them to form new and healthy cells or tissues or do the assign job(Volarevic et al., 2018). It is also possible to induce stem cells already present in the body to work overtime and produce new cells or tissue.

Potential Treatment by Stem Cells

Stem cells in therapeutics is at the nascent stage and costly with the noteworthy exception of bone marrow transplantation. Medical professionals and researchers have presumed that the embryonic and adult stem cells will soon be able to treat cancer, diabetes type1, diabetes type2, Alzheimer's disease, Parkinson disease, Huntington's disease, celiac disease, heart failure, muscular dystrophy and neurodegenerative diseases and many more (Tran & Damaser, 2015; Watt & Driskell, 2010). They have advocated that before the application of stem cell therapeutics in clinical trials, more research should be done to understand the behavior of stem cells in transplantation and the mechanism of interaction of stem cells with the diseased or injured microenvironment of the tissue(Gattazzo, Urciuolo, & Bonaldo, 2014; Tenney & Discher, 2009).

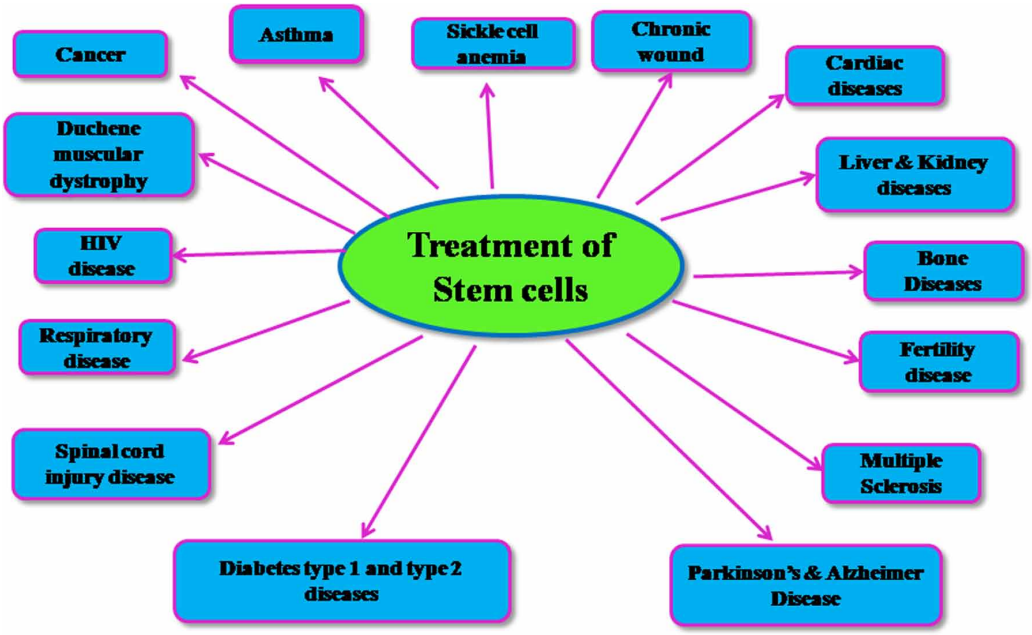
In the clinical application of stem cell transplants, bone marrow transplantation is a well-known therapy. After high doses of chemotherapy and radiotherapy, BMT can repopulate the marrow and restore all different types of cells in the blood in order to the elimination of endogenous cancer cells. Nowadays, for other clinical applications, also the isolation of further needed stem and progenitor cells is now being developing and is under investigation. Here, we enlightened the various types of possible treatments by stem cells in several diseases which are described below (Fig. 3):

Utilization of Stem Cells in Cancer Treatment

Cancer results from unregulated cellular proliferation or failure to induce cell death. All cancers result from changes in the DNA sequence of our genome. These changes occur throughout life because the genome within our cells is exposed to mutagens like UV radiation and accumulates mistakes during replication. These changes result in a progressive, subtle divergence of the DNA sequence from the original copy from the fertilized egg.

Occasionally, one of these mutations alters the function of a critical gene, providing a growth advantage to the cell in which it has occurred. This means that this cell and its offspring divide at a faster rate than that of their neighbors. The result is tumor formation, and subsequently, break away from their home tissue (Fig. 4). The metastasizing cells become attached to the wall of a blood vessel or lymph vessel. They secrete digestive enzymes into it. Then they cross the wall at the breach. Cancer cells creep or tumble along inside blood vessels, then leave the bloodstream the same way they got in. They start new tumors in new tissues. The whole process invasion of surrounding tissue and eventually 'metastasis', or spread of cancer to other parts of the body.

Figure 3. The figure depicts possible treatment by stem cells in different types of diseases



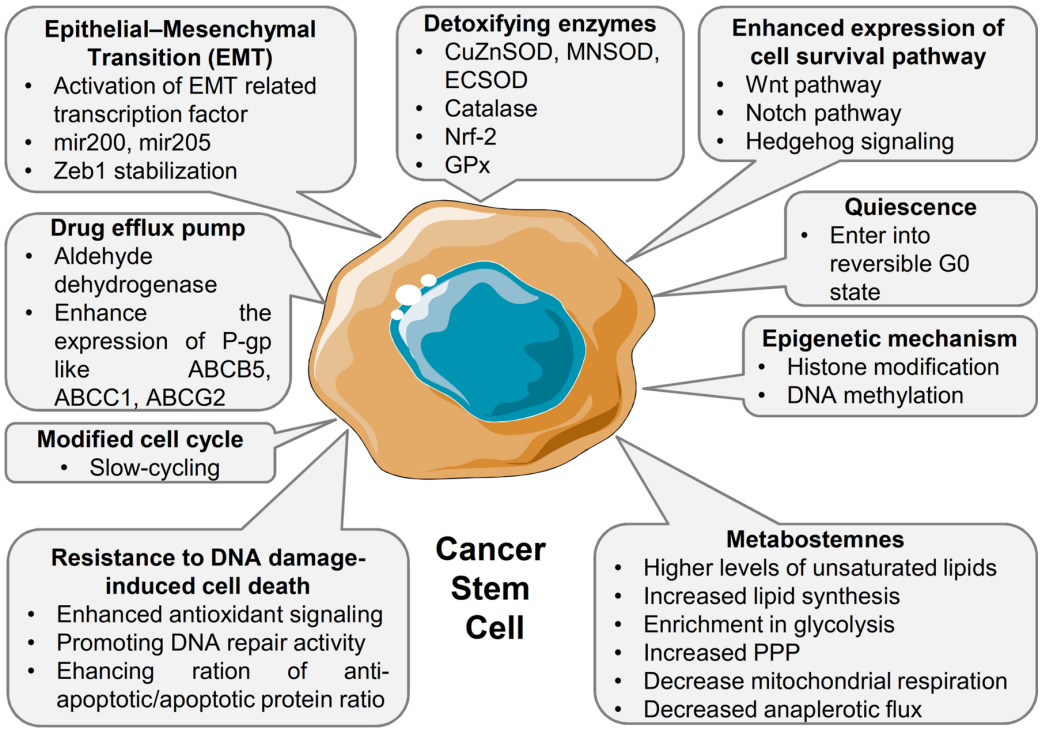
Stem cells are known to directly participate in cancer initiation, progression, and metastasis through dedifferentiation or epithelial to the mesenchymal transition process(Koury, Zhong, & Hao, 2017). It is also responsible for the development of therapeutic resistance and tumor relapse as they are the most resistance against chemotherapy and radiotherapy. Yet the same cells can be also re-educated to target cancer. Stem cells in cancer therapeutics is at the nascent stage and costly with the noteworthy exception of bone marrow transplantation.

History of Bone Marrow Transplantation in Cancer

The bone marrow transplantation was first done in a dog kennel by in Cooperstown, NY, during the 1950s(Vriesendorp & Heidt, 2016). RBCs could be successfully transfused from compatible donor to needy recipient. While transfusion of marrow cells from different donor is challenging task as it is treated as foreign invaders and thus host immune system destroy them without any mercy(S. A. Patel, Sherman, Munoz, & Rameshwar, 2008). However, rejection of graft vs. host reaction can be avoided by many ways, such as by immuno-suppressive drugs, transplantation only between MHC compatible individual and autologous stem cell transfer. In autologous stem cell transfer host receive own cells taken from different body part(Weiskopf et al., 2016).

E. Donnal Thomas conceived the idea that new and healthy bone marrow cells are essential for curing leukemia(Eskew, 2018). He tested many transplantation techniques in dogs and then applied in patients with late-stage leukemia. However, every patient who underwent transplantation died during the procedure or shortly thereafter, resulting in halting the human trial after four years. Eight years later, he identified genetic histocompatibility marker present in WBCs, and transfusion between closely matching HLA of donor and recipient does not result in lethality in dogs. This led to the resumption of human trials.

Figure 4. Metabolic traits of cancer stem cells, driving the resistance against host defense and therapy



Later he received Nobel Prize in 1990 for the seminal contribution in bone marrow transplantation for treatment of leukemia(Qiao, 2018).

Enzyme/Prodrug Therapy

Neural stem cells and mesenchymal stem cells are known to carry inherent tumor tropic properties(Ho, Tu, & Too, 2019). Additionally, they are also very weak immunogenically. Therefore, they can be engineered to express enzyme that converts a non-toxic prodrug into a toxic drug after homing to tumor tissues. It is also known as suicidal gene therapy. Cytosine deaminase is a central enzyme employed in prodrug therapy, that convert 5-fluorocytosine, into the more toxic, 5-fluorouracil at the targeted location. Herpes simplex virus-thymidine kinase is another enzyme that is used in targeted delivery of the anticancer drug using stem cells. It converts prodrug, monophosphorylateganciclovir, to cytotoxic triphosphate ganciclovir, that integrates into the DNA of dividing cells, leading to cell death via DNA polymerase inhibition.

Secreted Agents

Stem cells can be employed for controlled delivery of the drug at the desired location. It can be also used to overcome the inefficient delivery, drug short self-life, and toxic side effect associated with anticancer drugs(Kamalabadi-Farahani et al., 2018). The delivery of TNF-α-related apoptosis-inducing ligand, IFN-βetc. are shown to help in reducing tumor size and enhancing animal survival(Jung et al., 2019;

Kamalabadi-Farahani et al., 2018). TNF-α-related apoptosis-inducing ligand is known to induce tumor cell apoptosis; however, short life is a major barrier in the successful clinical application of TNF-α-related apoptosis-inducing ligand. Therefore, engineered stem cells expressing TNF-α-related apoptosis-inducing ligand is promising as it could continuously release the ligand and help in tumor elimination.

Viral Therapy

Oncolytic viruses are attenuated viruses that preferentially infect and kill tumor cells by a process known as oncolysis(Martinez-Quintanilla, Seah, Chua, & Shah, 2019). The oncolysis further releases new infectious virus particles that again infect the cancer cells, and the process goes on till the last tumor cell eliminated. Stem cells are used to deliver the oncolytic virus at local tumor site.

Nanoparticle Carriers

Nanoparticles are emerging as promising technology in targeted drug delivery systems as they can be tailored for the specific need of the experiment, including targeted controlled and stimulus-based drug-delivery(Dang & Guan, 2020). However, the major problem associated with nanomedicine is leaching and degradation before reaching destination, leading to inefficient delivery of the drug at the desired location. Stem cells offer a gateway to overcome the previous shortcoming associated with the nanoparticles-based drug delivery. Stem cells can easily uptake the nanoparticles, protect them from the harsh biological system, including host immunosurveillance and directly reach to the tumor side. In many studies, it has shown the efficient delivery of many anticancer drugs at the local tumor site, thus offering a big hope in cancer treatments(Gao, Zhang, Hu, & Sun, 2013).

Regenerative Medicine

Stem cells offer the potential benefit of using undifferentiated cells to repair or replace badly damaged cells or tissue(Onoshima, Yukawa, & Baba, 2015). Chemotherapy is known to wipe out actively dividing cells such as cancer cells. During the process, it also destroys bone marrow, including stem cells that eventually mature into cells of the blood and immune system. The death of progenitor and stem cells make organisms weak against many opportunistic pathogens, loss the organ regenerative capacity, and become lethal. Transplanting stem cells has been widely used to promote hematological recovery after radiotherapy or chemotherapy(Wang et al., 2020). This is also used to reconstitute the hematopoietic system by bone marrow transplantation in aplastic anemia and other blood cell genetic diseases. Hence combination of autologous stem cell support during high dose chemotherapy has employed for reducing the chemo-associated toxicity.

Immunotherapy

The immune system is the body's defense system, under the task to protect the body against germs or degenerated cells (like cancer cells). The immune system is very complex. It has two components: the cellular immune defense (for example, "scavenger cells" and "killer cells") and the complement system (for example "antibodies")(Taefehshokr, Baradaran, Baghbanzadeh, & Taefehshokr, 2019). While cancer is a multistep process that results from the alterations in normal proliferation, differentiation and/or cell

death mechanisms and has recently been associated with energy metabolism reprogramming and the evasion from immune destruction. Thus, incidence of cancer may be higher when there is a concomitant reduction in the functionality of the immune system.

Immune surveillance function executed by the immune system seems to represent an effective tumor suppressor mechanism, thus contributing to the reduction in cancer incidence and progression. Tumor-associated antigens are released by cancer cells either naturally and after coming in contact with chemical or radiation. The immune system has the ability to eliminate tumor cells, but some cells survive becoming variants of previous existing cells, being poorly immunogenic and able to enter a steady-state phase. During the steady-state, cells of the adaptive immune system (CD4+, CD8+), as well as effector molecules [e.g., IFN-y, interleukin (IL)-12], are primarily responsible for preventing and inhibiting tumor development. One related with the immune system that may become "exhausted", losing the ability to eliminate cancer cells, meaning that they may proliferate actively without control.

Traditionally T cells are employed in immunotherapy, where person's own T cells are taken out and reeducated in ex-vivo condition to attack tumor cells(S. J. Patel, Yamauchi, & Ito, 2019). However, the problem is that many time T cells inefficiently multiply; therefore, stem cells offer a useful choice in Tc-based therapy. Stem cell immunotherapy is effectively used to grow new cellular and immunological-based methodologies for the treatment of patients with malignancy and hematological issues. Adult stem cell has been effectively utilized for a long time in bone marrow transplants to treat leukemia and related bone/blood tumors. Then again, umbilical cord stem cells can be utilized as a part of hematopoietic cell transplants for patients lacking an appropriate donor. There are many avenues to generate tumor targeting T cells through reprogramming. One approach is to convert mature Tc cells into iPSC by inducing the expression of c-Myc, SOX-2, OCT-4, and KLF-4. SuchiPSC grown into large number and again reconverted to Tc cells that have all feature possess by original Tc cell.

Another approach is to collect naïve T cells and reprogrammed them to iPSC by c-Myc, SOX-2, OCT-4, and KLF-4(Caraballo Galva, Cai, Shao, & He, 2020). Afterword, reprogrammed cell transduced with tumor specific recombination receptor or chimeric antigen receptor. Then it grown into large number and then differentiated to Tc cell and injected into cancer patient. The advantage of this method is 1) capability to induce a precise response from naïve T cells; 2) can be easily scale-up for therapy purpose; 3) easily compliant in the clinical setting as no animal material is used.

Dendritic cells are immune cells that play a central role in recognizing foreign antigens and presenting it to Tc cells(Hashemi et al., 2020). Utilizing dendritic cells to train Tc cells against tumor specific antigens is promising in cancer immunotherapy and then Tc mount an immune response against tumor. In earlier studies, dendritic cells have been helped in tumor regression through immunological destruction. The dendritic cells expressing high level of CD141 and XCR1, have only observed to perform better.

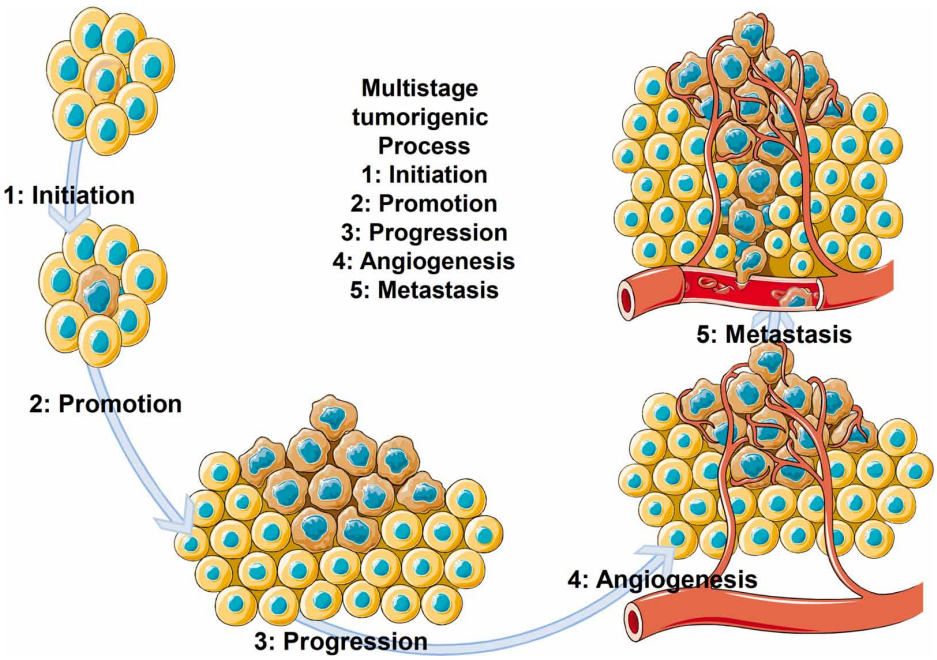
As a Carrier of Various Drug

Stem cells can be used as a carrier of drug, gene or even living microorganism by exploiting the property of tumor tropism and immune-privilege(Hadryś, Sochanik, McFadden, & Jazowiecka-Rakus, 2020). MSCs cells are shown to have capacity to infiltrate into tumor site through chemotaxis operated through tumor secreted-β, IL-8, EGF, HGF, FGF, and PDGF(Stamatopoulos et al., 2019). NSCs has also shown similar effects like MSCs. Stem cells has been used to deliver different cytokines like IL-12, TNF-α, tumor necrosis factor related apoptosis induced ligand, IFN-γ and many other anti-angiogenic factors.

Cancer Stem Cells and Vaccines for Cancers

Tumor is heterogeneous in nature due to genomic instability, and selection pressure. Therefore multi-modality often used in treatment but still some cells especially cancer stem cells (CSCs) able to resist most therapies and soon grow into mass(Guo & Dou, 2015). CSCs also evade immune system, therefore dendritic cells expressing CSC-associated antigens or CSCs marker glycan's can be used to mount an Tc specific immune response for destruction of CSCs (Fig. 5). Tumor specific antigen can be also isolated from tumor cells lysate and use to generate tumor specific vaccine.

Figure 5. Schematic representation of typical multistage nature of tumor



Targeting Cancer Stem Cells (CSCs)

CSCs are multipotent, long-lived, and display quiescent and responsible for relapse and drug resistance(Garcia-Mayea, Mir, Masson, Paciucci, & Lleonart, 2020). The CSCs hypothesis is an attractive model to explain the functional heterogeneity that is commonly observed in solid tumors. It proposes a hierarchical organization of cells within the tumor, in which a subpopulation of stem-like cells is responsible for sustaining tumor growth(Garcia-Mayea et al., 2020). The first evidence for CSCs came from acute myeloid leukemia. There is now increasing evidence for CSCs in a variety of solid tumors (both mouse and human). The frequency of CSCs in solid tumors is highly variable, reflecting biological variation as well as technical challenges. The CSC phenotype could be acquired by normal tissue stem cells, progenitor or even normal differentiated cells through acquiring the transforming mutations, which activate/deregulate certain signaling pathways. CSCs are believed to be, in part, responsible for

therapy resistance as they are generally more resistant than the cells that constitute the bulk of the tumor through multiple mechanisms as shown in figure 5.

The chemo resistant phenotype of CSCs is believed to be due to overexpression of drug efflux pumps, alterations in apoptosis proteins, overexpression of anti-apoptotic genes and members of the inhibitor of apoptosis protein, increased telomerase expression and increased antioxidant capacity/enhanced resistance to oxidative stress. It is believed that chemotherapeutic regimens are not able to effectively eradicate CSCs (but only the cancer cells that constitute the bulk of the tumor) and that this will ultimately be responsible for recurrence. Therefore, it is crucial to effectively target and eradicate these cells in order to improve the outcome of cancer patients. Targeting these cells by targeted therapy has shown better outcomes in multiple studies.

Anticancer Drug Screening

Stem cells can be also used for drug screening(McGivern & Ebert, 2014). It can be used for screening and optimization of enzyme/prodrug therapy, secreted agents, nanoparticle therapy, immunotherapy, etc.

FACTOR INFLUENCING STEM CELL THERAPIES

Since stem cells are physiologically similar, therefore is expected that they should behave in the same passion, which is not true in reality. The main factors influencing the differences are passage number, expansion medium, genotype, culture conditions, donor age, and stem cell source, etc.(Rojewski et al., 2019). Among NSCs and MSCs, MSCs show better outcome than NSCs.

Route of Transplantation

The route of stem cells graft also plays an important role in success of graft. The selection of a safe and efficacious route of administration is paramount importance in efficacy of the treatment(Mueller & Kramer, 2017). The contralateral injection had shown better results in glibostome than any other route. However, the intracranial route is invasive and not suitable for multiple times; therefore, intranasally injection may offer better option as it reduces the intravascular delivery-related obstacles, such as pulmonary embolism, obstruction by the BBB, and infarctions.

Graft Cell Number

The optimum cell number is also required for the successful removal of the tumor or mitigation of the therapy associated side effects(Chander & Gangenahalli, 2020). The excess number may enhance the risk of teratoma formation or leads to ectopic engraftment. While there would be no any detectable response after going below to a certain number. Thus, number of cells in the graft should be carefully examined before administration.

Supporting Graft Material

The supporting graft material is also taken into consideration during engraftment(La & Tranquillo, 2018). Semisolid substrate has shown better result in enhancing the graft survival compared to cell suspension injections, since the former provides mechanical support and thus reduce the metabolic stress.

Time of Administration

The timing of administration is also important; however, generally engraftment before cytotoxic therapy has shown improved survival and lower therapy-associated side effect than post-therapy administration.

CHALLENGES IN STEM CELL THERAPY

Treatment Durability

The major impediment in stem cell therapy is the tumor relapse irrespective of response. Therefore, multiple modalities must be used to successfully overcome tumor heterogeneity or drug resistance. It also minimizes the risk of tumor relapse.

Tumorigenicity

Stem cell has unique capacity to produce unaltered daughter cells (self-renewal), generate specialized cell types (potency), and undergo epithelial-to-mesenchymal transition similar to cancer stem cell therefore whether the cells have tumorigenic potential is hard to define since even established cancers can be difficult to grow with functional in vitro or in vivo assays. Many studies have documented the potential contribution of grafted stem cell in tumorigenesis, however multipotent NSCs, MSCs, and HSCs seems to better grafting option in terms of safety than ESCs and iPSCs.

CONCLUSION

Stem cell therapies give hope to those patients who normally not receive any treatment to cure their diseases. Medical professionals and researchers observed that the stem cells show promising anticancer cell therapy in the form of a bone marrow transplant, drug delivery, gene therapy, immunotherapy and living microorganism delivery but with the exception of bone marrow transplant, most are still in early stages and some undergoing clinical trials. The therapeutic efficacy and potential side effects of most stem cells used in the treatment of diseases are still not clear Henceforth, uniform regulation of stem cell therapy is required for the use on large scale rather than the use of surgery, radiotherapy and chemotherapy. Therefore, there is an urgent need to ascertain the behavior of different types of stem cells with their surrounding environment, regulation of biological processes such as cellular proliferation, apoptosis, growth and development by stem cells in order to improve in stem cell therapeutic performance and applicability in many different diseases. In nutshell, stem cell therapy has a high potential to

treat numerous diseases. Though, a more detailed associated risk assessment and potential hazards are a *prima facie* before their broader clinical application.

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Chapter 17
Cancer Stem Cells and Advanced Novel Technologies in Oncotherapy

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ABSTRACT

Self-renewal is the most important property of stem cells. Parallel to this, cancer stem cells (CSCs) have an indefinite proliferative ability that drives tumorigenesis. The conventional treatment of cancer includes chemotherapy, radiotherapy, and surgery, which decreases the tumour size. Contrary, targeted therapy against CSCs initially does not shrink the tumour but ultimately causes tumour degeneration. Nanobiotechnology, RNA interference, microRNA are emerging fields with a vital role in targeted therapy against CSCs. The non-protein encoding microRNAs has a major role in cancer treatment since they regulate gene expression during post-transcription. RNAi technology can silence the gene of interest with potency and specificity inhibiting tumour growth. In nanoparticles-based RNA interference, nanocarriers protect RNAi molecules from immune recognition and enzymatic degradation. The cancer cell gene expression profiling using next-generation sequencing helps in understanding the underlying cancer cell mechanisms. The current chapter deals with novel concepts in the treatment of cancer.

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INTRODUCTION

The human body consists of trillions of cells estimated to be 10^{14} and there are 250 types of specialized cells committed to a unique and distinct function. Normal cells divide in a highly controlled way and they succumb once their function is over or damaged. Further, new cells replace the old and carry out their special assigned chores. In case of cancer, the cells divide uncontrollably and keep on growing rapidly in an unregulated pace. Cancer cells contain genetic alterations and epigenetic modifications which crowd out normal cells, these are the parts where cells modify and generate cancer. Cancer cells are also often able to evade the immune system, a network of organs, tissues, and specialized cells that protects the body from infections and other conditions. Metastasis is the condition where cancer spreads to a different body part from where it started. Cancer is a devastating genetic disease, it is one of the major non-communicable diseases (NCD) in developing as well as developed nations, the leading causes of mortality worldwide (Lekha et al., 2018). The term cancer was derived from the Greek word Karkinos (Crab or Crayfish) (Lekha et al., 2018 and Prasad et al., 1982). The Greek physician Hippocrates (460-370 BC) who is considered as the "Father of Medicine", was the one who used the term "carcinos" and later Greek term translated it to cancer by the Roman physician, Celsus (28-50 BC) (Manohar, 2015). Generally, the normal functional cell transformed into a cancer cell mentioned as carcinogenesis, where two kinds of genes are involved in this process: (i) Oncogenes, which supports cell growth and differentiation (ii) tumour suppressor genes, which inhibits cell division (Pierouli et al., 2019). Various studies revealed that stem cells were involved in both normal development and carcinogenesis (Ciurea et al., 2014). Carcinogenesis or cancer development is a multifaceted phenomenon involves accretion of an arrangement of genetic, epigenetic, histological, and biochemical changes ultimately leading to the progression of pathological manifestations (Khan et al., 2019). The stem cells are present in many different somatic tissues, these cells are unique because it has three important properties a) self-renewal b) undifferentiated c) extensively proliferated. The primary and distinctive property of self-renewal is mainly notable due to more relevant to oncogenesis and malignancy. Then cancer stem cells (CSCs) are found in tumour or cancer possesses characteristics similarity with normal stem cells especially the capability to give rise to all cell types (Chen et al., 2011). The origin of the cancer stem cell (CSC) remains an enigma. After enormous clinical studies, the researchers have discovered that adult stem cells can turn into cancer stem cells with specific surface markers. The leukaemia CD34+/CD38− is one of the best-known examples of the surface marker of CSCs (Ciurea et al., 2014).

CSC plays a major role in tumour growth, so it is an utmost compulsion to develop targeted therapy against CSCs. In this chapter, we will discuss cancer stem cells, their origin, how it is involved in tumour growth and therapeutic strategies with new technologies including Nanobiotechnology, RNA interference (RNAi), microRNA, and Cancer cell gene profiling. Additionally, we will discuss about Cancer vaccines as well as CAR-T-cells involved in cancer treatment.

A NEW PERSPECTIVE OF CANCER STEM CELLS (CSCs)

The most adventurous research in cancer therapy is targeting of CSCs. In 1997 cancer stem cells were first reported by Bonnet and John Dick. Stem cell biologists isolated a subpopulation of acute myeloid leukaemia (AML) cells that expressed surface marker CD34 but lacked CD38 surface marker. This CD34+ /CD38− subpopulation could initiate tumour. In human AML the frequency of this CSCs is less

than 1 in 10,000 (Bonnet and Dick, 1997). The findings of AML cancer stem cells had prompted the identification of CSCs in other cancer types which includes brain, lung, breast, colon, pancreas, ovary, and prostate (Chen et al., 2011) head and neck, oesophagus, liver, testis (Moharil et al., 2017). Few of the organ-specific CSC surface markers summarized in table 1. Because of self-renewal and differentiation properties, CSCs has great potential to generate tumour. The conventional therapy includes hormone therapy, radiotherapy, chemotherapy fails due to their failure in recognition of CSCs. Conventional anti-cancer treatment can decrease tumour size but persistent CSCs would re-initiate the tumour formation. The targeted therapy against CSC using an efficient alternative as displayed in figure 1 (Asghari et al., 2019). It could improve the quality and survival of life of cancer patients particularly in metastatic cases and would cause lesser side effects (Chen et al., 2011). The "tumour -initiating cells" (TICs) is a term frequently described with CSCs capacities, yet there is confusion between two terms like cancer stem cells and tumour -initiating cells. The CSC is derived from a normal stem cell that requires the necessary genetic mutations for malignant transformation, whereas "tumour -initiating cells" are derived from the tumour itself. There is a crystal-clear evidence that the CSCs or tumour -initiating cells change as the disease progresses (Vermeulen et al., 2008). Even though the conventional therapies improved patient's survival yet they have several limitations includes drug resistance, excessive toxic effects, non-specific targeting, and undesired side effects (Gurunathan et al., 2018). The CSCs are responsible for the tumour relapse as they have the confinement to get resistant with conventional therapeutic options in cancer therapy including chemotherapy and radiation (Ambasta et al., 2011).

Figure 1. A schematic illustration showing targeted therapy against CSC

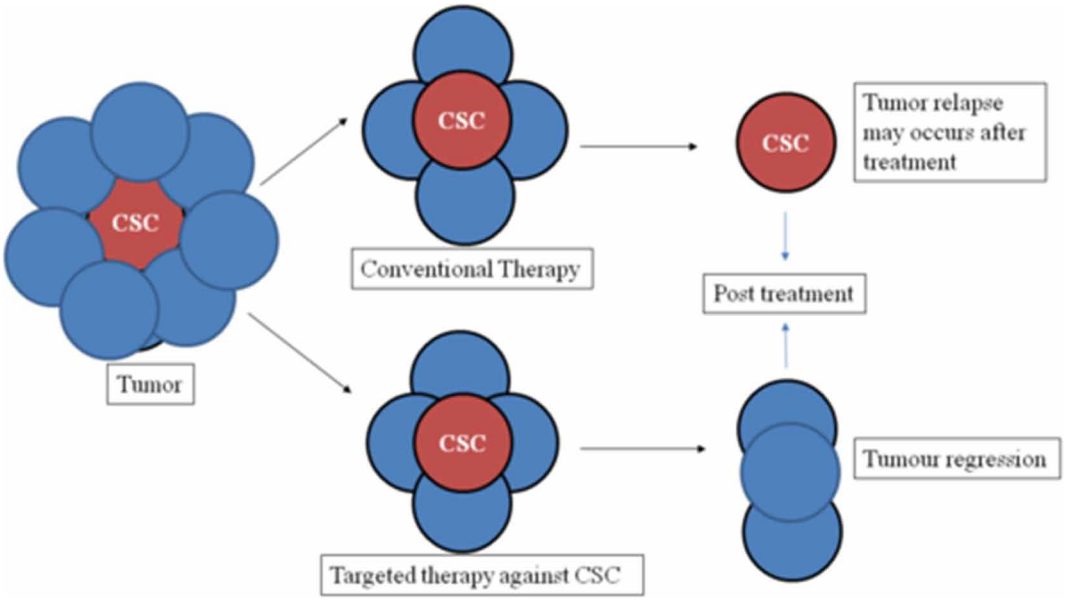


Table 1. List of CSC surface markers

Cancer type	CSC surface markers	Reference
Brain cancer	$CD133^+$	(Beier et al., 2007 and Singh et al., 2003)
Breast cancer	$CD44^+/ESA^+/CD24^-$/ALDH1	(Ricardo et al., 2011 and Croker et al., 2009)
Lung cancer	$CD133^+$	(Eramo et al., 2008)
Liver cancer	$CD133^+/CD90^+$	(Ma et al., 2007 and Yang et al., 2008)
Pancreatic cancer	$CD44^+/CD24^+/ESA^+$	(Li et al., 2007 and Hermann et al., 2007)
Colon cancer	$CD133^+$	(Vermeulen et al., 2007 and Kong et al., 2011)
Prostate cancer	$CD44^+/CD133^+$	(Collins et al., 2005 and Liu et al., 2011)
Ovarian cancer	$CD133^+/CD44^+/CD117^+/CD24^+$	(Gao et al., 2010 and Silva et al., 2011)
Multiple myelomas	$CD138^-$	(Peacock et al., 2007 and Asghari et al., 2019)
Leukaemia	$CD34^+/CD38^-$	(Wang et al., 2005 and Kong et al., 2011)

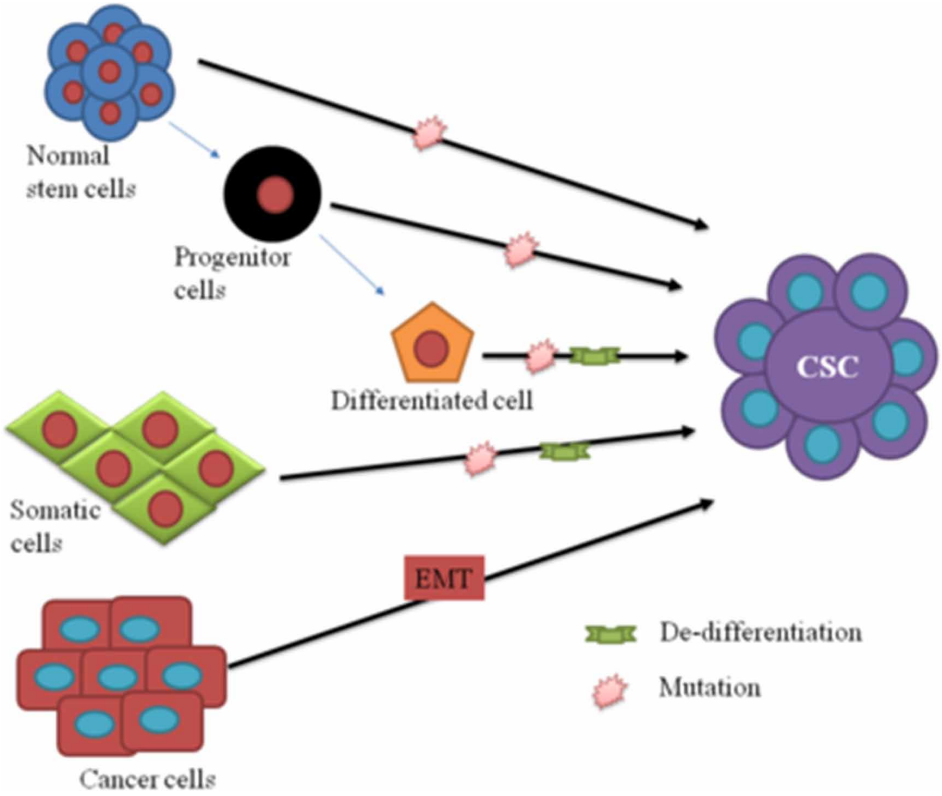
The Origin of CSCs

The fundamental characteristics of CSCs similar to the normal stem cells are the self-renewal property to form indistinguishable daughter cells through cell division and differentiate into many types of progenies (Eun et al., 2017). There are two main existing postulates regarding the origin of the CSCs that they originate either from a somatic cell or derived from embryonic or adult stem cells (Allegra and Trapasso, 2012). Normal stem cells may turn into CSCs that undergoes mutation. Another process where CSCs develops from restricted progenitors and differentiated cells that have assimilated the property of self-renewal as a sign of genetic or epigenetic mutations (Zhang et al., 2012). Furthermore, CSCs can progress from dedifferentiated somatic cells. Likewise, CSCs originate from cancer cells itself that undergoes epithelial-mesenchymal transition (EMT) (Khan et al., 2019) shown in figure 2.

Role of Epithelial-mesenchymal Transition (EMT) in CSCs

Epithelial-Mesenchymal Transition (EMT), a conserved evolutionary process that has been documented not only as a normal physiological mechanism for the embryogenesis, tissue homeostasis, tissue development, wound healing but also recognized as a pathological mechanism in the progress of several diseases including fibrosis, inflammation, and cancer(Li et al., 2011 and Thiery et al., 2009). Hence, the multistep event of EMT during cancer progression and metastasis closely resembles the embryological development (Iwatsuki et al., 2010). Greenburg and Hay were the first to analyse the EMT related changes in cell phenotype and also mesenchymal states in embryonic and adult epithelia in the 1980s (Greenburg and Hay, 1982). Progression of most carcinomas is associated with the acquisition of EMT, which results in the loss of expression of epithelial markers (E-cadherin and Zonula occludens-1 (ZO-1), Desmoplakin, Cytokeratin) and gains the expression of mesenchymal markers (vimentin, metalloproteases, fibronectin and N-cadherin)(Kong et al., 2011 and Kiesslich et al., 2013)and increases of "stemness" genes (e.g., Oct-4 and Nanog)(Van de Stolpe, 2013) leads to cell motility and invasion (Hollier et al., 2009) as summarized in table 2.The metastatic cells effectively undergo EMT i.e. CSC separate from the primary tumour and invade into the extracellular matrix (ECM), known as intravasation, survive in

Figure 2. An overview of the origin of the cancer stem cells (CSCs)



the circulating blood and then disseminate into distant organs known as extravasation, endure reverse mesenchymal-epithelial transition (MET), settle and eventually form micro metastases and outgrowth of clinically secondary cancer lesions(Yang et al., 2015). Outstandingly, findings of EMT cells that exhibited an increased drug resistance-related with CSCs signatures (Gupta et al., 2009).

The greatest event of EMT is the loss of cell-cell adhesion with reduced expression of E-cadherin which is mediated by diverse transcriptional repressor factors, e.g. ZEB1(zinc finger E-box binding homeo box 1), twist and snail1which later initiate a stem cell transcriptional program (Van de Stolpe, 2013). The E-cadherin coded by the gene CDH1 has two functions in epithelial cells that act as a cell-cell adhesion molecule and as the canonical WNT signalling cascade which negatively regulate central mediator of β-catenin (Schmalhofer et al., 2009). Additionally, EMT is influenced by an epigenetic mechanism including histone modification and DNA methylation and also regulated by microRNAs (miRNAs) (Kiesslich et al., 2013).

Table 2. List of loss of expression of epithelial markers and gains the expression of mesenchymal markers

Downregulation of epithelial markers	Upregulation of mesenchymal markers	Upregulation of nuclear localization or transcriptional factors
• **E-cadherin** • **Desmoplakin** • **Cytokeratin** • **Occludin**	• N-cadherin • Vimentin • Fibronectin • matrix metalloproteinase (MMPs)	• β –catenin • Smad-2/3 • NF-κB • Snai1/2, twist

CSC Niche

CSC exists in a distinctive microenvironment called a niche, plays a key role in CSC maintenance/enrichment, immune surveillance, and angiogenesis activation, preservation of phenotypic plasticity, differentiation/dedifferentiation, and invasion/metastasis. The tumour microenvironment or niche is the main factor that extrinsically impacts tumour heterogeneity. Niche is comprised of endothelial cells, immune cells, stromal cells, and cancer cells as well as connective tissue components, cytokines, and growth factors (He et al., 2017). The normal stem cell niches tumour-suppressive consisting of numerous activated signalling pathways to arrest cell growth. On the contrary, CSC niches comprise of tumour micro environmental cells including cancer-associated fibroblast that induce CSC growth and differentiation via activation of survival pathways through cell-cell interactions or growth factor secretion (Khan et al., 2019).The tumour niche/microenvironment not only provide growth-promoting signals but also contributes to therapeutic resistance by defending cancer cells from the therapy-induced damages (Eun et al., 2017).

CSC Signalling

The Wnt/β-catenin, Hedgehog (Hh) and Notch signalling pathway (Asghari et al., 2019) have critical roles in the maintenance and reappearance of CSCs (Khan et al., 2019). The Wnt/β-catenin is a conserved signal transduction pathway dynamically involved in both initiation and regulation of a numeral biological feature, for example, stem cell self-renewal, cell survival, cell growth, polarity, calcium homeostasis, migration, and organogenesis. Decontrolled functioning of the Wnt/β-catenin pathway is related to the pathogenesis of most human diseases including cancer (breast and colorectal cancer), neurodegenerative, and inflammatory, endocrine, and bone disorders (Khan et al., 2019). The Hedgehog (Hh) signalling pathway may regulate numerous cancer stem cells including glioblastoma, breast cancer, pancreatic adenocarcinoma, and chronic myeloid leukaemia (CML), multiple myeloma. The Hh signalling plays multiple roles in development, homeostasis, and disease by activating the Smoothened (Smo), which is a 7-pass transmembrane protein sending an intracellular signal. Owed to that, the most innovative types of Hh antagonists are targeted against Smo (Asghari et al., 2019). The Notch signalling pathway is additionally highly conserved well-known signalling pathway for the regulation of angiogenesis, cell proliferation, differentiation, stem cell fate determination, and development. The recent report revealed that deregulated Notch signalling is critically associated with the angiogenesis, proliferation, differentiation, maintenance, and migration of CSCs in human cancers. Therefore, targeting the Notch signalling pathway and its regulator might be of great significance in the eradication of CSCs and cancer management (Khan et al., 2019).

ADVANCED TECHNOLOGIES INVOLVED IN CANCER THERAPY

Cancer remains the second global cause of death (Mansoori et al., 2014). The International Agency for Research on Cancer (IARC) reported that worldwide there were 8.2 million deaths by cancer in 2012 and predicted to surge by 2030 up to 13 million (Gurunathan et al., 2018). The cancer is represented as a group of heterogeneous diseases well-characterized by uncontrolled growth and spread of abnormal cells ultimately leading to death. The treatment of cancer is not simple, it has some of the major issues which

are narrow therapeutic window, multidrug resistance (MDR), and side effects, limitations of available anticancer drugs. MDR is a major impediment that contributes to the failure of chemotherapies in various types of cancers including lung, breast, gastrointestinal, ovarian, and haematological malignancies (Gurunathan et al., 2018).

NANOTECHNOLOGY

Nanotechnology refers to an emerging field of science that includes synthesis and development of various nanomaterials, extremely small particles range of 1-1000 nm (1μm) (Ambasta et al., 2011). The prefix word "nano" means miniature size, nano is a Greek word which means "dwarf". Nanotechnology defined as the treatment of individual atoms, molecules, or compounds into structures to produce nanomaterials and nanodevices with distinctive properties. There are two approaches of synthesis of nanomaterials, top-down or the bottom-up involves altering discrete atoms and molecules into nanostructures (Nikalje, 2015). Nanotechnology applied in the medical field known as nanomedicine has opened a window for the development of inorganic and organic drug carriers as recognized as nanoparticles. The source material for nanoparticle includes lactic acid, chitosan, phospholipids, dextran, cholesterol, silica, carbon, polyethylene glycol (PEG), and some metals. Further, the surface of the nanoparticles modified by covalent conjugation with small functional groups for increasing their ability of precise targeting. Functional groups that improve nanoparticle specificity including antibodies, aptamers, folate, and tripeptide Arginine-Glycine-Aspartic acid (RGD) (Diaz et al., 2013).

Successively nanoparticles were utilized for diagnosis and treatment which are known as theragnostic carriers such as liposomes, dendrimers, carbon nanotubes, polymeric micelles and quantum dots that have been utilized for the delivery of drugs in cancer therapy summarized in table 3. In 1978, Widder and group were the first to propose drug delivery using nanoparticles. The basic principle behind is either the attachment or encapsulation of drug within the nanoparticle, these particles have a polymer or metal coating with magnetic cores. It helps to attach or immobilize a drug or an antibody on the nanoparticle surface and target into the desiderate site. The nanoparticle which is synthesized by the gold shell and iron core has been used to carry drug-like Doxorubicin, binds to an amino group in the gold shell (Ambasta et al., 2011). The nanotechnology applications in medicine including six various areas including i) diagnosis and imaging; ii)molecular changes detection in diseases; iii) drug delivery; iv) combined therapeutic and diagnostic (theragnostic) applications; v) report of therapeutic agent efficacy; and vi) applications of nanotechnology in scientific discovery and basic research (Asghari et al., 2019).

NANOPARTICLES USED IN CANCER THERAPY

Liposomes

In 1961, Bangham was the first to describe liposomes nanoparticles applied in medicine. Liposomes are spherical lipid vesicles with a structure of phospholipid bilayer. Nanoliposomes are nanometric versions of liposomes range 30–100 nm formed by phospholipids such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, and cholesterol, naturally occurring major components of the bilayer cell membrane. Nanoliposomes offer superfluous advantages of the

capability to modify surface and size, low toxicity, biodegradability, and biocompatibility. To diminish the recognition of nanoliposomes by macrophages, it can be coated using polyethylene glycol (PEG), biocompatible polymers identified as PEGylated or stealth liposomes. Example: Liposomes + drug (the Herceptin/trastuzumab antibody) which targets the Her-2 antigen expressed by assured breast cancer cells (Diaz et al., 2013).

Polymeric Nanoparticles

Polymeric nanoparticles are solid colloidal particles range 50-300 nm primed from biodegradable polymers like collagen and chitosan or non-biodegradable polymers like poly lactic-co-glycolic acid (PLGA) and polylactic acid (PLA). To improve the stability of PLGA nanoparticles further it can be coated with PEG. The natural polymer, Chitosan gained by the partial N-deacetylation of chitin, is a second most plentiful polysaccharide in nature (Diaz et al., 2013).

Polymeric Micelles

Polymeric micelles are prepared by amphiphilic block copolymers, for example, poly (N-isopropylacrylamide)-polystyrene and poly (ethylene oxide)-poly (β-benzyl-L-aspartate). Micelles, less than100 nm gathered with a hydrophilic shell and hydrophobic core make micelles effective nanocarriers for unwell water-soluble anticancer drugs, such as docetaxel and paclitaxel (Diaz et al., 2013).

Dendrimers

Dendrimer nanoparticles are globular macromolecules range vary from 5-10 nm with well-defined branching synthetic architectures. Dendrimers are composed of three different parts; a focal core, monotonous units of several interior layers, and several peripheral functional groups for further modification. Dendrimers are useful for the controlled release of the drug (Diaz et al., 2013).

Carbon Nanotubes (CNTs)

The carbon nanotubes (CNTs) are an allotrope of carbon, the most fascinating nano vectors presently under exploration. Functionalized CNTs had documented prodigious capacity as novel drug delivery systems based on their capability to cross biological barriers. Even though the specific mechanism of internalization (needle-like penetration or endocytosis) is not yet fully revealed, it is commonly documented that CNTs can enter cells, self-governing of cell type and functional groups present on their surface. Additionally, their high surface area offers numerous attachment spots for molecules, permitting for polyvalent derivatization. The utmost investigated anti-cancer drug in this framework are anthracycline doxorubicin (Dox), with PEG functionalized single-walled carbon nanotubes (SWCNTs) or copolymer-coated multi-walled carbon nanotubes (MWCNTs). Anti-tumour immunotherapy studied with CNT-based employs tumour cell vaccines (TCV). The TCV is made up of dendritic cells presenting tumour antigens or inactivated cancer cells to stimulate the immune response of the long-suffering patient against the tumour (Fabbro et al., 2012)

Quantum Dots (QDs)

Quantum dots (QDs) are 2–10 nm in size, nanocrystals of semiconducting materials containing a semiconductor inorganic core (e.g., CdSe), and an aqueous organic coated shell (e.g., ZnS) to increase optical properties. It can fluoresce while stimulated to light (Bhatia, 2016).

Table 3. Various example of drugs that can be delivered through nanomaterials

Therapeutics	*Nanocarriers*	*Target*	*References*
Cisplatin	Aptamer-PEG-PLGA	Prostate cancer	(Bhatia, 2016).
Vincristine+ Verapamil	PLGA Polymeric nanoparticles	Hepatocellular carcinoma	(Bhatia, 2016).
Interleukin-2	Protein liposome	Lung cancers	(Bhatia, 2016).
Doxorubicin	2,2 bis(hydroxymethyl) propanoic acid-based Dendrimers	Colon carcinoma cells of rat	(Bhatia, 2016).
5-Fluorouracil	ZnS Quantum Dots	Breast cancer	(Bhatia, 2016).
Methotrexate	MWCNs	Breast cancer	(Bhatia, 2016).
Gemcitabine	SWCNTs	Ovarian cancer	(Bhatia, 2016).
Folic acid	Gold nanoparticle	Ovarian cancer	(Asghari et al., 2019)
SPIONs-PEG-Ab	Monoclonal Ab A7	Colorectal carcinoma	Asghari et al., 2019)

Instances

There is a novel strategy that inhibits angiogenesis near CSCs. Hence new tumour can't grow and the existing tumour is unable to metastasize. Angiogenesis is an important process of the formation of new blood vessels from foregoing vasculature, controlled by pro-angiogenic and anti-angiogenic factors in the human body. The regulatory angiogenic factors including VEGF, PDGF, EGF, FGF, Placental growth factor, Angiogenin, Angiopoietin-1, and Interleukin-8.The anti-angiogenic factors including Angiostatin, Vasostatin, Prolactin, Endostatin, Angiopoietin-2, Fibronectin, Interferonα and γ Platelet factor-4, and Interleukin-12 (Fayette et al., 2005).It is believed that in the absence of the blood vessel formation, the tumour size can't exceed beyond 1-2 mm and also primary tumour can't escape and metastasize since blood delivers nutrients and oxygen for the survival of cells. The hypoxic condition enhances angiogenesis through activating the production of VEGF which binds to VEGF receptor (VEGFR) leads to the formation of a blood vessel and oxygenation of tumour ultimately turns into increase the tumour mass shown in figure 3 (Ambasta et al., 2011).The twin nanoparticle, gold nano shell encompassing with superparamagnetic iron oxide nanoparticles abbreviated as SPIONs approximately 0.4 to 0.5 nm thickness is used. The VEGF monoclonal antibody tagged with the iron and cancer stem cell marker tagged with the gold. It can be mobilized to a tumour site by applying the external magnetic field, and released VEGF antibodies in the tumour cells lead to blockage of the angiogenic pathway. This method is targeted to the destruction of CSCs and VEGF mediated angiogenesis pathway shown in figure 4 (Ambasta et al., 2011).

Figure 3. Tumour growth pathway: Cancer stem cell (CSCs) triggers tumour growth in hypoxic condition releases VEGF signal, initiate endothelial cell proliferation and blood vessels formation to vascularise the growing tumour

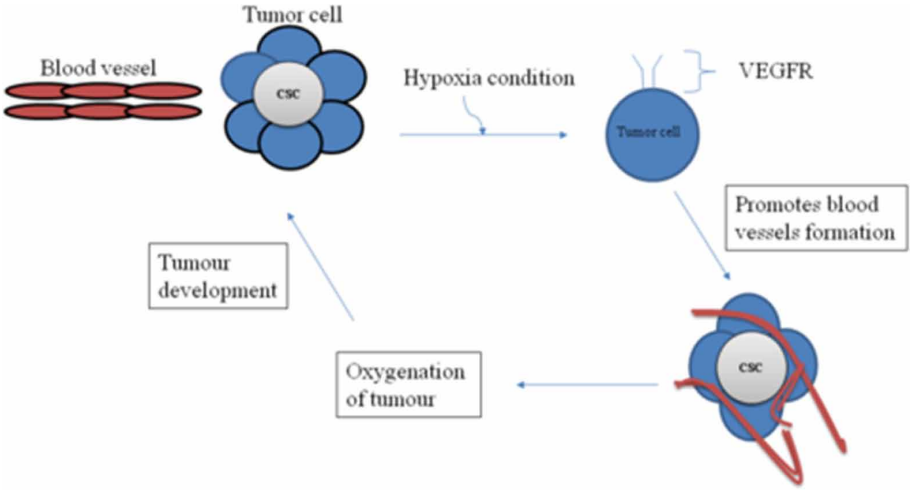
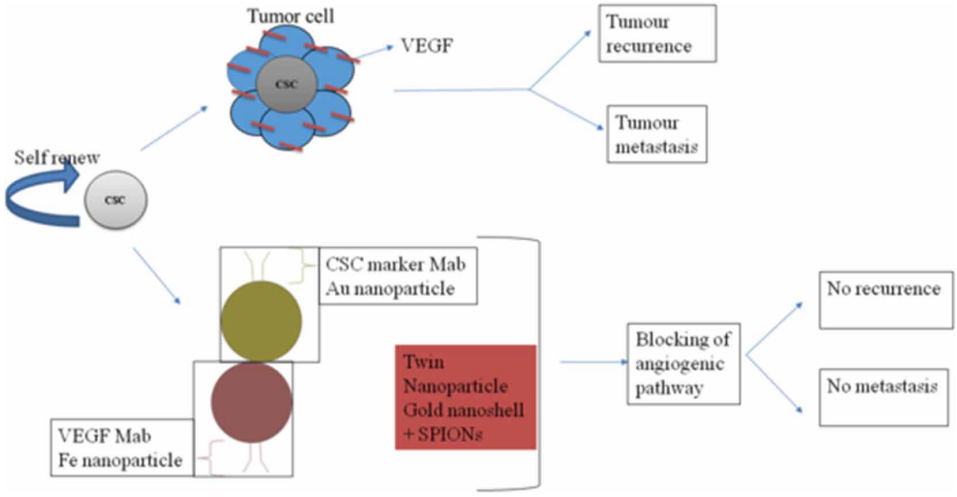


Figure 4. Blocking of angiogenic pathway using Gold, Super paramagnetic iron oxide nanoparticles (SPOINs), VEGF and Cancer stem cell marker monoclonal antibody tagged nanoparticles for targeted destruction of CSCs and VEGF mediated angiogenesis pathway



Advantages

- Nanotechnology is a convenient tool for combination therapy.
- Decreasing MDR by targeting various metabolic and physiological characteristics
- Improving plasma half-life, bio-distribution, and bio-availability of drugs
- Enhanced permeability and retention (EPR) effect
- The sustained controlled drug release

Disadvantages

- Non-biodegradable nanoparticle causes high tissue accumulation leads to toxicity.

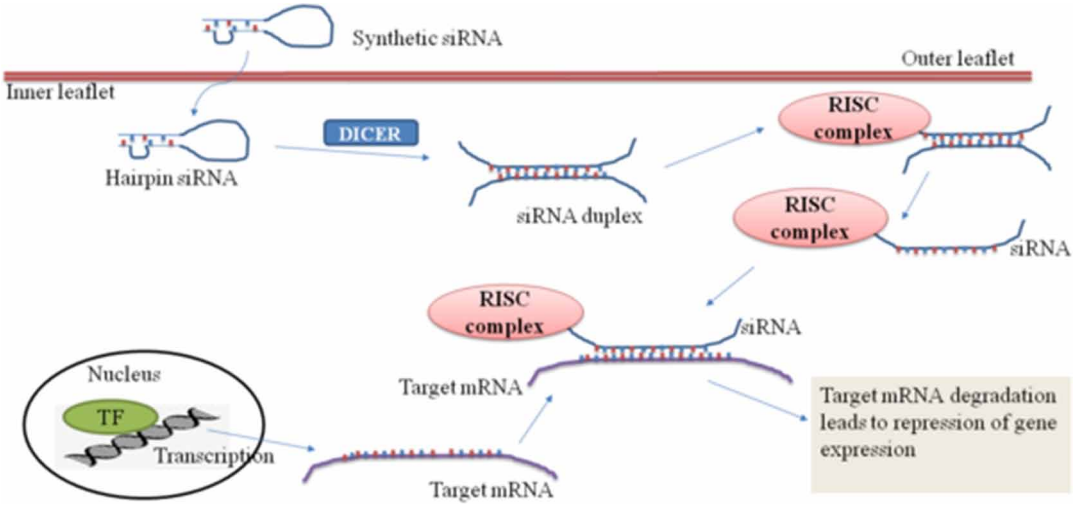
RNA INTERFERENCE-BASED THERAPY

In 1998, RNA interference (RNAi) molecules discovered by Fire's and Mellos'sin the *Caenorhabditis elegans* (*C. elegans*, a round worm) (Fire et al., 1998) and were awarded Nobel Prize in 2006 for their contribution in retrospective work of RNAi molecule research (Uchino et al., 2013). RNAi refers to the member of non-coding RNA (ncRNA). The ncRNAs are not translated into protein, despite doesn't mean it has no role (Mansoori et al., 2014). Studies have been shown only 2% of the mammalian genome is transcribed into mRNA further translated into protein, whereas preponderance of the mammalian genome is transcribed into ncRNA. The non-coding RNA is broadly divided into two categories, which are: i) Small regulatory RNA and ii) Long non-coding RNA. RNAi is a portion of a small regulatory RNA, includes small interfering (siRNA) and miRNA (Khvorova et al., 2003).RNAi based therapy is a powerful tool, used for treating cancer and there is evidence for its use in the treatment of various human diseases including respiratory syncytial virus (RSV) infection, age-related macular degeneration (AMD),and neurodegenerative disorders and single-gene disorders(Uchino et al., 2013). In cancer therapy, various kinds of small synthetic RNAs including siRNA, bish RNA, and shRNA are used. The considerate targets for gene silencing by RNAi based therapy for cancer are mutated tumour suppressor genes, oncogenes, and some other genes involved in tumour progression. The studies manipulated on robust animal models explored that targeting the precise protein in the cell cycle, like polo-like kinase (PLK1) and kinesin spindle protein (KSP) via siRNA displays an effective anti-tumour activity (Mansoori et al., 2014).

Mechanisms of Gene Silencing by siRNA

siRNA is formed in two stages i.e. starting and effecting stages. Primarily, the starting stage, along with double-stranded RNA (500-200 bp) is sliced into fragments by Dicer with a length of 23-21 nucleotides, and siRNA is formed. Another, the effecting stage where double-stranded siRNA is detached by helicase. Then the sense strand is delineated by endogenous endonucleases and the anti-sense strand is directed to the RNA induced silencing complex (RISC). This complex (i.e., antisense strand and RISC) is then directed to the target mRNA. A member of RISC, Argonaute has ribonuclease activity that degrades the target mRNA from the piwi region. Target mRNA degradation by RISC occurs in two pathways. Former, they might be fragmented by ribonuclease. Additionally, they may be associated with the homologous strand, and RNA polymerase forms double-stranded RNA leading to the continued interference pathway. By degradation of the target, mRNA leads to suppression of the target gene expression (figure 5), which is acknowledged as post-transcriptional gene silencing (PTGS) (Mansoori et al., 2014). Several kinds of delivery systems have been engaged for delivering siRNA such as antibody conjugates, peptides, micelles, natural polysaccharides, and synthetic cationic polymers. Yet, some lipid-based formulations such as niosomes, liposomes, and stable nucleic acid-lipid particles (SNALPs) have shown to be effective drug delivery systems as hopeful strategies for in vivo siRNA delivery is summarized in table 4 (Chalbataniet al., 2019).

Figure 5. Target gene suppression by siRNA; long double stranded RNA fragmented by Dicer leading to suppression of targeted gene



Advantages

- High specificity and lack of side effects compared to chemotherapy
- Low cost compared to other methods of gene therapy
- Targets multiple genes of different cellular pathways involved in tumour progression
- Developing a personalized drug for a specific patient is possible

Disadvantages

- The systemic delivery to tumour targets other than the liver has resulted in other problems.

Table 4. The current clinical status of RNAi therapeutics- siRNA based drug delivery in cancer therapy

siRNA drug	Target	Indications	Delivery system	Phase	References
CALAA-01	M2 subunit of ribonucleotide reductase (RRM2)	Solid tumours	Rondel® Nanoparticles (CD)	I	(Wang et al., 2011, Tatiparti et al., 2017, and Chalbatani et al., 2019)
siRNA-EphA2-DOPC	EphA2	Advanced solid tumours	Lipid-based nanoparticles	Pre-clinical	(Tatiparti et al., 2017)
DCR-MYC	Myc	Hepatocellular carcinoma	Lipid nanoparticles	I	(Chalbatani et al., 2019)
Atu027	PKN3	Advanced solid cancer	siRNA-lipoplex	I	(Mansoori et al., 2014)
ALN-VSP02	KSP, VEGF	Solid tumour s	SNALP	I	(Wang et al., 2011, Tatiparti et al., 2017, and Mansoori et al., 2014)
ATN-RNA	Tenascin-c	Astrocytic tumour	Necked	I	(Mansoori et al., 2014)

MicroRNA IN CANCER: BEGINNING WITH RESEARCH TO THERAPY

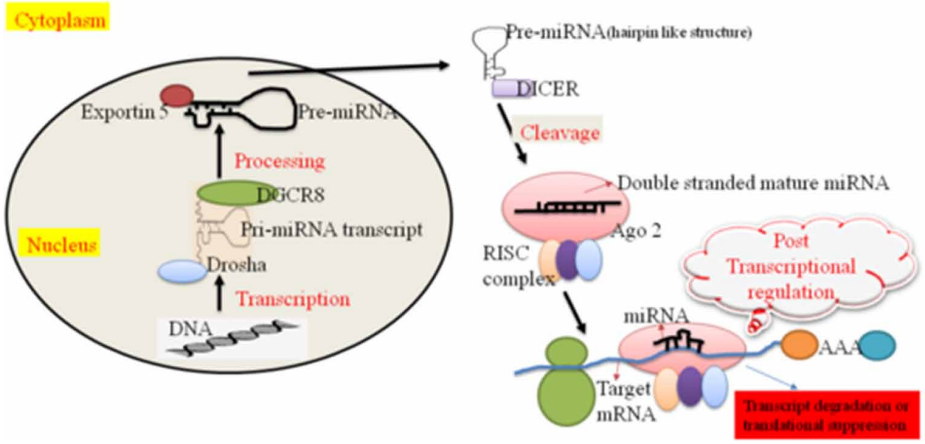
MicroRNAs or miRNAs/miRs are endogenous, tiny (20–24 nucleotides) non-coding RNAs discovered in 1993 (Kota et al., 2010) characterized in *C. elegans* (Kwok et al., 2017), lin-4 and let-7 miRNA (Khan et al., 2019). They were helpful for new findings in the phenomenon of RNA interference (RNAi) in 1998.The large family of non-coding RNAs encompasses miRNA, small interfering RNA (siRNA), nat-siRNA, tasiRNA, rasiRNA, tncRNA, scnRNA, piwi-interacting small RNA (piRA), and so on (Kota et al., 2010). miRNA is a very stable biological material. Human serum when exposed to diverse extreme states including pH, temperature, and freeze/thaw cycles still resulted in quantifiable miRNAs through RT-qPCR (Kwok et al., 2017). The miRNA genes are positioned in all chromosomes in humans except the Y chromosome. Approximately 50% of known miRNAs found in clusters and transcribed as polycistronic transcripts (Negrini et al., 2007). The human genome produces greater than 1,500 miRNAs(Kim et al., 2018).There are scientific reports demonstrated that miRNA negatively regulates the gene expression associated with several biological phenomena including development, proliferation, differentiation, homeostasis, and apoptosis through different mechanisms such as binding to the 3' untranslated regions (3' UTRs) or open reading frame of specific genes causing a suppression in translating mRNA or leading to the mRNA degradation via initiation of Argonaut (AGO) proteins which targets the 3' UTR. Surprisingly, a single miRNA able to regulate the multiple gene expression (Khan et al., 2019). Moreover, miRNAs are tissue-specific expressed in both animals and plants, and the dysregulation of miRNA directly related to the pathological origin of cancer (Zhang et al., 2019).

Biogenesis of miRNA, Novel Regulators of Gene Expression

The first step of the biogenesis of miRNA is transcribed into primary miRNA (pri-miRNA) transcripts with the help of RNA polymerase II. Next pri-miRNA further processed (spliced, capped, polyadenylated) by RNase III endonuclease enzyme Drosha and its cofactor protein (Pasha, or DGCR8) generates precursor-miRNA (pre-miRNA) in the nucleus approximately 70nucleotideswith a hairpin-like structure. Later, Exportin-5complex transports pre-miRNAs from the nucleus to the cytoplasm, then they were established into mature microRNA (miRNA) duplexes (do not contain loop) 22 nucleotides by Dicer, another RNase III enzyme assembled with transactivating response RNA-binding protein and protein activator of PKR. In conclusion, one strand, a guide strand of the mature miRNA duplex, is merged into the Argonaute 2 (Ago 2) containing RNA-induced silencing complex (RISC), which prompts either cleavage or translational suppression of targeted mRNAs based on their sequences (figure 6). When the miRNAs are unstable or their functions are disturbed, they indulge in the initiation and development of fatal human disorders, including cancer (Uchino et al., 2013, Kota et al., 2010 and Rolle, 2015). The scientific report says that in 70% of patients affected with chronic lymphocytic leukaemia (CLL), a mutation or deletion in chromosome 13q14 leads to significant downregulation of miR-15 and miR-16 (Uchino et al., 2013).

First pri-miRNAs are transcribed by RNA polymerase II and cleaved by Drosha into 70 nucleotides as pre-miRNAs, hairpin like structure. Next pre-miRNA exported to the cytoplasm by Exportin-5 and cleaved to double-strand RNAs by Dicer. These duplexes are associated with Ago2 and one strand is removed. RISC containing the guide strand triggers post-transcriptional regulator of target mRNA depending on the seed sequence of niRNA.

Figure 6. The miRNA as novel regulator of gene expression



OncomiRs

The OncomiRs are overexpressed miRNAs, play a role as an epigenetic regulator causally interrelated to disease initiation, development, progression, and invasion in cancer. There are few reported microRNAs most commonly overexpressed in tumours and their target genes. For example, Mir-155 is an overexpressed oncomiR in the breast, colorectal, and pancreatic cancer which is involved in invasion, angiogenesis, and DNA repairing by targeting core VHL, RAD51, MMR proteins (hMLH1, hMSH2, and hMSH6), and the suppressor of cytokine signalling SOCS1. Mir-21 is overexpressed in many solid tumour types, such as brain, breast, lung, liver, stomach, colon, pancreas, prostate and it's intricate in tumour growth, apoptosis, and proliferation by targeting the tumour suppressor PTEN, TPM1 (tropomyosin 1) and the Programmed Cell Death 4 (PDCD4) (Tessitore et al., 2016).

Tumour Suppressor miRs

Mir-34 family members are controlled by p53, and able to prompt apoptosis and cell-cycle arrest. Amongst their targets are MYCN, YY1, a transcription factor which negatively controls p53, Notch1, the receptor tyrosine kinase AXL, playing an important role in angiogenesis, cell survival, autophagy, migration, quite a few genes involved in growth factor signalling (ARAF, PIK3R2) or cell cycle regulation (cyclins D3 and G2, MCM2, PLK1, MCM5, SMAD4), Fra-1, with consequential MMP-1/9 downregulation, PDGFR, MDM4, and MET (Tessitore et al., 2016). Some of the tumour Suppressor miRs are listed in table 6.

miRNAs as Therapeutic Targets

In the current scenario, miRNA have been explored as either tumour suppressors or oncogenes in a variety of tumours (Kota et al., 2010). Cancer tissues are noticeable by specific up-regulation or down-regulation of definite miRNAs compared to normal tissues. The miRNA targeting approach encompasses two types of miRNA therapy including; (i) miRNA reduction or inhibition therapy, use of anti-miRNA oligonucleotides (antagomiRs) against oncogenic miRNAs that upregulated in tumours and (ii) miRNA

Table 5. Most commonly deregulated OncomiRs in cancer

OncomiRs	Type of the cancer	Target	Reference
Mir-155	Colorectal Cancer (CRC)	MMR	(Valeri et al., 2010)
	Breast cancer	RAD51	(Gaspariniet al., 2014)
	Breast cancer	VHL	(Kong et al., 2014)
	Pancreatic cancer	SOCS1	(Huang et al., 2013)
Mir-21	Hepatocellular Carcinoma (HCC)	PTEN	(Meng et al., 2007 and Bao et al., 2013)
	Non-small cell lung cancer (NSCLC)	PTEN	(Zhang et al., 2010 and Liu et al., 2013)
	Gastric cancer	PTEN	(Zhang B.G et al., 2012)
	CRC	PTEN	(Xiong et al., 2013)
	Squamous cell carcinoma	PTEN, TPM1	(Li et al., 2009)
	Prostate cancer	TPM1	(Li T. et al., 2009)
	Renal cancer	PDCD4	(Li et al., 2014)
	Gastric cancer	PDCD4	(Cao et al., 2012)
	CRC	PDCD4	(Chang et al., 2011)
	HCC	PDCD4	(Qiu et al., 2013)
	Glioblastoma	PDCD4	(Chen et al., 2011)

replacement or restoration therapy for tumour suppressor miRNAs that are downregulated precisely in cancers(Kota et al., 2010 and Chakraborty et al., 2018).

(i) The miRNA Reduction or Inhibition Therapy

In this technique, miRNAs act as oncogenes. Usually, some of the miRNAs are overexpressed or upregulated in particular cancers. The miRNA reduction or inhibition therapy can inactivate those miRNAs, especially in tumours. The most collective strategies used to block or inhibit oncogenic miRNAs is accomplished by miRNA inhibitory agents like antisense oligonucleotides (ASO), locked nucleic acids (LNA, e.g., Miravirsen), an anti-miR oligonucleotide (AMO), and antagomiR sand microRNAs sponge therapy (Chakraborty et al., 2018 and Tessitore et al., 2016). Nanotechnology-based approaches have been developed for the above-mentioned molecules. Drug delivery system based on viral (adeno-associated virus (AAV) and lentivirus systems) or non-viral (polymers, liposomes) strategies to create the efficient activity of molecules to reach target cells in living animals are currently under investigation (Tessitore et al., 2016). The miR-21 is overexpressed in lung, breast, colorectal, and pancreatic cancer, leukaemia, lymphoma, glioblastoma, and neuroblastoma (Tan et al., 2018).

MicroRNA sponge therapy

miRNA sponge therapy is a potent approach for overexpressed miRNAs. The ultimate objective is to attain miRNA's functional loss via the inactivation of miRNAs. It has been established that logically occurring non-coding RNAs may function as miRNA sponges in animals, humans and plants. A unique example of a miRNA sponge effect is exhibited in the Hepatitis C virus (HCV) infection. The miR-122

Table 6. List of few major microRNAs with tumour suppressor activity

MicroRNAs	Type of the cancer	Target	Reference
Mir-34	Neuroblastoma	MYCN	(Wei et al., 2008)
	Neuroblastoma	YY1	(Chen et al., 2011)
	Glioblastoma	Notch1	(Li W. et al., 2011)
	Breast	AXL	(Mackiewicz et al., 2011)
	Colon, CML	ARAF, PIK3R2, cyclins D3 and G2, MCM2, MCM5, PLK1, SMAD4	(Lal et al., 2011)
	Colon	Fra-1	(Wu et al., 2012)
	Lung, breast, colon	MDM4	(Mandke et al., 2012)
	Gastric	PDGFR, MET	(Peng et al., 2014)
Let-7 family	Lung	RAS	(Johnson et al., 2005)
	Lung	HMGA2	(Lee and Dutta, 2007)
	Breast	ER-α	(Zhao et al., 2011)
	Ovarian	PRGMC1	(Wendler et al., 2011)
	Neuroblastoma	MYCN	(Buechner et al., 2011)

binds to the 5' UTR of HCV genomic RNA, with viral protein translation encouraged and the HCV genome is protected from degradation. This perfect mechanism is targeted as therapy with miravirsen, miR-122 inhibitor which is currently in clinical trial phase II for treatment naïve and non-naïve patients (Kwok et al., 2017).

(ii) The miRNA Replacement or Restoration Therapy

In this method, miRNAs function as tumour suppressors. Generally, some of the miRNAs are deleted or downregulated in specific tumours are exchanged by exogenous administration of artificial miRNAs (i.e. pre-miRNAs or mature miRNAs). For example, let-7 is a verified tumour suppressor miRNA that is downregulated in numerous cancer types including lymphoma, melanoma, breast, lung, ovarian, prostate, and pancreas and outwardly also in CSC. The latest research showed that exogenous delivery of let-7 to establish tumours in a mouse model of non-small-cell lung cancer leads to the reduction of tumour burden (Kota et al., 2017). The first commercial miRNA replacement or restoration therapy is MRX34, is a liposome-based intravenously injectable miR-34 mimic. It is presently being investigated in clinical trials Phase I for advanced hepatocellular carcinoma (HCC) patients (Chakraborty et al., 2018). The miR-34 is a straight target of p53, a tumour suppressor frequently down-regulated in a diversity of cancers, including gastric cancer, medulloblastoma, pancreatic cancer, and human glioma and targets Notch, Bcl2, and HMGA2. The introduction of miR-34 imitator (oligonucleotide)in human gastric cancer cells by lentiviral-mediated delivery ensued in cell cycle arrest at the G1 phase and impairs cell growth, and reduced tumour sphere formation (Kota et al., 2017).

Advantages

- Specific delivery of lethal anti-cancer drugs and oncolytic viruses to cancer cells.
- Safe and cancer-tissue-specific treatment modality.
- Aids the detection and prognosis of the disease.

Disadvantages

- Low RNA stability has been illustrated in vivo.
- Sometimes, targeted delivery molecules could be trapped in the first-pass metabolism with speedy localization of small molecules delivered to the liver and kidneys.

CANCER CELL GENE PROFILING

Agreeing to the eukaryotic central dogma of molecular biology, making protein from gene the entire practice known as gene expression, vital role in determining cell's nature, developmental stage, and both health and pathological functions. Primarily the gene expression process includes replication of DNA, then transcription of DNA to mRNA, further mRNA translates into proteins. The process of the genes expressed in a cell measured at a definite time acknowledged as gene expression profiling. Gene expression profiling is a beneficial tool for modern biosciences that can differentiate between normal and cancer cells. Most widespread technologies used for gene profiling are DNA microarrays, RNA sequencing and qPCR. The most crucial step in gene expression profiling is cDNA construction. The preliminary stage of cDNA making is the extraction of total cellular RNA from the cancer cells followed by isolating a particular type of RNA which is converted into cDNA by the process of reverse transcription.

High-throughput next-generation sequencing (NGS) has modernized technique in gene expression profiling, provides the capability of massively parallel short-read DNA sequencing using cDNA known as RNA sequencing. The data attained will be used to create FASTQ format files. The qPCR (quantitative real-time PCR) is a technique used to quantify gene expression and also be able to monitor the process of polymerase chain reaction (PCR) driven DNA amplification in real-time. Thermostable DNA polymerase enzyme used in PCR to synthesize new strands of DNA from the template (especially cDNA). PCR necessitates nucleotides, will act as building blocks and primers, will specify the gene of interest to be amplified. The PCR reaction continues to form repetitive DNA amplification cycles (35-40 cycle). Each cycle comprises of three obligatory steps: denaturing, annealing, and extending. In qPCR there are two standard methods used to detect and quantify the amplified product, includes fluorescent dyes (e.g. SYBR green dye) that non-specifically interpolate with double-stranded DNA or sequence-specific DNA probes (e.g. TaqMan probes)fluorescently labelled that is complementary to the DNA and allow to detect after hybridization only (Pierouli et al., 2019).Nowadays, tumour molecular profiling can provide evidence about prognosis, diagnosis, and prediction to therapy response, which can help clinical decision making (Del vecchio et al., 2017).

NGS

Various types of sequencing experiments can be achieved via NGS including target gene sequencing, transcriptome sequencing (RNA-seq), whole-exome sequencing (WES), and whole-genome sequencing (WGS), according to the complexity of the analysis and the information to be attained (Caccaro et al., 2019).

In 2005, Roche released the first NGS technology with 454 Genome Sequencer. The abundant initial success can be credited to its key point: the relationship between emulsion PCR, and pyrosequencing (Del vecchio et al., 2017).

In 2006, the first Solexa sequencer, genome analyser was launched, which is a single run can analyse 1 Gb data. The company was acquired by Illumina in 2007. Illumina technology is based on the sequencing by synthesis (SBS) using cyclic reversible termination (CRT) (Del vecchio et al., 2017).

In 2010, Ion Torrent Thermo Fischer, a semiconductor-based technology, commercialized by Life Technologies was hurled. The Ion Torrent throughput can read sequences starting from 10 Mb to the current maximum of 15 GB as continuous improvements (Del vecchio et al., 2017).

SOLiD sequencing technology works by the hybridization ligation executed in oligonucleotide ligation and detection. This machinery was formerly industrialised by Applied Biosystems. The scheme was shared with 454 Roche sequencing technology, the emulsion PCR amplification as the first step during the DNA library preparation (Del vecchio et al., 2017).

In 2010, Pacific Biosciences sequencer was launched based on the new methodology of single-molecule real-time sequencing (SMRT). The present error rate is 0.1% only. Regarding read length, it shows excessive-performance up to 20,000 bp read length or 10 GB (Del vecchio et al., 2017).

In 2012, Oxford Nanopore broadcasted new equipment that can directly sequence a DNA fragment by evaluating the change in current flow, owing to the passage of such a molecule via the nanopore entrenched within a membrane. Min ION is a small (100 g weight), portable, USB powered device provided by a flow cell with 2048 independently addressable nanopores which are controlled by application-specific integrated circuit (ASIC) (Del vecchio et al., 2017).

NGS in Colorectal Cancer

Colorectal cancer (CRC) is a very heterogeneous type of cancer, discussed as tumour occurs in colon and rectum and signifies the third most common category of tumours around worldwide. The risk factors of CRC are obesity, red meat, alcohol, and smoke abuse. Physical exercise and diet are two principal points for the prevention of CRC(Del vecchio et al., 2017).A scientific report that investigated metastatic CRC (mCRC) affected patients treated with panitumumab implementing a Roche GS FLX, massive multigene NGS sequencing were analysed particularly 9 genes in 320 samples and detected mutations in K/NRAS, AKT, CTNNB1, PTEN, TP53, BRAF, PI3KCA, EGFR genes(table 7) (Peeters et al., 2013).

Advantages

- Suitable for efficient and rapid sequencing of complex genomes with consequent cost and time reduction.
- By using a very low amount of nucleic acids we can do multiple gene analysis.
- Method to acquire high-throughput data employing sensitivity and specificity.

- NGS techniques provide valued data about transcriptomics, copy number variations, mutational status, and epigenetics with the chance to combine available single genetic tests into a unique test able to detect manifold variants.

Disadvantages

- Need to use extra powerful computers with innovative algorithms to achieve analyses, with consequential problems in terms of economic and human possession for minor medical units or laboratories.

Table 7. NGS to detect actionable genes in CRC

Sample	NGS platform	Multigene analysed	Therapy	Reference
320 metastatic CRC (mCRC)	454 GS FLX	K/NRAS, BRAF, PI3KCA, TP53, PTEN, EGFR, AKT, CTNNB1	Panitumumab	(Peeters et al., 2013)
68 CRC (77 KRAS analysed)	GAIIx Illumina	KRAS	Anti-EGFR	(Kothari et al., 2014)
182 mCRC KRAS ex2 wt.	Ion Torrent	KRAS (29/182 patients) frequently mutant: TP53, RAS, PI3KCA, BRAF	Folfiri/Cetuximab	(Ciardiello et al., 2014)

CANCER VACCINES

Vaccines have made a marvellous contribution to global health; elimination of smallpox was a complicated mission until the vaccine has discovered. In 1967, Georg Klein discovered the idea of developing vaccines against cancer as therapeutics. The traditional whole-pathogen based vaccines against contagious or infectious diseases have given fruitful outcomes whereas cancer vaccines have presented unsatisfactory clinical outcomes, due to various biological barriers, the immunosuppressive tumour microenvironment, and inherently low tumour antigen immunogenicity. There are several modules of cancer vaccines that include cell-based, DNA, mRNA, and peptide/protein.

DNA vaccines were first introduced in the early 1990swhen scientists originate that plasmid DNA was able to induce effective antibody reactions against an encoded antigen. However, in 2010, the first DNA vaccine for cancer (ONCEPT®) approved by the United States Department of Agriculture. One major benefit of the mRNA vaccine above DNA vaccines is that the mRNA vaccine doesn't cross the nuclear membrane barrier to prompt protein expression. At present two types of mRNA vaccines are commonly utilized that is self-amplifying and non-replicating. Even though the self-amplifying mRNA vaccine is generally used in prophylactic for infectious diseases whereas, most mRNA cancer vaccines use non-replicating mRNA vaccines. Peptide vaccines are chemically manufactured owing to their small length, which is both cost and time-effective. In dissimilarity, protein vaccines are habitually obtained by using more multifaceted recombinant protein expression methodologies. The distinctive gain of both peptide and protein vaccines is a high level of security, which has been revealed in several pre-clinical and clinical studies. The foremost target cell-based vaccines are DCs, which are indispensable for originating anti-tumour immunity (Zhang et al., 2018).

CAR-T CELL THERAPY FOR CANCER

Chimeric antigen receptor (CAR) is a fusion protein encompassing extracellular target binding domain typically derived from the single-chain variable fragment(scFv) of spacer domain, a transmembrane domain, intracellular signalling domain and antibody holdingCD3related with zero or one or two co-stimulatory particles such as CD28, CD134, and CD137. Through gene transfer technology, T-cells were engineered to express CAR for specifically identifying their target antigen via the scFv binding domain, ensued in T cell activation in a major histocompatibility complex (MHC). Since past few years, clinical trials are going on to assess CAR-modified T cell (CAR-T cell) therapy for B cell malignancies including B cell acute lymphoblastic leukaemia (B-ALL), chronic lymphocytic leukaemia (CLL), B cell non-Hodgkin's lymphoma (B-NHL), and Hodgkin's lymphoma (HL) have proved auspicious outcomes by targeting CD19, CD20, or CD30 (Wang et al., 2017).

CONCLUSION

Ensuring a complete treatment for all kinds of cancer remains a task today, and finding effective anti-cancer drug modalities is one of the foremost focuses of cancer research worldwide. The cancer stem cells (CSCs) have specific features includes drug resistance, self-renewal, high migration capability, and aberrant differentiation which establish the tumour heterogeneous population. In recent years the studies on CSCs have paid attention to potential therapeutic applications. RNAi based therapy is one of the most adaptable knockdown tools in modern biotechnology, and the perspective of RNAi therapeutics expending miRNA for cancer treatment has been rapidly intensifying. Nanotechnology carries many benefits to deliver a drug specifically at a particular site, the nanocarriers act as a fantastic delivery system not only for anticancer agents and also for siRNA and miRNA-based drug. Finally, NGS is the most powerful and helpful system for gene expression profiling studies of cancer, can provide a prognosis, diagnosis, and prediction to therapy response.

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Chapter 18
Recent Research and Development in Stem Cell Therapy for Cancer Treatment: Promising Future and Challenges

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ABSTRACT

Cancer is the most prevalent and dangerous disease, and it leads to millions of deaths worldwide. Generally, metastatic cancer cells are not eradication by conventional surgical operative or chemotherapy-based treatment. New pathways have been established in various arenas such as unique biology, modulators regulatory mechanism, directional migration, self-renewal, etc. The individual pathways can be employed as therapeutic carriers, specific drug targeting, generation of acquiring nature immune cells, and re-generative medicine. The present scenario, stem cell therapy, focused on a promising tool for targeted cancer treatment. Stem cells also utilized as viruses and nanoparticles carry to enhance the primary therapeutic application in various dimensions such as cancer target therapy, regenerative medicine, immune-modulating therapy, and anticancer drugs screening. Furthermore, the rapid development in next-generation sequencing techniques and cancer genomics and proteomics analysis approaches are making therapeutics targeting organ-specific cancer more precise and efficient.

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INTRODUCTION

Cancer is a group of diseases, which is characterized by the uncontrolled rapid growth and spread of abnormal cells. If the spread is not controlled, it can result in death. Although the causes of cancer are not completely understood, numerous factors are known to increase the disease's occurrence, including many that are modifiable (e.g., tobacco use and excess body weight) and others that are not (e.g., inherited genetic mutations). The latter process is called metastasizing and is a major cause of death from cancer. A neoplasm and malignant tumour are other common names for cancer (WHO, 2020). These risk factors may act simultaneously or in sequence to initiate and/or promote cancer growth (American Cancer Society; 2020). Cancer is a leading motive of dying in each developed and growing nation (Siegel et al., 2019). Cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths, or one in six deaths, in 2018. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervical and thyroid cancer are the most common among women (WHO, 2020). The cancer burden continues to grow globally, exerting tremendous physical, emotional, and financial strain on individuals, families, communities, and health systems, due to rapid population growth and aging, etc. (Siegel et al., 2019). Many health systems in low- and middle-income countries are least prepared to manage this burden, and large numbers of cancer patients globally do not have access to timely quality diagnosis and treatment (WHO, 2020). Cancer is mainly treated by the usage of radiotherapy, chemotherapy and solid tissue removal by surgical procedure. The procedure of therapy preferably based on the nature of cancerous (Siegel et al., 2019). In fact, the most metastatic cancers cannot be operated by using contemporary techniques. Scientists and academic societies are constantly working towards innovation and indeed the creation of new techniques for treatment for cancer (Zhang et al., 2017). Conventional treatment has only effectual for specific malignant cancers (Sun, 2015).

Tissues such as the intestinal epithelium and the hematopoietic system continuously self-renew through the activity of a dedicated population of tissue-specific stem cells, also known as adult stem cells (Clever, 2013).

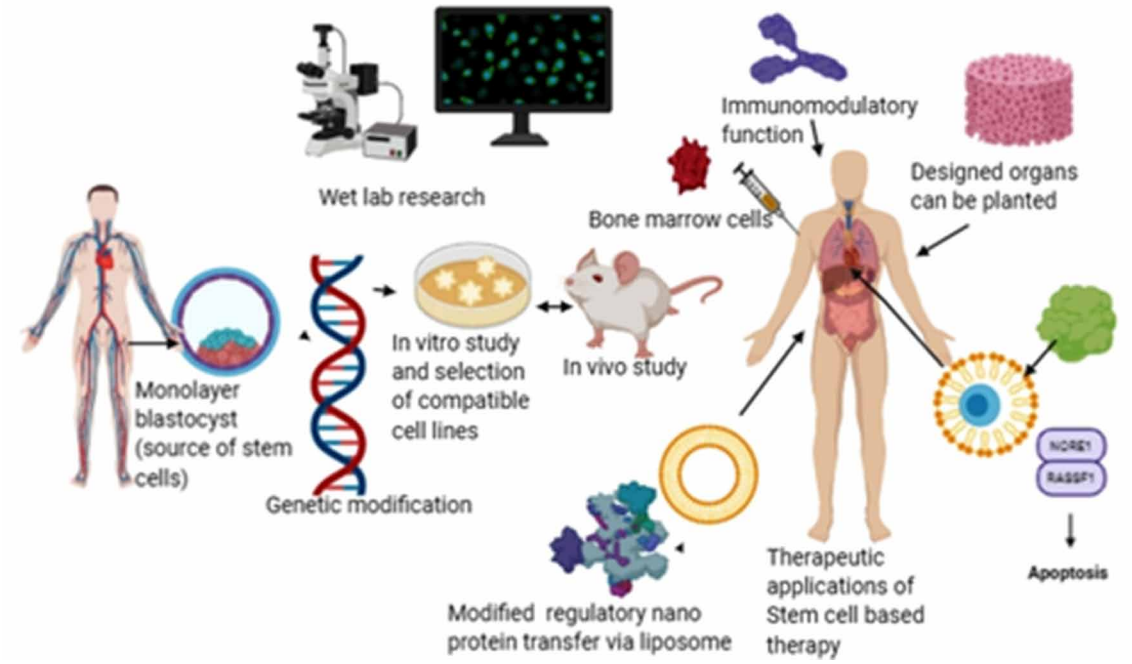
PROPERTIES AND SOURCES OF STEM CELLS

1. Normal Stem Cells

The stem cells in different tissues share two common properties, the ability to self-renew, for example, to divide and form at least one new stem cell, as well as to differentiate into the mature cells of the organ in which it resides (Zhang et al., 2017). Although some studies suggested that plasticity allowed stem cells from different tissues such as the brain or blood system to trans differentiate and form mature cells of many different tissues, it is now clear that such plasticity is frequently the result of a rare fusion of the stem cell or its progeny with a cell of another organ (Wang et al., 2003). The ability of stem cells to expand in number is under tight genetic constraints. This is not surprising since unlimited stem cell expansion, coupled with the ability of the stem cells to enter the circulation (essentially metastasize), would result in a cell with a phenotype similar to that of a cancer cell. All that would be lacking would be the property of tissue invasion (Al-Hajj and Clarke, 2004). Recent evidence has demonstrated that cancers can be viewed as an abnormal organ in which tumour growth is driven by a population of can-

cer stem cells (CSCs), which can give rise to both more CSCs as well as non-tumorigenic cancer cells. In marked contrast to the CSCs, these latter cells have either no or a markedly diminished capacity to form new tumours (Singh et al., 2003). This observation has implications for the biology of tumour formation as well as the diagnosis and treatment of cancer. To treat cancer effectively, the CSCs must be eliminated. Otherwise, the tumour will rapidly reform if the therapy eliminates non-tumorigenic cancer cells but spares a significant population of the CSCs (Al-Hajj and Clarke, 2004). Stem cells having a different property and exhibit unique feature. According to their originating sites, differentiation pattern, migration and varying capacities of the rate of proliferation, etc. These functions might be utilized for development and determine of antitumor therapeutic application (Figure 1).

Figure 1. The therapeutic approach for treatment to cancer by stem cell therapy



2. Embryonic Stem Cells

Embryonic stem cells (ESCs) are defined as pluripotent cells derived from the undifferentiated inner cells mass of embryo of the preimplantation embryo that can self-renew and generate all the cell types of the body in vivo and in vitro (Rossant, 2018). The isolation of human ESCs in 1998 generated tremendous interest in the possible use of ESCs for cell therapy. For example, genes could potentially be manipulated in ESCs to correct genetic deficiencies before therapeutic implantation. Besides, while most adult stem cells have limited proliferative capacity and can give rise to cell types within one particular lineage only, ESCs treated with the anti-differentiation cytokine leukaemia inhibitory factor (LIF) can proliferate indefinitely in cell culture and retain their potential to form all the tissues of the developing organism. Patient-specific ESCs could in theory also be developed by somatic cell nuclear transfer,

whereby a nucleus from a donor somatic cell is re-implanted into an enucleated oocyte to generate a cloned embryo, as was the case with Dolly the sheep. Although experiments in animals have shown that nuclear cloning combined with gene and cell therapy represents a valid strategy for treating genetic disorders (Hochedlinger & Jaenisch, 2003). For that reason, ESC used as a gold trendy in the comparison of all sorts of pluripotent stem cells, in desired cells will be developed from, except self-origin lineage. The application of ESCs in scientific research and scientific trial of stem cells are restricted.

The Yamanaka factors invention, which is related to induce pluripotent nature of stem cells (iPSCs) derived from somatic cells culture, was remarkable discovery in cellular biology research (Takahashi & Yamanaka, 2006). This is unlikely to be an efficient approach in humans. Some of the extracellular signals and a number of the molecular pathways required for differentiation of ESCs have been identified using both in vitro and in vivo systems. However, although moderate success has been achieved for differentiation of ESCs into ectodermal and mesodermal tissues, progress has been somewhat limited for differentiation of ESCs into endodermal tissues, such as in most gastrointestinal organ systems (Quante & Wang, 2009).

3. Adult Stem Cells (ASCs)

Adult stem cells have enabled to develop various types of tissues. In the segment, particular lineage cells have originated and developed a specialized cell such as hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and neural stem cells (NSCs), which employed for cancer treatment. Still, the mixture of HSCs, which derived from cord blood, so, this, is the only way to treatment FDA of some types of lymphoma, leukaemia, and multiple myeloma by stem cells which has recommended by the FDA (Copelan, 2006).

4. Induced Pluripotent Stem Cells (iPSCs)

iPSCs are the product of reprogramming a somatic cell into an embryonic stem cell (ESC)-like state, which is a providing a new approach to the generation of ESC-like cells. This pioneering method was first described in 2007 by Yamanaka and colleagues using mouse fibroblasts, in which the retroviral-mediated introduction of four genes encoding human transcription factors (octamer-binding transcription factor 3/4 [OCT3/4], SRY-related high mobility group box protein-2 [SOX2], the oncoprotein c-MYC and Kruppel-like factor 4 [KLF4]) induced pluripotency(Takahashi and Yamanaka, 2006). To date, iPSCs seem to be identical to ESCs, although the risks associated with using the oncogene *c-MYC* and retroviral vectors limit the use of iPSCs in a clinical setting. Another limitation has been the relatively low efficiency of generating iPSCs. However, many variations to this protocol have been described, including the use of nonretroviral vector approaches (adenovirus, plasmids, transposons, chemical compounds and the technique has been applied to several types of mouse and human somatic cells. Studies have shown that different combinations of other factors can substitute for the oncoproteins c-MYC and KLF4 and even produce iPSCs with as few as one factor (OCT3/4 or KLF4) in mouse neural stem cells (Kim et al., 2009). The finding that continued expression of the exogenously introduced genes is not required, and that the factors activate epigenetic reprogramming of somatic cells into an ESC-like state, offers hope that the methodology will continue to improve. In theory, all of the necessary factors could be introduced using one vector, which would be removed after reprogramming. The recent research revealed that the iPSCs and hESCs are having a significant role for initiation or induction of NK or effectors T cells, and

that contribute to development of anti-cancer vaccine for malignant cells (Ouyang et al., 2019). Thus, iPSC technology seems to be a viable method for generating iPSCs, without the controversy surrounding the use of embryonic cells. However, similar to the case with ESCs, much additional work is needed to define the methodologies necessary to achieve liver-specific and gut-specific differentiation.

5. Mesenchymal Stem Cells (MSCs)

MSCs have the capacity to self-proliferate and differentiate into oligodendrocytes, neurons, and astrocytes, etc. They have been usually employing to treat for brains, breast, lung, and prostate, etc. MSCs are found in many tissues and organs; essential studies have been performed on regeneration and repair of tissue etc. MSCs are simply isolated and cultivated in in vitro system and, similar to NSCs, is applied generally in the treatment of various cancer diseases (Zhang et al., 2017). MSCs have specific biological properties, thus extensively used to support other treatment processes or deliver targeted therapeutic agents for treating to diverse nature of cancers (Lin et al., 2019).

6. Hematopoietic Stem Cells (HSCs)

HSCs, the most abundant of blood lineage cells, are primarily found in the bone marrow, forming mature blood cells by gradually proliferating and differentiating lineage-restricted progenitors. HSC transplants have been working in clinical practice for over four decades (Zhang et al., 2017).

7. Endothelial Progenitor Cells (EPCs)

EPCs are the main drivers of vascular regeneration following transfection or pairing of antitumor or metastasis blockers a probable use beyond EPCs in cancer care. Recent research, indeed, has improved the attention on EPC roles in disease angiogenesis, and supposed benefits as part of therapeutic strategies. EPC findings are rare in cancer therapeutic tools (Goligorsky & Salven, 2013).

8. Disease Stem Cells (CSCs)

CSC, a stem-like tumour cell subpopulation, was derived from different types of tumour patient tissues and cell lines, focusing on cell surface markers. CSCs express stem genes, self-renew, differentiate from many other non-stem cancer cells, but resist traditional cancer medications. CSCs can cause major forms of cancer. Modern cancer therapies can destroy cancer cells but it cannot eradicate CSCs. Tumours normally occur at proliferation of the remaining CSCs and differentiation. Additionally, focused CSCs should resolve clinical problems such as drug resistance and recurrence (Dawood et al., 2014). CSCs are found in cancer tissues and play an important role in the growth, metastasis and recurrence of cancer (Chang, 2016). Targeting CSCs may indeed give promise to treating various kinds of cancers.

9. Neural Stem Cells (NSCs)

NSCs, presence in the central nervous system, have capacity of self-replicate and enable to develop new neurons and glial cells. As considered adult stem cells, NSCs originally derived from the brain, spinal cord, and retina also. It's also achieved from embryonic stem cells and induced pluripotent stem cells

(Stuckey and Shah, 2014). NSCs are exemplified by the articulation of nestin, Sox2, and other fantastic markers, as well as the development of culture media high in epidermal and fibroblast growth factors. NSCs used to treat of various types cancer such as breast, prostate and lung cancer etc. (Kanojia et al., 2015).

MOLECULAR REGULATION PROCESS IN STEM CELLS

Self-renewal and Proliferation are not the same process. Self-renewal is a specific cell division, have the capacity of one or both progenies to reproduce and differentiate is similar to those of the parental cell. Although a committed progenitor cell might have an extensive ability to proliferate, it is destined to eventually become terminally differentiated and stop dividing. For example, committed multipotent hematopoietic progenitor cells can give rise to mature blood elements for up to 2 months (Morrison and Weissman, 1994). However, with each cell division, the progenitor cell's progeny becomes progressively more differentiated and their proliferative capacity reduces. On the other hand, a self-renewing cell division of a hematopoietic stem cell (HSC) results in a cell that maintains its proliferative capacity and can reconstitute the blood system for the life of an animal (Hanson et al., 1999). Indeed, a single HSC or a progeny that arose from a self-renewing cell division can be serially transplanted several times and restore blood production in lethally irradiated animals. Most tumours develop over months to years and like normal tissues consist of heterogeneous populations of cells. In previous models of cancer, the unregulated growth of tumours was attributed to the serial acquisition of genetic events that resulted in: turning on genes promoting proliferation, silencing genes involved in inhibiting proliferation, and circumventing genes involved in programmed cell death. In the stem cell model for cancer, another key event in tumorigenesis is the disruption of genes involved in the regulation of stem cell self-renewal. Thus, some of the cancer cells within a tumour share with normal stem cells the ability to replicate without losing the capacity to proliferate. It is not surprising then that several genes initially identified as oncogenes have been implicated in normal stem cell self-renewal decisions. Genes that have been demonstrated to be involved in the regulation of self-renewal in normal stem cells from many tissues include Bmi-1, Notch, Wnt and Shh (Zhang et al., 2003). All of these genes were initially identified for their roles in tumour formation. Bmi-1 is a member of the polycomb family that functions to repress the transcription of its target genes via an epigenetic mechanism (Hanson et al., 1999) and in a mouse model of leukaemia, similarly, Lessard and Sauvageau (2003) showed that Bmi-1 is also needed for the self-renewal of the leukaemia-initiating cell (LIC). Since both CSCs and their nontumorigenic progeny share the same mutations that drive tumour formation, epigenetic events are likely responsible for the generation of at least some of the nontumorigenic cancer cells. Supporting this notion are studies that have examined the ability of oocytes to reprogram the nuclei of cancer cells. When nuclei obtained from medullo blastoma tumour cells arising in Ptc1 heterozygous mice were transferred into enucleated oocytes, cells from the resulting blastocysts were unable to form tumours when injected into mice suggesting that the oocytes were able to reprogram cancer cell nuclei via an epigenetic mechanism and suppress their tumorigenicity (Li et al., 2003).

Different pathways of signalling system involved in stem cells (SCs) and CSCs (Matsui, 2016). CSCs share common signalling pathways, such as JAK / STAT, Hedgehog, Wnt, Notch, PTEN / AKT / P13 K, NF-kB, MAPK / ERK, and SMAD. In CSCs these SC mechanisms are modified and are characteristic of the cancer types mentioned. The JAK/STAT pathway (Janus kinase/signal transducer and activator

of transcription) is mainly involved in glioblastoma development and breast CSCs (McCubrey et al., 2008). The Hedgehog pathways have effects on the patterning of the embryo but play a crucial role in the induction of myelogenous leukaemia. Blocking of the Hedgehog pathway decreases the quantity of CSCs in leukaemia, then representing an important target for cancer therapy (Zhao et al., 2009). The Wnt pathway is an essential regulator of SCs and CSCs regarding self-renewal, being perturbed in colon most cancers and leukaemia (Takeb et al., 2011). The Notch pathway is concerned with the improvement of breast tissue as a regulator of phone fate and differentiation. An extra in the activation of Notch ought to decide the aggressiveness of breast cancer (McAuliffe et al., 2012). The phosphatase and tensin homolog (PTEN)/protein kinase B (PKB or AKT)/phosphatidylinositide 3-kinase (P13K) signalling is a key regulator of self-renewal and maintenance of SCs and CSCs with an important function in the emergence of CSCs in prostate cancer. The NF-κB pathway is indispensable for leukemic cell survival and its inhibition impacts CSCs improvement in breast most cancers (Dubrovska et al., 2009). It has been seen that the extent of neural stem cells (NSC) proliferation is induced utilizing the activation of NF-κB, through the TNF-α sign transduction pathway, however, its aberrant legislation could lead to CSCs development in glioblastomas (Liu et al., 2010). Blocking the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) consequences in the increased inhibition of breast most cancers and the emergence of CSCs, sensitizing most cancers cells to chemotherapy (Yip et al., 2011). Gastrointestinal SCs can be perturbed, altering their plasticity and differentiation plausible via generating an aberrant response to TGF-β affecting the SMAD pathway and producing CSCs (Mishra et al. 2005). A hepatocellular carcinoma is an aggressive form of most cancers in which the TGF-β, Notch, and Wnt are deregulated, additionally having penalties in the SMAD proteins and changing SCs renewal, differentiation, and survival patterns (Ikushima and Miyazono, 2010). In grownup and CSCs systems all the noted pathways are frequent and conserved in the manipulate of SCs renewal, proliferation, and differentiation. Modern research equipment has helped divulge new molecular mechanisms of carcinogenesis. The development of malignancies is recognized to be a multistep manner involving innovative changes in the genome. The discovery of mutations leading to activation of oncogenes and inactivation of tumour suppressor genes was once an essential step in grasp the nature of carcinogenesis. Better perception of the ailment and rising contemporary technologies have led to the improvement of extra touchy diagnostic techniques and new treatment modalities. However, most cancers are nevertheless normally incurable when diagnosed in the superior metastatic degrees (Pesonen et al., 2010).

STEM CELL THERAPY FOR CANCER TREATMENT

1. Transplantation HSC

Transplantation of HSC primarily focused on the targeted treatment of specific cancer such as leukaemia, lymphomas, and another myeloma, etc., which have done previously around treated by chemotherapy (Copelan et al., 2006) (clinicaltrials.gov). At present, this methodology widely clinically tested in combinations with conventional approaches, for treatment of cancer such as sarcomas (NCT01807468), breast cancer (NCT00003927), and neuroblastoma, etc. the occurrence of graft-versus-host-disease (GVHD) when using allogeneic sources of HSCs remains a challenge, which is often treated with immunosuppressive drugs with less effectiveness and serious side effects (Casper et al., 2010).

2. MSCs Transplantation

The treatment of cancer procedure primarily leads to the removal of the invasive uncontrolled growth of cell layers or applying a high dose of physical or chemical radiation therapy for treated to cancer. Although, it leads to destroy the normal cellular system, as well as affect to hematopoietic system region. Thus, previous evidence revealed that the combination of MSCs transplantation therapy maintains the undifferentiated state and proliferation of HSCs, thereby enhancing the overall outcome of the treatment (Méndez-Ferrer et al., 2010).

In the approach of MSCs combination therapy, immune-modulatory effects might be strongly reduced. Furthermore, current experimental trials have exposed that the no adverse effect with transplantation of HSCs and MHCs, etc. Recently, a multi-centre trial has conducted (NCT02923375) for safety, tolerability, and efficacy of mesenchymo angioblast-derived MSC infusion in adults who have steroid-resistant GVHD. MSCs are also found to facilitate the recovery of injured organs and could enable body tolerance to high-dose chemotherapy that is to improve tumour-killing effects (Lee et al., 2011).

3. Stem Cell-Derived Exosomes as Therapeutic Carriers

Exosomes is a small size extracellular vehicles (EVs) having an endosome origin and diameter of 30−150 nm. It is actively secreted by some extensive cell types like endothelial cells, dendritic cells (DC), neural cells, erythrocyte, mesenchymal stem cells, epithelial and, tumour cells and oligodendroglial cells (Farooqi et al. 2018). The exosomes are widely circulated in the majority of physiological fluids, also containing in pleural fluid, bronchoalveolar lavage fluid, amniotic fluid, blood serum, breast milk, saliva, synovial fluid and urine (Masaoutis et al., 2018). Some synthetic polymers and liposomes are frequently using for drug delivery, because of having good quality as delivery vehicles. But, have some intrinsic drawback such as rapid phagocytizes, lower cycle stability, increased toxicity and poor biocompatibility. As well as exosomes can be delivered with some exogenous payloads such as in vivo or in vitro medium. They maintain cargo capacity during the movement; easily can be internalized into recipient cells, and discharge the drugs in the target site, thus exosomes played an important role in drug delivery vehicles in treating cancer (Jiang et al., 2019). These natural carriers having numerous benefits over other synthetic nano particulates, including stability, high capacity of cargo loading, unique biocompatibility, stability, and enhanced internalization capacity into infinite cells (Fuhrmann et al., 2015). Furthermore, they can be easily functionalized with specific and unique proteins or ligands on their surface to enhance and stable targeting effect to the tumour microenvironment (Wang et al., 2017). Some genetic materials, such as anti-cancerous siRNAs or mRNAs, were effectively packaged into stem cell-derived exosomes through the conventional transfection method. Katakowski et al. (2013) they collected exosomes derived from miR-146b-expressing bone marrow stromal cells. He depicted in a rat model of primary brain tumour, direct-injected in exosomes with materials into cancer cells, and found that amazing declined the growth of glioma xenograft. Similarly, exosomes derived from miR122 expressing MSCs, radically enhanced the antic cancerous effect on hepatocellular carcinoma tumour model (Lou et al., 2015). Also, MSCs secreted exosomes effectively transported siRNA to bladder tumour cells for targeting to the silencing of the polo-like kinase 1 gene (Greco et al., 2015). Alike, some small molecules of drugs possibly will be encapsulated into exosomes by two different approaches.

Primary it was observed that subsequently exogenous materials with priming, then competent to stem cells uptake, effectively package particular agents into exosomes. The exosomes efficiently inhibit the

tumour growth of myeloma and leukaemia cell lines (Bonomi et al., 2016). Similar, some other drugs used for priming to MSCs such as cisplatin, gemcitabine and doxorubicin etc. (Cocce et al., 2017). The content of drugs in exosomes also affects the priming rate, concentration, cellular uptake process and incubation periods, etc. Whereas, the other therapeutic drugs have been laden into exosomes by the post-loading method. After the separation from the culture medium of stem cells, exosomes were impeded for encapsulating drugs through employing the methods of electroporation, extrusion, dialysis, or saponin-assisted process (Fuhrmann et al., 2015). This facilitates loading both hydrophobic and hydrophilic drugs, whereas to control the drug cargo capacity, more accurately and improved its encapsulation competence. Human placenta-derived MSCs (hP-MSCs) secreted exosomes integrated with chitosan hydrogel might boost the maintenance and constancy of exosomes in *in vivo* and thus, further enhance their therapeutic efficiency in the tested model on hind limb of ischemia as exposed by firefly luciferase imaging of angiogenesis pattern. The strategy used to incorporate with highly facilitated the development of easy and effective approaches for enhancing the assessment of reduced the growth of tumour (Zhang et al., 2018).

4. Viral Therapy

One experimental strategy with an increasing amount of clinical evidence is oncolytic viruses, which replicate preferentially in tumour cells by taking advantage of cancer-specific cellular changes. Viral therapeutic also prospect for dealing with cancer treatment. Oncolytic viruses (OVs), instead of traditional attenuated viruses, provisionally proliferate in cancer cells. OVs have greatly increased spread within the host body and defended against the immune system. OVs combined with NSCs are competent to house cancer cells, and OVs delivered by NSC have shown enhanced anticancer effects as opposed to viruses alone against in vivo GBMs (Wollmann et al., 2012). The combination therapy enhances the potential of viral therapy. After the radio and temozolomide therapy, NSC-delivered OVs have increased continued existence in glioma bearing mice (Tobias et al., 2013). Previous clinical trials for anti-glioma gene therapy based on adenovirus vectors depicted the sufficient tolerability without serious adverse effects. Similarly, clinical trials for anti-glioma gene therapy based on adenovirus vectors depicted the sufficient tolerability without serious adverse effects. Similarly, MSCs derived viruses delivered to target a specific site and competent to kill cancer. The reduced viral potency having a measles virus combines with MSCs, delivered to the target region, the result depicted as a huge antitumor impact against hepatocellular carcinoma (Ong et al., 2013). In manner ways, carrying measles virus-infected MSCs homed to cancer implanted orthologous form such as the liver and transported MV infectivity to tumour cells by hetero fusion, they reduce the growth of cancer proliferation. MSCs mediate oncolytic herpes simplex virus (HSV) transported in to a model of GBM resection mice which elevated the anticancer effects of the virus. In this procedure, HSV derived via MSCs vigorously contaminated to GBM cells and killed to most cancers cells in vivo and in vitro. Mixed oHSV with TRAIL may also lead efficiently to avoid resistance in cancer. MSCs with oHSV / TRAIL have efficiently triggered most cell cancers via apoptosis and have cumulative survival time in mice with oHSV and TRAIL-resistant GBMs (Duebgen et al., 2014). In the series, modified Ad5 viruses enclosed with Ad35 fibres (Ad5/35) used CD46 used a receptor for infection of cells, which resolve the difficulty of low CAR potentiality, as many cancers have been revealed to express high levels of CD46. The employ of neural stem cells (NSC) has been a proposal for future therapy, targeted to intracranial glioma, and improved intratumoral microenvironmental distribution have seen in comparison to the virus through injection delivery approach (Tyler et al., 2009). Moreover, tumour cells have been recommended a useful cancer-targeting vehicle,

and homing to metastasis was demonstrated previously by the intravenous injection delivery approach (Garcia-Castro et al., 2005). Serotype of components approx. 35 also protect genetically modified vector from unspecific virus sequestration by blood components, as well as coagulation factor X, etc. (Liu et al., 2009). Besides, "targeted evolution" has been employing to develop potent oncolytic adenoviruses tools against colorectal cancer. Similar, ColoAd1, a combination of Ad3/Ad11p chimeric virus, preliminary oncolytic virus, which derived by this procedure and found to be 100 times more discerning and targeted for colon cancer cells in contrast to ONYX-015(Kuhn et al., 2008).

5. Stem Cell Resource for Production of Immune Cells

Present scenario immunotherapy has emerged as a significant research dimension in the field of clinical management of cancer. The tumour immunotherapy has been demonstrated for potential targeting for a variety of tumours, both in vitro and in vivo experimentation. The result depicted that considerable therapeutic potential for treating to verities of cancer. Immunotherapy has developed a gene adapted to T-cell receptors (TCRs) or chimeric antigen receptors (CARs) for cancer-related antigens; which develop HSCs attractive for use in cancer treatment (Gschweng et al., 2014). Similarly, patient-specific iPSCs might be prospective advantaged for the development of immunotherapy approaches (Serwold et al., 2010). The modified TCR gene is retained in T lymphocyte, which is originated from human iPSCs. Further it can be triggered to differentiate into functionally lively T cells (Brown et al., 2010). The cancer-specific T lymphocytes were successfully established and developed by reprogramming of desired T cells into iPSCs using in vitro, which further differentiate T lymphocytes direct administration into patients. However, the health, precise target and efficient functioning of T-cell-derived human iPSCs needs to be further validated (Zhang et al., 2017). Chimeric antigen receptor (CAR) T cells and natural killer (NK) cells effectively useful for treated to cancer as anticancer immunotherapy. The following immune-competent cells isolated from the patient body and activated by genetically modification with CAR assembled and directly infusion to the patient (Miliotou et al., 2018). The term application of immunotherapy for cancer, have some issues such as regulating the quality and quantity of immunotherapy approaches, including the matter of patients' concern who undergone the level of chemotherapy and older ages, etc. (Dolnikov et al., 2014). As a result, there are necessities for the generation and development of CAR cells from other derived sources. Similarly, human stem cells, such as ESCs, and iPSCs have potential resources for immunotherapy (Iriguchi and Kaneko, 2019). The different progression regulations have to lead to the development of stem cell proliferation in the cultured medium for initiated to T cell-initiating cytokines and NK cells etc. Also, to promote bone marrow stromal cells (OP9), hESCs, and T cell differentiation were cultured in the medium containing with FLT3L, IL-7 and SCF. Thus, the induction of CAR on HSCs revealed more advantages in treating cancer. The transplanted CAR-HSCs would be grafted in the bone marrow and constantly develop a variety of CAR-expressing immune cells, like as neutrophils, monocytes, NK cells, and T cells, etc. (Dolnikov et al., 2014). Microspheres encapsulated natural killer NK-92MI cells secreted granzymes and perforin, the developed immunotherapy efficiently kill to tumours cells in in vivo and in vitro system. Along with having a complete lack of side effects (Wu et al., 2019). The recent studies suggested that the combined result of these cells would be more strong immunity and potentially kill cancer.

6. Nanoparticles (NPs)-Carrying Stem Cells

The existing treatments procedures for cancer comprise therapies such as chemotherapy, radiation therapy, surgery, and immunotherapy, etc. While effectual impact against cancer, they unfavourably influence the quality of life of patients. In modern times, electrospun polymeric nanofibers have attracted significant attention in drug delivery having some benefits like a cost-effective fabrication route, simple, high surface to volume ratio, and capable a maximum drug loading capacity and efficiently deliver the drug in target regions. Some researcher reports that an electrospun nanofibrous piece integrated in Doxorubicin with silica nanoparticles and ZnO nanospheres laden with numerous cancer drugs has been used for treatment to postsurgical cancer etc. (Jain et al., 2016). Drug delivery systems based on riders of nanoparticles (NPs) typically include large concentration, insoluble chemotherapy agents; which protected from degradation in a biological environment. Stem cells have varieties of challenges such as inefficient dissemination in solid tumours, target micrometastatic lesions, and other challenges that are resolved by the nanoparticle delivery system. Moreover, stem cell nanoparticles have other benefits such as less unrestricted uptake by mononuclear cells and also protect therapeutic reagents from the host immune system (Auffinger et al., 2013). Jain et al. (2016) reported that phytocomponent having a good property of anticancer. They assayed in vitro of blend fibres PCL/GEL (50:50 by weight), found the reduced viability and growth of HeLa and MCF-7 cancer cells on the piperine eluting nanofibers, and depicted anticancer activity. Furthermore, the propagation of noncancerous cells such as NIH3T3 cells and human mesenchymal stem cells was affected to a noticeably very lesser extent in the same treatment. MSCs competently internalized NPs and capable of efficiently delivered in brain tumours (Roger et al., 2010). MSCs cell membranes loaded with doxorubicin containing porous silica, targeted to tumour tropic microenvironment. Another dimension of drug delivery tool, the amphibolic drug conjugates (ADCs) can self-assemble into micro bubbles (MBs)or nanoparticles (NPs) for targeted to drug delivery by efficient trafficking, less the net amount of excipient, physical integrity, high drug loading capacity, prevent premature leakage, chronic blood circulation periods, drug combination and also efficiently managed drug release kinetics (Liu et al., 2009). NPs can be intentioned delivery to cancer-specific regions, through connected with stem cells, some functional groups (amine and thiol etc.) directly interacted with targeted cells surface, exhibited a potent response (Cheng et al., 2010). The up taking or specific delivery property of NPs depends on the modified conjugated functional materials (Layek et al., 2019). Despite, rapid progress and development in therapeutic cell engineering, there has been no clinical trial that utilizes stem cells as potent carriers for targeting NPs to cancer treatment.

SIDE EFFECTS AND POTENTIAL RISKS OF STEM CELL THERAPY

1. Adverse Effect in Allogenic HSC Transplantation

The allogeneic HSC transplantation turns into a positive manner to treat hematologic and lymphoid cancers etc.

Though, must be set off long periods tested results wide ranges of patients, like suffering from chronic GVHD, organ failure, recurrence, immune reaction related infections and other types of secondary cancers etc. The continued existence of extension has no longer the sole goal for treatment; however, it should include the whole recovery of health, as well as social relationships (Liu et al., 2009). Furthermore, to

improve the outcomes, clinical studies have to issue about transplantation of HSC. Thus, it's recognized that the use of umbilical blood could be minimizing the prevalence and severity of chronic GVHD. So, some scientific trials have been revealed the efficiency of MSCs with co-transplantation on controlling and prevalence of GVHD, as nicely as different facet effects related to HSC transplantation (Auffinger et al., 2013).

2. Tumorigenesis Features

Normal stem cells and CSCs have displayed analogous biological signalling keys pathways etc. Thus, alternation of the microenvironment of stem cells might be possible to unfavourably extrinsic situation and as a result, regular stem cells may additionally change and convert into CSCs. However, transplanted stem cells that are exposed to external prerequisites at some point in the convention before transplantation may want to exchange their genomic expression. Though this phenomenon is still controversial, it is necessary to understand the cultural situation of the cells. PSCs are located greater tumorigenic than ASCs. However, it ought to distinguish between "teratoma" and "teratocarcinoma" development. The term "teratoma" refers to the inherent traits of PSCs to form "normal tumour", whereas the "teratocarcinoma" simply defined "irregular human tumour". Furthermore, many regulations keys have responsible prevent tumorigenesis of pluripotent stem cells. In addition, stem cells have facilitated the rapid growth, with higher multiplication rate, thus responsible for metastasis tumours. For instance, metastatic breast cancer cells have observed that reinstate the movement property after the co transplantation with MSCs in experimented in mouse subcutaneous region (Karnoub et al., 2007).

3. Medicine Toxicity and Resistance

The efficacy and utilization of stem cells as a drug or gene transport carriers, directly reliance on the number of specific cells that are localized within the tumour. Just about ~2–5% of total stem cells were reached to tumour tissue region after their systemic injection, they reflect to be secure over time of commentary. Most of the intravenously injected cells have been at the beginning entrapped in the lung parenchyma (involved in gas exchange), then later on travelled on organs such as lymph node, spleen and liver to liver, spleen, and lymph nodes, and finally cleared out from the body (Albarenque et al., 2011). Thus, it could raise some problem, like some nonspecific targeted therapeutics tool, which have used in higher-level doses, which may directly be affected by normal tissues and organs. Another challenge has in terms of 'insufficient mechanistic control', because therapeutic tablets have incapable of reducing the expansion of tumours (Chu et al., 2020). The research and development scenario focus and attempts on new combinatorial therapeutic methods and maximizes validation for enhancing targeted drug delivery on-site, along with transplanted stem cells to tumours affected parts, with less risk and higher productivity of therapy.

4. Increased Immune Responses and Autoimmunity

The utility of allogeneic stem cells are derived from unrelated donors may additionally elicit severe host immune responses (Lohan et al., 2017). The robust experimental techniques have demonstrated that the development of every specific cellular T cell response and humoral B cell-mediated response respective with donor antigens. In spite of the fact that the previous underlying infusion of these cells to the affected

person, thus pre-existing reaction develop memory response, would injury the following transplant and prevalence for graft rejection (Griffn et al., 2012). In some different perspectives, the danger of autoimmunity would happen with the utilization of autologous iPSC-based entire cell immunizations. These immunizations incorporate each CSC-explicit and normal tissue-related antigen. In this way, it is practical to initiate insusceptible reactions towards normal tissues. Be that as it may, further examination should be done to confirm the safety and validation of the methodology.

5. Prosperity of Viral Infection

Viral transfection is a typical and huge strategy to control stem cells for quality conveyance as gene delivery carriers. Notwithstanding, it perhaps presents the risk of viral contamination to the receiver. The primarily related difficulties are viral durable immunogenicity that should inspire negative immune reactions, causing toxins discharge, removal of transduced cells, compelled transgenic capacity size, and even passing of cells. Along these lines, viral vectors should be carefully changed to delete sequences that responsible for toxicity in a recipient, whereas introducing targeted sequences for anti-cancer impact (Nayerossadat et al., 2012). Additionally, enormous preclinical assessment is quintessential to check the safety and viably of viral vectors before interpreting the treatment into a clinical setting.

CONCLUDING OUTCOMES AND FUTURE PERSPECTIVE

Stem cells have obstacles in the destiny of pioneering clinical and scientific advances continually. Stem cell treatment already has a giant affect many factors of life; the scale of the method is every one of a kind challenge. Future stem cell remedy plans may additionally moreover is an extremely good barrier.

Stem cells transplant therapeutics; emerge as a cutting-edge tool, having next-generation tools and modern cancer biology, certify and validate to curing a variety of cancers. Thus, necessitate developing an atmosphere for innovation, skilled hand, policy for enforcement and the new dimension of stem cells such as regenerative medicine, personalized medicine, gene medicine would be possible with collaboration tasks in the global label. The selection of desired stem cells forms the host body still has challenges, due to presence in fewer numbers. As for all maximum cancers treatments, responses to oncolytic virus remedies vary from one character to some other and the top fine outcome is probably finished by way of manner of growing in my view personalized oncolytic virus medicine. For instance, pre-treatment tumour biopsies ought to be used to have a look at the results of 1 of kind viruses ex *vivo* to discover the maximum suitable virus for tumour eradication. A huge effort has been lately made for the characterization of molecular markers or biomarkers, to be used in optimized, validation, tailored remedy regimens and finally, lab to product development, etc. (Roock et al., 2009).

Furthermore, the effect of a profitable operation could be immediate, and the affected character would keep far away from chronic pharmacological therapy. The consequence of future treatment seems to be massive (Zakrzewski et al., 2019). In destiny pioneering clinical and medical advancements, stem cells have boundaries that have to be strictly regulated to make certain positive they may be preference given to human welfare or ethical restriction or overcome to policy and embedded alternative therapy, etc. There have already been infinite challenges about stem cells. Next, the most vital component is to recognize the procedure via which stem cells next characteristic in animal models. There can be no stopping this step. The subject as to the unknown appears to be the best challenge to be resolved, for the widespread,

worldwide adoption of the procedure. Further development and flexibility of stem cells may additionally also motive the lessen of the medical burden for humans. The affected person would be able to utilize the stem cellular remedy. There is now overwhelming evidence to guide the lifestyles of CSCs in many cancer types, however, our draw close of the cell hierarchies found in tumours is nevertheless in large part fashioned through observations fabricated from sorted, xeno transplanted tumour cells. The implementation of new technologies, together with CRISPR–Cas9, tumour organoids, and intravital imaging, opens up avenues for examining CSCs of their intact environment. Emerging research the usage of that device has commenced editing our perceptions of the elements and behaviour of CSCs. Paralleling the converting view on the nature of healthy stem cells, it is becoming obtrusive that CSCs may additionally not necessarily need to be rare, quiescent, and hardwired (Batle and Clevers, 2017).

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Chapter 19
Utilization of Bio-Imaging in Cancer Studies

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ABSTRACT

Biological studies have always relied on visual data and its precise interpretation. Bio-imaging is an integral part of cancer research as well as the diagnosis and treatment of various cancers. Cancer research employs the various bio-imaging techniques of fluorescence microscopy like confocal microscopy, FRET, FRAP, TPEF, SGH, etc. to study the complexity and characteristics of different cancer cells. The development of live-cell imaging has also helped in understanding the important biological processes which differentiate cancer cells from their environment. Advancement in the field of cancer diagnosis has taken place with the development of sophisticated radiology techniques like MRI, CT scans, and FDG-PET. Also, the development of novel nanotechnology-based probes has improved the quality of both cancer research and diagnosis. In this chapter, the authors summarize some of the bio-imaging techniques which are being used in the field of cancer studies.

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INTRODUCTION

"Seeing is believing" is an old saying used to justify the necessity of visual evidence for a hypothesis. Science itself would not have evolved without the inventions of microscopes and telescopes. Today's science is highly dependent upon visual shreds of evidence. Biology and its branches employ most of the technology for visualization of not only the problems but also in unsheathing the solutions to those problems and this had lead to the emergence of a whole new science called bio-imaging. Biomedical Imaging or bio-imaging can be defined as a process of acquiring the biological information in the form of images or visual effects while least affecting the concerned biological process (Vadivambal R., & Jayas D. S. et al., 2016).

Initially, only light microscopy was prevalent but it was after the 1960s that the real advancement started with the emergence of wide-field fluorescence microscopy till the early 1990s. During the decade of the 1990s further dye-based fluorescent techniques were developed such as monodensylcadaverine for autophagy detection. However, with the dawn of the new century, new horizons were explored in the field of live cell imaging (Vadivambal R., & Jayas D. S. et al., 2016; Ghamsari M. S. 2018). Many new techniques were then developed to generate high-resolution images some of which will be discussed in this chapter. Today the conventional techniques are empowered with tools such as machine learning and pattern recognition to better interpret the data and draw conclusions from radiology scans, histopathology slides, etc. (Bizzego A. et al., 2019). The field of bio-imaging is vast, thus we will limit our focus to the techniques which are extensively employed in cancer research and diagnosis.

BIO-IMAGING IN CANCER RESEARCH

The development of immuno-fluorescence techniques boosted cancer research by helping the scientists to understand the histo-chemistry and complexity of tumor organization. Confocal fluorescence imaging is very often used to study the nature of cancer cell cultures (Jogalekar M. P. et al., 2018; Le Roux L. et al., 2008). The concept of confocal microscopy is to scan a section of the specimen point by point in a given plane of focus and assembling the image after scanning the entire field. Thus, it allows the optical sectioning of the specimen by scanning the specimen in X,Y and Z axis just by changing the focal plane (Reynaud K. et al., 2001). The technique and nuances of confocal microscopy have been discussed in depth by (Semwogerere D. et al., 2005; Rai V. et al., 2011). The new technological advancements in confocal imaging include live-cell imaging (Dailey M. E. et al., 2006) and endo-microscopy (Belykh E. et al., 2019). These fluorescence techniques require a source of illumination which could be a laser or any monochromatic incandescent lamp which could bleach the specimen if overexposed. Sometimes photo-bleaching causes hindrance in studying the interaction between two molecules. To overcome this problem Forster resonance energy transfer or fluorescence resonance energy transfer (FRET) was invented. FRET implies the transfer of energy from one molecule (donor) to another molecule (acceptor) which happens to be a fluorescent molecule and within proximity of the donor (Hussain S. A., 2009). It is used to study the protein-ligand interactions, the intracellular distribution of molecules, etc.. Another modification of fluorescence microscopy is the fluorescence recovery after photobleaching (FRAP). This technique took a boost after the development of fluorescent tags which could be genetically attached to the cloned proteins. FRAP implies to the permanent or irreversible photo-bleaching of a pool of fluorophore and recovery of fluorescence by the diffusion/movement of intact probes from the surroundings

(Ishikawa-Ankerhold et al., 2014). This technique is readily employed to study the intracellular transport of proteins. Though photo-bleaching is used in FRAP, it also results in serious defects in studying other aspects of the cell. Also, the uses of high energy radiations like LASER or UV-radiations damage the specimen. To overcome these side effects two-photon excitation fluorescence (TPEF) microscopy was developed. In this technique, the fluorophore is simultaneously excited by two low energy photons instead of one high energy photon where the first photon excitation creates an intermediate excited state of fluorophore which is further excited by the second photon causing it to release energy and fluoresce (So P. T. et al., 2000; Alonzo C. A. et al., 2016). This technique is less damaging while providing higher penetration in 3D specimen. Another modification in fluorescence microscopy is the second harmonic generation (SHG) microscopy which is specific to tissues having non-centrosymmetric structures such as microtubules and collagen fibers; these are excited by two photons and convert the energy into a light of different wavelength (Chen X. et al., 2012; Bueno J. M. et al., 2016). SHG is widely used in studying tumor tissue architecture and the defects in ocular tissues (Bueno J. M. et al., 2016).

BIO-IMAGING IN CANCER DIAGNOSIS

Detection of tumors in their early stages gives a chance to prevent cancer. However not all tumors are physically observable, they are detected only after the manifestation of secondary effects. Cancer diagnostics employ many bio-imaging techniques to diagnose and categorize the stages of tumors and/or cancers. Among other techniques, magnetic resonance imaging (MRI) previously known as Nuclear magnetic resonance imaging, is the most widely used technique in cancer diagnostics. It offers less invasive procedures for cancer diagnosis. The principle behind MRI is the polarization of certain atomic nuclei by absorption of radio frequencies in presence of strong magnetic field and the atomic nuclei present in the vicinity of that atom. The nuclear polarization in terms generates a radio signal in resonance to the pulsating magnetic field (McRobbie D. W. et al., 2017). These resonance signals are then used to generate images of the tissue densities. MRI scans generated for biological samples usually represent the hydrogen nuclei present in the water and fats within the tissue. MRI gives a better soft-tissue resolution and information regarding pH and temperature which helps not only in diagnosis but also in categorizing the stages of cancer and designing the treatment strategies (Lu J. et al., 2013). Previously other radiology techniques like ultrasound and X-ray imaging were also employed in cancer diagnosis. Their evolution coupled with artificial intelligence lead to emergence of computerized tomography (CT) scan, where multiple scans are done at varying angles and the images are then superimposed and assessed using algorithms and reference scans with the help of high-end computer programs (Cantatore A., & Müller P., 2011). This technique has its advantages as well as disadvantages such as exposure to high energy radiations like X-rays. New techniques have emerged as modifications and upgrades to these techniques. Positron emission tomography (PET) or 2-deoxy-2-[fluorine-18]fluoro-D-glucose (FDG) PET is another such technique which is coupled with either with CT Scan or MRI or both. FDA is a radioactive analogue of glucose due to the presence of F^{18} that gives off positron emission which could be read as scintillation. FDG-PET is based on the principle of higher glucose uptake and glycolysis in cancer cells (Almuhaideb A. etal., 2011) thus the higher accumulation of FDG could pinpoint the distribution of cancerous cells and help in precise staging if cancer. FDG-PET/CT combined data gives a better readout of metabolic discrepancies and lesions within the body (Almuhaideb A. et al., 2011; Murray J. F. & Nadel J. 1987).

Now adding to the arsenal of cancer bio-imaging nano-technology based diagnostic techniques are being developed. They not only aim to diagnose cancers but also to precisely deliver drugs to the cancer site.

NANO-TECHNOLOGY IN CANCER BIO-IMAGING

Nanotechnology could be currently a developing innovation within the field of medical science. Nanoparticles mimic different biomolecules due to their comparative scale, organic interaction, and potential flag balance (Hussain S. et al., 2014). There are different types of nanoparticles (synthetic and nonsynthetic) that offer promising applications in bio-imaging and treatment of diabetes, neurodegeneration, respiratory diseases, and cancers (Baetke S. et al., 2015). Some of the examples of nanoparticles currently used in cancer bio-imaging are explained here. Some of the major nanoparticles used in cancer bio-imaging are described below:

Quantum Dots

Many tests have been exploited for detecting cancer biomarkers in different malignancies. In any case, the outflow of disease biomarker(s) seems, by all accounts, to be very low, along with these lines proper detection requests more sensitive optical imaging methods. While optical recognition utilizing traditional fluorophores regularly die out due to photobleaching issues, quantum dots (QDs) offer stable optical imaging in vitro and in vivo (Mashinchian O. et al., 2014). QDs are semiconductor nanocrystals. Their core consists of special semiconductor materials. They are typically composed of atoms from groups II-VI (of the periodic table) (e.g., CdSe, CdS, CdTe, ZnSe), III-V (InP and InAs) and IV-VI (PbSe) (Michalet X. et al., 2005). QDs are spherical and vary from 2nm to 10nm in diameter excluding polymer encapsulation. The encapsulation technique makes the QDs size 5-20 nm. They are used for optical imaging and fluorescence imaging and detection of a number of physiological and pathological conditions. It helps to diagnosis and treatment of diseased cells. The important biomedical utility of QDs is clinical imaging and the detection of diseased cells (Fang M. et al., 2012). The mixture of second-harmonic technology (CdTe)-associated QDs is used for the find out about tumor biology, cell motility, and metastatic motion of cancer antigens (Chen J. et al., 2016).

Gold Nano-particles

Gold nanoparticles were discovered by Faraday as colloidal dispersions (Faraday M., 1857). With the increasing importance of nano-medicine, the applications of gold nanoparticles in cancer cell imaging have gained the attention of the scientific community worldwide (Shi J. et al., 2017). These are based on the property of surface Plasmon resonance of gold which changes with the particle size and shape due to which they are prepared in the forms of spheres, rods, wires, and stars (Hutter E. et al., 2011). Gold nanoparticles have also lead to the development of novel Plasmon resonance energy transfer (PRET) based molecular imaging (Choi Y. et al., 2009).

Dendrimers

Dendrimers are micellar nanostructures with a high density of symmetrical branches and are of the order of 20nm size with multiple groups (Muthuraman A. et al., 2018). Formulations for dendrimers are made from the polymers of polyamidoamine, polyethyleneimine, polypropyleneimine, polyethelene glycol, poly-L-glutamic acid, melamine, and chitin (Madaan K. et al., 2014). Due to their enhanced permeability and retention, they are used for targeted tissue imaging for cancer diagnosis (Kompella U. B. et al., 2013).

SUMMARY

Technology has advanced a great deal since the development of preliminary bioimaging platforms such as linear microscopy and fluorescence microscopy. Not only new techniques have been developed but also the existing techniques have been upgraded to compete with the new techniques. The development of techniques like MRI and PDG-FRET has made noninvasive imaging and diagnosis of cancers more feasible. Novel nanoparticle-based probes for targeting and stable signal production had made imaging techniques more sensitive and reliable. Conventional fluorescence microscopy now has applications in live-cell imaging and invivo bio-imaging due to the evolution of confocal microscopy, FRET, FRAP, TPEF, etc..

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Chapter 20
Chemopreventive and Therapeutic Potential of Phytopharmaceuticals Against Oral Cancer:
Evidence-Based Reports From Preclinical Studies in Animal Models

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ABSTRACT

Oral cancer is a major public health problem in both developing and developed countries. It is believed to be the eighth most common cancer considering a major risk factor of worldwide morbidity and mortality. Major risk factors of this deadly disease are lifestyle (consumption of smoking and smokeless tobacco, alcohol, betel quid, etc.), unhealthy food, and poor dental care and viral infections. These factors are responsible for mutations in the DNA leading to the initiation of carcinogenesis. Oral carcinogenesis is a multistep process having three distinct phases: initiation, promotion, and progression. Modern cancer treatments (chemotherapy, surgery, radiation therapy, and immunotherapy) are associated with lots of side effects. Thus, phytopharmaceuticals are being used as alternative medicines in the prevention of oral carcinogenesis. Phytopharmaceuticals (such as resveratrol, sulforaphane, quercetin, etc.) have immense potential to prevent cancer development in every phase of carcinogenesis and more importantly, these compounds have fewer side effects.

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INTRODUCTION

Induction of oral carcinogenesis is a complex process that takes place when epithelial cells are subjected to genetic changes. It is a serious public health issue and an important cause of morbidity and mortality in South Asian countries especially in India and Bangladesh (Sharma et al., 2018; Kumar et al., 2019). Oral cancer is considered as the eighth most common cancers in the world and may occur in the lips, mouth, cheeks, and tongue. According to the most recent report of GLOBOCAN 2018 estimate, there are 3,54,864 new cases of oral cancer (lip and oral cavity) and out of which 1,17,384 death cases were reported worldwide (Bray et al., 2018). In India, it is the third most common type of cancer and 20 cases per 1,00,000 individuals have been recorded (Tanaka and Ishigamori, 2011; Danaraddi et al., 2014; Scrobota et al., 2016). Despite the development in this area in the last 20-30 years, the 5-year survival of the disease is ~50% (Bodhade and Dive, 2013; Bhavana and Lakshmi, 2014). International agency for research on cancer (IARC) predicted that worldwide new cases of oral cancer will increase up to 1.7 million by 2035 (Coelho, 2012).

Oral carcinogenesis, like other cancers, is a sequential multistep process involving three common stages- initiation, promotion, and progression. Initiation involves genetic mutations that cause irreversible alteration of a normal cell. Promotion is generally involved in increased proliferation of initiated cells that lead to progressive dysplasia. Progression is associated with the accumulation of mutated cells that help in the transformation of progressive dysplasia into malignant or invasive phenotype. These genetically mutated cells evade the cell cycle checkpoints that regulate cellular proliferation.

Oral cancers are of three types based on the clinic-pathological perspective- carcinomas of the lip vermilion, carcinomas of the oral cavity proper, and carcinomas in the oropharynx. Cancers of the oral cavity may be premalignant or malignant. The most common premalignant lesions are Leukoplakia, Erythroplakia, and Lichen planus. These premalignant (precancerous) lesions have a higher risk of malignant transformations. The malignant lesions of the oral cavity include squamous cell carcinoma (SCC), Lymphomas, Salivary gland adenocarcinomas, Sarcomas, and Melanomas. Approximately 90% of the oral cancer is SCC.

The modern cancer treatment includes chemotherapy, surgery, radiotherapy, targeted therapy, and immunotherapy (Barhoi et al., 2020). However, the results of these cancer interventions are not promising and are associated with side effects and normal cells also get affected. Therefore, development of alternative medicines with fewer side effects is in constant demand in the field of cancer biology for their therapeutic potential.

Herbal medicines or phytotherapy is gaining importance due to its diversified chemical constituent having medicinal property and more importantly, herbal medicines have very fewer side effects. More than 80% of the world's population primarily depends on herbal medicines for curing various diseases including cancer (Ekor, 2013; Carmona and Pereica, 2013). Scientific evidence supports that vitamins and herbal medicines play a significant role in cancer prevention and increases the quality of life of cancer patients (Albabtain et al., 2018).

Chemoprevention by using phytopharmaceuticals is a promising strategy to control the progression of the carcinogenesis process. Chemoprevention can be defined as the use of natural or chemically synthesized drugs or biological agents to prevent suppress or reverse carcinogenesis at its initial phases (initiation and promotion) or prevent the invasive potential of malignant cells (Ranjan et al., 2019). In the progression phase, chemoprevention ends and chemotherapy starts on. Clinical and epidemiological studies suggest that several phytopharmaceuticals like resveratrol, curcumin, garlic, etc. have chemopreventive

potential (Bhavana and Lakshmi, 2014). Phytopharmaceuticals are the plant-based enriched fractions of secondary metabolites of vitamins, carotenoids and polyphenols that can be used in the treatment of various ailments including cancer (Nooreen et al., 2018). According to the Drug and Cosmetics Act. (1940), a phytopharmaceutical drug can be defined as "purified and standardized fraction with defined minimum four bioactive or phytochemical compounds (qualitatively and quantitatively assessed) of an extract of a medicinal plant or its part, for internal or external use of human beings or animals for diagnosis, treatment, mitigation or prevention of any disease or disorder but does not include administration by parenteral route" (Gazette, 2015). Pharmaceuticals of plant origin with a broad spectrum of structural types not only act as a chemo-preventive agent in carcinogenesis but also reduce the tumor growth against established tumors.

RISK FACTORS FOR ORAL CANCER

Several etiological factors have the potential to promote oral carcinogenesis. Among them, lifestyle factor is being considered as the most efficient factor for triggering oral carcinogenesis. Lifestyle factors such as alcohol consumption, betel nut chewing, rampant consumption of smokeless tobacco (SLT), habits of smoking tobacco like cigarette, bidi are the major risk factor for oral cancer. Besides these, viral infection, unhealthy diet, and poor dental care are also considered as the risk factors for oral cancer.

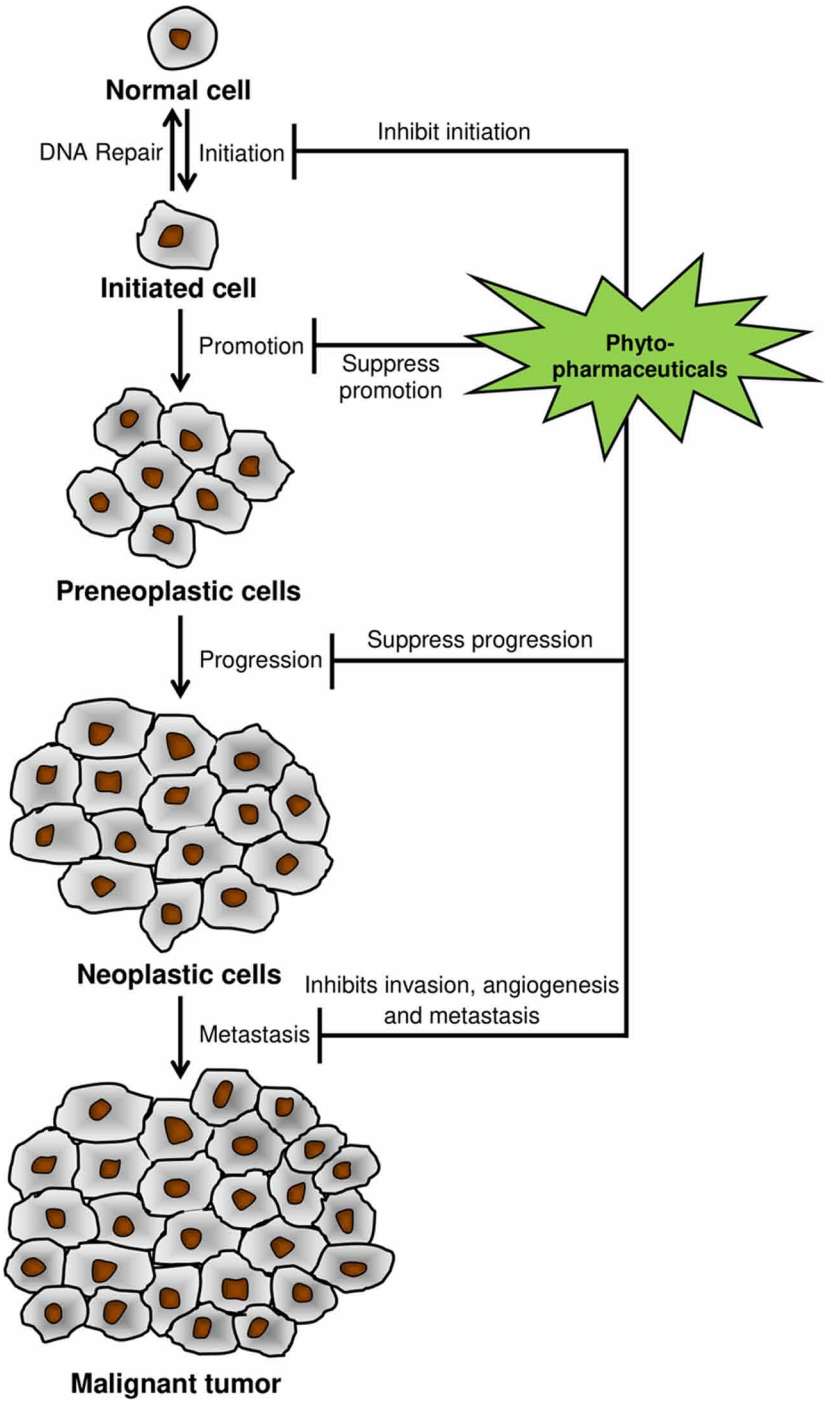
Viral infection is one of the major etiological risk factor for oral cancer. There is a report that infection of human papillomavirus (HPV), hepatis C virus (HCV), herpes group viruses, and adenoviruses are associated with oral carcinogenesis (Gupta and Medgut, 2013). Among these, HPV and herpes group viruses showed synergistic role in oral carcinogenesis and are referred to as "synergistic viruses" (Chan et al., 2001). Epstein-Barr virus (EBV), human herpes virus-8 (HHV-8) and cytomegalovirus (CMV) are the herpes group viruses that are often associated with oral cancer.

To date, there exists inadequate evidence regarding the potency of a singular factor in initiating carcinogenesis (Scrobota et al., 2016). And oxidative stress may be the crucial molecular mechanism behind the initiation, promotion, and progression of oral carcinogenesis.

PRECLINICAL MODELS FOR STUDYING ORAL CARCINOGENESIS

A better understanding of mechanistic pathways associated with initiation and progression of oral cancers will assist the scientist/clinicians around the globe to improve its prognosis. To improve the treatment strategy, biologically and clinically relevant animal models are indispensable for preclinical studies to investigate the progression of oral carcinomas and for proper diagnostic/therapeutic practice. Experimental animal models precisely indicate the cellular and molecular changes linked with initiation and progression of oral carcinogenesis and therefore are of crucial significance. Several animal models have been prepared and used for preclinical studies for *in vivo* evaluation of oral cancer. These oral cancer models are prepared by exposing them to chemical carcinogens, transplanting carcinoma cells, genetically modified and transgenetically altered mice co-treated with carcinogens

Figure 1. Mechanism of carcinogenesis and chemopreventive role of phytopharmaceuticals in cancer progression



Chemically Induced Animal Models for Studying Oral Cancer

Chemicals with carcinogenic potential are being used for the preparation of oral cancer models. Chemically induced oral cancer animal models are frequently being used in cancer research. To characterize numerous chemical carcinogens' role in oral cancer, there was a strong reason to prepare animal models of oral cancer. To resort to animal models is a nearly unavoidable prelude to understand the development of neoplasm and to evaluate the usefulness of new therapeutics. It is understood that animal models are just an alternative to understand the possible mechanism of any carcinogen inducing cancer in human subjects.

DMBA Induced Hamster Cheek Pouch Model

DMBA (Dimethyl-1,2 benzanthracene) is a polycyclic hydrocarbon having the immense mutagenic potential. DMBA induced hamster cheek pouch model was successfully prepared for the first time by John J. Salley in 1955. Painting of DMBA solution (dissolved in benzene or acetone) on to the cheek pouch of hamsters for three times per week for 16 weeks successfully induced squamous cell carcinoma (SCC). Later, Morris (1961) has produced a hamster cheek pouch model by treating 0.5% DMBA solution dissolved in acetone and reported maximum tumor yield with no morbidity (Morris, 1961). Further, 100% tumor incidence was observed when the pouch was painted with DMBA three times per week for eight weeks along with the painting of arecaidine six times per week for four weeks (Lin et al., 1996).

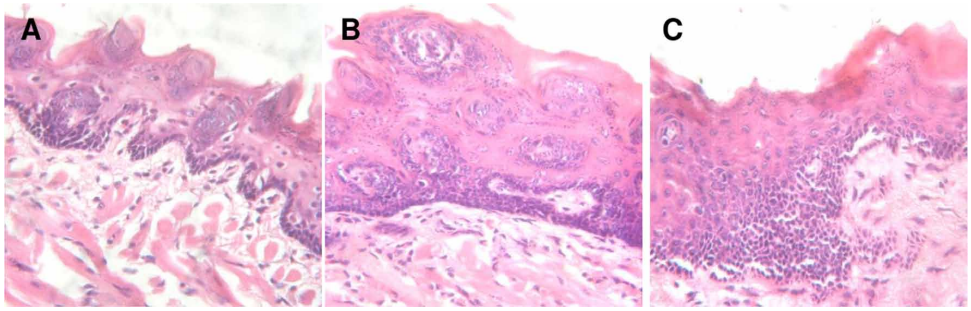
DMBA potentiates mutagenesis in mice by the formation of a DNA adduct (Ishida et al., 2017). Administration of arecaidine along with DMBA acts as a promoter of initiation of carcinogenesis. It is suggested that under the synergistic effect of arecaidine, DMBA showed 100% malignancy (Lin et al., 1996).

4NQO Induced Oral Cancer Model in Mice and Rat

4NQO (4-Nitroquinolone oxide) induced oral cancer model was introduced in rat oral cavity in the late 1970s by Wallenius and Lekholm. 4NQO is a potent carcinogen with mutagenic potential like other carcinogenic chemicals of cigarette smoke. 4NQO induce carcinomas in the tongue and esophagus and its exposure produces 100% carcinomas in all experimental animals. Oral squamous cell carcinoma (OSCC) is a multistep process and histologically comprises of oral precancerous lesions to invasive SCC. The oral carcinogenesis induced by 4NQO mimics the OSCC in human and constituted of increasing grades of hyperplasia to dy splastic alterations to SCC (Figure 2).

Wallenius and Lekholm (1973) produced the rat oral cavity cancer model by exposing the animals to water-soluble 4NQO for 7 months. 4NQO exposure for such a long time in rat induced palatal and gingival carcinoma. Carcinoma on the dorsum of the tongue is also prominent. Later, Steidler et al. (1985) treated the rats with the same dose but for a relatively shorter period (4 weeks). They observed that 4NQO induced oral carcinogenesis showed progressive changes of lingual epithelium showing mild to severe epithelial atypia before SCC developed which is evident from their histopathological studies. Later on, the researchers produced 4NQO induced rat oral cancer model by modulating the exposed dose and time (Nauta et al., 1996; Makita et al., 1996; Liu et al., 1999; Ribeiro et al., 2007; Kanojia et al., 2012).

Figure 2. Histological sections of 4NQO induced oral carcinogenesis in mice tongue showing progressive changes of the oral epithelium to hyperplasia and dysplasia
(A) Normal epithelium (B) Hyperplasia and (C) Severe dysplasia



4NQO induced oral cancer model was also established in the mice test system. Hawkins et al. (1994) established the mice oral cavity model, exposing the animals with 4NQO (5 mg/ml) for 16 weeks. This cancer model was characterized by progressive histological changes from atypia to hyperplasia to severe dysplasia. The tongue and palate of the oral cavity were also characterized by invasive SCC. 100% induction of 4NQO induced oral carcinogenesis in mice depends on the mode of exposure and exposure time. In a study, it was observed that painting of 4NQO (5 mg/ml) for three times per week for 16 weeks showed papilloma to only 20% of animals and 5% of animals were characterized by invasive SCC (Tang et al., 2004).

However, 4NQO exposure with 100 µg/ml through drinking water for 16 weeks produced 100% papilloma and invasive SCC in the tongue (Tang et al., 2004; Chen et al., 2019) of CBA and C57BL/6 mice. Hasina et al. (2009) exposed CBA mice with 50-100 µl/ml 4NQO through drinking water for 8 weeks and established the mice oral cancer model characterized by keratosis, dysplasia and Head and Neck Squamous Cell Carcinoma (HNSCC) with low morbidity and mortality rate.

Table 1. Chemically induced oral cancer model in animals for preclinical studies

Animal model used	Treatment	Feature(s)/observation(s)	Reference
Hamster-Pouch cheek model	DMBA treatment, 3 times a week for 16 weeks in a rat	The mucosa characterized with histological grades of hyperplasia, papilloma, carcinoma in situ and SCC	Salley, 1954
	Painting of oral mucosa with 0.5% DMBA solution dissolved in acetone, 3 times a week for 15 weeks	The mucosa showed the development of SCC with maximum tumor yield and no mortality	Morris, 1961
	DMBA treatment, 3 times a week for 8 consecutive weeks along with arecaidine, 6 times a week for 4 weeks	100% malignancies with distinct histological grades of oral carcinogenesis were observed	Lin et al., 1996

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Table 1. Continued

Animal model used	Treatment	Feature(s)/observation(s)	Reference
Rat-Oral cavity model	Repeated application of water-soluble 4NQO for 7 months	Experimental rats showed palatal carcinoma, carcinomas on the dorsum of the tongue and gingiva	Wallenius and Lekholm, 1973
	4NQO painting into the hard palate of rat mucosa, 3 times a week for 22 weeks	At 22 weeks of prolonged exposure of 4NQO showed highly differentiated SCC	Stenman, 1981
	Repeated exposure of 4NQO for 4 weeks	Histopathological changes in the lingual epithelium were progressive showing mild to severe epithelial atypia before SCC development	Steidler et al., 1985
	4NQO exposure to palatal mucosa in Wistar rats, 3 times a week for 2-26 weeks	Experimental animals showed dysplastic alteration from 2 weeks and at 26 weeks of treatment, SCC was successfully developed	Nauta et al., 1996
	4NQO administration (20 ppm) through drinking water in male F344 rats for 8 weeks	60% of exposed rats showed tongue squamous cell carcinoma and 27% rats showed squamous cell papilloma	Makita et al., 1996
	4NQO treatment (0.002%) was given to SD rats through drinking water for 9-32 weeks	Histopathological observation revealed the progressive carcinogenesis from hyperplasia, mild dysplasia, severe dysplasia and carcinoma in situ in SD rats	Liu et al., 1999
	4NQO through drinking water (50 ppm) for 4, 12 or 20 weeks in male Wistar rats	At 12 weeks, hyperplasia and dysplasia were prominent. At 20 weeks of 4NQO treatment, gingival squamous hyperplasia and SCC was observed	Ribeiro and Salvadori, 2007
	12 µl 0.25% 4NQO exposure, 3 times a week for 10-200 days into the buccal mucosa of male Sprague Dawley rat	Histological observation showed prominent dysplasia at 80 days of treatment and 200 days of treatment showed growth of papilloma in the buccal mucosa	Kanojia et al., 2012
Mice-Oral cavity model	5mg/ml 4NQO exposure, 3 times a week for 16 weeks in CBA mice	4NQO exposure for 16 weeks causes both gross and histological changes starting from atypia to massive SCC in tongue and palate	Hawkins, 1994
	Benzo [a] pyrene (B[a]P) treatment with 5, 25 and 100 ppm for 2 years in B6C3F1 mice	High dose treatment of B[a]P developed distinctive tongue lesions with papilloma and carcinomas of the tongue at an incidence rate of ~48%	Culp et al., 1998
	4NQO (5mg/ml) was painted on the tongue, 3 times a week for 16 weeks in CBA mice	20% of experimental animals showed papilloma and only 5% showed invasive SCC	Tang et al., 2004
	4NQO exposure (100 µg/ml) through drinking water for 16 weeks in CBA and C57B1/6 mice	Both the mice strain showed prominent papilloma and invasive SC in the tongue of experimental mice	Tang et al., 2004
	4NQO administration of 50 or 100 µg/ml through drinking water for 8-32 weeks in CBA mice	CBA mice treated with 100 µg/ml 4NQO for 8 weeks showed the incidence of keratosis, dysplasia, and Head and Neck squamous cell carcinoma (HNSCC) at 8 weeks with minimum morbidity and mortality	Hasina et al., 2009
	Dibenzo [a,l] pyrene (DB[a,l]P) exposure with a dose of 3, 6, 12 and 24 nmol into the oral cavity of B6C3F1 mice	DB[a,l]P exposure of 24 nmol showed 31% neoplasia in the oral cavity of treated animals	Guttenplan et al, 2012

Transplanted Mouse Model for Oral Cancer

The application of the transplanted mouse model was first established by Rygaard in 1969. He successfully transplanted human malignant tumor cells into nude mice and transplantation of neoplasmic cells to induce tumors is commonly used in scientific research. In the transplanted tumor mouse model, allogenic or xenogenic tissues or cells are commonly being transplanted in immunodeficient and nu/nu mice.

Braakhuis et al. (1984) established a head and neck cancer (HNC) xenograft model in nude mice from 130 HNC cancer surgical specimens. After that scientists are using transplanted cancer mouse models in cancer research because of its advantage over chemically-induced oral cancer model (Srivastava et al., 2015; Kim et al., 2016; Yoshida et al., 2018). The advantage of xenograft models is that it develops lymphatic and pulmonary metastases that mimic human OSCC (Dinesman et al., 1990; Myers et al., 2002). However, the xenograft model has a major drawback because of the lack of interaction between tumor cells and the host immune system as xenograft models were prepared in immunodeficient mice.

Table 2. A genetically modified mouse model for oral squamous cell carcinoma

Model	Description	Characters	Reference(s)
L2D1^{+} p53$^{+/-}$ and L2D1^{+} p53$^{-/-}$	Human cyclin D1 (D1) expression under ED-L2 promoter and p53 homozygous and heterozygous knockout	Both the models developed invasive oral esophageal SCC	Opitz et al., 2002; Nakagawa et al., 1997
p53 R172H K5 CrePR1 and p53 flox/flox K5 CrePR1	K5 Cre inducible p53 expression and p53 knockout	In both, the skin of the case SCC was developed more than oral SCC and metastasis was also noted	Li et al., 2016
Tgfbr1/Pten 2cKO K14-CreER tam	K14 Cre-inducible Tgfbr and Pten knock out	Oral cavity tumors are visible with well-differentiated SCC	Bian et al., 2012
LSL-Kras^{G12D}, K5 or K14 CrePr1	The Kras^{G12D} oncogene expression under K5 or K14 promoter	Development of squamous papilloma in the oral cavity was prominent	Coulin et al., 2004

Genetically Modified Mouse Model for Oral Cancer

Genetically engineered mouse models for studying oral carcinogenesis are intricate and novel cancer models. Experimentation in this novel mouse model has advanced the understandings of tumorogenesis. The first genetically modified was devised to establish the oral-esophageal squamous cell carcinoma and it was generated by Opitz et al., 2002. This cancer model was modeled by crossing L2-cyclin D1 (L2D1+) mice with p53-knockout mice. Several strains of genetically altered mouse models have been prepared to date (Opitz et al., 2002; Li et al., 2016; Bian et al., 2012). Table 2 showed few strains of genetically modified mouse models which extensively being used in the field of cancer research.

Transgenic Animals Co-Treated with Chemical Carcinogens as a Model for Oral Cancer

In multistage carcinogenesis, a significant link has been established between carcinogen exposure and DNA mutation. Exogenous chemical carcinogens induced both initiation and promotion of carcinogenesis. Genetic modifications in the mice host cell synergize these exogenous carcinogens in the carcinogenesis

process. For example, the treatment of 1 mg of DMBA in p53-deficiency mice causes the progressive event of carcinogenesis and intrasubmandibular carcinogenesis (Ide et al., 2003a). Even if a haploid loss of p53 gene is sufficient enough to drive carcinogenesis in the mice test system. In a study, 100% malignancy was observed in $p53^{-/-}$ mice when treated with 1 mg of DMBA whereas in $p53^{+/-}$ mice, 70% malignancy was observed. However, in $p53^{+/+}$ mice, only 10% malignancy was observed with the treatment of DMBA (Ide et al., 2003b). Exposure to 4NQO in $XPA^{-/-}$ (deficiency in xeroderma pigmentosum group A gene) mice showed complete nucleotide excision repair deficiency (Ide et al., 2001). Exposure of 4NQO in several strains of genetically altered mice (Table 3) exhibit synergistic potential in carcinogenesis by affecting different pathways (Tseng et al., 2015; Chen et al., 2016; Tamura et al., 2017).

CHEMOPREVENTIVE ACTIVITY OF PHYTOPHARMACEUTICALS IN ANIMAL MODELS

The chemopreventive efficiency of plant-based pharmaceuticals has been extensively explored to halt, suppress or reverse the carcinogenesis process. Preclinical evaluation of phytopharmaceuticals against oral cancer provides valuable information about the progression of carcinogenesis and its management. Over a few decades, various chemical compounds isolated from plants were tested for its chemopreventive/anticarcinogenic activity against established oral cancer models. Many times, crude extract of plants with medicinal importance has also been used against oral cancer to assess its therapeutic efficacy. The anti-cancer potential of some known phytopharmaceuticals (Figure 3) tested in animal oral cancer models are given below-

Sulforaphane

Sulforaphane [1-Isothiocyanato-4-(methane sulfonyl) butane] is a naturally occurring isothiocyanate present at higher concentrations in cruciferous vegetables such as broccoli, cauliflower, brussels sprouts, and cabbages. Talalay and Zhang have first isolated sulforaphane from broccoli and demonstrated its anticancer potential in Sprague-Dawley rat (Yagishita et al., 2019). A study by Zhang et al. (1994) showed that treatment of sulforaphane with 75 and 150 μmol significantly reduced the tumor weight in DMBA treated female Sprague-Dawley rats. Recently, Sulforaphane mediated tumor growth reduction was reported in OSCC cancer stem cell (OSCC-CSC) transplanted nude mice (Liu et al., 2017). Sulforaphane treatment of 6 μmol three times per week for 16 weeks showed a lower incidence of SCC and tumor growth in 4NQO induced Hamster cheek pouch model (Bauman et al., 2016). Sulforaphane is a well known pleiotropic anti-cancer agent that restrains cancer cell proliferation, stimulates apoptotic pathways in cancer cells and also inhibits tumor and metastasis (Liu et al., 2017). Sulforaphane exhibits its anticancer potential by modulating numerous cell signaling pathways including Keap-Nrf2 signaling (Kensler et al., 2012), NF kappa β signaling (Xu et al., 2005), mitogen-activated protein kinase (MAPK) pathway (Keum et al. 2006) and modulation of cytochrome enzymes (Siemianowicz et al., 2018).

Apigenin

Apigenin is a flavonoid compound present in many vegetables, herbs, and fruits. High apigenin consisting dietary sources are fresh parsley, vine spinach, cereal seed, Chinese cereal, and dried oregano.

Apigenin is present in onions, tea, oranges, kumquats, and cilantro. Dried parsley and chamomile tea have extremely high levels of apigenin content (Wang et al., 2019). Naturally, apigenin present in plants in a glycosylated form such as apigenin-7-*O*-glucoside, apigenin-8-*C*-glucoside (vitexin), apigenin-6-*C*-glucoside (isovitexin), etc. Apigenin was reported to have anti-oxidant, anti-mutagenic, anti-carcinogenic, anti-inflammatory properties (Wang et al., 2019).

Silvan et al. (2011) established a Syrian hamster buccal pouch carcinogenesis model and showed that 2.5 mg/kg body weight/day apigenin treatment completely prevented the DMBA induced oral tumor. Further, 0.16 mg/day subcutaneous treatment of water-soluble derivative of apigenin, potassium apigenin showed chemopreventive potential in DMBA induced SCC model (Gomez-Garcia et al., 2013). Apigenin exhibit multiple therapeutic functions by modulating multiple pathways. Apigenin induces cell cycle arrest and apoptosis in tumor cells and increases antioxidant activities (Kashyap et al., 2018). Apigenin also regulates both intrinsic and extrinsic apoptosis pathways such as changing mitochondrial protein expression, the release of cytochrome c, and modulates Bcl-2, Bax and Akt protein expression (Salehi et al., 2019). Silvan et al. (2011) stated that apigenin reduced the oral tumor in hamsters by improving antioxidant defense mechanism and modulation the phase I and phase II detoxification of DMBA by hepatic enzymes.

Curcumin

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a natural polyphenol available in the rhizome of Curcuma longa and also in other species of Curcuma. Due to the presence of curcumin, Curcuma longa has been widely used in Asian countries as traditional medicine. Curcumin exhibits antioxidant, anti-inflammatory, antimutagenic, antimicrobial and anticancer activity (Hewlings and Kalman, 2017). Curcumin administration with or without other plant products not only reduced the SCC incidence (Li et al., 2002).

It also inhibited angiogenesis and papilloma in DMBA induced hamsters oral model. Chemopreventive potential of curcumin administration in 4NQO induced oral cancer (Siddappa et al., 2017) and tongue squamous cell carcinoma (TSCC) (Liao et al., 2018) had also been reported. Curcumin has a scavenging activity of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). It modulates the activity of several antioxidant enzymes such as glutathione sulphydryl (GSH), catalase, and superoxide dismutase (SOD), etc. (Menon and Sudheer, 2007).

The activity ROS generating enzymes- lipoxygenase/cyclooxygenase and xanthine hydrogenase/oxidase (Gheibi et al., 2019) are decreased following curcumin administration. Curcumin can modulate several signaling pathways involved in the carcinogenesis process. It has been established that curcumin down-regulates the expression of PD-L1 and p-STAT3. Liao et al. (2018) demonstrated that curcumin potentially changed the immunosuppressive tumor microenvironment and effectively promote an anti-tumor immune response in TSCC. Curcumin also displays anti-inflammatory response by modulating or blocking the expression of nuclear factor-kappa β (NF-κB) (Menon and Sudheer, 2007).

Piperine

Piperine, an alkaloid isolated in Indian medicinal plant *Piper nigrum* (black pepper). It is also present in high concentrations in other peppers such as long pepper and white pepper. This plant has many therapeutic potential starting leishmanicidal activity to anti-cancer potential. Piperine was first isolated by Hans Christian Orsted in 1819 from the fruits of *Piper nigrum.*

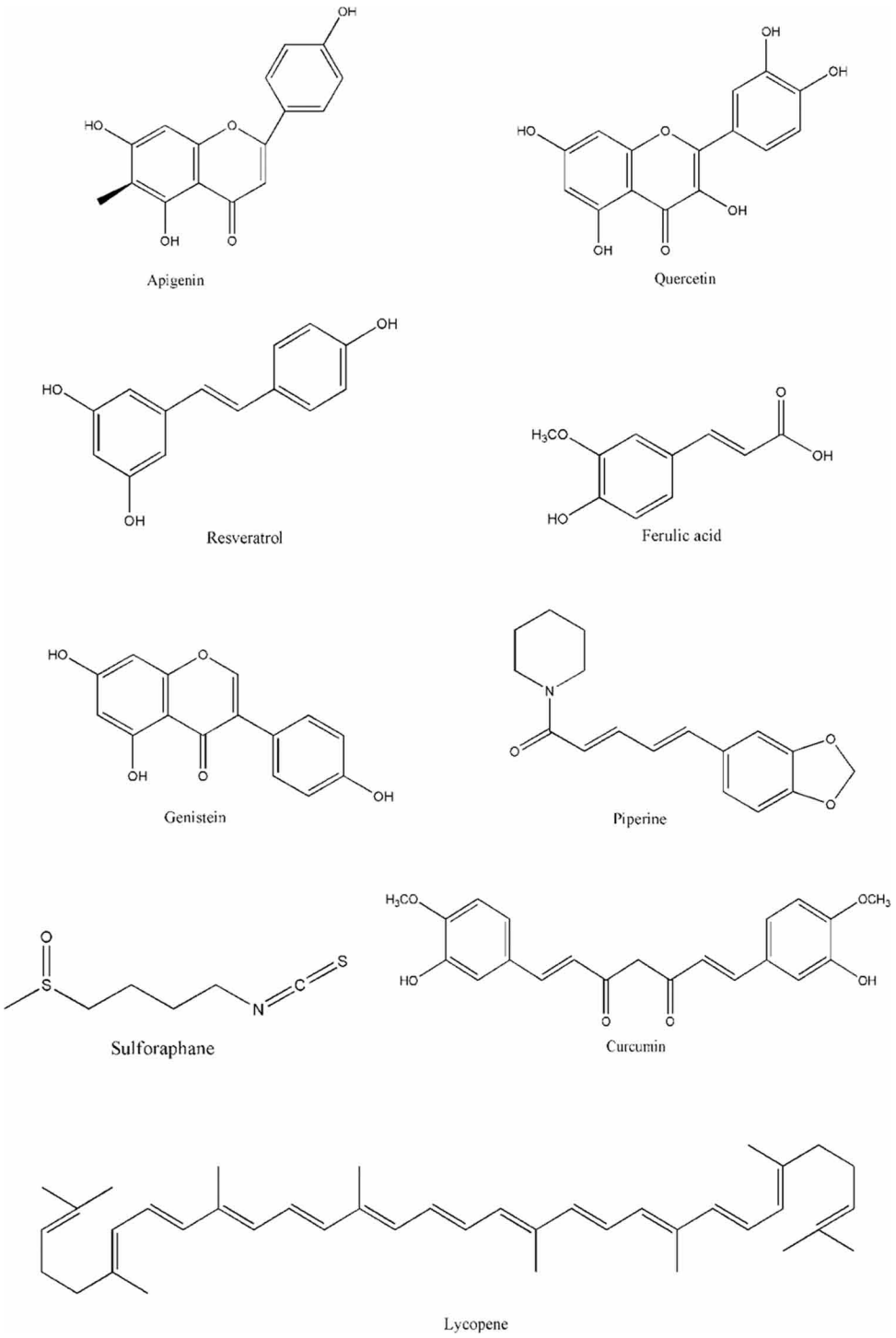
Table 3. Transgenic mice co-treated with exogenous chemical carcinogens for oral cancer model development

Mice strain	Description	Chemical carcinogen	Characters	Reference
p53+/-; p53-/-; p53+/+ mice	p53 mutated, knock out and normally expressed mice	DMBA- 1 mg single intra-submandibular injection	P53 genes knock out and mutated mice showed 100% and 70% SCC incidences respectively. However, the normal expression of p53 showed only 10% SCC.	Ide et al., 2003a
XPA-/-; p53+/- mouse	XPA deficient mouse carrying p53 mutated gene	4NQO- 10 ppm in drinking water for 50 weeks	4NQO treated XPA-/- p53+/- mice showed 100% SCC in 25 weeks	Ide et al., 2003b
L2D1+	Expression of human cyclin D1 protein under ED-L2 promoter	4NQO- 20-50 ppm through drinking water for 8 weeks	4NQO synergizes the susceptibility to oral dysplasia and SCC	Wilkey et al., 2009
Ndrg2+/-; Ndgr2-/- transgenic mice	N-myc downstream-regulated gene 2 (Ndrg-2) mutated and knockout mice	4NQO-50µg/ml in drinking water for 16 or 30 weeks	Accelerated tumor development with the larger size	Tamura et al., 2017
K14-EGFP-miR-211 transgenic mice	Expression of keratin-14 (K14) protein tagged with GFP and miRNA-211	4NQO- 100 µg/ml through drinking water for 16 weeks	miRNA-211 enhances the 4NQO induced oral carcinogenesis	Chen et al., 2016
K14-EGFP-miR-31 transgenic mice	Keratin-14 (K14) expression under the tagging of GFP and miRNA-31	4NQO- 100 µg/ml through drinking water for 16 weeks	Higher susceptibility to 4NQO induced oral and esophageal cancer	Tseng et al., 2015

Piperine (in its raw form- pepper) has been used by Indians as a traditional medicine in the treatment of cold & fever, muscular pain, analgesic, diuretic, strep throat, etc. Isolation of its pure compound led the researcher to assess its efficacy in preclinical studies. Piperine has found to possess anti-microbial, anti-fungal, anti-oxidant, anti-inflammation, anti-ulcer, anti-metastatic and anti-carcinogenic activity (Gorgani et al., 2016). The anti-cancer potential of piperine mostly carried out in animal models of different types of cancer.

The anticancer potential of piperine in the oral cancer model was first studied in 2009. Oral administration of piperine at a dose of 50 mg/kg body wt. in DMBA induced hamster buccal pouch cancer completely reduced SCC growth in the oral cavity (Manoharan et al., 2009). However, moderate keratosis, moderate hyperplasia and mild dysplasia in piperine treated animals were noticeable. The therapeutic potential of DMBA induced carcinogenesis in Swiss albino mice was also studied by Vellaichamy et al. (2009). The anti-carcinogenic property of Indian spice (black pepper) or piperine is due to its diverse mode of action in tumor tissues. In a study, piperine potentiates apoptosis in human squamous cell carcinoma (Siddiqui et al., 2017). Piperine operates multiple mechanistic approaches while curing cancer. Piperine activates the intrinsic and extrinsic pathways of apoptosis and also causes cell cycle arrest in cancerous cells (Siddiqui et al., 2017).

Figure 3. Chemical structure of selected phytopharmaceuticals showing potential chemo-preventive role against oral cancer progression
Source: PubChem database (Maitained by National Institute of Health, NIH)



Ferulic Acid

Ferulic acid [E-3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid] is a phenolic acid commonly found in whole grains, spinach, grapes, cereal seeds, parsley and mainly in wheat, tomatoes, oats, rye, and barley (Zdunska et al., 2018). This dietary antioxidant has a low toxicity profile and offers beneficial effects in the treatment of diabetes, neurodegenerative diseases, inflammation, microbial infection, and cancer. Epidemiological studies indicate that regular dietary intake of fruits and vegetables' containing a high amount of ferulic acid content significantly reduces the risk of many cancers (Batista, 2014).

There are only a few studies available on the chemopreventive potential of ferulic acid in the oral cancer mouse model and mostly carried out in skin and other tumor models in mice (Alias et al., 2009; Zhang et al., 2016; Min et al., 2018). Ferulic acid (40 mg/kg body wt./week) potentially and completely reduced the OSCC progression in mice model by changing p53 and Bcl-2 protein expression patterns (Balakrishnan et al., 2010). Ferulic acid inhibits the proliferative potential of cancer cells and promotes apoptosis by blocking the PI3K/Akt pathways (Wang et al., 2016) and increasing the expression of other apoptotic protein pRB, p21, and pERk1/2 (Thakkar et al., 2015).

Resveratrol

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a naturally occurring polyphenol with a broad range of biological functions. The natural sources of resveratrol are grapes, berries, peanuts, etc. It is also present in red wine at a significant concentration. Resveratrol has lots of therapeutic potential including anti-inflammatory, anti-tumorigenic, and antioxidant potential (Ramirez-Garza et al., 2018). Resveratrol also exhibits anticancer potential against various cancerous cells and *in vivo* tumor models by modulating multiple pathways associated with cell growth inhibition, cell-cycle arrest mechanisms, apoptosis, and suppression of transcription factors (Kim et al., 2018a; Kim et al., 2018b).

Resveratrol inhibits the progression and development of oral cancer evident from preclinical studies. Berta et al. (2010) reported the chemopreventive efficacy of resveratrol in DMBA induced carcinogenic model of hamster cheek pouch. Resveratrol also showed its antitumor potential in 4NQO induced C57BL/6 mice (Shrotriya et al., 2015). Resveratrol and grape seed extracts were found to be completely inhibited by the multiplicity and severity of preneoplastic and neoplastic lesions without any apparent toxicity. Oral administration of resveratrol decreases the tongue lesions including hyperplasia and dysplasia and also papilloma in the HNSCC tumor model induced by 4NQO (Sclafani et al., 2014). Resveratrol provokes the mitochondrial membrane potential and prevents the transitional state of Epithelial-Mesenchymal Transition and thus contribute a significant role in cancer chemoprevention (Kim et al., 2018a and Kim et al., 2018b). However, resveratrol also affects other pathways of carcinogenesis such as MAPKs, PI3K/Akt, IKK, PKC, etc. It can modulate the expression of p53, NFkβ, AP-1, HIF-1α, and STAT3 and exhibit anti-inflammatory, antiangiogenesis and antimetastatic activity (Siddiqui et al., 2015).

Nimbolide

Nimbolide is one of the major compounds of neem (*Azadirachta indica*) having potential anticancer activity. The other compounds with pharmacological benefits of neem are Azadirachtin, Salannin, Nimbin and Nimbic acid. Apart from its anti-cancer activity, nimbolide has antibacterial, anti-malarial

and anti-insecticidal activity (Bodduluru et al., 2014). Nimbolide is used as a traditional medicine in Indian Ayurvedic medicines to treat the wound, gastric ulcer, acne and infections (Wang et al., 2016b).

The anticancer activity of nimbolide has been established in tumor models of various types of cancers. Nimbolide also displays its chemopreventive role against carcinogen-induced oral cancer tumor models. Oral feeding of nimbolide and azadirachtin in oral cancer hamsters successfully reduced the incidence of SCC and dysplasia by preventing cell proliferation and inducing apoptosis (Kumar et al., 2010) and also blocks the invasive potential of tumor and angiogenesis (Bodduluru et al., 2014). Sophia et al. (2018) reported that the incidence of tumor growth in the Syrian hamster buccal pouch model was reduced by ~57% after oral feeding of nimbolide. Neem and its active compounds- nimbolide and azadirachtin, affect multiple pathways showing its chemoprevention against cancer. Nimbolide upregulates the anti-oxidant enzymes (SOD, catalase, GSH, etc.) and inhibits ROS-induced damage, cell proliferation and inhibits apoptosis (Vinothini et al., 2009). Nimbolide also modulates the metabolism of carcinogens by decreasing phase I enzymes and increasing phase II enzymes (GST, NADPH-diaphorase, etc.) (Subapriya et al., 2006). Induction of cell cycle arrest and apoptosis are also influenced by nimbolide and inhibit PI3K-Akt, MEK-Erk1/2 and JAK-STAT pathways (Bodduluru et al., 2014).

Genistein

Genistein [5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one] is an isoflavone occurring naturally in soybeans and other legumes such as chickpeas. Genistein plays a vital role in the prevention of diverse types of cancers. Epidemiological evidence supports the inverse relationship between dietary intake of genistein and cancer incidence. Genistein has antioxidant, anti-apoptotic, anti-inflammatory, anti-proliferative, and anti-metastatic potential against a variety of cancer models (Tuli et al., 2019). The therapeutic potential of genistein and its mechanism has been extensively studied in different cancer models. Genistein modulates the expression of cell cycle proteins, proteins associated with intrinsic and extrinsic pathways of apoptosis, angiogenesis, and metastasis (Ganai et al., 2015; Russo et al., 2016).

There are several reports that genistein inhibits the progression of the multistep carcinogenesis process of oral cancer. Ardito et al. (2017) reported that genistein has anti-cancer activity against tongue cancer. Studies on the hamster oral cancer model revealed that the administration of genistein reduces the oral incidence to ~40% (Yang et al., 2006). Administration genistein (0.5 mg/kg body wt.) in HSC-3 bearing xenograft mice could potentially reduce the invasive potential of cancerous cells and reduced CD31 expression (Myoung et al., 2002). Genistein affects the multiple pathways of the carcinogenesis process. Genistein modulates various proteins associated with apoptosis such as it increases the expression of Bax, Bak, Bad, caspase-3, and TRAIL-mediated apoptotic cell death factor (LC3-II and p62) and decreases PKL1 and MDM2 expression (Shafiee et al., 2016; Hsiao et al., 2018; Tuli et al., 2019). Genistein also targets other pathways that include NF-κB signaling, PI3K/Akt signaling, ERK 1/2, MAPK, and Wnt/β-catenin signaling pathway and showed its onco-preventive activity (Tuli et al., 2019). Besides these factors, genistein has shown to modulate the PPARɣ signaling cascade and induced endoplasmic reticulum stress and increases apoptosis of cancer cells (Banerjee et al., 2008).

Table 4. The chemo-preventive potential of phytopharmaceuticals in oral cancer animal models in vivo

Phytopharmaceutical	Animal model	Treatment	Observation	Reference(s)
Sulforaphane	C57BL/6 mice	6 µmol- 3 times a week for 16 or 24 weeks	Treated animals showed a lower incidence of SCC. Tongue tumor was significantly smaller than the 4NQO treated group	Bauman et al., 2016
	OSCC-CSCs transplanted nude mice	50 mg/kg body wt.	Sulforaphane reduced the tumor growth at 20th day by modulating miR200c expression	Liu et al., 2017
	Female Sprague-Dawley rat	75 and 150 µmol for 50 days	The incidence of the tumor was significantly reduced to 50% and sulforaphane treatment increases the survival of DMBA induced tumor animals.	Zhang et al., 1994
Apigenin	Hamsters- cheek pouch model	2.5 mg/kg body wt./day for 16 weeks	Completely prevented the incidence of SCC by improving antioxidant defense mechanisms and increasing phase I and phase II detoxification enzyme activity.	Silvan et al., 2011
	Hamsters- cheek pouch model	0.16 mg/day subcutaneously	Apigenin showed chemopreventive effect by reducing the incidence of moderate to intense dysplasia	Gomez-Garcia et al., 2013
Curcumin	C57BL/6 female mice	14.3 µg/mL for 8 weeks	At 25th week, curcumin administration significantly reduced the tumor volume	Siddappa et al., 2017
	Syrian golden hamster	10 mmol- 3 times per week for 18 weeks	Curcumin significantly decreased the incidence of SCC in DMBA induce oral cancer model	Li et al., 2002
	Hamsters- buccal cheek pouch model	80 mg/kg body wt.- 1 week before carcinogen treatment till 14 weeks	Curcumin completely reduced the OSCC progression, however, moderate hyperplasia and dysplasia was identifiable	Balakrishnan et al., 2010
Piperine	Hamsters- buccal cheek pouch modal	50 mg/kg body wt.- 1 week before carcinogen treatment till 14 weeks	Treated animals showed a complete reduction of tumor volume. OSCC progression was significantly reduced	Manoharan et al., 2009
	Male Swiss albino mice	50 mg/kg body wt- 3 times per week from 1 week before carcinogen exposure till 25 weeks	Oral administration of piperine significantly prevented the incidence of the tumor and reduced tumor volume in DMBA induced oral cancer model	Vallaichamy et al., 2009
Ferulic acid	Hamsters- buccal cheek pouch model	40 mg/kg body wt.- 1 week before carcinogen exposure till 14 weeks	Ferulic acid completely reduced the progression of OSCC by changing the p53 and Bcl-2 expression pattern	Balakrishnan et al., 2010
Resveratrol	C57BL/6 mice	0.25% w/w from 8 weeks after 4NQO exposure till 16 weeks	The tongue lesions including hyperplasia and dysplasia were reduced significantly	Shrotriya et al., 2015
	Syrian golden hamsters- buccal cheek model	150 µl resveratrol-mouthwash daily for 14 weeks	Mouthwash resveratrol treatment showed completely reduced incidences of dysplasia	Berta et al., 2010
Nimbolide	Hamsters- buccal pouch model	100 µg.kg body wt. from 4 weeks (when dysplasia was prominent) till 8 weeks (when SCC was prominent in the control group)	Administration of nimbolide completely reduced the tumor growth by ~57% and showed more prevention at early intervention	Sophia et al., 2018
	Syrian hamsters-buccal pouch model	10 and 100 µg/kg body wt. for 14 weeks	Completely reduced the SCC incidence and high dose treatment also exhibited a reduction of dysplastic lesions	Kumar et al., 2010
Genistein	HSC-3 bearing Balb/c (nu/nu) mice	0.5 mg/kg body wt. injected into the tumor site for 30 days	Genistein treated mice showed reduced invasiveness potential of cancer cells and reduced expression of CD31	Myoung et al., 2002
	Hamsters- buccal pouch model	10 mg/kg body wt. daily for 12 weeks	Oral cancer lesions were found to be decreased by ~40%	Yang et al., 2006

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Table 4. Continued

Phytopharmaceutical	Animal model	Treatment	Observation	Reference(s)
Lycopene	Hamsters- buccal cheek pouch model	2.5 mg/kg body wt.- 3 times per week for 14 weeks	Lycopene administration showed chemoprevention against DMBA induced oral carcinoma by modulating lipid peroxidation and enhancing the activity of enzymes involved in glutathione redox cycles	Bhuvaneswari et al., 2001
	Male albino rat	2.5 mg/kg body wt.- once per day for 32 weeks	Lycopene reduced the tumor growth and SCC and carcinoma in situ incidences were reduced by 90% in 4NQO induced oral cancer model	El-Rouby et al., 2011
Quercetin	Hamsters- buccal pouch model	2% quercetin for 10 and 16 weeks	Quercetin treatment significantly reduced the incidence of papilloma and SCC in DMBA oral cancer	Balasubramanian and Govinasamy, 1996
	F344 rat	500 ppm starting from 1-week prior carcinogen exposure till 10 weeks	At 32 weeks, quercetin treatment significantly reduced the incidence of papilloma and SCC	Makita et al., 1996
	Colon-25 transplanted transgenic mice	Quercetin derivative (quercetin chalcone) treatment at a dose of 0.8 mg/ml and 1.6 mg/ml for 20 days	High dose quercetin significantly reduced tumor size up to 65%	Hayashi and Lott, 2000
Epigallocatechin gallate	Balb/c (nu/nu) mice	10 and 20 mg/kg body wt. per day up to 45 days	EGCG administration showed ~6 fold reduction in tumor weight without apparent symptoms of toxicity	Chen et al., 2011
	Balb/c (nu/nu) mice	75 mg/kg body wt.- 2 times for 4 weeks	EGCG feeding in HSC-3 nude mice suppresses the tumor growth and tumor volume was reduced by ~45%	Yoshimura et al., 2019

Lycopene

Lycopene is a naturally occurring red colored carotenoid pigment found in many fruits including tomato, pink grapefruit, watermelon, papaya, and guava. Lycopene has exhibited a wide range of biological activities including antioxidant, immune system modulator, enhancing phase II enzyme metabolism, anti-inflammatory response, anti-proliferative and anti-angiogenesis activities, induction of apoptosis and anti-cancer (Kaur et al., 2017). The anticancer activities of lycopene were extensively studied in different cancer models of *in vitro, in vivo* and humans also. Anticancer activity of lycopene can be attributed to its antioxidant properties. However, other modes of action like overexpression of detoxification enzymes, induction of gap junctional communication, inhibition of cell proliferation and progression and modulation of signal transduction pathways were also reported (Camara et al., 2013).

Lycopene mediated onco-prevention in animal oral cancer model has been demonstrated by several researchers. Lycopene administration of 2.5 mg/kg body wt. suppresses the DMBA induced oral carcinogenesis in hamsters (Bhuvaneswari et al. 2001) by modulating lipid peroxidation and enhancing the enzyme activity involved in glutathione redox cycling. El-Rouby (2011) evaluated the chemopreventive efficacy of lycopene in tongue carcinoma. Oral administration of lycopene exerts protective effects against 4NQO induced tongue cancer reducing the proliferation of cancerous cells and enhancing cellular adhesiveness.

Quercetin

Quercetin (3,3',4',5,7-pentahydroxyflavone) is an important flavonol present ubiquitously in vegetables and fruits including apple, broccoli, onions, nuts, spinach, black and green tea, coffee, etc. Quercetin is the most extensively studied natural product exhibiting a broad range of biological actions. Quercetin has antioxidant, anti-inflammatory, antibacterial, antiviral, free radical scavenging and immune-modulatory properties (Kim and Park, 2018). Besides these biological functions, quercetin exhibits potential anti-cancer properties against many cancers proved by *in vitro* and *in vivo* studies.

Quercetin also displays the anticancer activity against oral cancer both in cell lines and in animal models. In animal models, quercetin administration reduced the proliferation of cancer cells in the hamster buccal pouch cancer model and thus reduced the progressive transformations of dysplasia to SCC (Balasubramanian and Govindasamy, 1996). Oral feeding of quercetin for 10 and 16 weeks significantly reduced the incidence of papilloma and dysplasia in DMBA induced oral cancer model. Quercetin administration (500 ppm) also reduced the numbers of animals with papilloma and SCC induced by 4NQO treatment (Makita et al., 1996). Hayashi et al. (2000) reported that the administration of quercetin derivative (Quercetin chalcone) significantly reduced the tumor size and volume up to 65% in colon 25 transplanted Balb/c mice.

The inhibitory effect of quercetin against cancer progression may be attributable to the free radical scavenging activity, inhibition of DNA synthesis; modulation of cytochrome P450 monooxygenase activity and inhibition of DMBA-DNA adduct formation (Balasubramanian and Govindasamy, 1996). The anticancer activity of quercetin is by activation of cell death domains leading to FAS and FADD activation that in turn activates caspase-8 mediated cancer cell death (Vargas and Burd, 2010). It was also interfering NFkβ pathway by modulating the expression of associated protein/factors and exerts in anti-inflammatory responses (Hashemzaei et al., 2017). Quercetin downregulates the expression of mutated p53, p21-ras oncogene, inhibits the activity of tyrosine kinase, and also the expression of VEGFR-2, mTOR, Akt and ribosomal S6 kinase (Pratheeshkumar et al., 2012) showing its anti-cancer and anti-angiogenic potential.

Epigallocatechin Gallate (EGCG)

Epigallocatechin gallate (EGCG) is a polyphenol mostly found in tea (*Camellia sinensis*). Tea also consists of other polyphenols, of course not limited to, epicatechin, epicatechin gallate (ECG), and epigallocatechin (EGC). EGCG is the major polyphenol in green tea (>40% of dry weight), four to five-fold more than black tea.

Studies showed that EGCG is an effective polyphenol in the reduction of cell growth, induction of apoptosis, and inhibition of angiogenesis in oral cancers (Ko et al., 2007). EGCG effectively inhibits the growth of cancers cell extracted from dysplastic leukoplakia and SCC (Khafif et al., 1998). In the animal oral cancer model, EGCG administration (10 and 20 mg/kg body wt) significantly reduces the tumor size (~6 folds) (Chen et al., 2011). Oral feeding of EGCG has chemopreventive potential against xenograft animal tumor model by changing Ki-67 expression and caspase-3 and caspase-7 activities (Yoshimura et al., 2019). In this model, the EGCG administration of 75 mg/kg body wt. twice per day for four weeks reflects a detectable reduction of tumor growth in Balb c (nu/nu) mice. EGCG potentially changes the expression of various proteins associated with carcinogenesis affecting the pathways of cell cycle regulation and apoptosis. EGCG administration suppresses the incidence of dysplasia and SCC

in 4NQO induced tongue carcinoma in Spraque-Dawley rats (Kono et al., 2014) by downregulating the expression of NFkβ, Ki-67, p65, and IKKα.

NANOPARTICLES OF PHYTOPHARMACEUTICALS: RECENT TRENDS

Chemoprevention of oral cancer showed promising results in preclinical studies. However, the results of phytopharmaceuticals in clinical settings showed limited success. Poor bioavailability of these phytopharmaceuticals and inefficient systemic delivery are the major limitations of most natural chemopreventive products (Iriti and Varoni, 2013). To overcome this problem, scientists around the glove introduced the concept of "nanochemoprevention" referring to the systemic delivery of encapsulated phytopharmaceuticals in biocompatible nanoparticles (Siddiqui et al., 2012).

Nanoparticles of phytopharmaceuticals increase the solubility and bioavailability and thus its efficacy as potential chemopreventive agents. Siddiqui et al. (2009) have demonstrated that encapsulated EGCG has higher anticancer activity in xenografted athymic nude mice. Sulfikkarali et al. (2013) showed that Naringenin-loaded nanoparticle completely prevented tumor progression in DMBA induced oral cancer in Syrian hamsters. Moreover, naringenin nanoparticles also lowered the expression of PCNA (proliferating cell nuclear antigen) and p53 in buccal mucosa of DMBA-exposed animals as compared to free naringenin.

Various phytopharmaceuticals have been formulated for oral administration with encapsulated nanoparticles. For example, liposome-encapsulated curcumin showed a significant reduction in the growth of HNSCC (Wang et al., 2008). Similarly, Wu et al. (2012) showed that propylene glycol assisted fenretinide nanoparticles are responsible for chemoprevention of oral cancer. Thus, it is evident that nanoparticles of phytopharmaceuticals have shown more promising results in preclinical models as their free counterparts. This technology was employed to overcome the low solubility and bioavailability of phytopharmaceuticals. Studies in nanochemoprevention are still going on to reach more conclusive results.

CONCLUSION

Phytopharmaceutical assisted cancer chemoprevention has immense therapeutic potential and is an exciting and challenging area of research. The bioactive phytochemicals play a key role in the prevention of different types of cancers including oral cancer. The preclinical studies regarding the chemopreventive potential of phytopharmaceuticals have been studied extensively by establishing animal cancer models *in vivo*. The establishment of oral cancer in animal models facilitates understanding the multistep progression of carcinogenesis and its better treatment strategy. Likewise, chemoprevention enables a better understanding of the potential of phytopharmaceuticals and their mechanism of action in cancer treatment before moving forward to clinical trials. Some of the phytopharmaceuticals (like resveratrol, lycopene, EGCG) have been used successfully in clinical trials. However, chemoprevention by phytopharmaceuticals in oral cancers is still inconclusive due to a lack of sufficient research data. There are enormous numbers of plants and their bioactive products available in nature. Isolation of their active ingredients and their evaluation against cancer is still a challenging task. However, preclinical evaluation of novel phytopharmaceuticals against cancer has a great contribution due to fewer side effects as compared to conventional drugs.

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Chapter 21
Nutrition and Cancer

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ABSTRACT

Cancer is the second biggest killer worldwide. It has been estimated that specific lifestyle and dietary measures can prevent 30–40% of all cancers. Consumption of nutrient sparse foods, such as refined flour products and concentrated sugars, consumption of red meat, low fibre intake, and disproportion of omega 3 and omega 6 fatty acids, contributes to cancer risks. Microbiological and chemical food contaminants as well as conventional and industrial food processing methods may further increase the carcinogenicity of diets while protective agents in a cancer prevention diet include folic acid, selenium, vitamin D, vitamin B-12, chlorophyll, and antioxidants such as the carotenoids, kryptoxanthin, lycopene, and lutein. Diet can also influence the gut microbes that may have positive or adverse effects on cancer risk. The authors summarize cancer prevention by functional foods and discuss the role of different dietary factors such as promoter or inhibitor in pathogenesis of different subtypes of cancer worldwide.

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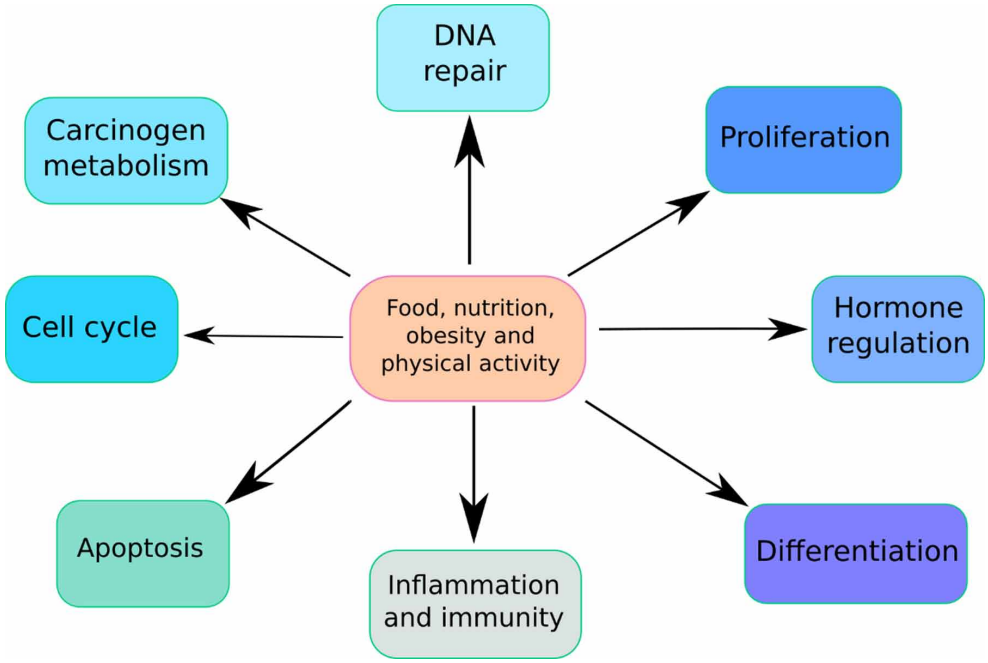
INTRODUCTION

The relationship between nutrition and cancer was initially recognized in 1940 by an experimental study, where progressive confinement of nutritional factors evidently reduced the prevalence of cancer in mice (Tannenbaum, 1940). Other advancements came after two decades depicting the relation between geographical impact and cancer incidence. This was suggestive of the difference in dietary habits and lifestyle (Doll, 1966; Doll, 1970). This was followed by protuberant number of case-control studies that led to the identification of cancer risk factors in diet. Macro and micronutrient components in food along with food patterns play etiological roles in incidence of cancer (Fig. 1). The effects of these components can be altered by lifestyle factors like exercise and physical activity. Besides increasing the body weight, physical inactivity is thought to contribute to cancer risk by negatively effecting the endocrine and immune system. Diets with low in vegetables, fruits and whole grains, are linked with a number of cancers. In this chapter, we discuss the significant role of dietary factors in promotion or inhibition of pathogenesis of different subtypes of cancer worldwide.

Figure 1. Effect of diet on different cellular processes



Over Consumption of Energy

Our diet is an essential fuel to sprint our body function smoothly and adequately, not only health but mental health as well. A common saying from Mark Twain states that the only way to keep healthy is to eat what we don't want, drink what we don't like, and do what we'd rather not." This quote consciously warns our mind of balanced diet.

Calories and Obesity

The World Health Organization (WHO) defines obesity as an 'abnormal or excessive fat accumulation that may impair health', and states that 'the fundamental cause of obesity and overweight is an energy imbalance between calories consumed and calories (WHO,2014; Schoeller, 2008). This modern evidence suggests that a major driver of increased caloric intake across diverse populations has been a decrease in the quality of diet: changes in the types and quality of foods consumed, which together influence long-term energy balance (Mozaffarian, Hao, & Rimm, 2011; Bertoia, Mukamal, & Cahill, 2015). Some of the key factors that characterize diet quality include carbohydrate quality; intakes of whole foods such as nuts, beans, fruits, and vegetables; specific fats and oils; and overall dietary patterns. For example, in Mexico, so-called traditional dietary patterns, characterized by the consumption of corn tortillas and maize-based preparations, beans, vegetables, fruits, and vegetable-based fats, have been associated with lower risk of obesity and diabetes (Rodrıguez-Ramırez, Mundo-Rosas2011; Romero-Polvo, Denova-Gutie´rrez, & Rivera-Paredez, 2012).

Notably, overall food quality and dietary patterns also have a major impact on metabolic risk independent of obesity. In other words, a person can remain obese and substantially decrease its metabolic risk with a healthy diet, while a lean person can develop substantial metabolic dysfunction with a poor diet. In addition to the obesity status and total calories, different dietary factors influence visceral adiposity and liver fat production (hepatic de novo lipogenesis). In particular, rapidly digestible carbohydrates activate de novo lipogenesis and influence adiposity, as do trans fats. Other controlled trials further suggest, at least to one month, that the quality of the diet may actually influence metabolic expenditure (Ebbeling, Swain, & Feldman, 2012). While longer-term trials are needed to confirm this last finding, these new results represent a potential paradigm shift – quality of the diet influences not only energy intake, but also energy output.

Glucose Metabolism

Changes in energy metabolism consisting of increased resting energy expenditure associated with alterations of glucose, lipid and protein metabolism are typical of cancer-related anorexia/cachexia syndrome (CACS).The tumor growth and the chronic activation of the immune system (to counteract the tumor growth) are responsible for an increased energy expenditure and thus for a continuous consumption of energetic substrates, especially glucose (Mantovani, Macciò, & Lai, 1998). In fact, the oxidation of glucose into CO_2 and H_2O through the Krebs' cycle is a well-known major source of energy and plays a key role in the biosynthesis of ATP, DNA, RNA and phospholipids. Glucose is also necessary for the pentose-phosphate pathway and the synthesis of reducing compounds such as NADPH (Mantovani, Macciò, Massa, & Madeddu, 2001).

Diabetes and Cancer

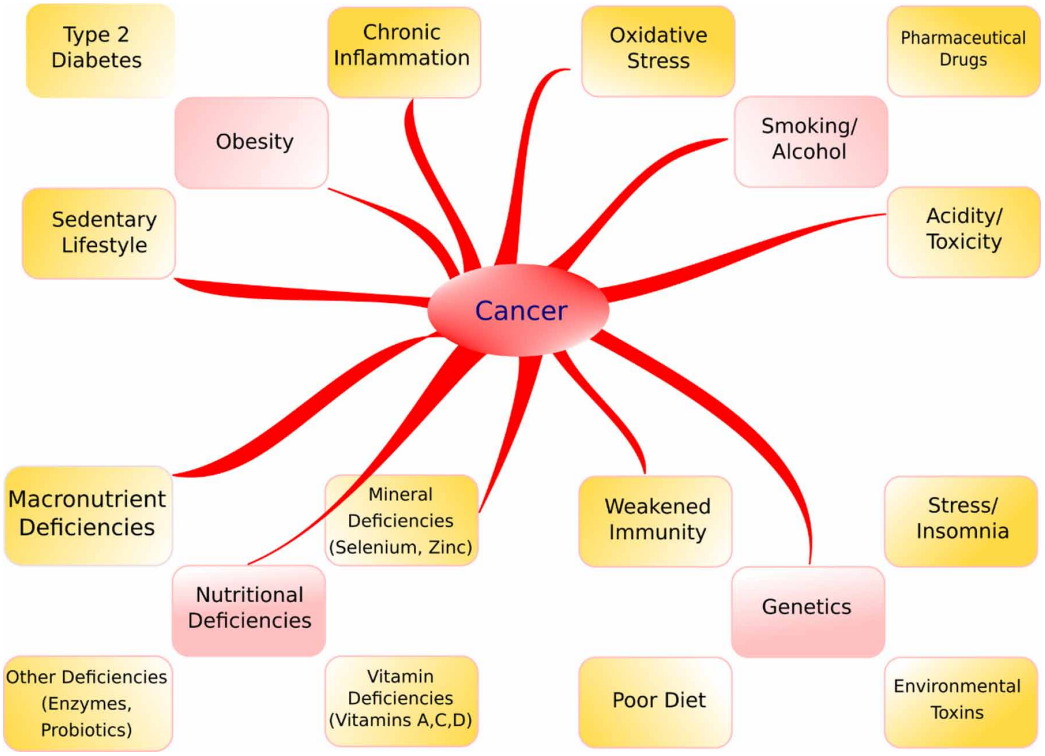
It is inappropriate to consider diabetic patients as a homogeneous cohort. In addition, a series of potential confounders directly related to the disease (obesity, quality of metabolic control, drugs employed for treatment, diet, etc.) and present in diabetic patients may influence the association between diabetes and cancer.

FOODS THAT MAY INCREASE THE LIKELIHOOD OF DEVELOPING CANCER

Sugar and Refined Carbohydrates

Carbohydrate intake has been hypothesized to modulate cancer risk depends on the amount and type consumed. Carbohydrates is a broad category of biomolecules and its monosaccharide forms are the preferred source of cellular energy (Leturque, Brot-Laroche et al. 2012). Apart from the crude function, carbohydrates cause different effects at the physiological, cellular, and ecological levels. Remarkable among these are microbial, epigenetic modulations, endocrine and systemic alterations resulting from their consumption that may potentially influence cancer risk and progression (Hullar and Fu 2014). Nutrition plays an important role in cancers (Fig. 2). Cancers may be prevented by appropriate diets, physical activity and by maintaining appropriate body weight (Food 1997).

Figure 2. Dietary relationship with increased likelihood of developing cancer



Dietary sugar stimulates insulin release and may predispose us to insulin resistance (Daly, Vale et al. 1997). Then the resulting chronic hyperinsulinemia may stimulate tumorigenesis as insulin is a mitogen of breast tumor cell growth in vitro (van de Poll, van Rotterdam et al. 2005). Carbohydrates with a low GI value are more slowly digested, absorbed, and metabolized, causing a lower and slower rise in blood glucose and insulin levels. In meta-analysis, greater consumption of foods with high GI is significantly associated with greater risk of breast cancer (Choi, Giovannucci et al. 2012, Mullie, Koechlin et al. 2016) (Gómez, Hernández-Prado et al. 2009).

CRC is the third most common cancer in men and the second in women worldwide. Given that these tumors develop adjacent to the intestinal lumen. It is not surprising that the link between diet and CRC risk is one of the strongest for any type of cancer. Higher consumption of grain and bread is associated with increased CRC risk. The study also shows, higher intake of whole grains and legumes is associated with lower risk for developing CRC. Researchers have reported that the risk for developing CRC was higher among individuals who consumed carbohydrates in the form of rice, white bread, and pasta as compared to the individuals who consumed carbohydrates as whole grain cereals (Haenszel, Berg et al. 1973, Franceschi, Favero et al. 1997).

Processed Meat

Meat is an integral part of human's diet and is a major source of protein and fat for populations all over the world. Although it is rich in saturated fats and cholesterol. Meat consumption has been increased considerably in recent years. Muscle meat from beef, lamb, horse, goat, veal, pork, and deer is defined as red meat, while as poultry meat is referred to as white meat. Processed meat includes all those types of meat products, such as sausages, cold cuts and other meats, which have undergone a process to extend their shelf life and which have been mixed with ingredients such as curing salt or salt. Although meat is consumed on regular basis by the majority of the population, there is concern raised by many studies that high intake of red and/or processed meat is associated with diseases like obesity, type 2 diabetes, cardiovascular disease, and a variety of cancers (Klurfeld 2015). Studies have shown that the higher intake of poultry and fish has an inverse associations for cancers of the esophagus, anus, rectum, liver, colon, pleura and lung were observed (Daniel, Cross et al. 2011). In this scenario, although red and processed meat consumption has been linked to the risk of various cancers and other health outcomes, the type of cancer that is best studied in relation to meat consumption is colorectal cancer (CRC).

Numerous case control and cohort studies have evaluated the relationship between meat consumption and associated cancer risk, and most of those studies indeed observed a positive association. In 1996, colon cancer panel of the world health organization have consensus conference on nutrition in prevention and therapy on cancer. They recommended that consumption of fish and poultry should be preferred over red meat (Scheppach, Bingham et al. 1999). More recently, in a summary evaluation of the studies published thus far, the World Cancer Research Fund in 2007 concluded that high consumption of red meat and processed meat were convincingly associated with CRC risk. So it has been recommended to restrict consumption of red and/or processed meat intake (Klurfeld 2015). Therefore, different international agencies and independent researchers recommended to decrease meat intake and as such reduce the risk. So to prevent the cancer, American cancer society advises to limit the intake of red and processed meat and recommended to choose fish and poultry instead of beef, pork or lamb (Kushi, Byers et al. 2006).

Over-Cooked Food

Studies have proved that overcooking of potatoes, toast, and other starchy food gets acrylamide, a chemical which is produced during cooking, and has been associated with cancer. The danger of high levels of acrylamide have been known for about a decade now (Gökmen 2015). Also, heterocyclic amine production from overcooked foods is extremely mutagenic in numerous invitro and in-vivo test systems. Likewise, deep-fried, overcooked foods and high-salted foods are coupled in a way to escalate gastric cancer incidences. Moreover, eating of food with small fiber ingredients and having animal fat increases

the threat of stomach and esophageal malignancies. High incidence of gastric cancer in the USA has been linked mainly with eating of red meat and diet having less fiber.

It is reported that cooking food at higher temperatures and for shorter durations than recommended (Gulland 2017). So, it is better not to overcook the food items as it generates harmful chemicals that would be harmful to people in long term. so better is to go for the golden yellow color or lighter to decrease the harmful chemical levels.

Low Fiber Food

In various potential health studies low fiber was not found to be a risk for breast cancer (Buttriss and Stokes 2008). It is possible that fiber measurements is just a surrogate measure for unrefined plant food intake. Scientists found an inverse correlation between vegetable, fruit and whole grain intake and CRC (Jacobs Jr, Pereira et al. 2000). It was reported that about five daily servings of vegetables were needed to reduce cancer risk and its effect was found stronger among older subjects. Studies have demonstrated less risk of colon cancer for populations with total high fiber diet then the high fat diet.

FOODS WITH CANCER-FIGHTING PROPERTIES

Vegetables and Fruits

Furthermore, consideration of the potential biological effects of various constituents of fruits and vegetables suggested plausible mechanisms for protective effects, such as by reducing oxidative damage of DNA or increasing the activity of enzymes able to detoxify carcinogens (Steinmetz and Potter, 1991). This view was consolidated by an expert panel report published in 1997, which stated that there was ‘convincing’ evidence that high intakes of fruit and/or vegetables decrease the risk for cancers of the mouth and pharynx, esophagus, stomach, colorectum and lung (World Cancer Research Fund/American Institute for Cancer Research, 1997).

Nuts

By definition, a nut is a dry fruit consisting of a hard or tough shell around an edible kernel. Nuts mainly contain monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA) and a very low content of saturated fatty acids (SFA), ranging from 4% to 16%. The percentage of MUFA and PUFA varies between different types of nuts: many nuts contain mostly MUFA (mainly oleic acid). Brazil nuts have similar proportions of MUFA and PUFA, whereas walnuts contain mainly PUFA, both linoleic acid and α-linolenic acid. Importantly, nuts are enriched in several phytochemicals and indeed their beneficial effects have been largely ascribed to the simultaneous presence of these micronutrients. Amongst the other micronutrients, nuts contain the B-vitamin folate, ranging from 22 μg in pecans and Brazil nuts to 145 μg in peanuts (per 100 g), as well as antioxidant vitamins (tocopherols) and phenolic compounds (23). Almonds are particularly rich in α-tocopherol, while walnuts are enriched in γ-tocopherol.

The first indications of a potential protective effect of nut consumption on cancer appeared already in the late 80s/early 90s. A case-control study of stomach cancer found a dose-response relationship for seven dietary items, including nuts. Similarly, a cohort study conducted on 14 000 Adventist men found

a statistically significant reduction of prostate cancer risk associated with increasing consumption of beans, lentils, peas, tomatoes, raisins, dates, and other dried fruits. The hypothesis that grains, cereals and nuts may be protective against prostate cancer was later supported by a study on prostate cancer in a Canadian population that discovered a 31% risk reduction, although in this study nuts were mixed with legumes and seeds. Evidence of a protective role of nuts on colorectal cancer in women also appeared with a prospective study conducted in Taiwan that recruited almost 24 000 people and followed them annually for 10 years. Beneficial effects of peanut consumption were observed in women, although the authors acknowledged some limitations of the study, including the lack of detailed information on other potential factors.

Olive oil

Olives (especially those that have not been subjected to the Spanish brining process) contain up to 16 g/kg typified by acteosides, hydroxytyrosol, tyrosol and phenyl propionic acids. Olive oil, especially extra virgin, contains smaller amounts of hydroxytyrosol and tyrosol, but also contains secoiridoids and lignans in abundance. Both olives and olive oil contain substantial amounts of other compounds deemed to be anticancer agents (e.g. squalene and terpenoids) as well as the peroxidation-resistant lipid oleic acid. It seems probable that olive and olive oil consumption in southern Europe represents an important contribution to the beneficial effects on health of the Mediterranean diet

Garlic

The antibacterial properties of garlic were first described by Pasteur as early as in 1858. Furthermore, this cytotoxic effect was highly specific against cancer cells but not the non-cancerous cells (Zhang, H., et al. (2014). Transferable antic. Despite the efforts in the last several decades, the anticancer effects of garlic still lack a piece of convincing evidence: a decisively curative result against aggressive cancer in the animal models or in humans.

Fish

The beneficial effects of omega-3 PUFA consumption are likely related to its anti-inflammatory and pro-resolution effects, mainly due to the inhibition of nuclear factor kappaB (NF-κB) and the production of pro resolution mediators, such as resolvins, protectins, and maresins.

Omega-PUFAs play essential roles in cell signaling and in the cell structure and fluidity of membranes. They participate in the resolution of inflammation and have anti-inflammatory and antinociceptive effects.

OTHER DIETARY FACTORS

Folic Acid

When comparing the highest to lowest intake of folate, higher intake was associated with a nearly 50% decreased risk for squamous cell carcinoma of the head and neck (HNSCC) ; 35% reduced risk for oral

cavity and pharyngeal (OPC) ; 41% reduced risk for all histological types of esophageal; 34% reduction in pancreatic; and 16% reduction in bladder cancers .

Antioxidants

One of the consequences of chemotherapy and radiation therapy is the generation of ROS which via its direct and indirect effects on tumor cells, induces DNA damage and/or affects DNA replication machinery, leading to aberrations in several cellular signaling pathways resulting in chemotherapy- or radiation therapy-induced cell death.

ROLE OF NUTRITION IN CANCER

Cancer of Oral Cavity and Pharynx

Several studies have confirmed that dietary patterns particularly intake of fruits and vegetables have shown a pertinent role in the reduction of risk of oral and pharyngeal cancer to about 50 per cent. This is chiefly attributed to the presence of micronutrients such as carotenoids. (La Vecchia et al., 1991; Zheng et al., 1993). Also, a relation has been seen between retinol, an indicator of meat intake and risk of oral cancer (La Vecchia et al., 1991). Moreover, a direct association has been found with pork, eggs, animal fat, and sausages, with oral cancer (Franceschi et al., 1991; Marshall and Boyle, 1996), probably because of the carcinogens in broiled meat (de Meester and Gerber, 1995). Tobacco and alcohol consumption have far more likelihood of developing oral cancer (Marshall et al., 1992). Overall, a high intake of saturated fats, meat, cholesterol, etc. elevate the risk of cancers of the pharynx and oral cavity and intake of healthy foods like fruit and vegetables possibly lessens the risk of oral cancer.

Cancer of Respiratory Organs

Lower β-carotene levels have been prognostic of increased lung cancer prevalence. Prospective studies have also shown that intake of diary product might surge the risk of lung cancer (Axelsson et al., 1996; Nyberg et al., 1998). Another study has reported a strongly increasing trend in lung cancer risk with dietary cholesterol in men and women (Goodman et al., 1988). A corelative association has also been seen between saturated fat consumption and lung cancer (Michael et al., 1993). Furthermore, a positive dose–response relation has been pragmatic between the risk of lung cancer and consumption of dairy foods, processed meats like bacon and spam, eggs, and particular desserts like cakes and custard. The dose–response relation showed more strong association among intense smokers (Goodman et al., 1992).

Esophageal Cancer

There are studies that suggest that the use of uncooked vegetables and fruits, chiefly citrus fruit, could decrease the risk of esophageal cancer (Ziegler et al., 1981; Cheng et al., 1992; Hu et al., 1994). This is ascribed to the presence of Vitamin C in citrus fruits that has a role in slowing down the formation of carcinogens (Ziegler et al.,1981; Tuyns et al.,1987, Hercberg et al., 1998). Many studies support the fact that soup and beverages enhance the risk of esophageal cancer (De Stefani et al., 1990; Cheng et

al., 1992; Hu et al., 1994). Other studies see a positive relation between cereals and esophageal cancer (Yu et al., 1988; Tzonou et al., 1996). Several studies suggest that the cooking method in the preparation of barbecued and fried meat could be associated with esophageal carcinogenesis (Yu et al., 1988; De Stefani et al.,1999). Vitamin A has also been shown to play a key role in defensive injured esophageal epithelial cells (Poulain et al., 2009). Some studies have provided sturdy confirmation that selenium and zinc can be preventive factors for incidence of esophageal cancer (Cai et al., 2006; Lu et al., 2006; Wei et al., 2004).

Gastric Cancer

Research based evidence suggests ditto be the most important component of gastric cancer. A human model has been developed for gastric carcinogenesis leading from superficial gastritis to carcinoma. It has been extensively studied on the basis of multistage process in which dietary elements act on the mucosa at various stages (Correa et al., 1988). A positive relation has been shown with diet rich in meat-derived food and gastric carcinogenesis (Buiatti et al., 1989; Wu-Williams et al., 1990; De Stefani et al., 1998), possibly because of the enhanced tolerance to DNA damage underlying the lesser mismatch repair (MMR) genes activity (Buermeyer et al., 1999). A case–control study has suggested a higher danger of gastric cancer due to higher intake of salt and pickled as well as smoked food because of the possible formation of intragastric nitrosamines (Ramón et al., 1993). Excessive consumption of vegetables and beans have shown a reciprocal association with risk of gastric cancer whereas frequent consumption of sweets have seen to increase the risk of gastric cancer by 70 percent (Ward et al., 1999).

Colorectal Cancer

Several lifestyle factors, including nutrition, have been associated with a higher risk of colorectal cancer. Studies suggest that calcium and phosphate in the diet increase the bile acids and levels of free ionized fatty acids in the colon (Newmark et al. 1984). High consumption of vegetables and fruit with less intake of refined sugar have seen to reduce the risk of colon cancer. The underlying hypothesis suggests hyperinsulinemia to promote colon carcinogenesis. Also, elevated consumption of red meat, beef, pork etc. sturdily correlated with higher risk of colon cancer or adenoma. It is because fat from red meat may be less digested or absorbed, due to its high stearic acid content.

Breast Cancer

Dietary factors stand out to be the most modifiable risk factors in breast cancer. Consumption of vegetables and fruit have been associated to a diminished risk of premenopausal breast (Gandini, 2000). Also, it has been observed that intake of total lignin precursors can result in a significant reduction in breast cancer risk among premenopausal women (McCann, 2004). Prospective studies have evidenced the decrease in the risk of breast cancer by ingestion of olive oil (Trichopoulou et al., 1995). Also, a meta-analysis has shown an increased risk of the disease related to meat intake (Boyd et al., 2003). The ingestion of red meat has also been linked with a raised risk of hormone receptor–positive breast cancer but not with hormone receptor–negative cancer risk (Cho et al., 2006) in a population of pre-menopausal women. Another study has established an optimistic association of breast cancer with ingestion of red

meat particularly deep-fried in pan drippings with a brown or black crust (Dai et al., 2002), indicative of the consequence of heterocyclic amines or additional carcinogens generated at high temperatures.

Skin Cancer

It has been established that people who consume more levels of vegetables and fruits have a reduced risk of SCC as low as 54 percent (Ibiebele et al., 2007). Decreased risk of melanoma has been pragmatic with higher intake of vitamins D, C, A, cryptoxanthin, α- and β-carotene, lycopene and lutein (Millen et al., 2004). Regular eating of pomegranates and celeriac has also been linked with a significantly reduced risk of SCC and BCC (de Vries et al., 2012). Vitamins E and Cact as antioxidants and guard against skin cancer (Table 1). Vitamin C, important for hydroxylating proline and lysine in the synthesis of connective tissues proteins, may alter the development of tumor. Vitamin E acts as an intracellular antioxidant and averts lipid peroxidation (McNaughton et al., 2005). Retinoic acid, the variant of vitamin A, is extremely vital in skin cell differentiation, maintenance and proliferation. It also helps in raising the epidermal thickness thereby lessening the amount of ultraviolet (UV) light reaching the underlying layers of the skin (Siegel et al., 2012).

Table 1. Nutritional risk factors for selected cancer

Site for Cancer	Low risk factors	High risk factors
Oral cavity and pharynx	Fruits and vegetables	Saturated fats, meat, cholesterol
Respiratory organs	Green vegetables, carotene	Cholesterol
Esophageal cancer	Vegetables and fruits	Alcoholic beverages
Gastric Cancer	Dairy products, raw vegetables, fruits	Salty food, hot drinks, irregular meals
Colorectal Cancer	Fiber rich diet	Cholesterol, high fat diet
Breast Cancer	Olive oil, lignin precursors	High fat/calorie diet
Skin Cancer	Vitamin A, D C, E and carotenoids	UV light

Nutrition During Cancer Treatment

The available options of cancer treatment like radiation, chemotherapy, surgery, hormone therapy, immunotherapy, clinical trials etc. have never been so satisfactory. All these treatment options are associated with side effects which can be combatted only with suitable diet therapy. Proteins, water, carbohydrates, fats, minerals and vitamins are the main nutrients to focus on during the course of cancer treatment. Protein needs frequently surge for sustenance of muscle strength and maintenance and also for recovering from illnesses, resisting infections, and repairing tissues. Carbohydrate sources are also crucial, taking complex carbs and whole grains over sweets and empty carbs promote nutrient-dense foods and sustainable energy. Removal of “unhealthy” saturated and trans fats and addition of “healthy” mono- and polyunsaturated fats in the diet promote cholesterol management and heart health. Fluid needs are not limited to water and may include broth, soup, milk and gelatin. Mineral and vitamin needs may also increase, especially if facing reduced appetite, making multivitamins or mineral supplements appropriate substitutes for sources of the essential nutrients required by the body systems to produce energy.

CONCLUDING REMARKS

The pathogenesis and etiology of cancer is a complex relationship of environmental and genetic factors. Nutrient supplements and food intake play a significant role in cancer development. The quality and quantity of diet nutrients are closely linked with cancer incidence and pathogenesis. Dietary recommendations to reduce the risk of developing cancer, including lower meat consumption, less intake of high glycemic foods, intake of organic foods, limiting consumption of salt, coffee and alcoholic beverages, excessive use of fruits and vegetables in diet have shown a noticeable difference in cancer development. What we eat and drink, and how we live can surely help short-circuit the cancer process and adapt the risk of cancer development, particularly in genetically susceptible individuals.

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Chapter 22
Functional Mechanisms of Green Tea Polyphenols and Their Molecular Targets in Prevention of Multiple Cancers

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ABSTRACT

Cancer is portrayed as a group of disease characterized by alteration in the normal regulation of cell growth by the successive acquisition of genetic, somatic, and epigenetic alteration. Synthetic drugs are single targets while natural products are multi-targeted to prevent cancer. NF-κB is persistently active in a number of disease states, including cancer, and therefore has a critical role in cancer development and progression. It also provides a mechanistic link between inflammation and cancer and is a major controlling factor resistant to apoptosis in both pre-neoplastic and malignant cells. Importantly, NF-kB and the signaling pathways that mediate its activation have become attractive targets for the development of new chemopreventive and chemotherapeutic approaches. Natural antioxidants have been shown to possess chemopreventive and chemotherapeutic potential via targeting NF-κB signaling, among which tea polyphenols have been studied extensively. In this chapter, the authors summarize the regulation of NF-κB pathway by green tea polyphenols in different cancer types.

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INTRODUCTION

Cancer is a very complex disease involving gradual accumulation of germ line, somatic and epigenetic mutation that cause the alternation in homeostasis that controls normal cellular proliferations and potential to spread or invade other parts of the body (Hanahan & Weinberg, 2011). After cardiovascular, cancer is one of the major ailment effecting humankind and still remains as one of the leading causes of mortality worldwide, for instance, more than 10 million new patients are diagnosed with cancer every year and over 6 million deaths documented and roughly 12% deaths worldwide (Nagai & Kim, 2017). Near about 15 million new cancer cases are estimated to be diagnosed by the year 2020which is alarming that cancer cases will increase over 20 million by 2025(Siegel, Miller, & Jemal, 2020). The progression of cancer is a multistep and multifactorial process that may be caused by external factors like environmental pollutants, tobacco, infectious organisms and aflatoxin contaminated food product or internal factors such as genetic mutations (inherited/acquired), hormonal imbalance and immune conditions can act together/singular to cause the incipience of cancer (Hanahan & Weinberg, 2011). As cancer is linked with high morbidity and mortality worldwide, therefore it is an urgent need to develop the ways to manage this dreadful disease, however, the existing treatment modalities includes chemotherapy, surgery, radiotherapy, hormonal therapy, targeted therapy, gene therapy and stem cell therapy (Arruebo et al., 2011). Natural products, mainly derived from plant sources have great interest to cure the ailment and according to WHO report more than 60% population worldwide rely on plant product as a medicinal source (Veeresham, 2012) In addition to this, according to the National Center for Complementary and Alternative Medicine (USA), 38% of adults in the United States opted for complementary and alternative medicine over conventional drugs. Synthetic drugs are single targets while natural products are multi-targeted to control cancer (Chamberlin et al., 2019). By and large, existing synthetic drug has major side effects over the natural product derive especially from plants. Among natural compounds, green tea and its polyphenolic derivative display a meaningful role in different cancer prevention and cure which was extensively studied worldwide against in vitro and in vivo model (H. Wang et al., 2012). Green tea leaves have different components and each component display a diverse role that can be beneficial to the mankind worldwide (Prasanth, Sivamaruthi, Chaiyasut, & Tencomnao, 2019). One of the green tea polyphenols flavonols is catechins which are found in greater amount and demonstrated the diverse pharmacological properties (Khan & Mukhtar, 2019). However, (-)-epigallocatechin-3-gallate (EGCG) near about 59% of the total catechins from the leaves of the green tea, 19% (-)-epigallocatechin (EGC), 13.6% (-)-epicatechin-3-gallate (ECG) and 6.4% (-)-epicatechin (EC) (V. Nair, Bandyopadhyay, & Kundu, 2013). The chemical structural and functional difference between these catechins are of hydroxyl group present on the B-ring as well as the presence /absence of a moiety of galloyl (Botten, Fugallo, Fraternali, & Molteni, 2015). It was observed that among of all catechins, EGCG is the most studied and demonstrated the vital role in cancer-preventive and therapeutic. A large number of studies were evaluated to demonstrate the effects of EGCG on different in vitro molecular targets and in vivo molecular targets as potential cancer chemoprevention as well as therapy (Singh, Shankar, & Srivastava, 2011). We observed that majorities of these studies showed that EGCG regulated large array of anticancer molecular targets and specially targets NF-kB associated signaling pathway (L.-X. Wang et al., 2019). Despite the tremendous study on EGCG, its applicability and validation to human model has met with limited success for many reasons like the inefficient systemic delivery and bioavailability (Siddiqui, Adhami, Ahmad, & Mukhtar, 2010). To overcome these limitations, researcher adopted various approaches, including nanoparticles-based delivery, surface modification, addition of additional adjuvant and combination therapy to enhance the

role as cancer chemoprevention (Pucci, Martinelli, & Ciofani, 2019). Green tea and its polyphenols demonstrated the anticancer activity against multiple cancer type via modulating the level of the NF-κb pathway and associated genes/proteins, which is summarized in this review.

NF-κB: STRUCTURE AND FUNCTION

NF-κB was first discovered by Baltimore and coworkers in 1986 as a factor in the nucleus of B-cells of immune system that binds to the enhancer of the kappa light chain of immunoglobulin genes (Sen & Baltimore, 1986). It has since been shown to be expressed ubiquitously in the cytoplasm of almost all cell types, from *Drosophila* to man. NF-κB transcription factors comprise five different types of proteins Class 1: Rel A (p65), cRel, Rel B, and Class 2: p50/NF-κB1 and p52/ NF-κB (Ghosh & Karin, 2002). In the absence of stimulatory signals, NF-κB resides in the cytoplasm in the form of a heterodimeric complex with its inhibitory proteins IκBα. Stimulation of the cells with various stimuli activates the IκB kinase (IKK) complex, which is composed of two catalytic subunit (IKKα and IKKβ) and a regulatory subunit (IKKγ/NEMO (Rothwarf & Karin, 1999). Activated IKK phosphorylates NF-κB bound IκB proteins, and targets them for polyubiquitination and rapid degradation by creating binding site for SCF βTRCP ubiquitin ligase complex (Karin & Ben-Neriah, 2000). After releasing from its inhibitory protein, IκBα, NF-κB translocate to the nucleus where it regulates the transcription of various target genes involved in inflammation and carcinogenesis (C. Chen, Edelstein, & Gélinas, 2000).

Involvement of NF-κB in Carcinogenesis

Experimental evidences suggest that sustained or constitutive activation of NF-κB is prevalent in cell lines and tumor tissue specimens and contributes to malignant progression and therapeutic resistance in most of the human cancer (Van Waes, 2007). It has been shown that NF-κB may regulate the production of prostaglandins via the pro-inflammatory gene cyclooxygenase-2 (COX2), which has been shown to be overexpressed in a variety of cancers including colorectal cancer and mesothelioma (Kalgutkar & Zhao, 2001). Other investigations have revealed that NF-κB regulates the expression of pro-inflammatory genes including tumor necrosis factor (TNF), interleukin-1 (IL-1) and inducible NO-synthase (iNOS) (Ahn & Aggarwal, 2005). Activation of NF-κB leads to over-expression of cell adhesion molecules such as intercellular adhesion molecule (ICAM)-1, endothelial- leukocyte adhesion molecule (ELAM)-1, and vascular cell adhesion molecule (VCAM)-1 which help in the migration of cancerous cells [10]. It has been shown that NF-κB also activates the transactivation of vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) which are involved in angiogenesis and metastasis (Bargou et al., 1997). Constitutive activation of NF-κB has been reported in breast (Sovak et al., 1997), cervix (A. Nair, Venkatraman, Maliekal, Nair, & Karunagaran, 2003), prostate (Suh et al., 2002), lung (Mukhopadhyay, Roth, & Maxwell, 1995), colon (Kojima et al., 2004), pancreas(W. Wang et al., 1999), head and neck (Allen, Ricker, Chen, & Van Waes, 2007; Ondrey et al., 1999), esophagus and (Abdel-Latif et al., 2004), gastric carcinomas .Studies have shown that NF-κB is over-expressed and activated in various types of cancers, especially in the poorly differentiated cancers(Karin, 2006).It has been demonstrated that blockage of constitutive active NF-κB suppresses the growth of Hodgkin's tumor cell and multiple myeloma cells (Hideshima et al., 2002). Therefore, it

is becoming clear that inhibition of NF-κB activity is highly desirable in the prevention and treatment of various types of cancer.

Therapeutic Application of Inhibition of NF-κB Signaling in Cancer

Many inflammatory agents and tumor promoters activate NF-κB, whereas chemopreventive agents suppress activation, suggesting a strong link of NF-κB with carcinogenesis (Bharti & Aggarwal, 2002). It has been shown that NF-κB activation is involved in different stages of carcinogenesis such as transformation, initiation, promotion, angiogenesis, invasion, and metastasis. Zhou et al demonstrated that activation of NF-κB blocks apoptosis and promotes cell proliferation (Zhou, Gu, Zhu, Woods, & Findley, 2003). Other studies have shown that NF-κB activation induces resistance to apoptosis induced by various chemotherapeutic agents (Van Antwerp, Martin, Kafri, Green, & Verma, 1996). Most chemopreventive agents appear to suppress the activation of the NF-κB which sensitizes the tumors to chemotherapeutic agents via abrogation of NF-κB activation suggesting that NF-κB is an ideal target for chemosensitization. Various studies indicate that the benefit of conventional chemotherapy could be enhanced by natural and synthetic NF-κB inhibitors. Therefore, targeting NF-κB is a novel chemopreventive and chemotherapeutic strategy against various types of human cancers. Because many of the currently employed chemotherapeutic agents pose significant adverse effects, screening of naturally occurring diet-based NF-κB inhibitors could prove a safer way to treat various malignancies.

Natural anti-oxidants that have been shown to inhibit the NF-κB activation in different types of cancers are summarized in Table 1.

GREEN TEA AND ITS POLYPHENOLS

Tea is produced from the leaves of the plant *Camellia sinensis*. Next to the water, tea is the most consumed beverage in the world. To produce green tea, freshly harvested leaves are rapidly steamed or pan-fried to inactivate enzymes, thereby preventing fermentation and producing a dry, stable product. Tea polyphenols usually account for 30% to 42% of the dry weight of the solids in brewing green tea (Balentine, Wiseman, & Bouwens, 1997). Among all polyophenols in green tea, EGCG is the major polyphenol which account for 50% to 80% of the total polyphenols present in tea. Other polyphenols like Catechin, gallocatechin, epigallocatechin digallates, epicatechin digallate, 3-*O*-methyl EC and EGC, catechin gallate, and gallocatechin gallate are present in smaller quantities.

GREEN TEA AND CANCER

In recent years, green tea has gained considerable attention as an agent that could reduce the risk of several cancer types. The cancer-chemopreventive effects of green tea appear to be mediated by the polyphenolic constituents present in the green tea. Epidemiological and laboratory data from all over the world suggest that green tea and its polyphenols have chemopreventive and chemotherapeutic potential against various types of human cancers. Accumulating evidence indicates that consumption of tea, especially green tea, is good for preventing cancer. To elucidate the cancer preventive mechanisms of green tea, much effort has been devoted to investigating the anticancer effects of EGCG, the major component of

Table 1. Natural Antioxidant and their Molecular target

Molecule	Target	Reference
α-Tocopherol	Decreases adhesion of activated monocytes to endothelial cell by inhibiting NF-κB.	(Islam, Devaraj, & Jialal, 1998)
Apple juice/extract	Protects Cr (VI)-induced cellular injury and reduce its carcinogenic potential; downregulates NF-κB signaling	(Davis et al., 2006; Shi & Jiang, 2002)
Carnosol	Suppresses the nitric oxide (NO) production and iNOS gene expression by inhibiting NF-κB activation; targets MMP-mediated cellular events in cancer cells through down-regulating NF-κB.	(Lo, Liang, Lin-Shiau, Ho, & Lin, 2002)
β-Carotene	Function as a potential inhibitor for redox-based NF-κB activation; inhibits tumor-specific angiogenesis by altering the cytokine profile and inhibits the nuclear translocation of transcription factors in B16F-10 melanoma cells	(Bai et al., 2005; Guruvayoorappan & Kuttan, 2007)
Curcumin (Diferulolylmethane)	Inhibits NF-κB activation pathway at a step before IkB-α phosphorylation but after the convergence of various stimuli	(Teiten, Eifes, Dicato, & Diederich, 2010; Tong, Wang, Sun, & Suo, 2016)
EGCG	Decreases the activity and protein levels of iNOS and iNOS mRNA through prevention of the binding of nuclear factor-kappaB to the iNOS promoter; down-regulation of TNF-a gene expression by blocking NF-κB activation	(Lin & Lin, 1997; Yang, De Villiers, McClain, & Varilek, 1998)
Proanthocyanidins from green tea	Inhibition of COX-2 and iNOS via the down-regulation of TAK1-NF-κB pathway	(Hou et al., 2003)
Fisetin	Suppresses NF-κB via inhibition of IκB-α phosphorylation and degradation. mediates antitumor and anti-inflammatory effects through modulation of NF-κB	(Park et al., 2007)
Lupeol	Anti-skin tumor-promoting effects in CD-1 mouse and inhibits conventional as well as novel biomarkers of tumor promotion; decreased tumor volume and suppressed local metastasis, which was more effective than cisplatin alone	(T. K. Lee et al., 2007; Saleem, Afaq, Adhami, & Mukhtar, 2004)
Magnolol	Significantly suppresses the TNFα-induced expression of VCAM-1, reduction in the amount of NF-κB/p65 in the nuclei of HAECs. LDL; Attenuated the ox LDL-induced ROS generation and subsequent NF-κB activation inhibits cell proliferation in G1 to S phase cell cycle progress and MMP-9 expression through the transcription factors NF-κB and AP-1 in TNF-a-induced VSMC	(Y. H. Chen, Lin, Chen, Ku, & Chen, 2002; H.-M. Kim et al., 2007; Ou, Chou, Sheu, Hsu, & Lee, 2007)
Quercetin	Inhibits hydrogen peroxide (H_2O_2)-induced NF-κB DNA binding activity and DNA damage in HepG2 cells; Inhibits inducible nitric oxide synthase, cyclooxygenase-2, reactive C-protein, and down-regulation of the NF-κB pathway in Chang Liver cells; inhibits TNF-induced NF-κB transcription factor recruitment to proinflammatory gene promoters in murine intestinal epithelial cells; inhibits expression of inflammatory cytokines through attenuation of NF-κB and p38 MAPK in HMC-1 human mast cell line; inhibits endotoxin LPS-induced IL-6 expression and NF-κB activation in macrophages.	(García-Mediavilla et al., 2007; B. H. Kim et al., 2007; Ying et al., 2009; X.-A. Zhang, Zhang, Yin, & Zhang, 2015)
S-allyl-cysteine	Inhibits activation of NF-κB in human T cells	(Geng, Rong, & Lau, 1997)
Silybin	Silybin and silymarin--new and emerging applications in medicine	(Feher & Lengyel, 2012)
Strawberry extracts	Inhibitory effect on activator protein-1, NF-κB, and cell transformation	(S. Y. Wang, Feng, Lu, Bowman, & Ding, 2005)
Tomato peel polysaccharide	Inhibits NF-κB activation in LPS-stimulated J774 macrophages.	(De Stefano et al., 2007)
UDN glycoprotein	Regulates activities of manganese-superoxide dismutase, activator protein-1, and NF-κB stimulated by reactive oxygen radicals in LPS-stimulated HCT-116 cells.	(S.-J. Lee & Lim, 2007)

green tea. It has been revealed that EGCG restrained carcinogenesis in a variety of tissues via targeting NF-κB, topo-isomerase I, matrix metalloproteinases and other potential targets. Therefore, EGCG is a multipotent anticancer agent, which not only provides solid evidence to support the anticancer potential of green tea, but also offers new clues for discovering multiple-targeted anticancer drugs. Interestingly, it has been reported that EGCG and tamoxifen synergistically induced apoptosis and cell growth inhibition of MDA-MB-231 human breast cancer cells through NF-κB inactivation (Stuart, Larsen, & Rosengren, 2007). Zhang et al reported that EGCG increases accumulation of doxorubicin in the solid tumors of human carcinoma xenograft model, suggesting that it restores the sensitivity of doxorubicin (Q. Zhang, Wei, & Liu, 2004).

Modulation of NF-κB Signaling by Green tea in Prostate Cancer (PCa)

NF-κB has been hypothesized to contribute the PCa progression by regulating the expression of a number of target genes involved in cell proliferation, anti-apoptosis, angiogenesis, and metastasis. PCa cells have been reported to have constitutive NF-κB expression due to increased activity of the IκB kinase (IKK) complex (Suh et al., 2002). It has been shown that NF-κB promotes the growth of PCa cells by regulating the c-myc, cyclin D1, and IL-6 genes expression. Another study demonstrated that NF-κB inhibits apoptosis in PCa cells via activation of anti-apoptotic gene Bcl-2 (Song, Sneddon, Heys, & Wahle, 2006). It has also been investigated that NF-κB regulates the genes involved in angiogenesis (IL-8, VEGF), invasion and metastasis (MMP9, uPA, uPA receptor) in PCa. Constitutive activation of NF-κB signaling was observed in PCa growth and progression in autochthonous transgenic adenocarcinoma of the mouse prostate (TRAMP) model, which perfectly mimics the development of human PCa(Shukla et al., 2004). The recent finding suggests the association of NF-κB activation with prostate carcinogenesis in noble rats (Yatkin, Bernoulli, & Santti, 2006). Constitutive NF-κB activation was also observed in human prostate adenocarcinoma, which correlates with disease progression (Ismail A, Lessard, Mes-Masson, & Saad, 2004). Another study has shown the up regulation of NF-κB in the lymph tissue of human PCa metastasis which correlates with another finding of NF-κB nuclear localization in different grade of human prostate tumor tissue (Fradet et al., 2004; I. Siddiqui et al., 2008). Another research group has shown that EGCG induces apoptosis in androgen dependent human PCa LNCaP cells via inhibition of NF-κB activity. In this study, we observed that EGCG had a concurrent effect on NF-κB, causing a change in the ratio of Bax/Bcl_2 in a manner that favors apoptosis. This altered expression of Bcl_2 family members triggered the activation of initiator caspases 9 and 8 followed by activation of effector caspase 3 (Hastak et al., 2003). Proteasome inhibitors are able to induce tumor growth arrest. The inhibition of proteasome by EGCG in LNCaP cells results in accumulation of two proteasome substrates, p27/Kip1 and IKBα, an inhibitor of transcription factor NF-κB, followed by growth arrest in the G1 phase of the cell cycle (Nam, Smith, & Dou, 2001).Recently, we have shown the chemopreventive effect of green tea in TRAMP mice and observed its potential chemopreventive activity in these mice via inhibition of NF-κB signaling and its associated events (I. A. Siddiqui et al., 2008).

Modulation of NF-κB Signaling by Green tea in Skin Cancer

Activation of NF-κB has been proposed as an event that promotes melanoma tumor progression (Huang, DeGuzman, Bucana, & Fidler, 2000b; Payne & Cornelius, 2002; Richmond, 2002). In human melanoma, a number of NF-κB-regulated chemokines are constitutively expressed at high levels: CXC

ligand 8 (CXCL8), interleukin-8 (IL8), CXCL1 (Melanoma growth stimulatory activity (MGSA), CCL5 (regulated on activation, normal T expressed and secreted (RANTES), and CCL2 (monocyte chemotactic protein-1 (MCP1) (Son et al., 2014). These NF-κB-regulated chemokines are thought to enhance melanoma progression through autocrine and paracrine loops. *In vitro* and *in vivo* studies have shown that NF-κB activity is upregulated in dysplastic nevi and lesions of human melanoma when compared with human nevi or melanocytes in normal skin (Dhawan & Richmond, 2002). Inhibition of NF-κB in highly metastatic melanoma xenografts in nude mice resulted in a decrease in angiogenesis as measured by microvessel density, which correlated with a decrease in the level of CXCL8 expression (Huang, DeGuzman, Bucana, & Fidler, 2000a). Another study has revealed that EGCG protects skin from photoaging via inhibiting NF-κB in guinea pigs, SKH-1 hairless mice and human dermal fibroblast cultures (Chan et al., 2008). Xia et al in 2005 reported that EGCG attenuated UV-induced NF-κB activation and expression of IL-6 in cultured human keratinocytes (Xia, Song, Bi, Chu, & Wan, 2005). A study has shown that EGCG inhibits TPA-induced activation of NF-κB in mouse skin via inhibition of IκBα (Κυνδυ & ΣΥΡΗ, 2007). Our laboratory has also shown that EGCG inhibits UV-induced activation of NF-κB in normal human epidermal keratinocytes and in SKH1 hairless mice via inhibition of IκBα and IKKα therefore, protects skin against the adverse effects of UV radiation (Afaq, Adhami, Ahmad, & Mukhtar, 2003; Singh et al., 2011). We have investigated the differential response of EGCG regarding modulation of NF-κB. EGCG inhibited NF-κB in human epidermoid carcinoma (A431) cells, but not in normal human epidermal keratinocytes (NHEK) (Ahmad, Gupta, & Mukhtar, 2000).

Modulation of NF-κB Signaling by Green tea in Colon Cancer

Accumulating evidence indicate that carcinogenesis of colon involves the upregulation of COX-2 expression which plays a key role in the inflammation of colon and therefore has been linked to the development of colon cancer. It has been reported that NF-κB pathway and COX-2 expression is upregulated in stromal myofibroblasts surrounding colon adenocarcinomas compared to normal colon. Induction of COX-2 expression is primarily induced by NF-κB. It has been shown that EGCG inhibits COX-2 promoter activity in human colorectal cancer cell lines HT-29 and HCA-7 via inhibition of NF-κB activation suggesting anti-proliferative effect of EGCG in human colon cancer (Peng, Dixon, Muga, Smith, & Wargovich, 2006; Saldanha, Kala, & Tollefsbol, 2014). Other study has depicted that EGCG induces apoptosis via inhibition of LPS induced NF-κB activation in human HT-29 colon cancer cell line (Jeong, Kim, Hu, & Kong, 2004). This study has demonstrated that EGCG inhibits NF-κB activity via inhibition of IKBα phosphorylation

Modulation of NF-κB Signaling by Green tea in Pancreatic Cancer

Evidences have suggested that NF-κB plays an important role in pancreatic cancer development. It has been reported that NF-κB is constitutively activated in 70% of human pancreatic cancers and in human pancreatic cell lines viz. BxPC-3 (Chandler, Canete, & Callery, 2004), PANC-1 (Sclabas et al., 2005) and MIA PaCa-2 (Liptay et al., 2003), but not in normal pancreatic tissues or in immortalized, non-tumorigenic pancreatic epithelial cells (Y.-W. Wang, Wang, Zhou, Pan, & Sun, 2012). Activation of NF-κB has been observed in animal models of pancreatic cancer and in human pancreatic cancer tissues (Sclabas et al., 2003). A study has shown that suppression of NF-κB DNA binding restored apoptosis in pancreatic cancer cells, whereas treatment with various NF-κB inhibitors or transfection of the IκB

super-repressor strongly enhanced the apoptotic effect of etoposide (VP16) or doxorubicin in resistant pancreatic cancer cells (Arlt et al., 2001). It has been suggested that by promoting proliferation and inhibiting apoptosis, NF-κB maintain the balance between proliferation and apoptosis toward malignant growth in pancreatic tumor cells [63]. Since green tea and its polyphenol EGCG have been shown to decrease NF-κB activity in various types of cancers, we speculate that green tea by inhibiting NF-κB may serve as a potent chemopreventive agent against pancreatic cancer.

Modulation of NF-κB Signaling by Green tea in Osteosarcoma

It has been demonstrated that using dominant negative approach to inhibit NF-κB activity can results in reversion of malignancy in human osteosarcoma cells (Mori et al., 2007). We recently demonstrated that green tea polyphenol (GTP) effectively decreases NF-κB DNA binding activity and its nuclear translocation, which correlated with growth inhibition and apoptosis in osteosarcoma SAOS2 cell (Hafeez et al., 2006).

Modulation of NF-κB Signaling by Green tea in Breast Cancer

NF-κB has been implicated in the lobuloalveolar development of the mammary gland. It has been reported that NF-κB is over-expressed in a majority of breast cancer samples and breast cancer cell lines (Bhat-Nakshatri, Sweeney, & Nakshatri, 2002). Nakshatriet al in 1997 have shown that NF-κB is activated in the majority of human breast cancer cell lines and correlated with the conversion of breast cancer cells to hormone-independent growth, a characteristic of more aggressive and metastatic tumors (Nakshatri, Bhat-Nakshatri, Martin, Goulet, & Sledge, 1997). Another study suggests that the overexpression of mouse NF-κB is associated with the development of mammary tumors in animal models (Romieu-Mourez et al., 2003). It has been documented that a NF-κB antagonist blocked epidermal growth factor-induced NF-κB activation, and caused apoptotic death in ER-breast cancer cells (Biswas, Cruz, Gansberger, & Pardee, 2000). They also showed that the NF-κB antagonist inhibited the growth and caused extensive regression of estrogen responsive (ER) -mouse mammary epithelial tumors. Other investigators have demonstrated elevation of NF-κB-regulated gene transcripts in breast tumors compared to adjacent normal tissues (Cogswell, Guttridge, Funkhouser, & Baldwin, 2000). Activation of NF-κB is observed before malignant transformation of mammary gland in rat models, suggesting NF-κB may be responsible for mammary tumor initiation (Kim et al., 2000). Metastasis of breast cancer occurs primarily through the lymphatic system, and the extent of lymph node involvement is a key prognostic factor for the disease. VEGF is secreted from breast tumor cells, acting as a potent lymphangiogenic factor favoring tumor growth and metastasis (Saharinen, Eklund, Pulkki, Bono, & Alitalo, 2011; Skobe et al., 2001). NF-κB has been reported to up-regulate VEGF transcript in breast cancer cells, supporting its role in angiogenesis and breast cancer metastasis (Shibata et al., 2002; Tsai, Shiah, Lin, Wu, & Kuo, 2003). Tumor cells resistant to the cytotoxicity of chemotherapeutic agents and ionizing radiation may limit the effectiveness of adjuvant therapy in breast cancer treatment. Chemotherapeutic agents such as taxol, doxorubicin, tamoxifen, and cisplatin have been linked to NF-κB activation in cancer cells (Pahl, 1999). Likewise, γ-irradiation, used in the treatment of cancer patients, has also been found to activate NF-κB (Patel et al., 2000). As mentioned, activation of NF-κB causes resistance to apoptosis, which may explain chemoresistance. Overexpression of the epidermal growth factor receptor family member Her-2/neu in breast cancer leads to autophosphorylation of the receptor and induction of multiple downstream

signaling pathways, including the Akt kinase to NF-κB cascade that is associated with poor prognosis of breast cancer. Pianetti et al in 2002 has showed that EGCG inhibits growth of NF639 Her-2/neu-driven breast cancer cells via reducing receptor autophosphorylation and downstream Akt and NF-κB activities (Pianetti, Guo, Kavanagh, & Sonenshein, 2002). In another study they further reported that resistant cells have losttyrosine phosphorylation on the Her-2/neu receptor and displayed elevated NF-κB activity, and inhibition of this activity sensitized cells to EGCG (Guo, Lu, Subramanian, & Sonenshein, 2006). Another study suggests anti-proliferative and anti-angiogenic activities of EGCG in breast cancer. They have shown that EGCG inhibits VEGF signaling via inhibition of constitutive NF-κB activation in MDA-MB-231 breast carcinoma cell lines (Masuda et al., 2002). Therefore, it is important to assess whether the green tea polyphenol EGCG can be used in combinatorial treatments of breast cancer to enhance the effectiveness of therapeutic regimens in the clinic.

Modulation of NF-κB Signaling by Green tea in Human Lung Cancer

NF-kB activation leads to upregulation of TNF-alpha gene expression which is considered to be an endogenous tumor promoter. TNF-alpha is also known to be a central mediator in chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis. TNF-alpha transgenic mice, which overexpress TNF-alpha only in lungs, serve as an animal model of human idiopathic pulmonary fibrosis which also frequently develops lung cancer. Since expression of TNF is regulated by overexpression of NF-KB we hypothesize that blocking NF-κB activation by green tea in lung carcinoma could be a beneficial measure against preventing lung cancer. Yang et al in 2005 illustrated that EGCG represses the invasive potential of highly metastatic human lung carcinoma 95-D cells via inhibition of NF-κB and its downstream target gene MMP9. Another study has demonstrated the effect of EGCG on focused cDNA array of 588 cancer related genes in human lung cancer cells PC-9 (Okabe, Fujimoto, Sueoka, SUGANUMA, & FUJIKI, 2001). They observed that EGCG downmodulates 12 genes, among which NF-κB inducing kinase (NIK) was significantly downregulated which is known to be involved in activation of NF-κB via phosphorylation of IKK.

SUMMARY AND PERSPECTIVES

This review summarizes the anti-cancer effects of green tea and its polyphenol in the prevention and treatment of cancer especially those which are associated with overexpression of NF-κB. In addition, we propose other putative cancer targets which are associated with enhanced NF-κB and associated factors and where green tea might find its potential applications both in prevention and treatment.

As the consensus is developing among scientific world that there exists a link between inflammation and cancer, NF-κB has been a molecule in question in target. The fact that NF-κB is activated by many inflammatory signals and other tumor promoting agents in different types of cancers further warrants an urgent need for the development of effective measures to inhibit its activation as a potential chemopreventive and chemotherapeutic intervention. The need to inhibit NF-κB signaling further stems from the fact that its overexpression is associated with bypass of apoptosis and therapy failure. In these terms blockade of NF-κB signaling by green tea holds commendable promise since it has been shown that green tea has a multifaceted mode of action in repressing the activity of this oncogenic transcription factor. It has been shown that in order to inhibit NF-κB activation green tea could lead to the preferen-

tial degradation of its activation subunits enhance the cellular levels of its repressor IκB expression and decreasing the levels of IKK which leads to stabilization of NF-κB inhibitory protein IκBα and hence inhibit the activation of NF-κB. In addition to these direct inhibitory effects we have found that NF-kB activated downstream signaling could be attenuated by the green tea and the polyphenols contained therein (unpublished data). This is evident from the reports that green tea inhibits the activity of NF-κB target proteins such as COX2 and TNF-a, both of which have been implicated in the development and progression of colon and lung carcinoma respectively. This indicates that green tea and its polyphenol could at least prolong the carcinogenesis in these organs. Since we have now deciphering the role of NF-κB in PCa, the cell growth inhibiting effects of green tea on prostate carcinogenesis could be due to the interference of green tea with the constitutively active NF-κB signaling in many clinical cases of prostate and also in breast cancer. We suggest that green tea could be potentially employed along with the standard first line therapy for major NF-κB related cancer as an adjuvant to decrease the mortality and morbidity associated with various cancers.

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Chapter 23
Natural Product Compounds for Breast Cancer Treatment

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ABSTRACT

Breast cancer is the primary cause of cancer death in women. Although current therapies have shown some promise against breast cancer, there is still no effective cure for the majority of patients in the advanced stages of breast cancer. Treatment with present synthetic drugs may lead to a number of adverse effects. Consequently, research into natural product compounds may provide an alternative pathway to determining effective against breast cancer. This chapter reviews molecular targets of breast cancer treatment as well as bioactive compounds sourced from bibliographic information such as Medline, Google Scholar, PubMed databases. The authors hope that this book chapter contributes significantly to previous and ongoing research and encourages further investigation into the potential of natural product compounds in breast cancer.

INTRODUCTION

Cancer is characterized by the uncontrolled presentation of growth and division in the cell cycle, which is mainly caused by a gene mutation in the nucleus of tumor cells. It is a gene events-related sequential progression that can be seen in a single clone of cells. Two cancerous types are malignant and benign tumors. Breast cancer is a malignant type occurring in breast cells and is known as the second cause of cancer-related mortality in both women above 50 years of age and younger women. Age, economic conditions, race, dietary iodine insufficiency as well as high concentration of hormone are the major risk factors associated with breast cancer (Sun, Zhao et al. 2017).

Breast cancer is predominantly induced by inherited mutations of BRCA1 and BRCA2 genes (Filippini and Vega 2013). In addition, the development of breast tumors is also contributed by other factors, including an increase in breast tissue density, obesity, alcohol consumption, physical inactivity and the treatment by hormone therapies such as estrogen, progestin. The pathogenesis of this disease is considered to target two major molecules: estrogen receptor alpha (ERα), a receptor of steroid hormone as well

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as a transcription factor, and epidermal growth factor 2 (*ERBB2*, previously *HER2* or *HER2/neu*) that belongs to family of the epidermal growth factor receptor as a tyrosine kinase-associated transmembrane receptor. In cells of breast cancer tissues, ERα is stimulated by the presence of estrogen, leading to the activation of oncogenic growth pathways. Moreover, ERα signaling is also marked by the expression of steroid hormone-related progesterone receptor (PR). Hence, the fundamental treatment for ER-positive or PR-positive individuals is based on the downregulation of ER signal pathway by using endocrine agents (Mishra, Kumari et al. 2020).

Various treatments are employed for breast cancer management like surgery, radiation therapy, endocrine therapy and chemotherapy. Despite remarkable influences on normal cells, radiations have more effects on damage to cancerous tissues, which exhibit stronger growth, accompanied by the lack of rapid repairable ability. Chemotherapy for patients with cancer is characterized by oral and intravenous administrations of several medicines, however, it also induces severe adverse effects as well as don't use for some breast cancer individuals (Waks and Winer 2019).

Therapeutic agents are employed for breast cancer treatment are including alkylating agent such as cyclophosphamide; anti-metabolite: 5-fluorouracil, methotrexate, capecitabine; hormone and antagonist: tamoxifen, letrozole & anastrozole; miscellaneous: trastuzumab, lapatinib and natural product such as paclitaxel, vinorelbine, doxorubicin. The therapeutic agents used for breast cancer treatment have many adverse drug reactions and these adverse reactions discourage patient adherence to the therapy (Waks and Winer 2019).

In this chapter, we review the up-to-date understanding of natural promising bioactive compounds that present in chemo effective potential against breast cancer. The bioactive compounds have anti-inflammatory, antiangiogenic, antiproliferative, antimetastatic, and anticancer properties. We focus on the possible mechanisms of these bioactive compounds on breast cancer progression.

Curcumin

Curcuma longa Linn., a perennial, tropical herb belongs to the ginger family, is widely cultivated in Asia. Its rhizome is extensively consumed for providing colors and flavors of foods as well as medicinal purposes. Curcumin is commonly used as a natural pigment obtained from the root of *Curcuma longa,* possessing anti-inflammatory, anticarcinogenic, and antimetastatic properties. It was reported that the administration of curcumin resulted in the modifications in the actions and expressions of various important proteins associated with the survival and proliferation of tumors, including enzymes, cytokines, gene-products and transcription factors. Banerjee et al. conducted a study to investigate the significant effects of curcumin on apoptosis, G2/M arrest and decrease in proliferation of cancer cells through assembly dynamics-related inhibition of microtubules as well as activation of mitotic checkpoint in MCF-7 cells (Banerjee, Singh et al. 2010). Another study reported that curcumin had effective ability to induce congregation of cells in the G1 phase, accompanied with zeste homolog 2 (EZH2) down-regulation by activation of three essential enzymes belonging to the mitogen-activated protein kinase (MAPK) pathway, namely p38 kinase, c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) (Hua, Fu et al. 2010). In addition, it was showed that curcumin might be able to affect the association between abnormal expression of signals via the Wnt/β-catenin pathway and breast cancer development by the suppression of slug, β-catenin and cyclin D1 in both MDA-MB-231 and MCF-7 cell lines (Prasad, Rath et al. 2009). Furthermore, prevention of p53 mRNAs, Ki-67 and proliferating cell nuclear antigen (PCNA) expression in breast cancer cells, whereas Bax mRNA expression-related

stimulation with the p21 mRNA down-regulation were presented in human mammary epithelial cells treated by curcumin (Ramachandran and You 1999). Curcumin was also responsible for significantly decreasing in the number of Sp1 and NF-YA transcription factors, which was associated with the signaling activation of AKT-mTOR and PKC-p38-ERK-cFos pathway (Chatterjee, Ghosh et al. 2019). Several important events occuring in cancer cells such as invasion, adhesion as well as migratory and proliferative activities because of NF-κBp65 expression-associated down-regulation were inhibited by the curcumin administration (Chiu and Su 2009). Curcumin further reduced NF-κB expression, MAPK, Akt phosphorylation as well as HER2 oncoprotein in both SK-BR-3-hr and BT-474 cell lines (Lai, Chien et al. 2012). Moreover, curcumin suppressed breast tumor angiogenesis (Chakraborty, Jain et al. 2008). p53 known as a cancerous suppressor gene, that is related to different cellular activities like apoptosis, DNA repair and cell cycle arrest, and thus, its mutations resulted in lowering survivability as well as resisting typical therapies. Activation of p53 gene and regulatory activities of other apoptotic proteins causing apoptosis was observed in breast cancer cells treated with curcumin. Besides, curcumin induced the overexpression of MCL-1 and TRAP3 apoptotic genes together with the downregulation of AP13 and TRAIL genes in cells of breast cancer tumors (Talib, Al-Hadid et al. 2018). Doxorubicin is a typical chemotherapy agent against breast cancer development, but the treatment with these medications for some patients in the long-term is limited by doxorubicin resistance. Interestingly, it was revealed that a combinative treatment with curcumin showed remarkable enhancement of doxorubicin sensitivity to MDA-MB-231 and MCF-7 cell lines due to inhibitory capability for ATPase activity of ABCB4 (Wen, Fu et al. 2019). In summary, curcumin has therapeutic potential against breast cancer.

Resveratrol

Resveratrol, a phytochemical, is found in various natural products for foods, abundantly in grapes. An experiment was conducted to demonstrate the significant potential of dose-dependent treatment with resveratrol in breast cancer via inhibitions of tumor growth in ER-positive MCF-7 cell lines as well as E2 stimulation-induced progesterone receptor gene expression. Additionally, resveratrol was responsible for the suppression of insulin-like growth factor I receptor mRNA and transforming growth factor- α as well as the overexpression of transforming growth factor β2 mRNA in MCF-7 cells (Lu and Serrero 1999). Moreover, resveratrol had a proteasome inhibitory effect on Δ16HER2 accumulation, resulting in the production of Δ16HER2/HER3 heterodimers, which played an important role in triggering tumor growth and proliferation via stimulating the downstream mTORC1/p70S6K/4EBP1 pathway (Andreani, Bartolacci et al. 2017). An *in vitro* study revealed that the use of resveratrol for MDA-MB-231 breast cancer cells was found to remarkably increase apoptosis while reducing extracellular numbers of vascular endothelial growth factor VEGF. Another *in vivo* experiment showed that resveratrol caused transcription through ERalpha and ERbeta, which was associated with reductions of cancer growth and angiogenesis as well as an increase in the apoptotic index in MDA-MB-231 cell lines (Garvin, Öllinger et al. 2006). Autophagy known as a pathway has responsibility for realizing the metabolic conditions of the cells itself as well as some organelles renewal and plays opposing. Hence, autophagy-associated regulation is considered as an important target for cancer treatment, and p62 controlled by constitutive autophagy is the main factor in this pathway. The administration of resveratrol for cancer prevention exhibited the inhibition of Nrf2 and mTOR activation induced by stimulating autophagy and accelerating p62 degradation (Tian, Song et al. 2019). Furthermore, the anti-breast cancer effect of resveratrol as an estrogen agonist on tumor cells stably transfected with mutant ER (D351Y) and wild-type

ER(D351) lines was induced by activating TGFα mRNA. Besides, resveratrol was also found to target ER-independent pathways, which was demonstrated by different effects on the numbers of ER protein in both two cell lines, including down-regulation of wild-type ER levels and significant up-regulation of mutant ER levels. Generally, resveratrol is believed to act as ER agonist at low concentrations and stimulate ER-independent pathway leading to inhibitory potential for tumor growth (Levenson, Gehm et al. 2003). Azios et al., reported that resverstrol at a concentration of 50 μM exhibited an antiestrogenic ability to prevent breast cancer cell migration as well as resulted in comprehensive and persistent extension of actin structures. Additionally, treatment by resveratrol at 50 μM was found to inhibit Cdc42 and Rac activities, whereas the use at a dose of 5 μM resulted in Rac activation in breast cancer tumors. The intake of resveratrol at 5 μM induced invasion, tumor migration and increase in lamellipodia based on mechanisms like estrogen activities. Besides, lamellipodia response to resveratrol at a dose of 5 μM, or epidermal growth factor, was prevented in cells expressing dominant-negative Rac, suggesting that Rac regulates resveratrol (5 μM) targeting to the actin cytoskeleton (Azios, Krishnamoorthy et al. 2007). A study revealed that the antagonistic ability of resveratrol at a dose-dependent manner to promote the growth of 17-beta-estradiol (E2) was found in both cellular and molecular levels via cell growth as well as gene expression (Lu and Serrero 1999). Metastasis is known as a major cause of breast cancer-related mortality due to the association between neoplastic metastasis and cell migration and invasion. It was reported that the insulin-like growth factor (IGF-1) played important role in stimulating PI-3K/Akt signaling pathway leading to cell migration and expanding metastatic status in ER-negative breast cancer cells. Feng-Yao Tang et al., conducted an *in vitro* study on MDA-MB 435 cell line to investigate the significant impact of resveratrol on IGF-1 inhibition related to cell migration due to the inactivation of PI-3K/Akt signaling pathway. Matrix metalloproteinase-2 (MMP-2) mainly contributes to degrade extracellular matrices and the dysregulation of this enzyme is associated with breast cancer metastasis and invasion. Interestingly, resveratrol was found to significant suppress IGF-1-mediated MMP-2 expression, together with modification in tumor invasion (Tang, Su et al. 2008). Giulia Lanzilli showed that apoptosis was observed in MCF-7 cells treated by resveratrol in a time- and dose-dependent manner. Furthermore, resveratrol inhibited the growth of malignant tumors because of its pharmacological effects on cell death, cell cycle arrest at S-phase related to suppress telomerase activity. Apart from the inhibition of telomerase action, resveratrol also induced the down-regulation of hTERT nuclear levels as well as the reverse transcriptase subunit of the telomerase complex. Besides, the intake of resveratrol showed direct antiproliferative and pro-apoptotic effects. Resveratrol was endowed with additional suppressive telomerase function and intracellular hTERT distribution on critical tumor biological properties (Lanzilli, Fuggetta et al. 2006). Another study reported that the induction of apoptosis by resveratrol in MCF-7 cells might be based on an oxidative, caspase-independent mechanism through inhibiting PI3K signaling converges to Bcl-2, linked to calpain protease and NF-κB activity. Additionally, cell cycle arrest and cell death in MCF-7 breast cancer cells resulted from resveratrol treatment via interfering with the estrogen receptor (ERα)–dependent phosphoinositide 3-kinase (PI3K) pathway (Pozo-Guisado, Merino et al. 2005). Although cyclooxygenase-2 (COX-2) is considered as an anti-apoptotic agent linked to tumorigenesis, recent studies have ascribed COX-2 in association with proapoptotic activity. It was reported that COX-2 proteins accumulated in human MCF-7 and MDA-MB-231 cell cultures, which was induced by resveratrol via the stimulation of MAPK and activator protein 1-dependent. Moreover, COX had novel intranuclear colocalization with Ser15-phosphorylated p53 and p300 leading to increase cell death in breast cancer cells treated by resveratrol (Tang, Shih et al. 2006). Besides, cell cycle and anti-apoptotic proteins include Bcl-2, X-related agent against CDKs and apoptosis protein in breast cancer

cells, which were regulated by resveratrol resulted from the alteration of tumor-suppressive miRNAs such as miR-542-3p, miR-409-3p, miR-200c-3p, miR-122-5p and miR-125b-5p (Venkatadri, Muni et al. 2016). For the same purpose of stimulating apoptosis in tumors, Nakagawa, H., et al., conducted an experiment to demonstrate the suppressive ability of resveratrol at high doses (≥44 μM) to cell growth in both (ER)-positive (KPL-1 and MCF-7) and -negative (MKL-F) breast cancer cell lines. It was found that apoptosis in association with the presence of a sub-G1 fraction led to growth inhibition, which induced activation of caspase-3, up-regulation of Bak and Bax protein as well as down-regulation of Bcl-x_L protein. Interestingly, the administration of resveratrol at doses of 52–74 μM caused the antagonistic effect on activity of linoleic acid known as a breast cancer activator and growth suppression in both ER-positive and -negative cell lines (Nakagawa, Kiyozuka et al. 2001). Generally, resveratrol may be a promising candidate for preventing metastasis, proliferation and epigenetic alterations as well as inducing apoptosis and increasing sensitization of chemotherapeutic drugs (Sinha, Sarkar et al. 2016). These findings support the potential use of resveratrol as a chemotherapeutic agent in breast cancers.

Genistein

Genistein, a main isoflavonoid is found in several products from soybean, has been believed as a potential agent against breast cancer progression. It has the responsibility for inactivation of protein tyrosine kinase (PTK) resulting in apoptosis, oncogenesis and control of cell growth. The anti-cancer effects of genistein based on typical mechanisms including clonogenic ability, cell proliferation and oncogenesis are observed in both human and animal cells. Yiwei Li showed that genistein induced up-regulation of $p21^{WAF1}$ and Bax expression and down-regulation of p53 and Bcl-2 expression in MDA-MB-231 cancer cells. In addition, apoptosis via a p53-independent pathway, regulation of apoptotic gene expression as well as growth inhibition of MDA-MB-231 breast cancer cells were caused by genistein intake (Li, Upadhyay et al. 1999). It was also reported that MCF-7 cell lines treated by genistein at a dose-dependent manner exhibited inhibitory activity of cell growth-related to apoptotic and cytostatic effects (Pagliacci, Smacchia et al. 1994). Furthermore, the treatment by genistein with a physiological concentration range of 10 nM-20 μM produced anti-breast cancer effects as estrogen agonist and cell growth inhibitor (Zava and Duwe 1997). A study revealed that genistein may block mammary epithelial cell growth by interfering with signal transduction events stimulated by estradiol or growth factors. Additionally, the inhibition of cell growth caused by genistein was not related to suppress EGF-receptor PTK activity or stimulate ER- signaling pathways in breast cancer tumors (Peterson and Barnes 1996). Hsieh showed that genistein could act as an estrogen agonist in vivo and in vitro, resulting in the proliferation of cultured human breast cancer cells (MCF-7) and the induction of pS2 gene expression (Hsieh, Santell et al. 1998). Genistein inhibits tumor growth independent of the presence of the estrogen receptor (Peterson and Barnes 1991). Audrey King-Batoon also reported that tumorigenic development was diminished by the dietary treatment of genistein at low concentrations through alteration of promoter methylation linked to gene expression (King-Batoon, Leszczynska et al. 2008). Yanchen Liu showed the capability of genistein to induce differentiation of breast cancer stem cells based on activating MEK/ERK and PI3K/Akt signaling pathways via the release of amphiregulin from ER+ cells. Besides, genistein also induced effective promotion of the morphological modification mammospheres, up-regulation of differentiation-related cell markers of mammospheres in the co-culture system, accompanied with a decrease in the ratio of a subset of CD44+/CD24-/ESA+ cells (Liu, Zou et al. 2016). Hypoxia-inducible factor-1α (HIF-1α) is believed as a promising target for improving various diseases including breast cancer because of the

correlation between its inhibition and therapeutic effects. Mukund reported that genistein interacted with the FIH-1 binding site of HIF-1α protein leading to a reduction of the HIF-1α level in BC cells (Mukund, Saddala et al. 2019). Another study showed that MCF-7 cells treated by genistein presented in significantly reducing cell viability, together with increasing ROS formation. Additionally, genistein induced the alterative ability to inflammatory-related gene expression associated with both ERs and ERα/ERβ ratio, which was responsible for decreasing anti-inflammatory and activating pro-inflammatory gene expression in MCF-7 breast cancer cell lines (Pons, Vilanova-Llompart et al. 2019). Qi Xie reported that genistein decreased activity of DNA methyltransferase (DNMT) as well as DNMT1 expression through competitive inhibition with hemimethylated DNA binding to the catalytic domain of DNMT1. Moreover, DNA methylation in the promoter region of multiple tumor suppressor genes (TSGs) including adenomatous polyposis coli, mammary serpin peptidase inhibitor, ataxia telangiectasia mutated and phosphatase and tensin homolog was decreased by the effects of genistein (Xie, Bai et al. 2014). These previous studies suggested that genistein might contribute promising therapeutic potential to the treatment of human breast cancer.

Pristimerin

Pristimerin, an active component obtained from *Hippocrateaceae* and *Celastraceae* species, has been used for traditional medicine due to the anti-inflammatory effect. Interestingly, pristimerin also has the anti-growth effect on both cancer stem cells and cancer cells. An *in vivo* study was conducted to investigate cell growth inhibition of MCF-7 and MDA-MB-231-originated xenografts by the administration of pristimerin in NOD.CB17-Prkdc^{scid}/J mice. Besides, the stimulation of TUNEL staining and casp3 and/or casp7 as well as the cleavage of PARP leading to apoptosis were exhibited in mice treated by pristimerin (Cevatemre, Erkısa et al. 2018). Mu Xian-Min reported the dose-dependently suppressive ability of pristimerin to human chymotrypsin proteasomal activity in MDA-MB-231 cell lines. An *in vitro* study showed that the use of pristimerin induced inhibition of breast cancer cell migration, invasion and lamellipodia formation via up-regulation of RGS4 expression. Moreover, mouse models implanted breast tumors exhibited a decrease in cell viability and invasion after pristimerin treatment (Mu, Shi et al. 2012). Shihuan Cheng also reported that pristimerin suppressed breast cancer cell viability, cell cycle and migration while caused cell apoptosis. Also under pristimerin treatment, miR-542-5p was up-regulated whereas DUB3 was down-regulated (Cheng, Zhang et al. 2020). These findings further explain that pristimerin has the therapeutic potential for targeting breast cancer

Ginsenoside Rg3

Ginsenoside Rg3 is an extract from the natural product ginseng. Previous studies have shown Rg3 has anti-metastasis of cancer in vivo and in vitro. Rg3 treatment decreases the number of migrated cells and reduces the width of the scar in wound healing (Chen, Qian et al. 2011). Bo-Min Kim revealed that Rg3 resulted in apoptosis and proliferative inhibition because of an increase in the number of apoptotic cells and the ratio of proapoptotic Bax to antiapoptotic Bcl-2. In addition, the cleavage of poly (ADP-ribose) polymerase, the cytochrome c release from mitochondria as well as depolarization of the mitochondria membrane potential were observed in MDA-MB-231 cells treated by Rg3 (Kim, Kim et al. 2013). It was found that Rg3 was responsible for IKKβ inactivity, degrading IκBα and subsequently translocating the p65 subunit of NF-κB, which led to suppress NF-κB-related DNA binding and transcriptional activity.

Also, the use of Rg3 in MDA-MB-231 cell lines resulted in a reduction of the constitutive stimulation of Akt and ERK via phosphorylation. Additionally, the numbers of the mutant p53 were decreased by Rg3 dose- and time-dependent treatment. Besides, an increase in the connection between p53 and its negative regulator Mdm2 was exhibited in MDA-MB-231 cells treated with Rg3 (Kim, Kim et al. 2014). Triple-negative breast cancer is characterized by distant metastatic status and high ability to recurrence knows as an aggressive subtype of this disease. Zuguo Yuan et al., reported the chemosensitizing effects of Rg3 due to the stimulative responsibility for paclitaxel-caused apoptosis and cytotoxicity in xenograft and TNBC cells, which was associated with regulation of Bax/Bcl-2 expression as well as NF-κB signaling inhibition on triple-negative breast cancer (Yuan, Jiang et al. 2017). Moreover, Juyeon Ham showed that Rg3 induced a decrease in cell viability through alteration of the epigenetic methylation level leading to deregulation of tumor genes. Rg3 also caused late-stage apoptotic effect and cell proliferative inhibition up to 60%, accompanied with down-regulation of hypermethylated NOX4, TRMT1L and PSMC6 as well as up-regulation of KDM5A, ST3GAL4 and RNLS (Ham, Lee et al. 2018). With all previously published data, Rg3 should be considered as a potential agent for breast cancer treatment.

Furanodiene

Furanodiene, a bioactive terpenoid is extracted from Rhizoma Curcumae widely used in Chinese traditional medicine, has anti-tumor, and metastatic properties in various cancer cell lines. Zang feng Zhong, et al., revealed that the concentration-dependent treatment with furanodiene resulted in the proliferative inhibition and an increase in the release of LDH in both MCF-7 and MDA-MB-231 breasrt cancer cell lines, which was related to the induction of cell cyce arrest in G0/G1 phase. Additionally, furanodiene remarkably suppressed the protein expression of Akt, Bcl-xL, total CDK2, p-CDK2, total cyclin D1, p-cyclin D1, total Rb and p-Rb, whereas increased the expression of Bax and Bad, the proteolytic cleavage of caspase-7, caspase-9 and poly-ADP-ribose polymerase (PARP). Besides, an *vivo* study showed the suppression of cell viability in a dose- dependently furanodiene administration (Zhong, Dang et al. 2012). These authors also demonstrated that at low concentrations (5–25 μM), furanodiene inhibited adhesion, migration and invasion of breast cancer cells, but it did not induce cytotoxicity, apoptosis and cell cycle arrest. Furanodiene down-regulated the integrin αV expression, β-catenin expression, focal adhesion kinase (FAK) phosphorylation, Akt phosphorylation, and PI3 kinase p85 phosphorylation (Zhong, Tan et al. 2014). Another study reported that doxorubicin resistance in MCF-7 cells was improved by furanodiene treatment because of cell growth and proliferative inhibition together with cell cytotoxicity. Furanodiene also preferentially interfered with NFκB-independent and intrinsic/extrinsic-dependent pathways leading to apoptosis in these cell lines (Zhong, Yu et al. 2017). For the same anticancer purpose to doxorubicin-resistant MCF-7 cells, Zhong, Z.-F., et al., designed a study to show the alteration of mitochondrial function, apoptotic effect and reduction of ATP levels in MCF-7 cell lines with furanodiene intake. Moreover, these noticeable properties resulted from AMPK activation, which was caused by effects of furanodiene on the AMPK pathway intermediates and phosphorylation of AMPK (Zhong, Tan et al. 2016). Furanodiene also played its anticancer effects through antiangiogenesis and causing ROS formation, apoptosis and DNA strand damage. Furanodiene suppressed efflux transporter Pgp function and decreased the number of Pgp protein, but no impact on Pgp-associated gene (MDR1) expression (Zhu, Guo et al. 2019). These findings suggest furanodiene may be an essential agent incorporated in next-generation chemotherapy protocols.

Kaempferol

Kaempferol, a phytoestrogen is detected in various vegetables, has several potent effects on a decrease in the risk of many chronic diseases, including cancer. It was demonstrated that kaempferol induced an increase in the antioxidant defence leading to inhibit free radicals, and thus prevented cancer development. In addition, the modulation of many essential factors in cellular signal transduction pathways related to inflammation, apoptosis, metastasis and angiogenesis was also caused by the intake of kaempferol (Wang, Yang et al. 2019). Kaempferol at dose of 50 and 10 μM significantly prevented human breast carcinoma MDA-MB-453 cells growth. Kaempferol induced cell cycle arrest at the G2/M phase, along with down-regulated CDK1 and cyclin A and B in the G2/M-phase-related proteins. Besides, kaempferol-induced apoptosis was related to the up-regulation of p53 (Choi and Ahn 2008). Zhu Li, et al., revealed that kaempferol had the anti-proliferative effect on triple-negative BC (TNBC) MDA-MB-231 cell lines, which was stronger when compared with that in the estrogen receptor-positive BT474 cell lines. Moreover, kaempferol administration for 48 h induced cell cycle arrest in G2/M phases because of a significant reduction of cell population in G1 phase as well as a marked increase in the population of cells in G2 phase. DNA damage and apoptotic activity were presented in MDA-MB-231 cells treated by kaempferol. Additionally, kaempferol-treated group exhibited the overexpression of γH2AX, p-ATM, cleaved caspase 3 and cleaved caspase 9 in comparison with control group (Zhu and Xue 2019). Cláudia Azevedo, et al., reported that kaempferol was capable of anti-proliferation as well as cytotoxicities, which was attributed to inhibiting glucose uptake related to GLUT1 in MCF-7 cells (Azevedo, Correia-Branco et al. 2015). According to a study of Geum Lee et al., kaempferol inhibited triclosan or E2 (17β-estradiol), which were responsible for induction of invasion and migration in MCF-7 cells as well as reduction of protein expressions related to metastasis- activating genes. In addition, the kaempferol-induced inhibition of triclosan led to cell proliferation based on IGF-1R and nongenomic ER signaling pathway, which might result from increase in AKT, ERK, MEK1/2 and IRS-1 phosphorylation (Kim, Hwang et al. 2016, Lee, Choi et al. 2017). For the similar purpose to prevent cell proliferation in MCF-7 cells, Diantini reported that the concentration- dependent intake of kaempferol resulted in activating the caspase signaling cascade, including PARP, caspase-3 and caspase-9, and then causing apoptosis and proliferative inhibition (Diantini, Subarnas et al. 2012). Also, the down-regulation of Bcl2 expression was induced by kaempferol, leading to cell-growth inhibition (Yi, Zuo et al. 2016). Moreover, the anti-breast cancer potential of kaempferol was demonstrated by down-regulating phosphoinositide 3-kinase/protein kinase B signaling pathways as well as epithelial-mesenchymal transition (EMT)-related markers in cancer cells treated with kaempferol (Imran, Salehi et al. 2019). Many evidence supports the use of kaempferol for breast cancer prevention.

Epigallocatechin-3-gallate

Epigallocatechin-3- gallate (EGCG), the most active and abundant polyphenolic flavonoid isolated from gree tea, has been widely used because of anticarcinogenic, neuroprotective, anti-microbial and antioxidant effects (Chakrawarti, Agrawal et al. 2016). EGCG has been revealed to be potent against the initiation, progression, and invasion stages of multistage carcinogenesis. EGCG inhibited the cellular proliferation and cell viability in a dose-dependent and time-dependent manner in MDA-MB-468 human breast cancer cell lines. EGCG stimulated apoptotic activity through an increase in expression of tumor suppressor protein p53 as well as its phosphorylation at Ser 15 residue and proapoptotic protein

Bax, together with a decrease in antiapoptotic protein Bcl-2 expression. In addition, the ratio of Bax/Bcl-2 proteins increased by EGCG treatment led to increase in releasing cytochrome c from mitochondria into cytosols as well as activating poly(ADP-ribose) polymerase, caspase-3 and up-regulating expression of Apaf-1, which might be also responsible forapoptosis in these cells (Roy, Baliga et al. 2005). Karine Belguise et al., also reported the cell growth inhibitory effect of EGCG in soft agar of Her-2/neu- overexpressing breast cancer cells due to alteration of key regulator expression in the epithelial to mesenchymal transition (EMT) pathway, and thus, reduced invasive phenotype. Furthermore, up-regulation of the epithelial genes MTA3, E-cadherin and γ-catenin as well as down-regulation of the pro-invasive snail gene were observed after EGCG treatment. In matrigel, EGCG also suppressed invasion and branching colony growth. Similarly, the inhibition of branching colony growth associated with protein kinase CK2 and NFκB c-Rel was presented in mouse mammary tumor cells with EGCG treatment. Besides, ERα is believed to play an essential role in the prevention of mesenchymal transition and has a main transcriptional regulator called Forkhead box O (FOXO3a). Hence, in ERα-positive cells, EGCG- caused FOXO3a stimulation was found to reverse invasive phenotype (Belguise, Guo et al. 2007). In a clinical trial, EGCG in 400 mg capsules was orally administered three times daily to breast cancer patients undergoing treatment with radiotherapy. Compared to patients who received radiotherapy alone, those given radiotherapy plus EGCG showed significantly lower serum levels of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and reduced activation of metalloproteinase-9 and metalloproteinase-2 (MMP9/MMP2). MMP-2 and MMP-9 play an important role in the homeostasis of the extracellular matrix (ECM), and hence an imbalance in their expression or activity may have important consequences in various pathologies such as development and progression of cancer Moreover, the sera obtained from those patients to in vitro cultures of highly-metastatic human MDA-MB-231 breast cancer cells resulted to suppression of cell proliferation and invasion; arrest of cell cycles at the G0/G1 phase; reduction of activation of MMP9/MMP2, expressions of Bcl-2/Bax, c-Met receptor, NF-κB, and the phosphorylation of Akt (Zhang, Wang et al. 2012). Jiyoung Kim, et al., showed that EGCG decreased invasiveness and cell tumorigenic proliferation in an HBP1 transcriptional repressor -dependent manner. EGCG also prevented Wnt signaling by causing the HBP1 and suppressed aspects of invasive breast cancer (Kim, Zhang et al. 2006). Stefania Pianetti, et al., reported that EGCG had the inhibitory ability to basal Her-2/neu receptor tyrosine phosphorylation resulting in a reduction of signaling through the Akt kinase, phosphatidylinositol 3- kinase to NF-κB pathway, which induced growth suppression of mouse mammary tumor virus (MMTV)-Her-2/neu NF639 cells. Similarly, SMF and Ba/F3 2 + 4 cells treated with EGCG exhibited inbibition of basal receptor phosphorylation, and therefore, these beneficial effects of EGCG might be applied for the treatment of tumors with Her-2/neu overexpression (Pianetti, Guo et al. 2002). Another study was designed to show the anti-cancer potential of EGCG in both ER-negative MDA-MB-231 and ER-positive MCF-7 cell lines due to inhibition of human telomerase reverse transcriptase (*hTERT*) as well as the telomerase catalytic subunit with epigenesis-related mechanisms. Additionally, chromatin structures of the *hTERT* promoter were modified by EGCG via decrease in level of acetyl-H4, acetyl-H3K9 and acetyl-H3 to the *hTERT* promoter, which contributed to facilitation of interaction between the *hTERT* regulatory region and *hTERT* repressors like E2F-1 and MAD1 in both breast cancer cell lines (Meeran, Patel et al. 2011). Moreover, EGCG treatment inhibited the activity, mRNA and protein expression level of MMP-2 as well as the expression of membrane type-1-matrix metalloproteinase (MT1-MMP), vascular endothelial growth factor (VEGF), nuclear factor-kappa B (NF-kB) and focal adhesion kinase (FAK). EGCG treatment decreased the expression of integrin receptors α5, β1, αv and β3. These findings confirmed that EGCG played an

important role for inhibition of pro-MMP-2 activity and expression (Sen, Moulik et al. 2009). EGCG decreased cell viability of ERα-positive (T47D) cells as concentration- and time-dependently. EGCG also remarkably increased the genes of Casp9, Casp3 and PTEN, accompanied by decreased AKT approximately equal to tamoxifen. In gene expression, EGCG led to the increased ratio of Bax/Bcl-2 and reduced gene expression of *hTERT* in T47D cells (Moradzadeh, Hosseini et al. 2017). EGCG resulted in the migratory and invasive suppression of MCF-7 cells together with down-regulation of Rac1 activity and vasodilator-stimulated phosphoprotein (VASP) expression (Zhang, Han et al. 2009). Then all previous studies could conclude that EGCG potentiated efficacy against human metastatic breast cancer.

Genipin

Genipin, an aglycon was found in the extract of *Gardenia jasminoides* Ellis, has commonly been used in traditional medicine for improving inflammation associated with cancer and other diseases. Genipin has been reported to exert significant anticancer effects (Shanmugam, Shen et al. 2018). Genipin induced efficient apoptosis in MDA-MB-231 cells by the down-regulation of Bcl-2, up-regulation of Bax and proteolytic activation of casp3. Genipin also increased the activation of JNK and p38 MAPK. Importantly, genipin significantly inhibited invasive and migratory phenotypes of MDA-MB-231 cells (Kim, Jeong et al. 2012). The previous finding suggests that genipin may prevent or alleviate metastatic breast cancer.

Lycopene

Carotenoids contain important compounds abundantly found in various vegetables and fruits and have widely been consumed to enhance several diseases. Among them, lycopene, a red-pigmented carotenoid, has showed the inhibitory effects on breast cancer and other types of cancers with high consumption, which was demonstrated the anti-proliferative ability to mamary, endometrial, prostate and lung cancer cells in many studies (Sesso, Buring et al. 2005). Padma Uppala, et al., revealed that the treatment with lycopene caused alteration of heat shock protein as well as the expression levels and post-translational activity of cell cycle proteins such as cytokeratin 8/18 (CK8/18), CK19. In MCF-7 breast cancer cells, the inhibition of cell proliferation was also found after lycopene administration (Uppala, Dissmore et al. 2013). Another study was designed in two aggressive breast cancer cell lines including MDA-MB-231 and H-Ras-transformed MCF10A human breast epithelial cells to show the effective ability of lycopene to against cell proliferation, migration and invasion of both these cell lines. In addition, Peng et al., also reported the apoptotic and anti-proliferative effects of lycopene treatment on MCF-7 cell lines through the regulation of Bax and p53 expression (Peng, Li et al. 2017). Lycopene-induced anti-proliferative and/or anti-invasive/migratory effects in these cells may be involved in the ERKs and Akt signaling pathways (Min-Soo, Hwang et al. 2010). Lycopene significantly suppressed the growth of breast cancer and prostate cells at physiologically relevant doses above 1.25 μM. Lycopene also inhibited IκB phosphorylation in the cells and NF-κB transcriptional activity. Lycopene-treated cells exhibited significant inhibition of TNF-induced NF-κB p65 subunit nuclear translocation. Lycopene inhibited both activity of recombinant IκB kinase β and IKKβ from MDA-MB-231 cells. The anticancer effects of lycopene might occur via suppression of the NF-κB signaling pathway at the early stage of cytoplasmic IKK kinase activity, leading to decreased NF-κB-responsive gene regulation (Assar, Vidalle et al. 2016). Previous findings may provide useful information for the application of lycopene in establishing strategy to prevent the metastatic breast cancer.

Apigenin

Apigenin (4′,5,7,-trihydroxyflavone), a well-known flavonoid is rich in herbs, vegetables and fruits such as celery, celeriac, parsley, chamomile, thyme, oranges, lemon balm and onions, has been widely studied due to its potent anticarcinogenic, antioxidant and anti-inflammatory effects (Mohammad Nabavi, Habtemariam et al. 2015). Perrott, et al,. showed that apigenin resulted in ionization of radiation, replicative exhaustion, oncogenic RAS as well as constitutive MAPK in three fibroblast strains, which was capable of suppressing senescence-related secretory phenotype (SASP) leading to the formation and development of tumors. Besides, apigenin treatment also caused suppression of the SASP because of IL-1α signaling inhibition via IRAK4, IRAK1, NF-κB and p38-MAPK, conjugated with the secretion and expression of a SASP factor (CXCL10) (Perrott, Wiley et al. 2017). According to another study in MDA-MB-453 human breast cancer cell line, repression of clonogenic viability and cell proliferation was presented in these cells treated by dose- and time-dependent apigenin administration, which was attributed to apoptotic activity associated with increase in levels of Bax/Bcl-2 ratios, PARP cleavage and casp3. Apigenin also induced the cytochrome c release in cells as well as the cleavage of casp 6, casp 7, casp 8 and casp 9 (Choi and Kim 2009). Apigenin induced autophagy via the accretion of acidic vesicular organelles (AVOs) and the presence of autophagosomes in MDA-MB-231 cell lines. Apigenin could increase the processed form of LC3-I as well as the level of LC3-II. Apigenin has apoptosis- and autophagy-causing effects in breast cancer cells (Cao, Liu et al. 2013).The treatment by apigenin in breast cancer cells with HER2/*neu* overexpression also caused apoptotic induction and growth-suppressive activity in a concentration- and time- dependent manner, which were associated with inhibiting expression of HER3 and HER2/*neu* leading to AKT and PI3K inactivation (Way, Kao et al. 2004). Apigenin significantly inhibited the proliferation of MDA-MB-453 human breast cancer cells in a dose- and time-dependent manner. It was reported that apigenin used in a concentration-dependent manner could either act as estrogen or an estrogen antagonist. At low doses (under 1 μmol/L), the cell growth was inhibited in MCF-7 cells, however, this did not occur in MCF-7 cells with antiestrogenic resistance. The mechanism of apigenin at low concentrations was based on the activation of ERα via amplifying its coactivator in breast cancer-1, leading to enhance receptor transcription. In contrast, the apigenin treatment with high doses (above 10 μmol/L) had cell growth inhibitory effect on both two types of cell lines, which might be associated with down-regulation of ERα together with amplification in levels of breast cancer-1 expression, inhibition of ERα mobility as well as several protein kinases such as p38, AKT, MAPK and protein kinase A (Long, Fan et al. 2008). Hepatocyte growth factor (HGF) and its receptor, Met, play a critical role in control of cell invasive growth linked to the survival of individuals with breast cancer. The cell scattering and motility as well as invasion and migration induced by HGF were inhibited in MDA-MB-231 human cell lines treated by apigenin at a dose-dependent manner. Hepatocyte growth factor (HGF) and its receptor, Met, play a critical roles in control of cell invasive growth linked to the survival of individuals with breast cancer. The cell scattering and motility as well as invasion and migration induced by HGF were inhibited in MDA-MB-231 human cell lines treated by apigenin at a dose-dependent manner. In addition, apigenin affected HGF-caused signaling stimulation related to invasive growth by inhibiting the HGF-induced Akt phosphorylation but not Met, ERK, and JNK phosphorylation. Besides, MDA-MB-231 cells after apigenin treatment exhibited HGF-activated integrin β4 function including cell–endothelial and cell–matrix adhesion (Lee, Chen et al. 2008). These evidence promise apigenin as a new therapeutic agent against antiestrogen-resistant breast cancer.

Silymarin

Silybum marianum L. (Milk thistle), a member of Carduus marianum family, is an ancient medicinal plant which has been used for centuries for treatment of different diseases such as liver and gallbladder disorders, protecting the liver against snakebite and insect stings, mushroom poisoning and alcohol abuse Silymarin, a flavonoid obtained from milk thistle, has gained importance because of anti-angiogenic effect on several types of cancers, including prostate cancer, lung cancer, ovarian cancer, liver carcinoma, colon cancer, bladder cancer, cervical cancer and breast cancer. Silymarin induced a significantly high to complete suppression of both anchorage-independent and anchorage-dependent growth in human breast carcinoma MDA-MB 468 cells with a dose- and time-dependent treatment. The breast cancer inhibitory effect via anti-carcinogenesis was attributed to Cip1/p21 induction, leading to suppress the threshold kinase activities of CDKs as well as related cyclins, and thus, induce G1 arrest in cell cycle progression (Zi, Feyes et al. 1998). The overexpression of the plasma membrane transporter, P-glycoprotein (Pgp) is one of the main causes of multidrug resistance. The function of Pgp is an ATP-driven efflux pump, resulting in a decrease of intracellular anticancer drug concentration. Silymarin showed Pgp inhibitory activity as much as verapamil, a well-known Pgp inhibitor. The mechanism Pgp inhibitory activity of silymarin may be explained by binding to the Pgp substrate-binding site (Kwon, Jung et al. 2006). In clinical trial, silymarin gel showed the efficacy in prevention of radiodermatitis in patients with breast cancer. The median National Cancer Institute Common Terminology for Adverse Events NCI-CTCAE and Radiation Therapy Oncology Group RTOG scores were significantly lower in patients- treated silymarin at the end of the third to fifth weeks in the randomized, double-blinded, placebo-controlled clinical trial (Karbasforooshan, Hosseini et al. 2019). Taken together, these results suggest that silymarin may exert a strong anticarcinogenic effect against breast cancer.

Chalcones

Chalcones, precursor constituents contributing to the formation of flavonoid in plants, mostly occur in the hydroxylated forms and are believed to have potent properties for treating many diseases. It was shown that both MCF-7 and MDA-MB-231 cell lines were presented in apoptotic activity and inhibition of cell cycle progession at G2/M phase. The effect of chalcones on cell cycle arrest resulted from an increase in the expression of Cdc2 protein, cyclin A and cyclin B1, together with p21 and p27 expression in a p53-independent manner. An improvement in Fas/APO-1 and its two form ligands, soluble Fas ligand (sFasL) and membrane-bound Fas ligand (mFasL), led to apoptosis caused by chalcones. Additionally, chalcones induced the activation of mitochondrial apoptotic signaling through the reduction of Bcl-X_L and Bcl-2 as well as increase in the amount of Bak and Bax, and subsequent stimulation of caspase-9 in two cell lines (Hsu, Kuo et al. 2006). Chalcones are also had P-glycoprotein inhibitory activity, which the over-expression of efflux transporters such as P-glycoprotein is responsible for multidrug resistance. These compounds were shown to enhance the uptake of doxorubicin by MCF-7 cells that over-expressed Pgp (Liu, Tee et al. 2008). Taken together, chalcones may participate in the antiproliferative activity of chalcone in human breast cancer cells.

Terpenoids

Terpenoids are known as terpenes or isoprenoids and the largest group of natural components identified in different plants, serving a range of important physiological functions. Naoko Yoshida et al., revealed that some terpenoids like glycyrrhetic acid and (R)-(+)-citronellal, could act as Pgp inhibitors. They also reported that abietic acid and glycyrrhetic acid decreased the [^{3}H]$E_2$17βG accumulation into vesicles from Sf9 cells with MRP2-overexpression. Glycyrrhetic acid suppressed the [^{3}H]$E_2$17βG accumulation into vesicles from LLC-PK1 cells with BCRP-overexpression. These findings suggested that abietic acid and glycyrrhetic acid could effectively suppress BCRP- or MRP2- mediated membrane transport and might interact with their substrates in pharmacokinetic processes (Yoshida, Takada et al. 2008). Ateba Sylvin have reviewed various terpenoids such as tanshinone IIA, thymoquinone, cucurbitacin B, costunolide, celastrol, lycopene and triptolide against breast cancer and found that these terpenoids showed significant inhibition of metastasis proliferation, apoptosis resistance, migration and tumor angiogenesis in several breast cancer cells/tumors in vitro and in vivo (Ateba, Mvondo et al. 2018).

CONCLUSIONS

Breast cancer is a significant cause of death all over the world and its management is very challenging. Currently, chemotherapy still has mainly disadvantaged, such as heterogeneity of the cancer cells, normal tissue toxicity, rapid drug resistance. This chapter provides information on selected natural products for chemoprevention against breast cancer. Natural product compounds show inhibitory effects on tumor growth, angiogenesis, proliferation, invasion and metastasis. These natural products can be preventive agents that can reduce side effects and improve the effectiveness of drugs on human breast cancer while maintaining high selectivity and low toxicity. The identification of natural product compounds that can control or inhibit potential molecular targets will provide many opportunities for chemoprevention.

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Chapter 24
Cancer:
Clinical Trial Design and Principles

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ABSTRACT

Clinical trials are essential to govern the impact of a new possible treatment. It is utilized to determine the safety level and efficacy of a certain treatment. Clinical trial studies in cancer have provided successful treatment leading to longer survival span in the patients. The design of clinical trials for cancer has been done to find new ways to prevent, diagnose, treat, and manage symptoms of the disease. This chapter will provide detailed information on different aspects of clinical trials in cancer research. Protocols outlining the design and method to conduct a clinical trial in each phase will be discussed. The process and the conditions applied in each phase (I, II, and III) will be described precisely. The design of trials done in every aspect such as prevention, immunochemotherapy, diagnosis, and treatment to combat cancer will be illustrated. Also, recent innovations in clinical design strategies and principles behind it as well as the use of recent advances in artificial intelligence in reshaping key steps of clinical trial design to increase trial success rates.

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INTRODUCTION

A continuous increase in the global burden of cancer is primarily because of the factors like world population, pollution, food habits, aging, and adaptation of certain cancer-causing behaviors in developing countries. Based on GLOBOSCAN estimates in 2018, 18.1 million new cases of cancer were registered and 9.6 million deaths occurred worldwide. One in 6 women and one in 5 men during their lifetime develop cancer. Also, one in 11 women and one in 8 men die from cancer. (Latest global cancer data 2018) It has become a leading cause of death among people in economically developed countries second leading cause among people of developing countries. This makes cancer research and its drug discovery more prevalent than other diseases.

Clinical trials are essential to govern the impact of a new possible drug or treatment. Clinical trials are utilized to determine the safety level and efficacy of a certain drug or treatment. Clinical trial studies in cancer have provided successful treatments leading to longer survival span of the patients. The design of clinical trials for cancer has been done to explore new ways to prevent, diagnose, treat, and manage symptoms of the disease. In the mid-1950s, the first randomized clinical trial was carried out and within two decades it was proved as a useful way to detect the relative effectiveness of cancer treatments and substantial progress has been made to date. The clinicians and statisticians should cooperatively put efforts in the design and analysis of studies as well as there should be a synergistic effort between clinicians and patients too during the conduction of such studies (Edmund A. G., 1979)

HOW IS A CLINICAL TRIAL FOR CANCER COMPARED TO OTHER THERAPEUTIC AREAS?

Oncology is found to be complicated than other therapeutic areas to an extent. One major difference is the endpoints; like in clinical trials for other therapeutics, the aim is to test the efficacy and safety of a certain antibiotic against infection whereas oncology clinical trials try to extend the quality of life of the subject. Also, there is a difference, in how the severities of the events have been reported; in non-oncology clinical trials, the events are considered as mild, moderate or severe whereas in oncology trials the adverse events are divided into numeric grades according to National Cancer Institute guidelines: Common Terminology Criteria for Adverse Events (CTCAE). In some cases, mild corresponds to grade 1, grade 2 corresponds to moderate, grade 3 corresponds to severe, and grade 4 corresponds to life-threatening condition and to death.

TYPES OF ONCOLOGY CLINICAL TRIAL

Prevention

The prevention trials focus on ways of preventing cancer or recurrence. For example, the trial focusing on the use of vitamins, change in diet, the use of different medications or exercise for decreasing the chances of developing cancer. A related study was done considering a hypothesis that if the intake of dietary fat is reduced, the incidence of breast cancer will also reduce. It was found that total dietary fiber was associated with lower breast cancer risk in the early adulthood of women (Maryam S.F. et al., 2016).

Also, some of the FDA-approved approaches include; the risk of breast cancer by using raloxifene or tamoxifen and cervical cancer prevention using vaccines generated against papillomavirus.

Screening

In cancer, one of the biggest problems is to detect the disease at an early stage and lack of which makes it one of the deadliest diseases. The screening trials focus on detecting and identifying cancer in people who are not suffering from cancer. The screening trial includes three types of tests such as; imaging test-which produces pictures of areas inside the body, laboratory test- which checks urine, blood and other body fluids and tissues and genetic tests- which checks for inherited genetic markers which are linked to cancer (Black W. C., 2006).

Immunotherapy

Immunotherapy is a kind of new treatment that involves the strategies to help the immune system find and destroy cancer cells. Immunotherapy trains the immune cells to remember cancer cells and this "immunomemory" then results in longer-lasting remission. For example, the immunotherapy drug pembrolizumab was found to increase the survival rate of people with advanced lung cancer by five years (Heady D., 2019). Many immunotherapy drugs have been approved for use against cancer and many are under clinical trial. The clinical trial for testing new immunotherapeutic majorly involves two phases. Phase I determines and defines the maximum-tolerated dose (MTD) and dose-limiting toxicity. The dose is kept on increasing until significant toxicity occurs. Phase II estimates the effectivity of the treatment by analyzing the endpoints of the new active immunotherapy (Kissick, Haydn T., and Martin G. Sanda. 2017). Cancer immunotherapy is found to be effective in different types of cancer and does not cause the same side effects as in chemotherapy and radiation.

Treatment

Treatment trials are done to determine the efficacy of new treatments such as vaccines, drugs, therapies, and surgical procedures. The major part of clinical trials is performed for testing potential products for the treatment of cancer. There are clinical trials for every type of cancer while, many trials are based on late-stage disease, trials are also done to stop cancer from coming back, reduce side effects, and improve quality of life.

BASIC PROTOCOL OF CLINICAL TRIAL FOR CANCER

Since any scientific study needs designed protocols which need to be followed to perceive accurate results, clinical trials also require a set a procedure to be followed. A protocol includes all the information essential and related to the study. The usual elements of a clinical trial protocol involve:

I. Explanation and scientific background for the study;
II. Purpose of the study
III. Criteria for selection of patients;

IV. Proper design of the study
V. Adequate treatment programs
VI. Procedures followed in event of response, no response, and toxicity
VII. Required laboratory and clinical data
VIII. Criteria estimated for evaluating the effect of treatment on the patients
IX. Statistical considerations
X. Informed consent by the patients
XI. Properly arranged record forms
XII. Important references
XIII. Study investigator and telephone number.

PHASES OF ONCOLOGY TRIALS

In a nutshell, the clinical trial for cancer is divided into four phases;

Phase I

After a treatment has been approved for human studies, it is then tested in a small group involving patients suffering from different types of cancer.

Phase II

If the treatment safely passes Phase I, then enters Phase II, which is a larger study involving patients with one or more specific type or stage of cancer. The main aim of the study is to assess whether the treatment works and to determine the optimal dosing of the drug (Geller, N. L. 1990).

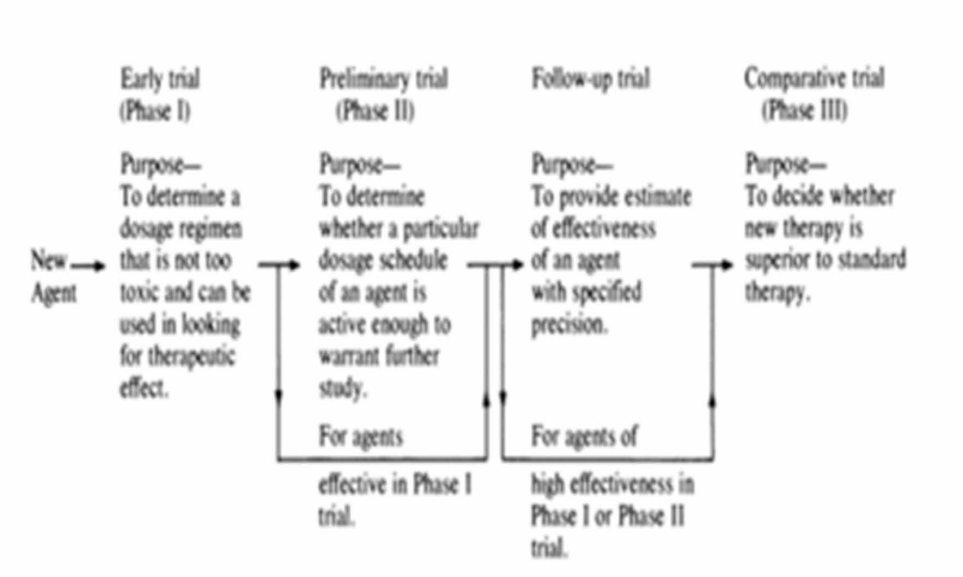
Phase III

Once the treatment shows good results in the previous trial, it is then tested upon an even larger number of patients involving around hundreds to thousands of patients with a specific type and stage of cancer (Evans, S. R. 2010). In most of the cases, the trials are randomized i.e., the patients are randomly assigned for taking the new treatment. This test is done to provide definitive evidence for FDA approval.

Phase IV

Once the treatment is approved by FDA, they are watched over for a long period as even after testing the treatment on hundreds or thousands of people, the full effects of the treatment might not be known. This is mainly done to monitor rare side effects that might show up a long time after the treatment.

Figure 1. Diagram depicting work-flow of oncology clinical trial
(Gehan, E. A. 1979)



Phase 0

In phase 0 small trials are done on humans and involving small doses of the new treatment tested on few patients. This study is done to explore how a treatment may work. It determines how the treatment acts in the human body, how it reaches the tumor, and how the cancer cells respond to the new treatment in the human body. One big difference between Phase 0 and other phase trials is that the patients volunteering in the study do not get benefits by taking part as the drug is too low to be toxic and is less risky for the patients as compared to Phase I patients. This study is just to assess whether the treatment is doing what is expected of it and whether it has been absorbed and reached its target properly. Thus, it is useful in avoiding the delay due to contemplation and finding out reasons for the treatment not acting as expected later in Phase II and Phase III. However, it is not performed more often. (Kinders, R. et al., 2007)

Phase I

The major objective of the Phase I trial is to determine an optimal dose that could be safely tolerated by the patients with minimum toxicity and better activity. It is also used to determine the route of administration of the treatment i.e., through the mouth, vein, etc. It is useful for elucidating the nature of the side effects of the drug both quantitatively and qualitatively. Few basic steps of Phase I involve selection of starting dose, methods for dose escalation, and selection of appropriate patients to receive the treatment. Previous studies done on animals are used to determine the starting dose in humans. Freidrich, et al. (1996), have shown that the maximum tolerable dosage in humans is comparable with five animals such as rat, dog, mouse, monkey, and hamster when the dosage is expressed in per unit of surface area in square meters. Therefore, the dosage levels are taken as 1/3 or less of the average value of the maximum tolerated dosage per unit surface area in the five animals. Also, the study stated that it

is a dangerous attempt to directly extrapolate from animal toxicity data to maximum tolerated dosage in humans (Freireich, E. J. et al., 1966).

The protocol for escalating the dosage should be highly standardized and also it is desirable to skip a few steps and obtain evidence of biological effect as low dosage neither benefits the patient nor it provides useful information. On the other hand, one must be cautious of escalating the dosage when side–effects are observed at a particular dosage. According to a general guideline, each dose should be given to three patients and should be observed until the period of acute side effects is anticipated. The sample of patients chosen for the study affects the conclusion obtained in a major manner. It depends on the sensitivity of the patient towards the new treatment being studied. If patients with advanced diseases are only put for the test then the dosage should be very low as they will have the disease at an earlier stage (Gehan, E. A. 1979).

Phase II

The main objective of Phase II is to determine the effect of the treatmenton cancer, how does the new treatment affect the body, and fights cancer and to assess whether the dosage chosen in Phase I is effective or not. Treatment would be considered certainly of interest if it is exhibiting more activity than the existing treatment or if it is found to be lesser toxic than the other treatment even if it has less activity. Figure 1 indicates the diagrammatic step-wise protocol of how the trials are done. The estimate of the effectiveness of a treatment can be properly determined in Phase II or in the follow-up trial which is conducted before the Phase III trial. The clinicians should reach at one of the following conclusions such as; the treatment is unlikely to be effective in a certain percentage of the patient, or; the treatment is found to be effective in a certain percentage of patients. There are many different approaches to determine the minimum size of the patient sample,one of the approaches is given by Gehan E. A(1969). Table 1 provides the minimum size of patients necessarily required in the Phase II trial to decide whether a treatment is worthy or not for further study in a certain percentage of patients.

Table 1. Table depicting the minimum size of patients required for Phase II study considering therapeutic effectiveness and rejection error (Gehan, E. A. 1979)

Rejection error β (%)	Therapeutic effectiveness (%)									
	5	10	15	20	25	30	35	40	45	50
5	59	29	19	14	11	9	7	6	6	5
10	45	22	15	11	9	7	6	5	4	4

Here, β is the rejection error which is defined as the chance of failing to approve a treatment for further study even when it should have been sent. Therefore, if one is interested in a treatment with 20% efficacy and can accept a 5% rejection error then the sample number of patients needed is 14. This data has been derived assuming or considering that the effectiveness of the treatment is 20% and by calculating that the chance of failure consecutively for 14 patients is less than 5%. If all 14 patients do not respond then the further trial of the treatment should be stopped as the chances of failure are less than 5% of the time if the treatment truly has the effectiveness of 20%. So, according to table 1, if all

the patients in the study fail to respond then further study on treatment is halted. If even one or more than one patient responds than the number of patients is increased to estimate the effectiveness of the treatment approximately (Seymour L. et al., 2010).

Phase III

It is also known as a comparative clinical trial involving two or more treatment and its purpose is of the treatment has been observed in Phase I and II trials. In Phase III, most commonly the effectiveness of the treatment is compared with the best standard treatment present to date for treating the disease. Phase III is considered as the most essential phase of cancer clinical trials as it helps the clinicians to ultimately determine whether the candidate treatment inherits any advantage from the already approved treatments or not and therefore every aspect of this phase should be properly and elaborately considered. However, the study should not be undertaken when the treatment is most likely to be modified very frequently. The number of patients is added to Phase III trial in this case. This suggests that the consideration for a treatment to be further passed on to the Phase III trial depends on whether the treatment has even small to moderate advance in effectiveness (Ocana A. et al., 2013). There is no real rationale for the trial if the treatment does not inherit any advantage over the already existing ones. If the new treatment depicts significant superiority than the standard treatment according to Phase I and Phase II study, then it would be quite unethical to select the patient in randomized fashion for new and standard treatment (Gehan, E. A. 1979).

Objectives of Phase III trial

The type of Phase III trial to be undertaken differs a lot based on the precise meaning of "determining the relative effectiveness of the treatments". The primary goal of the trial is to determine the better treatment among the two treatments and the secondary goal is to estimate the effectiveness of the treatment. In conclusion, as soon as we get to know that one treatment is superior to the other the study is stopped even if the estimation of effectiveness of the treatment is imprecise. Also, an alternative objective could be to select better treatment among the two and also have some precise information about the effectiveness of the treatment. With this combination of choosing better treatment and having knowledge about its effectiveness, it is also important to have an adequate amount of patient sample size to analyze the results accurately (Buyse M. 2016). A sufficient number of patients should be taken for the treatment for precise study. Patients might be further added to satisfy the selection requirement.

Considerations while planning a Phase III trial

Factors that need to be considered to plan clinical trials are; comparability among the group of patients, patient stratification, feasibility of the study, size of the patients, and retrospective versus prospective studies.

(i) **Comparability among a group of patients:** It is very essential to have a comparable group of patients in a controlled clinical trial (Hill A. B. 1962). It should be designed in a way where the only explanation about the difference between the treatments should be the outcome of the treatment and not because of the differences in types of patients taken the treatment. Therefore, comparabil-

ity of patients, when chosen for study, during the study and at the time of analysis of outcome, is highly important. Randomization is one of the methods used for the selection of patients which assures that the patients will be comparable on the average and considering factors influencing prognosis. In cases where patients are not comparable, different procedures for adjusting prognosis is utilized (Carter S. K. 1977). During the retrospective study, patients treated with new treatment are compared with patients treated in the past.

It is necessary to manage patients with treatment in the same way for achieving comparability. The decision for stopping the treatment or for removing patients is also taken in the same way by utilizing the same criteria. If an investigator has a preference towards one of the treatments so that the patients are kept for longer, is designated toxic only when the effect is very severe and the results can be biased towards the preferred treatment. The double-blind study could be stated as one of the solutions for this. In a double-blind study neither the physician nor the patients knowabout the treatment being administered. A double-blind study is not found effective in reducing biases as different types of treatment produce several kinds of toxicity that are known to physicians. The need for a double-blind study is less when the criteria for response and toxicity are more objective. This study also is not found suitable for various other studies such as radiotherapeutic and surgical procedures.

Patients in each treatment group should be compared according to the criteria design to analyze the study. For example, if the response is considered as a reduction by 50% in the diameter of the tumor then the same criteria should be considered in both groups. The evaluation of response can be evaluated by an investigator who does not know which drug has been administered. Each patient is evaluated by a committee of the cooperative group engaged in a clinical trial by utilizing the objective criteria. This diminishes the idea of biasness by a single investigator.

(ii) **Stratification of patients:** A patient suffering from any given type of cancer differ in prognosis due to differences in many factors such as age, bone marrow status, stage of the disease, and prior therapies undertaken. For example, in adult leukemia, patients are categorized based on their age, platelet count, infection status, hemoglobin value, and type of leukemia. Whether the prognostic characteristics are considered for stratification of patients according to which the patients are expected to be comparable in prognosis are still unknown and a topic of argument within the investigators. The patients could be randomized by randomizing patients to the treatment groups within each stratum. Also, randomization is performed without considering strata. The arguments for or against stratification is discussed in a recent paper (Brown Jr B. W. 1980). Some statements put forward in favor of stratification are; that the patients are more comparable than when the prognostic characteristics are ignored, groups inheriting lack of comparability can still be adjusted for analysis, and lack of stratification of patient's leads to study of the difference between treatments with less power and sensitivity. Also, against stratification, it was argued that randomization tends to balance the allocation of treatment within each stratification group even when prognosis factors are not utilized.

A method to achieve balanced patients in each treatment based on their prognostic factors is elucidated by Pocock and Simon (Pocock S. J., & Simon R. 1975) according to which when the patients from multiple institutions are assigned for treatments then the balance of patients should be done at institution level itself.

(iii) **Feasibility and size of study:** A clinical trial should be considered for the number of patients required, length of study, and feasibility. One important factor is the planning of th patients number entering for the study every year. An estimate of this number and the duration of the follow-up period for observation of endpoint in the average number of patients will determine the patient who needs to enter the study.The duration of the trials should not be too long as the motivation and interest of the investigators start fading with time.

The determination of the size of a comparative clinical trial is by considering the trial as a test for the null hypothesis versus an alternative. A proper number of patients is entered to study to produce an accurate response and test the hypothesis based on the significance level and power. For example, in a trial of A versus B, the null hypothesis would be that there is no difference in response of A and B whereas the alternative hypothesis implies that there is the real difference between the response of A and B. One-sided alternative tells about the direction of the difference. For example, the response rate of A is higher than B. In a two-sided alternative, it specifies that either A or B might have a higher response rate.

Designs for comparative studies in Phase III

Designs for comparative studies are rather easy from a statistical viewpoint. The complexity arises due to factors such as; from the multiple observations made on each patient, problems during following the protocol in some patients, and on deciding whether to terminate the study based on the results observed. Some of the designs made for performing comparative study are explained as:

(i) **Simple randomized design:** It is the simplest design in which the patients are randomized for two or more treatments without arranging patients in groups based on their prognostic characteristics. When the patients become available, they are assigned to one of the treatments in a randomized fashion. But the non-comparability of patients should be considered for the analysis of the response. It is the design of choice as the prognostic characteristic of the patient is unknown. Also, to assign each treatment to an equal number of patients the randomization is restricted so that only a certain number of patients are entered.

(ii) **Stratified random design:** When the patients are grouped into strata or prognostic categories and differences in outcome are expected among the strata itself then the stratification is defined and the patients are allocated with each treatment randomly. The simplest situation would be in the strata, are pairs of patients and each patient in pair receives either A or B treatment randomly. Moreover, too much stratification of patients should not be done. For example, during the study for acute leukemia age, platelet count, infection status, hemoglobin value, and type of leukemia based on prognostic characteristics and this would result in too many categories of patients to be defined. Therefore, as the number of strata becomes more, there would be only one in each stratum and the advantages of stratification will be finished. As a rule of thumb, not more than eight categories in stratification should be defined. Preferably only four characteristics should be defined as the difference among four prognostic characteristics would provide four major sources of variation and a sufficient number of patients will be available to be entered in each stratum.

(iii) **Cross-over designs:** This design is a combination of paired comparison design and simple randomized design in which each patient is used as their own control. In this, treatment is administered in sequence such as; first treatment A is administered, followed by B in half a number of patients

and administration of treatment B followed by A in the other half number of patients. A patient is randomly assigned to a particular sequence of administration of treatment. However, in cancer clinical trials, practical difficulties might be encountered as some patients might not survive for long to receive another treatment. Also, in some cases, the patient may give a very good response to the first treatment due to which longer time is needed before another treatment can be administered. Also, there is a chance of a crossover effect in which the response to the first treatment is related to the response for the second treatment. Similar studies have been performed stating and proving the above facts (Acute Leukemia Group B 1963; Frei III, E. et al., 1961).

(iv) **Factorial design:** A situation that commonly occurs in clinical trials is the number of factors that are to be studied for their relationship with the response. For example, in 2 X 2 factorial designs, two treatments administrated at two levels (high and low dosage) can be studied for their relationship with the response. This dosage-treatment combination defines four combinations for which patients are randomized. It is used to study possible interaction effect i.e. whether the difference in effectiveness occurs at both levels or not.

SUMMARY

The data collected regarding the information about the efficacy and safety of a new drug has to be analyzed thoroughly. Despite patients and physicians having a positive approach towards clinical trial participation, the potential barriers faced by the patients and the complexity of patient enrollment procedure converts a successful clinical trial into a rare event (Dignam J. J. et al., 2006). The clinical trial plays a key role in advancing cancer treatment from the research setting to a cancer care clinic. Therefore, it is also necessary to understand the nature of the trial enrolment pattern and resolve its complexity. Increasing accrual in clinical trials is highly important for multiple reasons. Faster accrual will lead to the quick conduct of the clinical trial. It is one of the most predominant reasons for the failure of clinical trials. Also, when trials stop due to failure to accrue non-financial cost increase, the research staff and patient might lose trust and morale. The more rapid completion of the trials will allow more development of new treatments (Giffin R. B. et al., 2010). Also, to increase the generalizability of the clinical trial outcome the clinical trial accrual should increase.The predominant type of trial is a Phase III trial in which comparative study is done. It measures the efficacy of new treatment from the standard one. However, the sometimes-observable difference is very low which proves that the new treatment is relatively safe as it has reached Phase III but it will not necessarily work better than previous other treatments. Phase I is found to be more dangerous as it is the first study done on humans after it has been tested on animals. Also, at Phase I the effectiveness and toxicity of a particular titerare not determined very well. Although a lot of statistically derived standard ranges have been made it is not always sure. Therefore, it is necessary for all the important aspects which affect oncology clinical trial should be considered while conducting it.Having huge number oncology-related treatments in the pipeline for getting approval, the next decade will see pharmaceutical companies and its research partners facing challenges to turn several promising scientific ideas into effective and safe medicine to treat and extend the survival span of the cancer patients.

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Chapter 25
Phytopharmaceuticals in Cancer Treatment

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ABSTRACT

Several modern treatment procedures have been received to battle malignancy with the point of limiting lethality. Phytopharmaceuticals are auxiliary metabolites of plant origin which exclusively contain one or more substances as active ingredients or might be a blend of them. Analysts have excitedly attempted to diminish the lethality of current chemotherapeutic agents either by consolidating them with herbals

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or in utilizing herbals alone. Synergy is a procedure where a few substances participate to reach a consolidated impact that is more prominent than the entirety of their different impacts. It may be viewed as a characteristic straight technique that has developed ordinarily by nature to acquire more efficacies at a low cost. This chapter aims to present the fundamental mechanism of the activity of phytochemicals in combination therapy. This chapter additionally features the remarkable synergistic impacts of plant-drug cooperation with an emphasis on anticancer strategies.

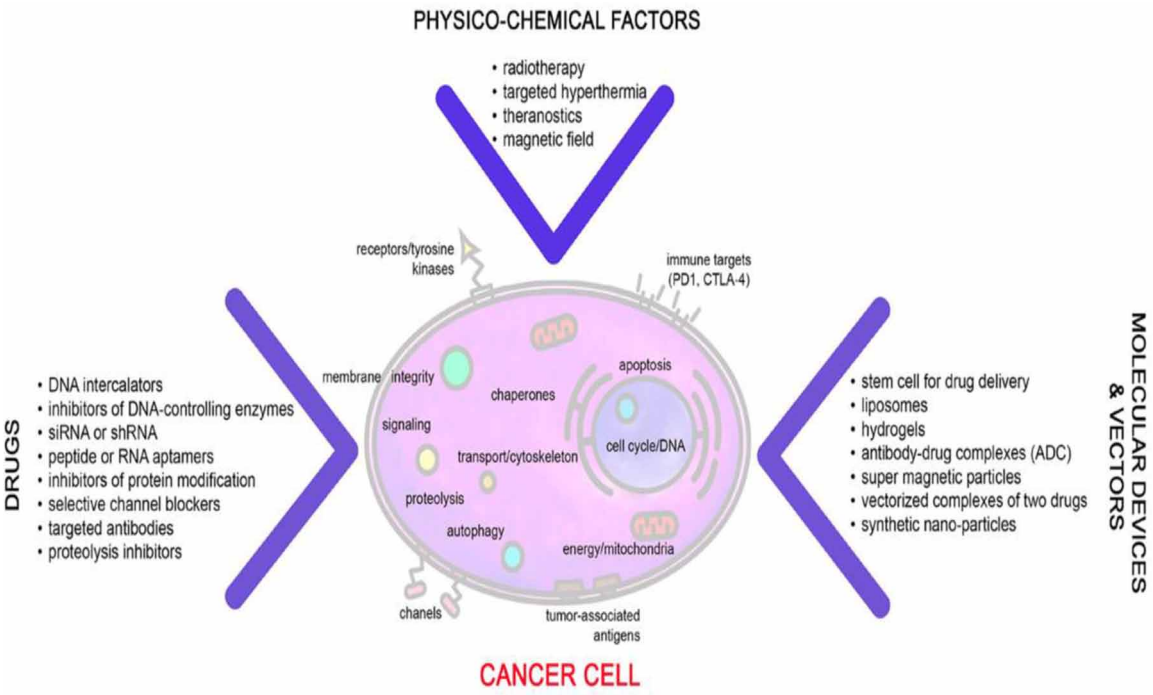
INTRODUCTION

Cancer is a complex clinical condition where various molecular pathways and cellular processes are adjusted. Each type of cancer possesses its own unique molecular finger impression and, at least one of the cancer hallmarks (Hanahan D. and Weinberg R.A., 2011), was altered. Additionally, all malignant growths have a typical common behavior based on uncontrolled expansion and invasion in spite of this heterogeneous situation. This obtrusive phenotype is the genuine clinical issue and, in most cases, still stays unresolved, leading to morbidity and mortality. Despite what might be expected, the molecular promiscuity of certain particles, particularly those from natural origin (Barrajon-Catalan E., et al., 2014), permits them to exert a potential multi-target system of action. Compound promiscuity is defined as specific interaction of small molecules with multiple biological targets representing molecular basis of poly pharmacology, an emerging theme in the field of drug discovery and chemical biology. Promiscuity isn't constantly because of a solitary compound however a blend of compounds, as occurs in some complex natural concentrates. These compounds can interact with various targets, adjusting various pathways or various steps of a similar signaling cascade in cancer. In these cases, each compound can collaborate with one or numerous targets, expanding the pharmacological promiscuity of the whole medication or drug. Also, natural concentrates or their fundamental component can be combined with customary chemotherapy, lessening the development of resistance to antitumor medications and harmful toxic impacts (Rejhová A., et al., 2018).

Cancer, with more than hundred distinct types, is considered as a complicated malady because of the uncontrolled wild expansion of tumor cells and the capability of attacking other tissue through the blood and lymphatic system (Sivin N., 1993). Conventional chemotherapy for treatment of malignant growths, albeit very viable, has been associated with toxicities to normal tissue and organs, which is also a significant dose limited factor. Besides, chemo-resistance is another significant deterrent for successful treatment of cancer (Castaigne S. et al., 1990). There is board disappointment with respect to surgical medical procedure, radiotherapy, and particularly chemotherapy and consequently, treatment of cancer is being re-examined worldwide. The conventional model believed so far that the malignant phenotype is driven by a predominant signal transduction pathway is getting progressively unacceptable. This is because of the presence of resistance to target-and mechanism-based drugs, and hence reflects the hereditary flexibility of the malignant cell genome as well as the redundancy in the pathways that govern kinase signal transduction networks (Warrell R.P. Jr et al., 1991). Based on this, the conventional mono-target chemotherapy protocol for cancer treatment is getting progressively inadequate and may lead cancer cells to develop acquired drug opposition because of the complex signaling pathways associated with cancer (Chen Z.X. et al., 1991). The multi component treatment, in which more than one drugs were utilized simultaneously, is the demonstrated cure for cancers (Compton M.M., 1992). The idea of combination of drugs, with comparable or various modes of action, attempts to bring about synergistic

or addictive restorative impacts, including expanded therapeutic effectiveness, diminished host lethality, and negligible or deferred drug resistance (Figure 1) (Mahmoud N.N. et al., 2000). Drugs that contain a several dynamic components have been being used quite a long time ago. Numerous traditional medications, including the Unani and Chinese medicine, have utilized blends of naturally occurring herbs or natural herbal extracts (Lee Y.J. et al., 2000). Cancer is a complex illness which includes distinctive signaling pathways and in this manner combination therapy in which one or more drugs are utilized simultaneously is the demonstrated remedy for cancer (Doos L. et al., 2014).

Figure 1. Figure depicting drugs, physico-chemical factors, molecular devices and vectors tested in combinatorial treatment approaches of cancer



Multidrug treatment is a useful methodology concentrated on the immediate blocking or killing of harming agents, for example, cancer cells or pathogens as well as on the actuation of human body defenses or repair mechanisms. The concept derives from a gradual withdrawal of the previously adopted dogma, for decades, of mono-drug treatment; pharmacological research was depended on the recognizable proof of a solitary dynamic guideline (Obodozie-Ofoegbu O. et al., 2012). With respect to phytotherapy research, recently the commitments of traditional Chinese, Unani medication, Ayurveda and traditional medication, western phytomedicine started to be scientifically affirmed and acknowledged. Besides, during the last 20 years, the world has experienced an expanding pace of the utilization of customary drugs joined with comparative and alternative medicine (CAM), which are represented not just by homeopathy, naturopathy, chiropractic, and energy medication, among others, yet in addition ethno pharmacology and phytotherapy (Katselou M.G. et al., 2014). It is turning out to be clear that numerous diseases have a multifaceted etiology, which could be dealt more effectively with a drug combination

system than a solitary administration. In Western nations, for multifactorial or complex disease treatment e.g., cancer, hypertension, metabolic and inflammatory illnesses, AIDS and infections, a compelling multidrug treatment therapy is normally adopted (Katselou M.G. et al., 2014). In this unique situation, phytotherapy and ethno pharmacology play a key role, as they discover their effectiveness from herbs or plants, which are "secundumnaturam", a complex pool of a huge number of molecules. Of note, human pharmacotherapy began with the utilization of plants in ancient times, most likely copying animal self-medication (Petrovska B.B., 2012).

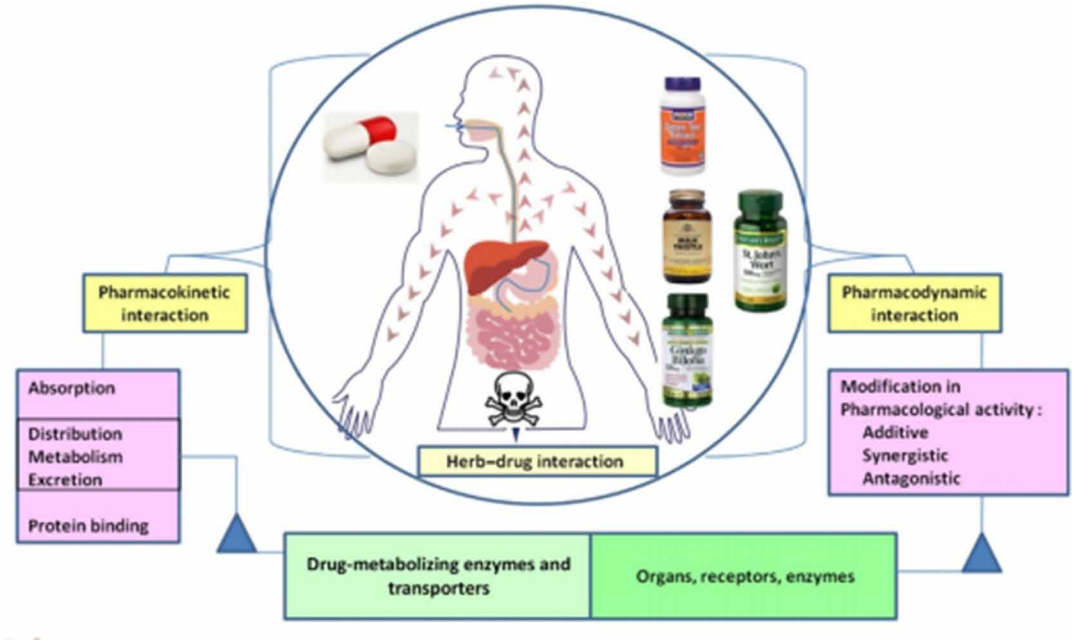
Historically, plants have given a wellspring of motivation for novel drug compounds and have demonstrated extraordinary guarantee in the treatment of ailments. The incredible varieties of auxiliary metabolites from plants have been sources of commercially significant pharmaceutical compounds. Some 41% of recently affirmed drugs between the period 1983–1994 had a characteristic natural product origin and this expanded to 60% while thinking about anti-infective and anticancer compounds (Cragg G.M. et al., 1997). At first natural products were utilized in unmodified form, as in the form of concentrated herbal concentrates. It turns into a complex issue when utilizing an herbal product, since its action isn't for the most part because of a solitary entity however because of a blend of different constituents. For instance, green vegetables and fruits were found to diminish the risk of malignant growth, widely because of the activity of a combination of polyphenols (Mertens-Talcott S.U., and Percival S.S., 2005). A wide variety of compounds may either improve (synergistic) or decline (antagonistic) the therapeutic action or lethality of drugs. Phytomedicine have been in human use since time immemorial. It has played a crucial role in therapeutics when employed in combination. (Dash B. and Junius M. et al., 1987). Thus, human require greater wisdom as the plants have multiple metabolites for their survival and longevity. The fundamental role of auxiliary metabolites in advancing plant survival. They serve in plant defense mechanisms and guarantee a reduction in the possibility of developing to pest and microorganisms. There is an expanded utilization of herbs along with conventional drugs rather than utilizing them in place of drugs, raising concerns for the investigation of interactions between the herbs and drugs. Four points can be put forward to clarify the expanded utilization of drug combinations. To start with, first is the expansion in the expense of medicinal services at health care, drug costs and the number of patients. Second is an increase in multidrug resistant strains has prompted a quest for alternative methods of treatment. Third, the diminished adequacy and treatment failure of current modern medications likewise favors the utilization of herbs. At last, because of the complex multiple interconnected nodes of the cell signaling system, it is imperative to utilize numerous modulating methodologies to achieve clinical progress. Phytomedicine can accomplish this strategy via exerting advantageous impacts through addictive or synergistic actions of a number of chemical compounds acting at single or multiple target destinations related with a physiological process.

Combined Therapies and Synergy

As defined by the Mosby's Medical Dictionary (2008), pharmacological synergy or synergism is a joint activity of two or more molecules (drugs) in such a way, that one improves the activity of the other to create an impact more prominent than that which might be acquired with either one of the molecules in proportional quantity or produce impacts that couldn't be obtained with any safe quantity of either molecule, or both. Potentiation, another term utilized in receptor pharmacology, is a synergistic activity where the impact of two medications given at the same time is more prominent than the entirety of the impacts of each medication given independently. The mechanisms involved in Herb-Drugs interaction

were graphically represented in Figure 2. Berenbaum was among the first to characterize pharmacological synergy and carried out spearheading work in the clarification of molecular potentiation between two drugs and drug collaborations (Berenbaum M C., 1984). He presented and developed the "isobole method" which is an important tool likewise in the investigation and study of Phytopharmaceuticals (Wagner H., 2011). Promoters of natural drugs have consistently been searching for synergies and were constantly stressing on the point that in phytotherapy the mixtures make the drugs. But, is the chemical composition of herbal medications or botanical drugs being addressed, and would one be able to be certain that nature gives us with the perfect ideal mixtures? Unmistakably, plants don't produce secondary metabolites to profit vertebrates but produce them to conceivably address the diverse environmental pressures, for example, microbial or predator attacks. Plants often react to a stressor by expanding the biosynthesis of various classes of molecules rather than an individual secondary metabolite (Shiojiri K. et al., 2010). This might be an intricate response to a particular predator attack (Kessler A., and Baldwin I. T., 2001). Or then again mixtures may essentially influence the solubility and distribution of the conceivably active constituents. Considering absorption and dissemination (how to get the toxic substance into the predator) mixtures can be ideal. For instance, the ichthy toxiclignans justicidin B and piscatorin in the Amazonian fish poison plant Phyllanthus piscatorum L. are promptly solvent in water when administered in the plant extract but almost water insoluble when purified (Gertsch J. et al., 2003).

Figure 2. Graphical representation of mechanisms involved in Herb-Drugs interaction



Synergy is comprehensively defined as the association or cooperation of two or more substances, organizations or different agents to deliver a combined impact more prominent than the sum of their separate segments (Tennakoon P.L.K., 2007). The word Synergy was derived from the Greek word "synergos", which signifies "working together." More accurately, synergism or synergy as per the

McGraw–Hill Concise Dictionary of Modern Medicine is demonstrated as "the cooperative interaction between at least two or more components of a framework, to such an extent that the combined impact is more noteworthy than the sum of each part".

In terms of anatomy, it is the joined activity of muscle groups which brings about a force more prominent than that which could be produced by the individual muscles, while in pharmacology, synergism is depicted as an approach to deal with multidrug-resistant bacterial infections or virulent malignancies, among others, in which the utilization of remedial agents may influence various pathways, making the treatment increasingly productive (Segen J.C., 2005). Hence, synergism or pharmacological synergy is the impact of combined components associating to generate new and unexpected impacts in comparison to singular components, referring ordinarily to the activity of entire plants, rather than the active constituents in isolation (Jonas W.B., 2005). As the above proclamations obviously appear, the meaning of synergy may overlap to potentiation, a term that implies a synergistic activity, wherein the impact of two medications directed at the same time is more noteworthy than the sum of the impacts of each medication given independently (Jonas W.B., 2005). Nevertheless, a concrete definition can be derived only from numerical methodology, showed and demonstrated by spearheading works of Berenbaum, which provided the basis to its utilization in pharmacology and phytopharmacology (Berenbaum M.C., 1953). The isobole strategy of Berenbaum is certainly not a unique technique that emerged from applied sciences; various procedures have been also developed to comprehend drug-drug collaborations, for example, the isobologram technique of Loewe (Leyden Webb J., 1963), the fractional product technique of Webb (Chou T.C. and Talalay P. et al., 1984) and the combination index technique for Chou and Talalay (Wagner, H. et al., 2009).

In view of the classic pharmacological dogma "one drug-one target", monotherapy has been the customary methodology, not exclusively to treat diseases, yet in addition to discover new dynamic medications or drugs against a chosen target. Nowadays, there is evidence bringing up that combined treatments are substantially more effective than single-drug-based medications. In this sense, combinational therapy is extended out to treat not only malignant growth but also different illnesses, for example, AIDS, bacterial contaminations, hypertension, metabolic or rheumatic disorders (Roell, K.R. et al., 2017). Combined drug therapy design is a hard and challenging task in which individual and combined activities must be portrayed and it some of the time requires new preclinical and clinical trials. Additionally, these different comparisons are sometimes hard to incorporate into those studies. Combined treatments are typically based on co-administration of at least two or more medications. These combined drugs can be based on the combination of unadulterated compounds, or can be accomplished by utilizing drugs dependent on mixtures of natural concentrates. Synergy is subsequently the most significant characteristic of combined therapies, including natural extracts. The primary driving force for synergy research originates from pharmaceutical legislation which demands the confirmation that each compound of a combined pharmaceutical preparation contributes to the claimed total adequacy (Roell, K.R. et al., 2017). In terms of pharmacology, synergy is the capacity of certain mixtures to be more potent than the sum of their individual parts. This definition is exportable to different disciplines and depends on the reciprocal as well as additive mechanism of action. Synergy isn't an absolute factor and the pharmacological interaction between the components of a mixture can be more or less synergic. This aspect is the primary distinction among additive and synergic behavior and is sometimes overlooked by analysts and clinicians. Another aspect to be investigated is that a single mixture can act in various manners relying upon the proportion of its constituent parts. Hence, various proportions of similar compounds could give various outcomes as far as synergy is concerned, not only in an absolute way, but also in terms of being more or less synergic

(Chou, T.C. et al., 2006). In this sense, a perfect proportion which gives the most noteworthy synergy results is always mathematically possible.

There are a number of different ways to create synergy studies, yet in all cases, a past and detailed design is required to acquire indisputable outcomes. Most synergy studies fail on account of a lacking design, both in qualitative and quantitative ways. There are various comprehensive and relevant reviews on synergy (Stoddart, M.J. 2011); however, some aspect about study design is a point of concern. First is the selection of drug of choice. The correct choice of the drugs for synergy studies is considered as the initial step to succeed. There are numerous accessible drugs for a solitary disease, yet all are not appropriate for a synergic study. The drugs must be selected considering various molecular targets. If not, antagonism or other undesired pharmacological collaborations can be acquired. These diverse molecular targets can be located in various molecules, in recognized epitopes of the similar molecules or even in molecules of various pathways. Second is the synergy study method. As referenced above, there are various strategies to study synergy between drugs. Quantitative strategies, for example, Combination Index (CI) (Johri, R.K. and Zutshi, U. et al., 1992) or Fractional Inhibitory Concentration Index (FICI) calculations are favored as they get better conclusions. Third is the Biological assay. According to the chosen strategy and method for synergy studies, a powerful, dependable and reliable biological assay must be chosen that permits testing a large number of samples with an enormous variability in composition. Endurance or viability tests are diverse and permit high throughput screening approaches (Ernst, E. and Pittler, M.H., 1999). They are generally utilized for anticancer compound research, yet additionally also for the other different areas of drug discovery where synergy is topical, for example, antimicrobial drug discovery. The fourth one is sample testing. Once the appropriate test is selected, a satisfactory design of the plates is additionally essential. The Check-board plate design is most likely the best methodology for pair-wise combinations utilizing multi-wells plates. This technique can be utilized not only for pair-wise combinations but also for three-drug combinations. Following these suggestions, the conclusive outcomes won't just be scientifically important, yet additionally comparable to other single medication or combination treatments. This will also permit specialists and clinicians to get better conclusions and contributes to the development of new remedial methodologies. References to synergic interactions between drugs in cancer investigation and research were bottomless in terms of the list of sources. In any case, focusing on natural extract synergy research, three primary groups of examples can be classified. The first group incorporates research covering complex concentrates whose components present synergistic interactions among them. The second groups incorporate instances of synergy between various concentrates and natural compounds of various origins. At last, the third group contains instances of anticancer approved drugs combined with natural compounds or concentrates. Some of the most pertinent instances of each category are listed below in Table 1.

Generally, synergistic impacts are considered as positive, in low doses they were perceived as an advantage, despite the fact that, there may likewise negative aspects also. Adverse reactions (ADR's) in general will be more evident with combination of herbs or interactions with recommended synthetic medicines, however clinical manifestations don't seem to be similar, which might be expected mostly due to an absence of reporting of ADR's for herbals. Significant positive interactions would incorporate those of Ayurveda, which utilizes many fixed combination formulae with "Trikatu" highlighting in a large number of them. This mixture contains dark pepper, Piper longum, and ginger, Zingiberofficinalis and albeit an ancient recipe, it is recently reported that this combination has been explored scientifically and reasons were set forward for its inclusion. Pepper contains the alkaloid piperine, which is known to increase the bioavailability of various drugs, for example, vasicine (otherwise called peganine); an antiasthmatic

Table 1. Examples of synergic interactions among compounds and approved drugs in relation with cancer research

Extract/Compound	Synergy	Experimental Model (Cell Line)	Effect	References
Pomegranate extract	Among their compounds	Oral cancer (KB, CAL27), colon cancer (HT-29, HCT116, SW480, SW620) and prostate cancer (RWPE-1, 22Rv1)	Antiproliferative, apoptotic and antioxidant	Wagner, H., Ulrich-Merzenich, G. et al., 2009
Grape extract	Among their compounds and with Ara-C and tazofurin	Leukemia (HL-60)	Antiproliferative and apoptotic	Chou, T.C., 2006
Rosemary extract	Among their compounds	Colon cancer (HT-29)	Antiproliferative	Johri, R.K. and Zutshi, U., 1992
Ginger extract	Among their compounds	Prostate cancer (PC-3)	Antiproliferative	Ernst, E. and Pittler, M.H., 1999
Graviola flavonoids	Among their compounds	Prostate cancer (PC-3)	Prostate cancer (PC-3)	Chou, T. C., 2006
Turmeric extract	With rosemary compounds	Breast cancer (MDA-MB-453, MDA-MB-468, and MCF7)	Antiproliferative, G1 cell cycle arrest	Webb J.L., 1963
Tea extract	With capsicum compounds C	Cervical cancer (HeLa) and breast cancer (4T1)	Antiproliferative	Chou T.C. and Talalay P., 1984
Resveratrol	With Cisplatin and doxorubicin	Acute leukemia (ML-2/DX30, AML-2/DX100 and AML-2/DX300)	Antiproliferative	Zutshi R.K. et al., 1984
Quercetin	With doxorubicin, cisplatin, arsenic trioxide and temozolomide	Neuroblastoma and Edwing's sarcoma cell lines, laryngeal cancer (Hep2), leukemia (U937 and HL-60) and astrocytoma	Antiproliferative	Zutshi R.K. et al., 1984
(-)epìgallocatechin-3- gallate	With doxorubicin, gemcitabine and cisplatin	Carcinoma doxorubicin resistant (KB-A-1 xenograft), cholangiocarcinoma (Mz-ChA-1 cell line and xenograft) and ovarian cancer (SKOC3, CAOV3 and C200)	Antiproliferative	
Curcumin	With 5-fluorouracil, oxaliplatin, cisplatin, etoposide, camptothecine and doxorubicin	Colon cancer (HT-29), ovarian cancer (2008 and C13) and human and rat glioblastoma cell lines	Antiproliferative	
Genistein	With cisplatin, 5-fluorouracil, arsenic trioxide, doxorubicin, gemcitabine camptothecine and hidroxi-camptothecine	Pancreatic cancer (BxPC-3 xenograft, COL-357 and L3.6pl) colon cancer (HT29), hepatic cancer (HepG2, Hep3B, SK-Hep-1, HEpG2 xenograft), cervical cancer (HeLa) ovarian cancer (OAW-42), bladder cancer (TCC-SUP) and lung cancer (ME-180pt, UMSCC-5)	Antiproliferative	
Carothenoids	With other phytochemicals	Prostate cancer LNCaP, PC-3 and DU-145) and breast cancer (MCF-7)	Antiproliferative	Mujumdar A.M. et al., 1999
Taxifolin	Andrographolide	Prostate (DU145)	Increase in cell cycle arrest at G2/M and apoptosis	Stoddart, M.J., 2011
5-Fluorouracil	combined with leuteolin or quercetin	Colorectal (CO115)	Activation of the apoptotic mitochondrial pathway	Zutshi R.K. et al., 1984
Curcumin	combined with cisplatin or oxaliplatin	Ovarian cancer cells	Increase induction of apoptosis	Shoba G. et al., 1998

alkaloid from Adhatodavesica (Chou, T. C., 2006). Undesirable interactions for instance would be the presence of tannins in an herbal drug, which may prevent the proteins absorption and alkaloids, or the induction of enzymes, for example, cytochrome P450 which may accelerate drug metabolism bringing about blood levels of actives unreasonably low for a restorative impact. This could have more genuine consequences, for instance in case of St. John's Wort (SJW), Hypericumperforatum extract, where interactions with oral contraceptives have been accounted, but inconsistently. For additional references negative interactions of herb drugs with synthetic medications has been reported by Ernst et al.,1999 (Webb J.L., 1963). Anyway, the synthetic drugs involved are generally notable for their capability to interact and patients taking them are cautioned not to combine their medicine with some other drugs, except if medical supervised. The most significant medications from this point of view are cyclosporine utilized as an immunosuppressant after transplantation, warfarin as an anticoagulant and the protease inhibitors used to treat HIV infections. Additionally, herbal products with reputed synergistic activity should not be utilized, if they are potent herbs utilized in conditions, where the dose is crucial factor. Foxglove, Digitalis, is certainly not an appropriate herb for congestive cardiovascular breakdown and heart insufficiency grade I and II as indicated by the NYHY, as the therapeutic index is so low, however hawthorn surely be used as a result of its progressively delicate, cumulative, and presumably synergistic effects.

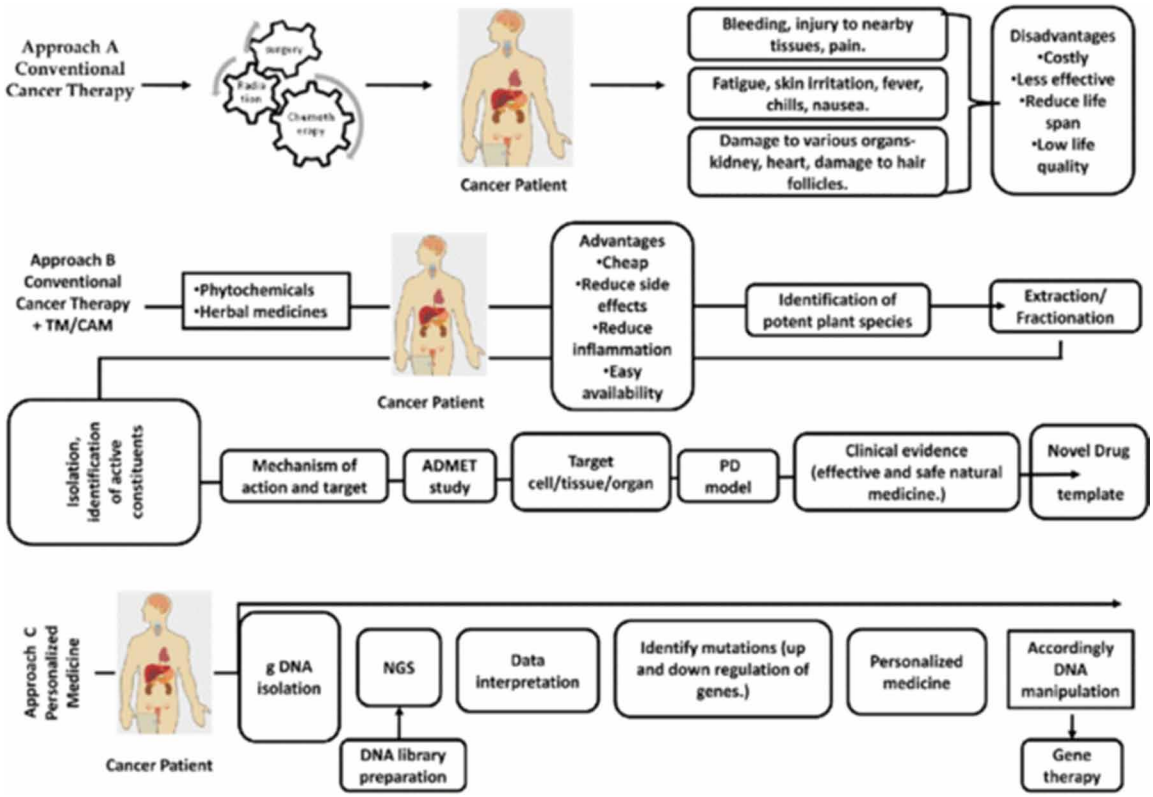
Plant and Drug Interaction

A new trend set by the cancer scientists of this era towards cancer research is combinatorial therapy utilizing two or more drugs regardless of their source. The simplified thought behind this treatment is that the combination of drugs (at appropriate concentrations) will create impacts that are more than the impacts anticipated from their summated singular potencies, characterized as synergy. In fact, synergy is a theoretical concept that refers to an outcome that emerges as a result of interacting processes. It can to be defined as the production of whole that is more noteworthy than the simple sum of its parts. The meaning can be given as working together. This can be best clarified by the graph called Isobologram. Isobologram is the graphical portrayal of two or more medications which gives the individual and combined effect of both the drugs. The hypothetical basis for the combination index (CI)- Isobologram equation that permits quantitative determination of drug interactions, where CI < 1, $= 1$, and > 1 show synergism, additive impact, and opposition or antagonism respectively. In view of these algorithms, computer software has been developed to permit robotized simulation of synergism and antagonism at all doses or affect levels (Chou T.C. and Talalay P., 1984). Synergy primarily relies upon the additive of two medications. This is appropriate when the two medications act upon the same system, so that most maximum response incited by both the drugs is same. The rule is that the natural response of combinations of two drugs equals to sum of two fractional responses minus their product. By chance the fractional response to sedate A alone at any specific dose is Fa, then for medicate B alone it is Fb. The additional response to drug B is the fraction Fb times the remaining possible response, which is 1-Fa, so the additional response because of medication B, in the presence of drugs A equivalents Fb*(1-Fa). Hence, the total response to a blend of two medications is Fa+Fb (1-Fa) = Fa+Fb-Fa*Fb. This equation assumes that the impacts of two drugs is additive. The proof of synergistic combinations and their ideal dose ratio depends on experimentation or on trial and error. An assortment of techniques was being utilized to examine the interaction of medications in combination, and for a similar set of data, the strategies can give discordant outcomes. The coexistence of a various techniques likewise doesn't imply that they are similarly legitimate. Various approaches in particular the isobologram technique (Leyden Webb J.,

1963), the fractional product method of Webb (1963) (Mertens-Talcott S.U. and Percival S.S., 2005) and the combination index strategy for Chou and Talalay 1984 and Roby C.A. et al., 2000, are commonly used to determine drug-drug associations. Most investigations have used quantitative techniques to identify modifications in cell cycle and proliferations, and apoptosis of the cells. These investigations depend on the expression of genes, for example, NF-κB, Ap-1 and other apoptotic genes; the caspases activity; DNA fragmentation and cytochrome-C analysis (Racker E. et al., 1986). Personalized approach using various phytochemical compounds provides a new dimension to the standard cancer therapy for improving its outcome in a complex and complementary way (Figure 3).

Combination of drugs can cause clinical issues. For instance, some herbal medicines may contain inorganic contaminants, for example, arsenic, lead, mercury or intentionally included pharmaceuticals, which can build the potential for antagonistic drug interactions. A large portion of the herbs contain active constituents that produce side-effects, for example, cardiotoxicity and hepatotoxicity, may create cancer-causing metabolites or alter the metabolism. For instance, the tea produced using the herb Larrea tridentate isn't suggested for the above said reasons. St. John's Wort, a broadly utilized herbal product, has been found to be metabolized by cytochrome P450 by expanding the CYP3A4 action (Poppenga R.H., 2002). The greater parts of the herbal formulations are known to improve the cytotoxic impact of drugs through unknown systems of mechanisms. Calcium channel blockers have been found to build up the toxicity of synthetically manufactured anticancer medications, for example, paclitaxel (Bano G.

Figure 3. Approach A: modern approach widely used by physicians including surgery, chemotherapy and radiation therapy



et al., 1991). Herbs like Angelica sinensis or Zingiberofficinale may expand the toxicity of anticancer medications since they are rich in calcium chain blockers (Mujumdar A.M. et al., 1999).

Approach B: hybrid therapy including modern cancer therapy and traditional/complementary alternative medicine (active secondary metabolite with chemotherapy); Approach C: customized medicine including potential opportunities for cancer therapy.

Numerous kinds of herb–drug collaborations can present genuine medical issues in patients. The interactions could be pharmacokinetic or pharmacodynamic in nature. Pharmacokinetic collaborations bring about altered absorption, distribution and elimination of the medication. The interaction can change the gastrointestinal motility, compete for plasma binding, restrain biotransformation and compete for renal tubular secretions. For instance, the cytochrome P-450 isoenzyme framework, the most well-known system of metabolism, can adjust the availability of theophylline, caffeine and so on, subsequently prompting a decline in the remedial response. Inhibitors of the cytochrome enzyme framework should be avoided when utilizing theophylline treatment. In the conventional Indian system of medication, piperine in 'Trikatu' is known to improve the bioavailability of drugs, for example, theophylline (Zutshi R.K. et al., 1984), oxyphenyl butazone (Lambert J.D. et al., 2004) and rifampicin (Shoba G. et al., 1998). It has been exhibited that 'Trikatu' meddles with the pharmacokinetic process in case of phenyl butazone (Lambert J.D. et al., 2004). Piperine restrains the glucuronidation of EGCG (epigallocatechingallate) in the small intestine as well as slowing back the gastrointestinal transit. This builds up its availability and residence time in the intestine allowing more prominent assimilation. The expansion in the bioavailability of EGCG in plasma may improve its cancer preventive activity in-vivo (Reddy L et al., 2003). Shoba et al., (1998) and De Vita Jr V.T. et al., (1975) demonstrated that co-administration of piperine and curcumin to humans and rodents upgraded the bioavailability of curcumin by 2000% and 154%, respectively, because of a inhibition of the glucuronidation of curcumin as the phenolics are heavily metabolized as glucoronide conjugates before arriving at the plasma (Kumi-Diaka J. et al., 2004). Pharmacodynamic interactions cause alterations in the way, a drug or natural medication influences a tissue or organ system. These interactions influence drug activity in a qualitative manner, either through an enhancing impact (synergistic or additive activities) or an antagonizing impact. The combinations are viewed as to be synergistic, if the viability is more noteworthy than the impact of either the agent alone or the sum of the impacts of the individual agents and antagonism is less than the expected impact of the combination (Grem J.L. et al., 1950). Combination treatment with genistein (isoflavone from soy) and β-lapachone (a simple plant product) likely involves various systems of activity or mechanisms in inducing apoptosis in human prostate adenocarcinoma PC3 cells.Caspase-3 is the primary target in genistein-induced apoptosis and NAD (P) H: quinine oxido-reductase (NQO1) in β-lapachone actuated apoptosis in PC3. Trial information reported that NQO1 is the principle target in β-lapachone-genistein combination incited apoptosis and is more effective in combination than single drug treatment (Boik J. et al., 2001). Interactions that happen at a similar receptor site are typically inhibitory, whereas interactions including distinctive receptor destinations may restrain; inhibit or potentiate the process. 5-Fluorouracil inhibits thymidylate synthase in the presence of 5, 10-methylenetetrahydrofolate. Leucovorin and 5-fluorouracil follow up on the similar target but the former increases the 5, 10-methylenetetrahydrofolate concentrations accordingly improving the cytotoxicity of the latter displaying synergy (Ackland M.L. et al., 2005).

Flavonoids and different antioxidants when utilized alone could produce useful, negative, or insignificant impacts in cancer patients, while if there are combined with other anticancer compounds (i.e., natural compounds or chemotherapy sedates), their efforts are bound to be helpful or else at least not harmful (Xavier C.P. et al., 2011). Mertens-Talcott et al., researched the combinational impact of

quercetin and ellagic acid on cell death in the MOLT4 human leukemia cell line. The two compounds together diminished more the proliferation and viability and enhanced the induction of apoptosis contrasted with each alone (Racker E. et al., 1986). In another study, the combination treatment of human gut (HuTu-80 and Caco-2) and breast malignant cells (PMC42) with quercetin and kaempferol was more powerful than the additive impacts of each flavonol (Tyagi A.K. et al., 2002). Other investigations have researched the impact of combination of chemotherapeutic medications with flavonoids. The treatment of colorectal tumor (CO115) with 5-Fluorouracil combined with leuteolin or quercetin expanded apoptosis with a huge impact for quercetin which included the activation of the apoptotic mitochondrial pathway (Raina K. and Agarwal R., 2007). In another investigation, the flavonoid silibinin strongly synergized the anti-proliferative impact of doxorubicin in prostate carcinoma DU145 cells. This combination was linked with an expansion in G2/M arrest and apoptosis contrasted with treatment of each compound alone (Montopoli M. et al., 2009). Silibinin likewise bears synergistic cytotoxic impacts when linked with chemotherapeutic drugs against breast and lung cancer cells (Zhang Z.R. et al., 2013). Curcumin was additionally demonstrated to be successful in combinational treatment. The blend of curcumin with either cisplatin or oxaliplatin expanded significantly the cytotoxic impact on ovarian cancer cells by expanding apoptosis (Gonzalez-Vallinas M. et al., 2013). In a recent study, the flavonoid Taxifolin synergized the impact of Andrographolide by expanding the cell cycle arrest and apoptosis in DU145 cells (Osiecki H., 2002).

Benefits of Combined use of Phyto-compounds for Prevention and Treatment of Cancer

Phytochemicals have been proved to be advantageous in treating or forestalling number of maladies including malignant growth due to their pleiotropic functions that empowers them to exert their activity in every stage of the diseased condition, for example, regulating cell cycle control, avoidance of apoptosis, autophagy, mitotic catastrophe, angiogenesis, invasion and metastasis, and genetic modulation. It is well studied fact they play crucial role in challenging different types of malignant growths both as individual molecule and in combination with conventional medications, for example, radiotherapy, chemotherapy, and photodynamic treatment (Kufe D.D. et al, 2003). In any case, unregulated utilization of such phytochemicals and their combination with chemotherapeutic agents may bring about number of antagonistic impacts that can possess hazard to the well-being of the patients (American Cancer Society). Hence, extreme consideration must be applied in the selection of proper combinations of phytochemicals to exploit their remedial advantages. In spite of enormous reports available on molecular mechanisms of phytochemicals, truthfully, only few molecules have been considered for clinical preliminary trials. A malignant growth cell is comprehensively considered as a biological entity described by constant uncontrolled growth and unusual proliferation. This sort of cell may create a tumor, an irregular mass of tissue, which might be solid or not, that can be differentiated as malignant or benign forms (Gupta D. et al., 2005). While the benign structure is generally non-dynamic and not harmful, malignant form may develop rapidly, invade and metastasize, and conceivably bring about death. Indeed, cancer is the second-driving reason for death in the USA and developed nations, pushing numerous specialists and doctors to endeavor to battle against malignant growth (El-Mowafy A.M., 2012). In this context, it is imperative to utilize multiple techniques to achieve clinical progress, in order to have impact on human survival and to improve life quality. One option is phytomedicine, which conceivably have advantageous impacts when appropriately associated with traditional drugs, both as a preventive methodology or target-

oriented strategy. The synergistic relation between plant-derived bioactive and conventional chemotherapeutic agents may lie in their mechanism of resistance, specifically the capacity of natural subsidiaries to antagonize drug opposition or to upgrade drug properties, or conceivably in the mitigation of side effects. Several plant-derived products have been researched in relationship with conventional medications to find a synergistic impact and it is noted that 20–30% of frequently utilized chemotherapeutics has been derived from plants (Torre L.A., et al. 2015). Synergistic combinations require the utilization of two compounds together, i.e., association in a similar analysis (mixing) of a chemotherapeutic agent and plant subordinates, and not just the comparison or the utilization of substances in parallel. The word synergy is derived from the Greek words synergia, which signifies 'cooperation', and synergos, which signifies 'working together'. Plant-derived agents may well elicit sole cytotoxic consequences on their own in cell-and animal based malignant growth models. Such envisions have been advanced and turn out to be broadly acknowledged, in clinical trials and Chinese-herbal medication, particularly with PM-optimization by means of recent omics and nanotechnology-based studies (Fitzgerald J.B. et al., 2006). The complexity of malignancy development and advancement hinders viable cancer treatment utilizing chemotherapy alone. Thus, looking for synergistic combinations of chemotherapeutic medications and different drugs can be a promising method to improve prognosis, quality of treatment and in general overall responsiveness to chemotherapy. Synergic combinations of chemotherapy with PMs or Chinese-herbal formulae that are known for anticancer potential are structured and expected for improving adequacy, lessening toxic impacts, optimizing anti-tumor immune reaction, or limiting cancer resistance to chemotherapy (Zhao L. et al., 2006).

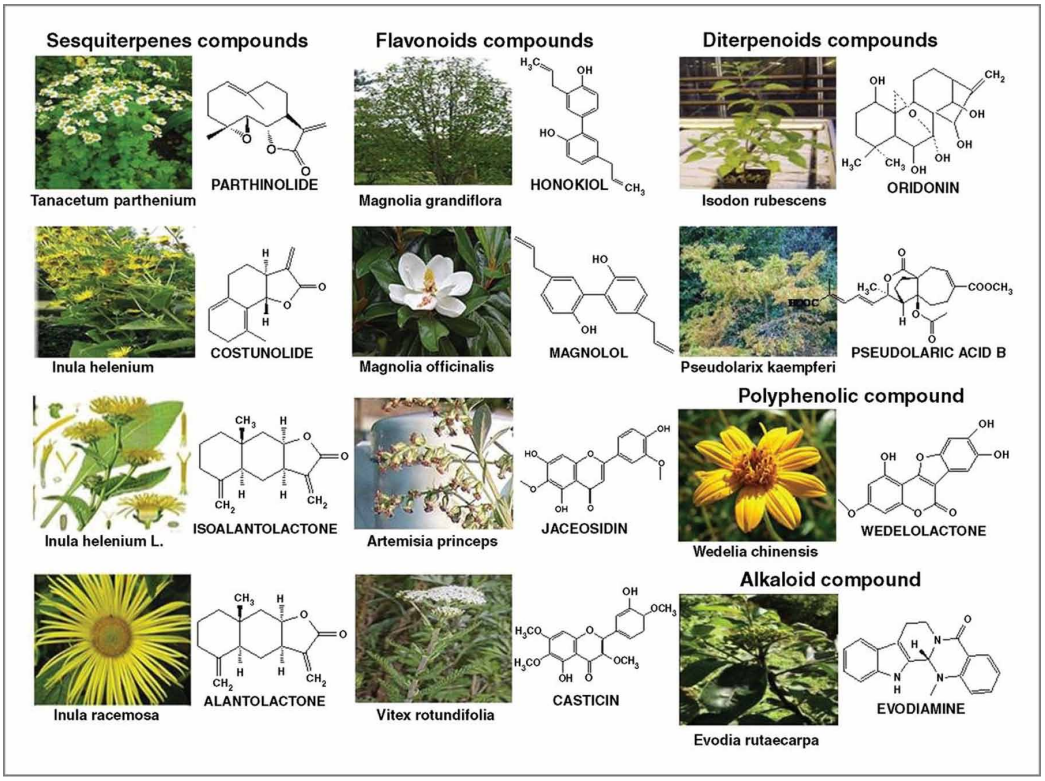
Combination or multi component treatment, where two or more medications are utilized together, typically has one or more of the following targets. First is to reduce the recurrence of acquired opposition and resistance which may emerge by combining drugs with minimal cross-resistance. Second, is to bring down the dosages of medications with getting a comparative remedial impact in order to achieve efficacy with less side-effects. Third is to sensitize the cells to the activity of one drug via the use of another drug (chemosenstization), this is often accomplished by adjusting the cell-cycle stage or development properties. Lastly is to accomplish an enhanced effectiveness through additivity, or even better, through synergism (Chou T.C., 2006). Assessment of the impact of drug mix is significant in every aspect of medication especially in cancer chemotherapy where combination treatment is generally utilized. The in vitro investigations are typically used to decide the nature and quantitative degree of drug combination (Tallarida R.J., 2001). The blend of two drugs can give synergism, antagonism or additive impact. Synergism implies that a blend of two medications produce a remedial impact more prominent than each of the two medications alone and more than additive impact (more prominent than the algebraic sum of the aggregate of the parts), while antagonism is an impact which is less than additive (Yu L.L. et al., 2011). The two techniques which are ordinarily utilized in the examination of drug combination impacts are the isobologram and the combination index (CI) where the CI strategy is the most generally used (Tallarida R.J., 2001). The isobologram technique depends on the Loewe additivity model which assesses the association at a chosen effect level and is subsequently valuable to examine the drug collaboration at the corresponding concentration, generally the median-effect concentration (Rahmani A.H. et al., 2014). However, the CI technique depends on the median-effect concentration inferred by Chou. The median-effect equation associates the drug portion and cytotoxicity or cytostatic effect (Roby C.A. et al., 2000). A software program to calculate combination indices (CI) is accessible and broadly used (Yu L.L. et al., 2011).

The standards of choosing drug combination have been based on drugs acting up on the same target however through various mechanisms, drugs acting up on different cell targets by means of a similar mechanism system; or drugs working on different molecules by means of various avenues. Various such combinations have been first endeavored among Chinese herbs that were accounted for in malignant growth cells, and indicated multifaceted promising results. For example, curcumin has been set up as a multi-targeting phytomedicine that shows mitigating, antioxidant, anti-inflammatory and chemotherapeutic impacts; while shows no or insignificant toxicity in animals, when utilized even at such elevated concentration of dosages (Cai Y.Y. et al., 2013). Moreover, curcumin tweaks the cellular levels of tumor silencer genes, apoptotic genes, articulation of oncogenes, and their respective effectors, for example, enzymes and receptors signal adaptors (Guo J. et al., 2013). In this way, numerous Chinese herbs have been combined with curcumin to look for synergy. When curcumin was utilized with triptolide, they advanced apoptosis in ovarian malignancy cells, impacts that were attributed due to deactivation of some heat-shock proteins; HSP27 and HSP70 (Masuelli L. et al., 2014). Furthermore, the combine utilization of curcumin and emodin has considerably diminished development and migratory/obtrusive ability of breast cancer cells (Dong J. et al., 2010). Additionally, resveratrol has been reported to conferred collaboration with the in vitro and in vivo cytotoxic impacts of Curcumin in head and neck carcinomas (Dai Z. et al., 2008). In a meta-investigation on around two thousand patients indicated that the efficiency of platinum-based chemotherapy was upgraded by intravenous infusion of the Chinese formula Shenqifuzheng (McCulloch M. et al., 2006). Another randomized and controlled clinical preliminary demonstrated that the infusion additionally raised the restorative impacts of "cyclophosphamide, epirubicin and 5-fluorouracil regimen" in local-advanced breast cancer patients (Guo L. et al., 2012). Likewise, an astragalus-based Chinese herb expanded the inhibitory impacts of cisplatin in advanced non-small-cell lung malignancy (Sin T.K. et al., 2015). Another perspective is the adverse events, like nausea, vomiting, and anorexia, regularly develop as secondary/side-effects to chemotherapy. In this way, reducing these untoward impacts is such a commendable objective to improve the quality of chemotherapy. In this context, the utilization of astragalus-polysaccharide particularly concealed fatigue, queasiness/vomiting, gastric-torment and loss of appetite connected with the use of the chemotherapeutic-drugs, vinorelbine and cisplatin, in patients with advanced NSCLC, thereby incredibly enhance the acknowledgment of treatment/therapy and improving patients' life quality (El-Mowafy A.M. et al., 2010). Quercetin, crocin and resveratrol were found to successfully diminish the cardiotoxicity evoked by doxorubicin (Chang C.Y. et al., 2008). The additive utilization of EGCG or resveratrol improved cisplatin-initiated renal lethality, aggravation and oxidative-stress (Sabová L. et al., 2010). Hence, while these studies hold some guarantee, still progressively satisfactory, fastidious/reliable studies need to be conveyed, especially to herb-drug combination, so as to definitely depict their combinatorial activities and exact clinical utility.

There is an expanding proof demonstrating the effectiveness of utilizing combined phyto- compounds for the treatment of solid tumors (Mahmoud N.N. et al., 2000).Chemical structure of some capable natural compounds and their natural sources has been depicted in Figure 4. A considerable number of herbs and herbal preparations are utilized by malignancy patients for their ability to stimulate immunity and well-being of patients. Mistletoe (Viscumalbum), ginger (Curcuma longa), garlic (Allium sativum) are the most important ones. Other than having anti-proliferative action such herbs were likewise accounted to potentiate the anticancer impact of different herbs or drugs. Antrodiacamphora extract, when joined with antitumor agents show adjuvant anti-proliferative consequences on hepatoma cells (in vitro) and on xenografted cell in tumor-implanted nude mice (in vivo) (Freudlsperger C. et al., 2010).

Mistletoe extracts have cytotoxic impact on Jurkat cells; the action is attributed to lectins present in the herb. It has been accounted that the concentrates in combination with doxorubicin exert the synergistic cytotoxic and apoptosis-prompting consequences on Jurkat cells (Ebrahimpour S. et al., 2010). Mistletoe lectin-1 is reported to increase anti-proliferative impacts of the peroxisome proliferator-activated receptor gamma (PPAR gamma) agonist rosiglitazone on human malignant melanoma cells (Ghosh D. et al., 2006).

Figure 4. Chemical structure of the promising natural compounds and major natural sources



Garlic, an herb, and naltrexone, a narcotic receptor antagonist, both have immune-modulatory and antitumor impacts. Recently, it has been indicated that aged garlic extract has synergistic impacts with naltrexone on restraint of tumor development and increment of survival time when tested on experimentally initiated fibrosarcoma tumor in BAL B/C mice (Ibrahim A. et al., 2011). The leaves of Azadirachtaindica are accounted to have immune stimulatory activity. It has been indicated that pre-treatment of mice with the extract of the herb diminished the degree of leucopenia and neutropenia in normal and tumor-bearing cyclophosphamide treated mice (Liu H.S. et al., 2011).

Curcumin, the dynamic constituent of turmeric (Curcuma longa) is a potential anticancer agent. It has been appeared to decrease viability of the highly cancerous, metastatic rodent mammary gland cell line ENUI564 in culture and diminish metastasis of these cells injected into nude mice (Manikandan R. et al., 2012). Curcumin has been demonstrated to initiate tumor apoptosis and repress tumor multiplication, invasion, angiogenesis, and metastasis by modulating various targets in different sorts of

malignant growth cells (Du Q. et al., 2013). There are several recent reports of curcumin demonstrating synergistic anticancer action with other natural products or drugs. Curcumin in combination with catechins, the polyphenolic compounds of green tea (Camellia sinensis) can synergistically restrain the expansion of HCT 15, HCT 116 of human colon adenocarcinoma and human larynx carcinoma Hep G-2 cells proficiently through induction of apoptosis (Siddiqui R.A. et al., 2013). Curcumin in mix with resveratrol is accounted to repress the multiplication of Hepa 1-6 hepatocellular carcinoma cells in a dose and time-dependent manner (Chen P. et al., 2013). The research data recommended that curcumin and resveratrol is a promising combination in treating liver malignant growth. Synergistic anticancer effects of curcumin with two dietary compounds docohexaenoic acid and an omega-3 unsaturated fatty acid present in cold-water fish have been shown in an in vivo model of DMBA-instigated mammary tumorigenesis in mice (Bueno Pérez L. et al., 2013).

There are several reports where an auxiliary metabolite from a plant has shown synergistic cooperation with anticancer medications. Emodin, which is a constituent of Rhamnusfrangula and Cascara sagrada, has indicated synergistic growth inhibitory impact with 3′-azido-3′-deoxythymidine (AZT) on adriamycin-resistant human chronic myelogenous leukemia (K562/ADM) cells (Kakar S.S. et al., 2014). The flavolignanhydrocarpin, which is a constituent of Berberis species, has been accounted to potentiate the impact of anticancer alkaloid vincristine in a sensitive and p-gp-expressing acute lymphoblastic leukemia cell line (Uesato S. et al., 2014). With a ferin A (Kitagawa R.R. et al., 2008), a bioactive compound isolated from Withania somnifera, in mix with cisplatin is reported to offer increasingly effective treatment for ovarian malignancy (Li Q. et al., 2014). Nobiletin from Citrus depressa when given along with a combination of paclitaxel and carboplatin produced a synergistic inhibitory impact against the proliferation of the human non-small-cell lung carcinoma cell lines A549 and H460 (Xu W.S. et al., 2014). Of the two chemotherapeutic medications, paclitaxel was responsible for the synergistic impact. Cyclamin, a 13, 28-epoxyoleanane type triterpenoid saponin from Ardisia japonica is a strong chemo sensitizer. A low cytotoxic degree of cyclamin synergistically upgrades the growth inhibitory impact of 5-fluorouracil, cisplatin, and epirubicin on Bel-7402, however not on HL-7702 cells (Kitagawa R.R. et al., 2008). Furanodiene, a terpenoid isolated from Rhizomacurcumae, a notable Chinese therapeutic herb is accounted to have anti-proliferative activities in a number of cell lines. Recently, it was reported that combined treatment of furanodiene with paclitaxel demonstrated anti-proliferative activities in 95-D lung cancer cells (Nurcahyanti A.D. and Wink M., 2015). It has been reported that the cytotoxic impact of a 1,4-naphthoquinone namely 5-methoxy-3,4-dihydroxanthomegnin isolated from Paepalanthus latipes gets potentiated by ascorbic acid (Yu J. and Chen Q., 2014).

Besides essential oil and polyphenol derivatives, numerous other natural mixes or complex mixtures may exert synergistic anticancer properties associated with conventional chemotherapeutic medications. For instance, l-canavanine, an anti-metabolite found in a several plants of the Fabaceae family, which is hardly lethal alone, potentiated the cytotoxicity of vinblastine and paclitaxel in two cell models i.e. HeLa and hepatocellular carcinoma cells (Yu J. et al., 2013). In addition, in an orthotopic pancreatic cancer mouse model with PANC-1 cells, β-carboline (an alkaloid from the plant Rauwolfia vomitoria)-enriched concentrate in combination with gemcitabine reduced tumor burden and metastatic potential in gemcitabine non-responsive tumor (Einbond L.S. et al., 2013). A similar plant, Rauwolfia vomitoria, in blend with carboplatin, had the option to expand chemosensitivity in ovarian disease cells (OVCAR-5, OVCAR-8, SHIN-3) and to inhibit tumor growth in a mouse model with intraperitoneal metastasis and massive ascites formation (Kapadia G.J. et al., 2013). Garcinia benzophenones (obtained from Garcinia species) were tested on HT29 colon malignancy cells in association with various chemopreventive

agents and exhibited an extraordinary ability to block cancer cell development (Shen F. et al., 1999). Another work demonstrated that red beetroot (Beta vulgaris) extract together with doxorubicin initiated synergistic anti-proliferative impacts against pancreatic (PaCa), breast (MCF-7) and prostate (PC-3) tumor cells (Weber G. et al., 1997).

Quercetin has been found to act synergistically with triazofurin in human ovarian carcinoma cells [OVCAR5] (Jayaram H.N. et al., 1982). Triazofurin therapy prompts the decrease of cellular GTP pool and diminished IP3 concentration (Suganuma M. et al., 1999). The latter is achieved by obstructing the S phase of the cell cycle (Suganuma M. et al., 2001). Since triazofurin and quercetin block extraordinary biochemical targets and arrest various phases of cell cycle, the combined treatment yields a synergistic decrease of IP3 concentration by 30% (Jayaram H.N. et al., 1982). Additionally, it is conceivable to utilize lower concentration of triazofurin during combination treatment consequently diminishing the side-effects. Sulindac is a non-steroidal anti-inflammatory drug that is utilized as a cancer preventive agent, yet the utilization of this is confined because of its adverse reactions and side-effects. High dosages of this medication restrain COX-1, which can lead to gastrointestinal bleeding. The apoptotic prompting activities of EGCG on lung malignant growth PC-9 cells has been found to be synergistically upgraded by other chemopreventive specialists, for example, sulindac and tamoxifen (Suganuma M. et al., 1999). Suganuma et al., (2001) revealed that the antitumor impacts of EGCG were synergistically expanded by sulindac and the tumor incidence diminished in mice with numerous intestinal neoplasia and apoptosis induced against colon carcinogenesis of rodents making it an appropriate candidate in combination with NSAIDs (Sen S. et al., 2005). Since EGCG additionally represses COX, it might be expected that both EGCG and sulindac inhibited tumor by emphatically obstructing the enzyme activity. Vinorelbine, a semi-synthetic vinca alkaloid is a compelling and less lethal chemotherapeutic specialist (Hwang J-T. et al., 2005). Pre-treatment with curcumin upgrades the apoptotic impact of vinorelbine by the mitochondrial pathway in H520 cells; by down regulating anti-apoptotic Bcl-2 and Bcl-XL; upregulating pro-apoptotic Bcl-xs and Bax and, actuating caspase-9 and -3. The supression of NF-κB and Ap-1 by both curcumin and vinorelbine may potentially clarify their combined anti-proliferative and apoptotic impacts (Tanos V. et al., 2002). The distinguishing proof of curcumin having potential synergy with standard cytotoxic drugs can lead to low therapeutic dosages of the medications, in this way, lessening their toxicity and long-term side-effects.

5-Fluorouracil (5-FU) is one of the widely utilized chemotherapeutic drugs targeting different tumors; however, its chemo resistance remains a significant hindrance in clinical settings. Natural products have also been believed to conquer drug obstruction in a couple of these cell lines. Hwang et al., (2011) concentrated on the combined cytotoxicity of 5-FU and genistein on HT-29 colon malignancy cells and reported that the combination brought about a decrease in the survival signal Glut-1 and a rise of pro-apoptotic p53 and p21. The combination likewise abolished the up regulated state of COX-2 and prostaglandin secretion caused because of only 5-FU treatment. A critical synergistic inhibitory impact was noted with the mix of genistein and tamoxifen on the growth rate of dysplastic and an additive anti-proliferative impact on malignant breast cells. This can be of noteworthy clinical application in treating mammalian dysphasia, since; currently there is no other chemopreventive treatment accessible. It can likewise be an effective adjuvant treatment in women with breast malignancy (Duarte V.M. et al., 2010).

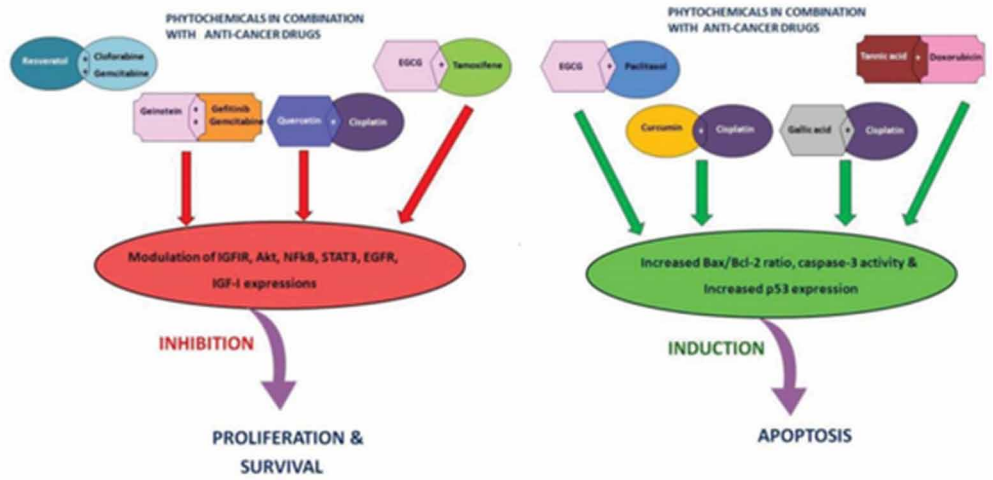
Phytochemical Combination Therapy in Preclinical Animal Studies

The positive cooperative synergy among phytochemicals and chemotherapeutic agents has additionally been exhibited in various preclinical animal models (Figure 5). In a mouse cervical multistage squamous cell carcinoma xenograft model developed utilizing 3-methylcholanthrene, the joined administration of CUR and paclitaxel brought about a synergistic decrease in occurrence of tumor as well as diminished the tumor volume to a more prominent degree when contrasted with the separately administered paclitaxel or CUR (Nautiyal J. et al., 2011). These outcomes proposed that an suboptimal dosage of CUR potentiates the antitumor activity of paclitaxel by down regulating the activation and downstream signaling of anti-apoptotic factors and survival signals, for example, NF-kB, MAPKs, and Akt, all of which play a significant role in multiplication, cancer cell endurance, angiogenesis, and metastasis. The inhibition of NF-kB activity by CUR is likewise liable for the expansion of the antitumor activity of cisplatin, eventually improving growth suppression in-vivo (Selvendiran K. et al., 2011). Recently, a preclinical examination uncovered that combination treatment with CUR and dasatinib was profoundly effective. The combination caused over 95% relapse of intestinal adenomas in Apc min/C mice, which might be because of the decreased proliferation and expanded apoptosis (Sartippour M.R. et al., 2008). Comparative remedial synergy has likewise been displayed by the CUR analog, HO-3867 by means of down regulation of the transcription factor, phosphorylated STAT3 (Stuart M.C. et al., 2007). This down regulation brings about the inhibition of the anti-apoptotic factors in the malignant growth cell consequently advancing apoptosis. Interestingly, a blend of green tea concentrate and Tmx altogether repressed proliferation of both ER-positive and ER-negative human breast cancer cell lines, namely MCF-7, ZR-75–1, T-47D, and MDA-MB-231. Green tea and Tmx improved malignancy cell development inhibition in a dose-subordinate way in MCF-7, ZR75, and T47D. They likewise repressed tumor development (116.5 § 31.9 mm3) contrasted with control animals (622.2 § 163.3 mm3) in xenograft models of MCF7 in nude mice (Zhang F.Y. et al., 2006). The outcomes likewise demonstrated that green tea combination adequately suppressed the translation interceded by estrogen response element; when contrasted with the individual agents in all the three cell lines. Activation of p44/42 MAPK, post-receptor signal transduction events, which add to the proliferative impact of estrogen in mammary cells, is additionally synergistically down regulated by the green tea and Tmx blend (Fukui M. et al., 2010). EGCG (25 mM) and 4-hydroxytamoxifen (4-OHT) (1 mM) synergistically potentiated the cytotoxic impacts on the breast malignancy cells, MDAMB-231. The investigation demonstrated the temperol appearance of cells in G1-phase arrest, which was, in any case, not linked with apoptosis. In addition, EGCG metabolism stayed unaltered since; 4-OHT is a powerless competitive inhibitor of microsomal glucouronyl transferases (UGT) activity (Zhang F.Y. et al., 2006). Therefore; the combination procedure augments the neutralizing impacts on breast malignancy cells headless of their ER status, and thus it might serve as a perfect means for breast cancer prevention.

Cytotoxicity of doxorubicin (DOX) to lung malignancy cells (A549), liver cancer (HepG2), and breast cancer cells (MCF7, DOX safe MCF-7) was enhanced on combination with naringenin (Salehi B. et al., 2018). This synergy was additionally evident in-vivo in a subcutaneous Lewis lung carcinoma tumor model (C57Bl/6 female mice) in contrast with treatment with DOX alone. Likewise, it was found that mice treated with this combination displayed less signs of toxicityin contrast to those treated with DOX alone. The outcomes showed that naringenin specifically enhanced inhibition of cancerous cell growth initiated by DOX in a cancer cell type dependent way.

DOX accumulation was expanded by 69% and 65%, separately, by naringenin in A549 and MCF-7 cells however; not in HepG2 and MCF-7/DOX cells; when contrasted with control. However, blend of DOX with verapamil expanded DOX accumulation intra-cellularly in HepG2 and MCF-7/DOX cells yet not in A549 and MCF-7 cell, when contrasted with DOX alone. This was additionally affirmed by flow cytometric investigation proposing that naringenin builds up the cellular DOX accumulation via repressing DOX efflux.

Figure 5. Dietary phytochemicals as synergists with anticancer drugs



Combination therapy with phytochemicals and several genera of therapeutic medications synergistically attacks two separate targets of cancer mainly cell proliferation and survival and cell apoptosis.

A scientific report has found that RES can essentially upgrade the anticancer efficacy of paclitaxel in human breast malignancy cells, MDA-MB-435s, MDA-MB-231, SKBR-3, and in xenograft (MDA-MB-435s cells infused) female athymic nu/nu mice. The study uncovered that RES-induced check point kinase-2 protein activation, which partially added to paclitaxel's efficacy by inciting S-stage cell cycle arrest. This combination-initiated apoptosis by decreasing the accumulation of intracellular reactive oxygen species (ROS) and down regulating Bcl-2 and Bcl-xL phosphorylation prompted by paclitaxel. The combination, in any case, didn't influence Bax levels. Predictable with these outcomes, RES (16.5 mg/kg body weight, thrice/wk/i.p) and paclitaxel (10 mg/kg body weight, once/wk/i.p) combination particularly diminished tumor development through advancement of apoptosis and diminished proliferation of cell nuclear antigen protein levels (PCNA) (Sharifi-Rad M. et al., 2018).

CONCLUSION

The complexity in cancer developmental biology requires generally multi-target treatment methodologies. Therefore, the deployment of synergic combination of conventional chemotherapeutic medications with plant-extracts has advanced curative impacts, decreased adverse effects and improved patient immunity,

ultimately aiming to upgrade quality of life and drag out patient's life expectancy. Plants have widely been utilized as medicines since hundreds of years; for the treatment of a wide assortment of infections. Individuals over the ages have been depended on customary herbal agents to meet their medicinal necessities. Despite of the presence of conventional medications, natural products despite possesses a spot in treatment owing to their wide scope of healing properties. Auxiliary metabolites acquired from plants are for the most part responsible for their therapeutic properties. Present day medications show a number of side-effects which might be overwhelmed by utilizing plant derived mixes. The immense capability of plants in malignant growth treatment still stays unexplored. The opportunity has already come to develop more up to date drugs; hostile to malignancy; from plant sources which may pave a way to a non-toxic method of cancer control. Phytomedicine and ethno pharmacology have expanded in popularity in the most recent decades, particularly while considering their application in the immense field of human diseases, including cancer. The ongoing studies have recommended that plant-derived compounds have a high effect as remedial operators, either alone or in combination with the conventional medications. Indeed, bibliographic research utilizing "cancer and herbal" and "malignant growth and plant" as catchphrases recovered 78 and 149 studies on the website of clinicaltrials.gov (December 2018), regardless of whether not carefully linked with synergy (U.S. National Institutes of Health). Additionally, it should be remembered that the developing utilization of natural extracts, either self-recommended or integrated with conventional medications, will eventually contribute to increasing frequency of plant-drug collaborations. As a consequence of this kind of completion strategy planned will defnetly have edge in designing the more meaningful combination of different herbal products or the phytochemicals so as to it benefit in the field of cancer therapeutics. It will provide a greater tool into hands of clinicians as potential boon for the patients who are suffering from this deadly disease of cancer.

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Chapter 26

Obstructions in Nanoparticles Conveyance, Nano-Drug Retention, and EPR Effect in Cancer Therapies

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ABSTRACT

In this chapter, the authors first review nano-devices that are mixtures of biologic molecules and synthetic polymers like nano-shells and nano-particles for the most encouraging applications for different cancer therapies. Nano-sized medications additionally spill especially into tumor tissue through penetrable tumor vessels and are then held in the tumor bed because of diminished lymphatic drainage. This procedure is known as the enhanced penetrability and retention (EPR) impact. Nonetheless, while the EPR impact is generally held to improve conveyance of nano-medications to tumors, it in certainty offers not exactly a 2-overlay increment in nano-drug conveyance contrasted with basic ordinary organs, bringing about medication concentration that is not adequate for restoring most malignant growths. In this chapter, the authors likewise review different obstructions for nano-sized medication conveyance and to make the conveyance of nano-sized medications to tumors progressively successful by expanding on the EPR impact..

INTRODUCTION

Cancer is a disease influencing a huge number of individuals around the world. For advanced staged-malignant growths in patients, treatments were often restricted to the chemotherapy or radiation. However, these treatment alternatives accompany their own set of disadvantages. Concerning chemotherapy, the toxicity and non-selective nature were significant disadvantages. Chemotherapeutic medications being vague in nature bring about critical damage to the non- cancerous tissues (Wakaskar, 2017a). Furthermore, dominant forms of the chemotherapeutic medications accessible in the market have a high pharmacokinetic volume of distribution and low molecular weight (Bharali et al., 2009; Ahmad et al., 2020). The low molecular weight of these medications makes it susceptible to fast discharge. Drug molecules that are circling in vivo might be fundamentally bound to specific proteins or even lipids which are common in plasma. This thought is crucial as it is a broadly respected phenomenon that only free drug molecules can show significant collaborations with the target that can evoke the necessary therapeutic impact, for e.g., a specific receptor. Tragically, there is a significant absence of logical research to give an in-depth outline of how these collaborations add to the in vivo adequacy of either hydrophobic or hydrophilic medications. Some in vitro measures, for example, the shift assay can foresee the compound concentration that is accessible to achieve the adequacy after explicit associations with the given target. These compounds which conquer the hindrance of in vitro testing are then chosen for advanced in vivo testing. A higher concentration of the drug is accordingly important to accomplish remedial advantages which simultaneously make toxicity unavoidable. Another characteristic of these drugs which isn't especially favorable is its low therapeutic index. It is important that the minimal effective concentration be reached for ideal treatment yet tragically frequently these levels are surpassed. Together, these outcome results in serious bothersome side-effects, for example, nausea, emesis, bone marrow suppression, alopecia and the sloughing of the gut epithelial cells (Luo & Prestwich, 2002). Under these conditions, tumor-targeted conveyance of chemotherapeutic medications is maybe one of the most significant steps for chemotherapy. Nowadays, there is incredible interest for the development of nano delivery frameworks for malignant growth therapeutics. By utilizing nanotechnology in development of drug and conveyance, specialists are endeavoring to drive nanomedicine to have the option to convey the medication to the targeted tissue, discharge the drug at a controlled rate, be a compelling and reliable drug conveyance framework and

circumvent clearance by bodily procedures. A perfect framework would encourage explicit targeting and subsequently upgrading the viability while limiting undesired side-effects (Ahmad et al., 2017a; Soni et al., 2018; Barkat et al., 2019).

Solid tumors frequently develop drug resistance because of various notable mechanisms, including alternative drug export pumps, modifications in gene articulation, changes in the metabolic pathways that influence the metabolism of cytotoxic medications, deregulation of DNA repair, and ensuing induction of apoptosis. In addition to these well depicted mechanisms, the tumor microenvironment assumes a significant role in drug obstruction by imposing hindrances that restraint conveyance of the drug to the tumor. Therefore, thorough understanding of the mechanisms of action, microenvironment, and obstructions are needed to deliver the issues identified with the constrained adequacy of cancer chemotherapy (Stylianopoulos & Jain, 2015). In building up a protected and powerful drug transporter that specifically conveys cytotoxic drugs to tumor cells two methodologies are mainstream. The principal approach normally alluded to as "passive targeting" depends on fundamental contrasts in the structural highlights of solid tumors (Wakaskar, 2017b). These distinctions lead to some degree of specific extravasations and retention of long circulating nanocarriers. In the other methodology, the surface of the nanocarriers is altered to explicitly recognize tumor cells. The administering guideline for this situation is specific interaction between ligand, for example, nucleic acids, antibodies, etc. on the carrier surface and receptors expressed in tumor environments. This methodology is alluded to as "active targeting" (Rizwanullah et al., 2019).

To address the precise methodical toxicity of chemotherapy, a sensible system is to encapsulate the non-selective chemotherapeutic medications in a vehicle that can convey the medication explicitly into the tumor. This "Magic bullet" idea was first proposed by Dr. Paul Ehrlich in 1906 (Strebhardt & Ullrich, 2008) and nanoparticles frameworks hold incredible potential to acknowledge it. Today, nanoparticles have found its way in a wide range of applications ranging from therapeutics and diagnostics to treatment supervise and assessment (Pushpavanam et al., 2016). Nano-scale materials display peculiar properties that have made them agreeable alternatives in these fields like large surface area of nanoscale materials allows for its modification for better stability, biocompatibility and inter- connection with specific cells (Wakaskar et al., 2015; Rizwanullah et al., 2017). In the field of biomedical, transportation of the drug to the target site is basic nanoparticles offer solutions to long standing difficulties, for example, nonspecific bio-distribution and targeting, lack of water solubility, poor oral bioavailability and low therapeutic indices. Nano-sized agents have various hypothetical favorable advantages over conventional low molecular weight operators including an enormous loading limit, the capacity to shield the payload from debasement, explicit targeting, and controlled or sustained release (Longmire et al., 2011; Rizwanullah et al., 2018; Saifi et al., 2020). Their features can be improved by changing certain characteristics, for example, size, shape, payload, and surface features (McNerny et al., 2010). Thus, the field of nanomedicine has been quickly advancing, especially in the field of diagnosis and treatment of cancer (Kobayashi & Brechbiel, 2005; Haider et al., 2020). However, nano sized drugs are, by definition, bigger than most drugs and, accordingly, leaks more slowly from capillary beds. The vasculature of strong tumors is described by leaky vessels with poor lymphatic drainage. When directed intravenously, nano sized drugs in general tends to circulates for an extensive stretch of time, if they are not small enough to be discharged by the kidney or sufficiently large enough to be quickly perceived and trapped by the reticulo-endothelial system (RES) (Maeda et al., 2016; Mahtab et el., 2019). Nano sized operators with long circulation times spills specially into tumor tissue through the permeable tumor vasculature and are then held in the tumor bed because of diminished lymphatic drainage. This phenomenon is known as the enhanced

permeability and retention (EPR) effect (Wakaskar, 2017c). The basis method for nanosized drug conveyance is accumulation of the agents inside tumors because of the EPR impact followed by release of their remedial therapeutic payloads. However, in any case, EPR impacts are moderately unobtrusive, offering not exactly a 2-fold increment in conveyance contrasted with basic typical organs (Wakaskar, 2017c). The more extended the drugs remains in the circulation, the more certain is to extravasate into the tumor through the EPR impact, but yet, the drug can also likewise extravasate into normal tissues at the same time albeit at a slower rate. In this way, strategies that even incidentally increment the local EPR impact inside the tumor are expected to improve the specific take-up of the drug inside the tumor, subsequently improving its remedial impact.

BARRIERS TO THE DELIVERY OF NANO-SIZED DRUGS

1. Abnormal Tumor Vasculature

When a malignant tumor develops to >2-3 mm3 in size, the conveyance of oxygen and supplements becomes diffusion-restricted and the formation of fresh blood vessels becomes fundamental in order to meet the consistently expanding demands of the quickly developing and proliferating cancerous cells (LaRocque et al., 2009). The fast multiplication of malignancy cells promotes the formation of neovasculature for their nutritional and oxygen supply. These recently shaped tumor vasculatures are typically irregular in structures and design (Gao, 2016). They are convoluted and irregular, with ineffectively aligned defective endothelial cells, wide fenestrations, and absence of a smooth muscle layer (Miao et al., 2015). This is achieved through the release of angiogenic factors by the neoplastic tissue leading to expansion of the microvasculature inside the tumor so as to sustain further development (LaRocque et al., 2009). The resultant balance of angiogenic factors and matrix metalloproteinases (MMPs) inside neoplastic tissues brings about exceptionally disorganized vessels, which are dilated, with various pores and wide gap intersections between endothelial cells (Cho et al., 2008). The perivascular cells and basement membrane are missing or defective (Iyer et al., 2006). Moreover, tumor vessels mainly lack the smooth muscle layer that ordinarily encompasses endothelial cells (Figure 1) (Skinner et al., 1990). The normal vasculature is invested with tight junctions that are impermeable to molecules measured >2-4 nm, thus keeping the nanoparticles inside the circulation; in any case, the leaky vasculature of neoplastic tissue permits macromolecules with a diameter of ≥600 nm to extravasate into the neoplastic tissues. Since tumors don't have a very much evolved lymphatic framework, these extravasated nanoparticles in general deteriorate inside the neoplastic tissue (Talekar et al., 2011). This phenomenon of leaky vasculature and impaired lymphatic drainage has been alluded to as the EPR effect (Maeda & Matsumura, 1989).

The leaky nature of tumor vasculature structures the physiological basis of the EPR impact, which is advantageous to nanoparticles to extravasate from vessels and gathered in tumors (Gao, 2016; Haider et al., 2020).Moreover, the enhanced penetrability leads to excessive blood constituent extravasation, while the impaired lymphatic framework leads to inefficient leakage of fluid from the tumor center, which mutually adds to the advancement of raised interstitial fluid pressure (IFP) and fluid viscosity inside tumor mass, and eventually blocks the movement towards tumor parenchyma (Egeblad et al., 2010a). Also, the perfusion of blood vessels inside tumor is very heterogeneous contrasted with those in ordinary tissues, leaving areas inadequately perfused or even without perfusion (Gao, 2016). To arrive at those areas, NPs need to take an extreme journey to cross the tumor interstitium, leading to hetero-

geneous NP distribution patterns inside strong tumors, and compromised treatment results. The tumor vascular system is described by dilated, convoluted, and saccular channels with haphazard patterns of interconnection and branching (Ziyad & Iruela-Arispe, 2011; Rizwanullah et al., 2020a). In contrast to the microvasculature of normal tissue, which has an organized and normal branching order, tumor microvasculature is disordered and lacks the customary hierarchy of blood vessels (Konerding et al., 1995). Arterioles, capillaries, and venules are not recognizable thusly, and rather, vessels are enlarged and frequently interconnected by bidirectional shunts (Jain, 2003). One physiological outcome of these vascular irregularities is heterogeneity of tumor blood flow (Jain, 1988), bringing about poor and heterogeneous perfusion in the tumor and raised interstitial fluid pressure from consistent extravasation of fluid, which eventually makes hypoxic and acidic intra-tumoral conditions. This condition forestalls the penetration of nanosized medicates deep inside the tumor and, therefore, adds to tumor development, metastasis, and drug resistance (Huynh& Zheng, 2015).

Figure 1. Endothelial barrier in normal and tumor vessels

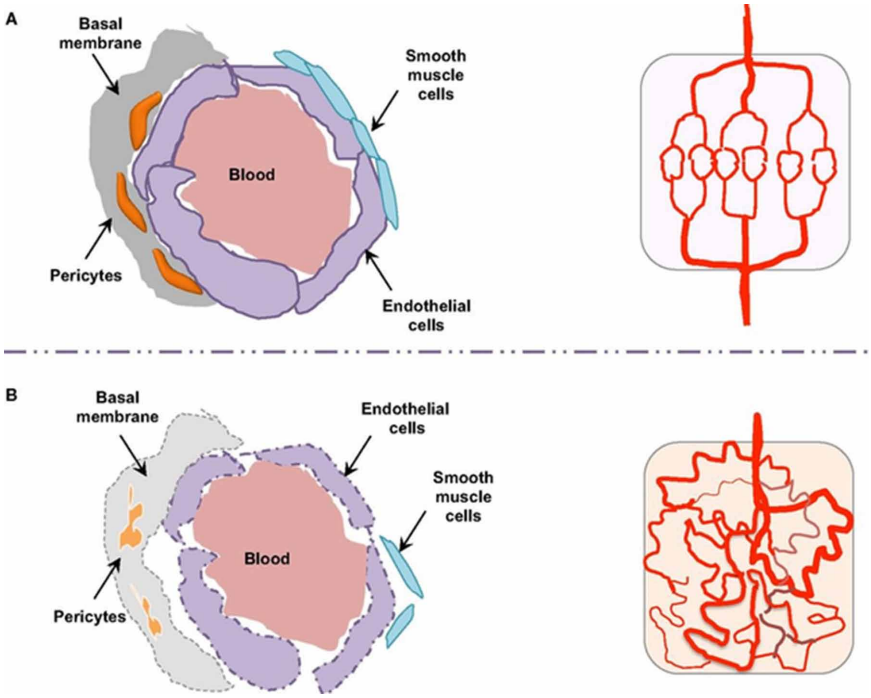
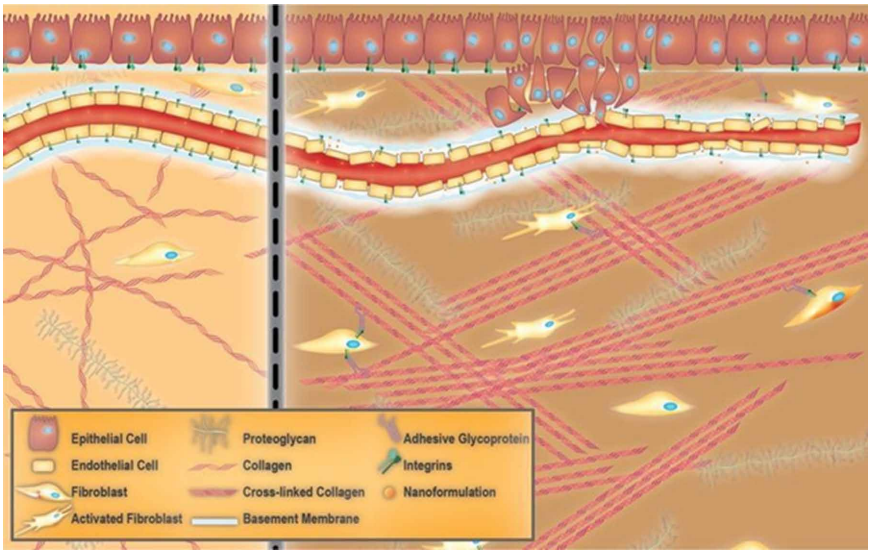


Figure A and B depict structural differences in normal and tumor blood vessels. In contrast to normal vessels, the vasculature pattern in tumor is extremely unsystematic and disordered with morphological and structural differences i.e. weak correlation between endothelial cells, abnormal shapes of pericytes, absence of smooth muscles as well as basal membrane alteration. (Adopted from Sandy A. et al., 2013).

2. Dense ECM

Another significant factor adding to the EPR impact is the stromal compartment, which can be subdivided into the extracellular matrix (ECM) and stromal cells. The latter incorporate endothelial cells, pericytes, (myo) fibroblasts, smooth muscle cells, dendritic cells, macrophages and other immune cells. The thickness of ECM components, for example, collagen and hyaluronic acids, strongly impacts nanomedicine accumulations, as it develops a boundary which forestalls the penetration of nanomedicine from the vessels to deep into the tumor interstitium, further leading to inhomogeneous distribution of medications and drug conveyance system (Yuan et al., 1994). In this unique situation, particularly the collagen content and the collagen distribution appear to play a significant role. ECM for the most part comprises of a cross-linked system of collagen, elastin filaments, proteoglycans, and hyaluronic acid (Figure 2) (Khawar et al., 2015).

Figure 2. Healthy ECM against aberrant tumor ECM (left and right sections, respectively)



Healthy ECM is described by the existence of an undamaged basement membrane, non-activated fibroblasts and random arrangement of collagen fibers (left). Abnormal tumor ECM features the tumor vessel basement membrane with a heterogeneous thickness that allows the dissemination of tumor cells as well as accretion of Nano formulations. The presence of collagen fibers that were aligned in a well-ordered manner and activated fibroblasts are additional characteristics of tumor ECM. (Hoda S. A. et al., 2020).

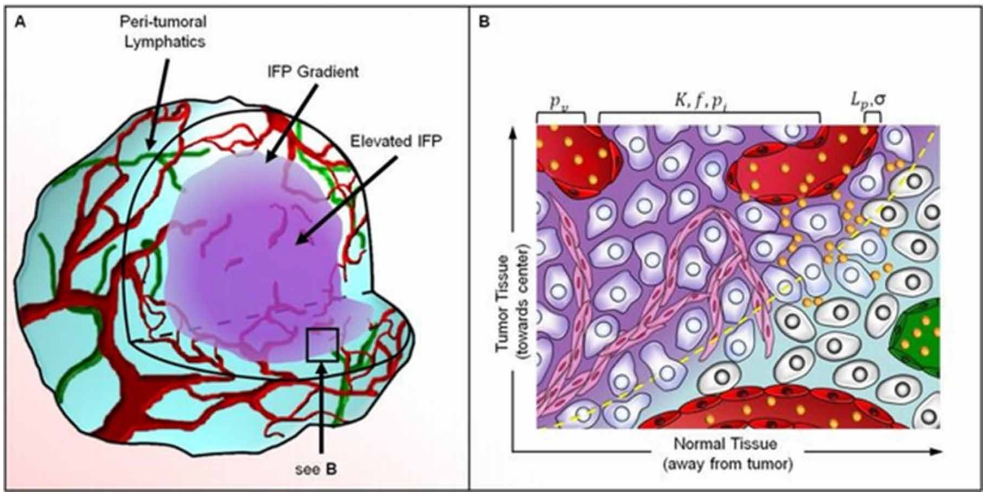
Tumor ECM is denser than the ordinary normal cells ECM because of the presence of high collagen content and expanded degree of lysyl oxidase (LOX) (Barua & Mitragotri, 2014). The high collagen level in the tumor ECM is a significant hindrance in the transport of NPs, while LOX could crosslink collagen and stiffens the ECM, which eventually breaks down the compelling transport and penetration of the nanoparticles. Moreover, the ECM network is made out of charged matrix polymers, for example, collagen and hyaluronan, which hinders conveyance of exceptionally highly charged NPs by virtue of

electrostatic interactions with these matrix polymers (Suzuki et al., 2017).The speculation that nano-medicine accumulation is undermined in collagen-rich tumors has been affirmed in a number of studies, indicating that thick fibrillar collagen forestalls large particles as well as standard chemotherapeutic agents from infiltrating deep into tumorous tissue (Yokoi et al., 2014). However, ECM decrease has a few limitations and systemic targeting of the ECM, which additionally influences healthy tissues, can induce antagonistic impacts like thromboembolism (Venning et al., 2015).

1. High Interstitial Fluid Pressure

Another boundary offering hindrance to the intra-tumor transport of NPs is the raised IFP (Figure 3) (Jain & Stylianopoulos, 2010). In typical tissues, IFP is marginally negative that is fundamental for tissue homeostasis, while tumors cells display significant interstitial hypertension as high as 50 mmHg in case of certain melanomas (Curti et al., 1993), which is brought about by the high permeability of tumor vasculatures in combination with the impaired lymphatic function in the tumor interstitial space. The IFP inside tumors can nearly be equivalent to the micro vascular pressure. However, it drops quickly to typical normal values at regions near the tumor margin, leading to a sharp outward pressure gradient. As a result, the transport of the NPs across the tumor interstitium is to a great extent debilitated, on the grounds that the principle system of mass transport is diffusion, especially for large particles. Even more terrible, the IFP can cause intravasation of NPs or therapeutic agents back to the blood supply (Liu et al., 2017). Moreover, the interstitial fluids outpourings from the tumor periphery into the surrounding tissue, conveying remedial NPs as well as cells and different development factors that may advance tumor progression and metastasis (Li et al., 2012).

Figure 3. An image showing high IFP and its relationship to the EPR effect



(a) Tumors experience elevated central IFP due to an increased trans-vascular fluid transport (Lp), reduced interstitial fluid transport (K), and absence of functional lymphatic vessels. Peri-tumoral lymphatic in general drains excess fluid at the tumor periphery leading to rise in IFP. (b) An image of

the peri-tumoral region where the yellow dashed line indicates the border between tumor and healthy tissue. Trans- vascular (pv{pi) and interstitial (+pi) pressure gradients drive the convective transport across blood vessels and through the tumor interstitium. This process occurs mainly along the tumor periphery where substantial trans-vascular and interstitial pressure gradients were present. Convection transports liposomes through large endothelial pores (s) and via the extra-cellular matrix (f) where they collect due to a lack of lymphatic clearance. In normal tissue, tight endothelial junctions limit liposome extravasation and functional lymphatic contribute to the clearance of the agent from the interstitium. (Adopted from Shawn S. et al., 2013)

In this manner, the raised IFP in tumors prevent effective NP transport across the blood vessels as well as in the tumor interstitium, consequently trading off the advantages of the EPR impact.

As tumor tissues possess high osmotic pressure, high interstitial fluid pressure (IFP) may upset satisfactory delivery of anticancer drugs (Jain, 1987). IFP is raised in solid tumors not just because of increased vessel permeability and hyper perfusion, in addition also because of poor lymphatic drainage which typically maintains fluid balance, as well as hyperplasia around blood vessels and increased production of extracellular matrix components (Jacobetz et al., 2013). In normal tissues, IFP is roughly 0 mmHg; though in tumors, IFP can reach micro vascular pressure levels with a range of 10−40 mmHg. High IFP limits the convection of nanosized drugs, while incomprehensibly advancing passive diffusion out of the tumor (Boucher & Jain, 1992). Diffusion is a slower trans-vascular process than convection, particularly for the transport of large sized nanosized drugs (Jain & Stylianopoulos, 2010). Moreover, stroma cells compress intratumoral blood and lymphatic vessels, further weakening blood flow, leading to blood stasis, loss of function, and further hindrance to nanosized drug penetration (Jain & Stylianopoulos, 2010). Finally, on account of the precarious drop in IFP on the edge of tumors, intratumoral liquid can escape from the tumor periphery into the surrounding tissue, accordingly spilling nanosized drugs from their planned target, the tumor, into surrounding tissues (Padera et al., 2004). Thus, high IFP represents a formidable obstruction to both the conveyance and efficacy of nanosized drugs. Numerous diverse vascular and micro environmental parameters add to heterogeneity in EPR- based nanomedicine accumulation. At the vessel level, these incorporate vascular penetrability, endothelial cell receptor articulation and vascular maturation. Stromal parameters which add to heterogeneity in EPR-based Nano-tumor targeting is the extracellular matrix, tumor cell thickness, hypoxia and the interstitial fluid pressure. These all pathophysiological parameters must be viewed as when intending to develop individualized and improved nanomedicine treatments.

2. Growth-Induced Solid Stress

Tumor development is related with the production of intratumoral mechanical forces, both liquid or fluid and solid, because of the unrestrained and quick tumor cell expansion in a restricted region. Solid pressure and mechanical forces are created when cellular and non-cellular segments of the tumor microenvironment interface with nearby noncancerous cells and parts of them

matrix. In certain tumor types with a less supportive stroma, strong stress-mediated vessel compression can happen due to multiplying tumor cells, upsetting the distribution of blood-borne anticancer drugs into tumor tissue (Stylianopoulos et al., 2012). Therefore, high tumor cell thickness is the foremost hindrance for penetration of nanosized sedates deep into tumor tissues (Lu et al., 2007). Solid stress caused either by malignant cells and stromal cell compression or deformation of vascular and lymphatic structures consequently adds to tumor progression. Cell compression simultaneously brings about changes

in gene expression, cellular expansion, apoptosis, invasion, and stromal cell- related extracellular matrix production and organization (Jain et al., 2014). Altogether, excessive development- incited solid stress compresses tumor vessels and decreases perfusion. Tumor overgrowth may likewise make an interstitial boundary for efficient medication penetration into tumor tissues. In this manner, alleviation of solid stress by focusing on the tumor or stromal cells may give an effective strategy for improving delivery of drugs (Khawar et al., 2015).

3. Solid Stress from Abnormal Stromal Matrix

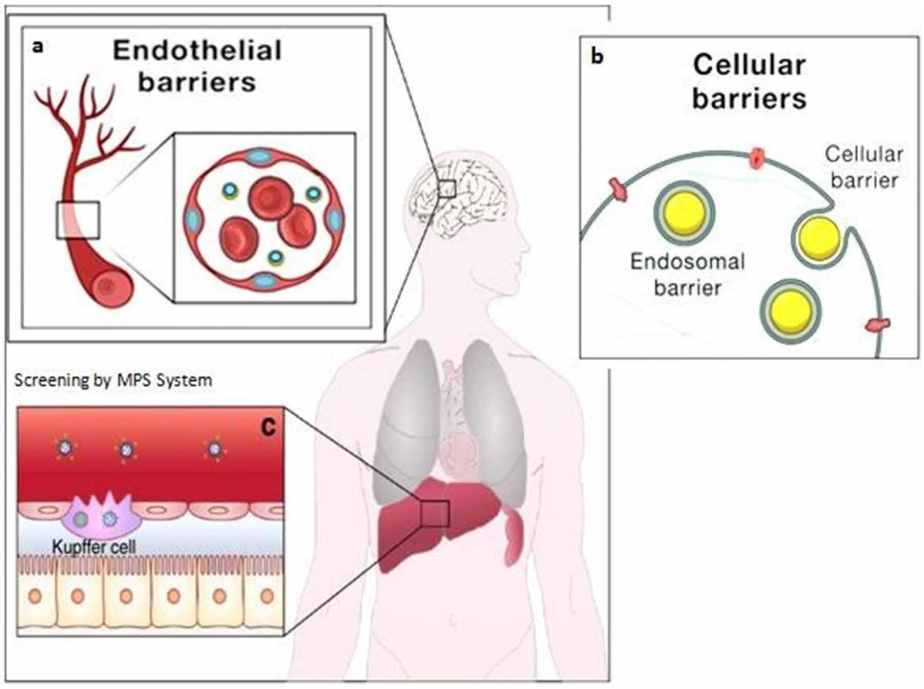
In typical normal tissues, the organization and structure of the extracellular matrix (ECM) is one of a kind and dynamic and functions to regulate cell growth. The significant ECM components are collagen, glycoprotein, proteoglycan, elastin, and hyaluronan. The ECM associated basic structural segments, catalysts, and development factors are essential for the guideline of cell proliferation and differentiation, eventually delaying cell survival and homeostasis. The ECM is localized at two distinct destinations namely the basement membrane and the interstitial space. For example, the matrix of the basement membrane layer contains exceptionally compact and less permeable structures contrasted with that of tumors formed by collagen (type IV), fibronectin, laminins, and other related proteins that help to connect collagen with other matrix proteins. Type I collagen, glycoproteins, and proteoglycans are abundant in the interstitial matrix and are all considered collectively responsible for tensile strength of normal tissues (Egeblad et al., 2010b). Apart from these normal stromal highlights, tumor stroma involves modified ECM attached to multifaceted stromal cells including fibroblasts, pericytes, endothelial cells, and immune cells (Maquart et al., 2005). Moreover, altered biophysical and biological qualities of the tumor ECM in a hypoxic microenvironment contribute to tumor progression and metastases (Gilkes et al., 2014). Fibrosis is a sign of numerous kinds of cancer; it develops because of extreme ECM production or restricted ECM turnover in tumor tissues (Frantz et al., 2010). Severe desmoplastic or fibrotic reactions are described by deregulated accumulation of different types of collagen networks (Egeblad et al., 2010), prompting extracellular matrix variations that in turn advance tumor progression through architectural and signaling interactions (Lu et al., 2012). Cancer-related fibroblasts (CAFs) assume to play significant role in extracellular matrix-interceded malignant changes, which incorporate up-regulated extracellular matrix synthesis, post-translational alterations, and matrix metalloproteinase (MMP)-initiated extracellular matrix remodeling, prompting the decrease in drug take-up in tumors (Egeblad et al., 2010). Resting fibroblasts are transformed into CAFs in response to tumor-related development factors, for example, TGF-β, stroma-derived factor (SDF-1), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) (Khawar et al., 2015). Thus, the abnormal matrix found in tumors further meddles with nanosized drug conveyance to tumor.

Drug Delivery in Cancer

The approaches for targeted conveyance of therapeutics in cancer normally includes fundamental administration of therapeutics packed in nanocarriers (NCs) or localized conveyance of therapeutics to the diseased tissue. Encapsulation of therapeutic molecules e.g., small molecules inhibitors, chemotherapy, RNAi, and so forth in NCs can improve their dissolvability and bioavailability, alter their bio-distribution, and can likewise encourage entry into the target cell (Akhter et al., 2020; Rizwanullah et al., 2020b). “Passively” directed NCs, which uses the enhanced permeability and retention (EPR) effect (Peer et al.,

2007), are the most widely investigated strategy for targeting malignant growth systematically. Moreover, only a small concentration of these NCs accumulates even in high-EPR xenografted tumors i.e. less than 1% as indicated by an ongoing meta-investigation study (Wilhelm et al., 2016). This could be because of numerous physiological obstructions and a high level of stochasticity linked with NCs extravasation through the tumor vasculature (Peer et al., 2007). A significant proportion of NCs are additionally cleared by the mononuclear phagocytic system (MPS); some get physically "stuck" in the sinusoids of the liver and others are taken up by hepatocytes and Kupffer cells (Blanco et al., 2015) (Figure 4). In spite of the fact that various passively targeted NCs have been approved over the past 20 years, none of their actively targeted counter parts have been progressed past clinical trials. Various ongoing reviews have condensed the current clinical status of effectively targeted NCs (Shi et al., 2017).

Figure 4. Schematic illustration of key physiological barriers faced by targeted NCs in drug delivery



A. NCs face endothelial barriers in the process of extravasation into the tumor tissue; blood–brain barrier illustration as an example. B. The main cellular obstacles are the taking up of NCs by the target cells and their escape from the endo-lysosomal network into the cytosols. C. Hepatic Kupffer cells, as an example of a mononuclear phagocytic system (MPS), is resulting in the clearance of systemically administered NCs, decreasing their half-life and effective dose (Adopted from Daniel R. et al., 2018).

More than 40,000 reports published in the last 10 years have concentrated on active targeting techniques and considerable advancement has been made toward our understanding of how NCs associate with cells and tissues. However, we still have not been able to conquer the difficulties and challenges introduced by physiological barriers, for example, tumor penetration, tumor heterogeneity, relative hypoxia, and endosomal escape, which have constrained the therapeutic advantage of actively targeted

NCs. Likewise, the administrative obstacles and the relatively complex scale-up of the manufacturing procedure of actively targeted NCs represent extra difficulties toward the translation of actively targeted NCs into clinical practice. Active cellular targeting strategies include using affinity ligands on the outside surface of NCs for specific homing, expanded retention at the target site, and take-up by the target cells (Saha et al., 2010). These ligands are chosen to binds to over express or clustered receptors on infected tissues and cell surfaces for e.g., HER2, folate receptor, CD44, etc. (Byrne et al., 2008). Additionally, actively targeted NCs should initially arrive at the target to take advantage of this expanded affinity and avidity. Effective passive targeting is thusly an essential prerequisite for NCs designed to systematically target tumor specific cells or the extracellular matrix (ECM). EPR-related phenomenon and its impact on NCs' accumulation and entrance into tumors is for the most part dependent on fast developing xenografted mice models with thick vasculature, which doesn't recapitulate most of solid tumors in humans (Bogart et al., 2014). A number of methodologies have therefore been proposed to augment, for example, TNF-α, angiotensin-II and sonoporation or bypass (loco-regional conveyance, targeting, and so on.), the EPR impact in low-EPR tumors or tumors in secluded organs like cerebrum, bone, ovaries, bladder, and so on respectively. Additional obstacle toward expanding the efficacy of NCs is pre-mature release of therapeutics. Several systems have been devised to overcome this issue, including the advancement of stimuli-responsive NCs. However, these highlights add another degree of complexity to the nanocarriers design strategy.

EPR Effect

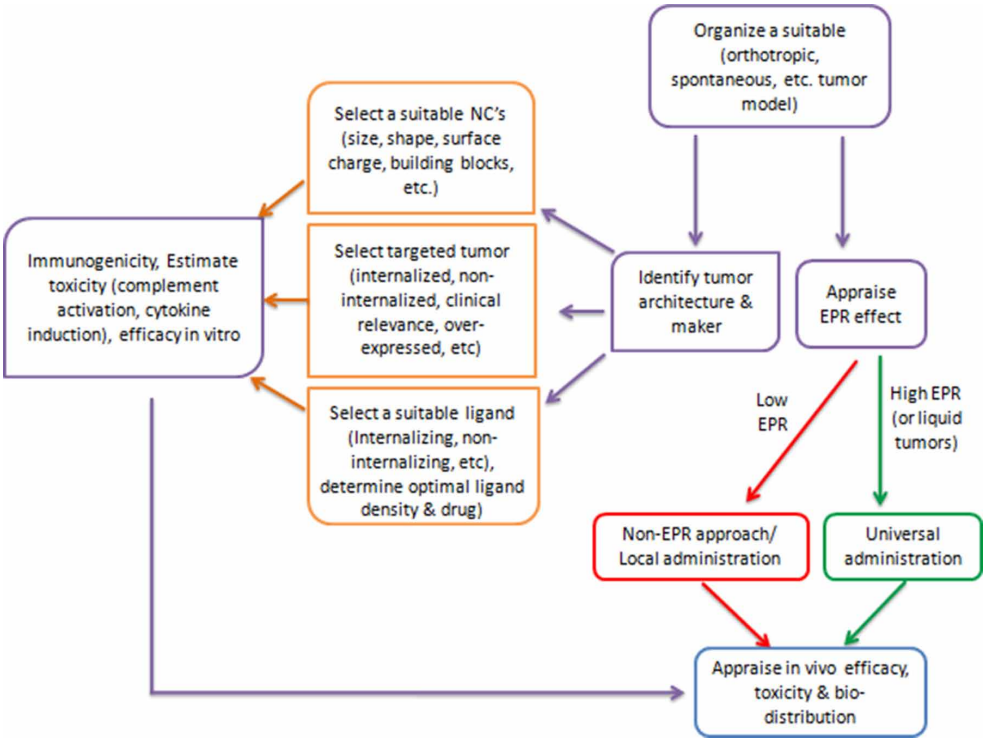
Cancers are not simply massing of harmful tumor cells; they resemble a complex organ where malignant cells build up an environment; so that the non-threatening cells like immune system cells support tumor development rather than tumor suppression. This interaction between malignant cells and non-malignant cells produces tumor microenvironment (TME) (Balkwill et al., 2012). The TME is well known to be associated with the various stages of malignant growth progression, especially local obstruction, immunoescaping and distant metastasis (Chen et al., 2015). Therefore, TME assumes a pivotal role, while tumor targeting is extremely essential in treatment of the disease. The majority of human malignancy research concludes non-malignant cells engaged with TME incorporates; the cells of the immune framework, tumor vasculature, lymphatic, as well as fibroblast, pericytes, and adipocytes (Balkwill et al., 2012). The non-malignant cell secreted proteins and stromal cells are additionally having a role in tumor progression and advancement (Mbeunkui & Johann, 2009). The vasculature system in tumor tissues is totally different as found in normal tissue blood vessels. Tumor blood vessels are known to be heterogeneous because of their organization, structure, and functions (Ruoslahti,2002). The vasculature framework which is produced in tumor tissue has distorted dynamics and they have characteristics like tortuosity, the nonappearance of a basement membrane layer, and hyper permeable in nature (Siemann,2011). The blood vessels are inconsistent in diameter, uneven shape, and abnormal bulges. At the point, when tumor tissues become diffusion constrained, they develop new vasculature for the nutrition intake, waste discharge, and oxygen supply. This procedure of neovascularization i.e. development of vasculature is called as angiogenesis (Byrne et al., 2008). The lack of vasculature supportive tissues intimates the formation of defective vessels, pores through endothelial gaps comprised of diameter between 100 nm to 2 μm relying upon size and type of tumor tissue.

Other than this, the tumor vasculature is portrayed by a poor lymphatic system which cripples the drain of the intratumoral parts resulting in aggregation of it into tumor tissues. This sort of phenomenon

of malignancy tissues is known as the EPR effect. EPR impact can be utilized for passive targeting of therapeutic and diagnostic molecules. The concept of passive targeting of remedial molecule starts with focusing on Poly (styrene-co-maleic Acid) NeoCarzinoStatin (SMANCS) to the tumor tissues. The nanoparticles which are having a diameter not as much as pore size, gets permeate in the tumor tissues through the leaky vasculature and retained for a longer drawn out time (Byrne et al., 2008). Previously, Maeda et al., reported that intravenous administration of Evans blue dye, which ties to plasma protein albumin, brought about the selective accumulation of color in tumor tissues. The amount of blue albumin in tumor tissue was ~10-fold higher than that of the blood after 145 h (Maeda, et al., 1986). This phenomenon was likewise also seen with radio labeled plasma proteins, including transferrin (90 kDa) and IgG (160 kDa), though, smaller proteins, for example, neocarzinostatin (12 kDa) and ovomucoid (29 kDa), didn't show their accumulation in tumor tissues (Greish, 2007). Another examination has affirmed that macromolecules with a molecular load beyond the renal threshold (40 kDa) will in general accumulate specially in neoplastic tissues after their intravenous administration. This special and preferential accumulation of macromolecules in tumor tissues occurred because of the EPR impact (Maeda, 2012). In a study sykes et al., reported delivery perfusion of gold nanoparticles via collagen fibers and its system into tumor tissues. They reported that perfusion of gold nanoparticles was interdependent on tumor size and size of gold nanoparticles (Sykes et al., 2016). From this examination they found that, gold nanoparticles having size $<$45 nm can undoubtedly permeate into the tumor without troubling tumor size, and these nanoparticles can be utilized as a viable tool for analytic or treatment therapy (Sykes et al., 2016). Therefore, in development of target NCs, various aspects in terms of design and biological consideration must be taken in order to build a suitable experimental system (Figure 5).

Conventional chemotherapy depends on low sub-atomic weight drugs (for the most part under 1000 Da) (de Jonge & Verweij, 2006). Because of their little size, chemotherapeutic agents, for example, doxorubicin, cisplatin or gemcitabine, have unfavorable pharmacokinetics and a problematic biodistribution, as exemplified by a short blood half-life and noticeable off-target aggregation in various multiple healthy organs. This, together with the unspecific system of activity of chemotherapeutic medications and their large volume of dissemination, causes extreme side-effects, for example, myelosuppression, mucositis, neurotoxicity, nausea, vomiting and alopecia (Harris et al., 2002). By expanding the size of systematically directed anticancer specialists to at least 5-10 nanometers in diameter i.e. surpassing the renal clearance threshold of ~40000 Da; kidney discharge can be decreased, blood half-life's prolonged, and target site accumulation improved (Figure 6). For instance, the encapsulation of doxorubicin into liposomes (Caelyx®/Doxil®) brings about an expansion in plasma half-life from 5-10 minutes for the free medication, to 2-3 days for the liposome- encapsulated drug (Gabizon et al., 1994). In this particular case, as in numerous other liposomal and micellar nanomedicine formulations, surface alteration with the secretive polymer polyethylene glycol (PEG) diminishes aggregation and opsonization with plasma proteins, adding to the prolonged circulation half-life (Park, 2010). By methods for improved circulation times, nanomedicine can accumulate in tumors by means of the Enhanced Permeability and Retention (EPR) impact, which was first portrayed by Matsumura and Maeda in 1986 (Matsumura & Maeda, 1986). EPR depends on explicit pathophysiological qualities of tumors versus sound tissues. In healthy tissues, low-atomic weight medicates effectively extravasate out of blood vessels, while nanomedicine can't do as such, because of their size. On the other hand, in tumors the abnormally wide fenestrations in the blood vessels lead to the extravasation of materials with sizes up to several hundred of nanometers. This, together with the absence of lymphatic seepage, prompts a moderately effective and specific aggregation of nanomedicine in tumors (Prabhakar et al., 2013).

Figure 5. Schematic illustration of the workflow proposed in developing actively targeted NCs dose
(Adopted from Daniel R. et al., 2018)

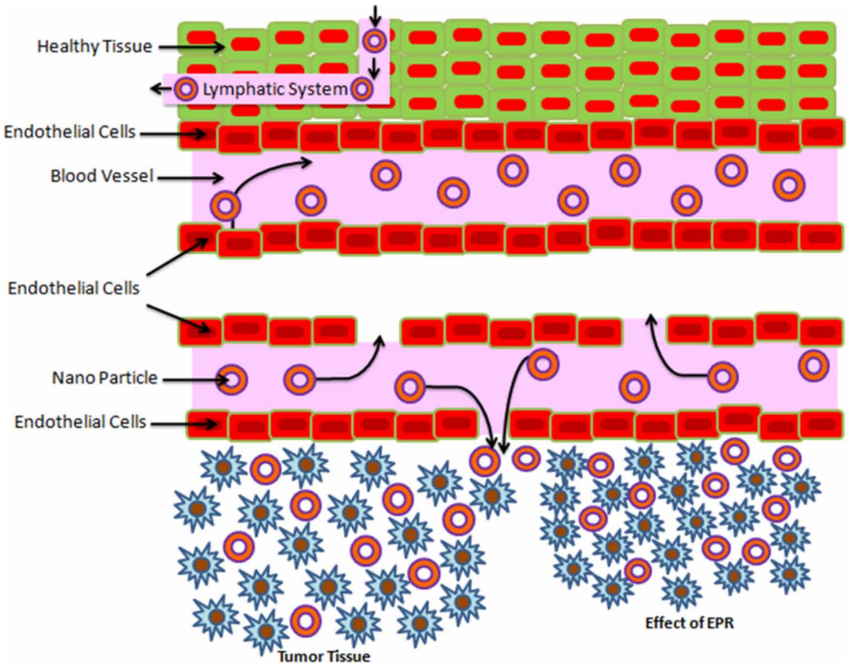


A successful cancer drug conveyance should accomplish high accumulation in tumor and spare the encompassing normal healthy tissues (Ahmad et al., 2015). The passive localizations of numerous medications and drug carriers because of their extravasation through cracked vasculature (named the Enhanced Permeability and Retention [EPR] impact) works very well for tumors. As tumor mass develops quickly; a system of blood vessels needs to be extended rapidly in order to accommodate tumor cells' requirement for oxygen and supplement. This anomalous and ineffectively controlled vessel generation (i.e. angiogenesis) brings about vessel walls with huge pores (40 nm to 1 um); these cracked/leaky vessels permit relatively huge nanoparticles to extravasate into tumor masses. As fast developing tumor mass does not have a working lymphatic framework, clearance of these nanoparticles is restricted and further upgrades the accumulation. Through the EPR impact, nanoparticles bigger than 8 nm (between 8-100 nm) can passively target tumors by freely passing through huge pores and accomplishes higher intratumoral accumulation. Most of current nanomedicine for solid tumor treatment depend on EPR impact to guarantee high drug accumulation and accordingly improving treatment adequacy. Without targeting cell types expressing communicating ligands of interest, this medication conveyance framework is called passive targeting.

Before reaching to the proximity of tumor site for EPR impact to occur, passive targeting needs drug delivery system to be long-circulating in order to permit absolute level of drug to the target area. To style nano-drugs which will stay in blood longer, one will "mask" these nano-drugs by modifying the surface with water-insoluble polymers such as polyethylene glycol (PEG). PEG is commonly used to develop water-insoluble nanoparticles to be water-soluble in several pre- clinical analysis laboratories.

PEG-coated liposomal doxorubicin (Doxil) is employed clinically for breast carcinoma offering advantage to passive tumor accumulation. As in vivo closed-circuit system for macromolecules (i.e., scavenger receptors of the reticuloendothelial system, RES) reportedly showed quicker uptake of negatively charged nanoparticles, nano-drugs with a neutral or positive charge were expected to possess an extended plasma half-life. Utilizing EPR impact for passive tumor targeting drug delivery isn't competently without problems. Although EPR impact is a distinctive hallmark in solid tumors; the central region of metastatic or larger tumor mass doesn't exhibit EPR impact, as a result of an extreme hypoxic condition. For this reason, there were strategies employed in the clinics to artificially enhance EPR effect via slow infusion of Angiogenin II to extend systolic blood pressure, topical application of NO-releasing agents to expand blood and photodynamic therapy or hyperthermia-mediated vascular permeabilization in solid tumors. Passive accumulation through EPR impact is the most acceptable drug delivery system for solid tumor treatment. However, size or relative molecular mass of the nanoparticles isn't the sole determinant of the EPR impact, different factors namely surface charge, and biocompatibility and in vivo closed-circuit system for macromolecules shouldn't be unnoticed in the course of designing of the nanomedicine for efficient passive tumor accumulation.

Figure 6. Diagrammatic representation of EPR effect



Normal blood vessels have surrounding smooth muscle-cell layer with tighten the cell to cell junctions difficult for macromolecular agents to extravasate. In contrast, in tumor tissues, blood vessels were always bearing loose cell to cell junctions, via which macromolecular agents can escape to tumor tissue. In addition, the defected lymphatic system in tumors leads to the macromolecular agent's retention in tumor tissues. (Adopted from Yin H, Liao L, Fang J (2014).

Intravenously injected nanosized medications were delivered into the pathological lesions via arterioles and released from capillaries (Barkat et al., 2020). Therefore, the key mediators of intra-tumoral delivery are small vessels, particularly capillaries (Armulik et al., 2011).Normal capillaries were lined up by a tightly sealed epithelial tissue, firmly connected and supported on the abluminal side by the stellate-shaped pericytes that were further enclosed in a skinny layer of the basement membrane (BM) (Armulik et al., 2011). In traditional normal tissues, pericyte coverage of the endothelial abluminal surface varies among different organs and blood vasculatures, with a general range between 10% and 70% (Sims, 1986). The vasculature BM, with major parts of type IV collagen, laminin, entactin (nidogen), and fibronectin, sometimes envelops blood vessels with a thickness scale ranging from100to150nm (Yokoi et al., 2014). Hence, so as to grow, tumor cells recruit a neovasculature to confirm an adequate supply of nutrients and oxygen. As tumors grow, they recruit new vessels or engulf existing blood vessels. Unlike traditional blood vessels, the tumor vasculature usually has incomplete epithelium lining inflicting comparatively large pores i.e. 0.1−3 μm in diameter, resulting in considerably higher vascular permeability and hydraulic conductivity (Danquah et al., 2011). Additionally, the extent of pericyte coverage on tumor vessels is usually diminished compared to normal tissues (Armulik et al., 2011). Both pericytes and basement membrane were loosely linked to the epithelium cells and occasionally penetrate deep in the tumor parenchyma, increasing the trans- endothelial permeability (Miao & Huang, 2015). However, perivascular smooth muscles commonly lack the tumor vessels, making them poorly reactive to normal vaso regulation (Chan et al., 1984).

Tumor cells were reported to exhibit pathophysiological characteristics totally different from that of standard cells. Passive targeting capitalizes on these variations to focus on delivery of the drug to the site of interest via what's commonly remarked as the enhanced permeability and retention (EPR) impact (Parveen et al., 2012; Gilani et al., 2018). EPR is a phenomenon where molecules of certain sizes accumulate to a greater extent in tumor cells than traditional cells. The accumulation is attributed to variations like hyper vasculature, lack of effective lymphatic drainage and enhanced production of permeability mediators (Maeda et al., 2000). Maeda et al., reported one of the first tumor targeted delivery of anti-tumor styrene-maleic acid copolymer-conjugated neocarzinostatin in 1979, eventually resulting in the introduction of the phenomena of EPR in solid tumors in the year1986 (Maeda et al., 1979&2000). Due to the quick and uncontrolled growing nature of the tumor, there's no lymphatic drainage system for tumors and the area or fenestration between the epithelium cells that line the blood vessel wall of the tumor vasculature is much larger than that in traditional tissues (20–150 nm vs. less than 10 nm). This development unremarkably referred to as the EPR impact of the tumor vasculature is the basis for passive targeting (Lu et al., 2015). Due to the EPR impact, nanoparticle systems of ˜20–150 nm can cross the blood vessel wall and preferentially accumulate within the interstitial area of tumor compared with traditional tissue. In contrast, chemotherapeutical medications were generally tiny molecules that are less than 10 nm in size and might cross the blood vessels of both tumor and traditional tissue to cause severe toxicity. Several nanoparticle systems have been developed to encapsulate anti-tumor drug with incontestable capability of reducing the general toxicity of chemotherapy, including both organic nanoparticle systems (e.g., liposome and polymeric nanoparticles) (Mundra, et al.,2015) and inorganic nanoparticle systems (e.g., silica and gold nanoparticles) (Heo et al., 2012). Matsumura and Maeda were the first to point out that nanoparticles were able to extravasate via the inherent leaky and loosely compacted vasculature to reach the tumor area and stay there as a result of the poor lymphatic drainage of tumors (Matsumura & Maeda, 1986). This phenomenon of EPR impact paved the approach for the passive targeting of tumors utilizing nanosized medication. However, drug delivery due to EPR remains

restricted; because the rate of leakage from the vessels is slow and also the drug may either be excreted or metabolized throughout the time it takes for the buildup to achieve therapeutic levels (Prabhakar et al., 2013). EPR effects were also modest, providing less than 2-fold increase in delivery in contrast with critical traditional organs. It is usually insufficient for achieving therapeutic levels inside the tumor, although side- effects are usually greatly reduced as a result of terribly low accumulation inside normal tissues lacking EPR (O'Brien et al., 2004).

Despite the potential that nanocarriers offer as therapeutic agents through EPR, it's important to pick out those with appropriate properties in order to enhance the period of circulation and to forestall immune response. Researchers have found that nanocarriers with a size range of 10–100 nm are ideal (Ahmad et al., 2017b; Akhter et al., 2018). This is because; kidneys filter out particles smaller than 10 nm and also the liver can capture particles bigger than 100 nm in size (Alexis et al., 2008). Another necessary consideration is the charge of the nanocarriers; neutral or anionic carriers are the best and escape renal elimination (Guasch et al., 1993). Oftentimes, the nanocarriers were also surface-coated to evade opsonization and phagocytosis by the reticuloendothelial system (RES) (Wakaskar, 2018). A standard surface coating used is polyethylene glycol (PEG) that is believed to reduce the protein interactions on the surface of the nanocarriers, preventing their binding to opsonin (Owens & Peppas, 2006). Also, this coating of PEG considerably imparts an in-vivo stealth nature to the nanoparticles by reducing the inter-particulate engaging forces and therefore rendering effective repulsive forces to incoming blood components like plasma proteins. As a result, this reduces clearance of these nanoparticles from the body.

Although EPR facilitates accumulation of nanocarriers, there remains a possibility for improvement with respect to target, highlighting some limitations to this approach. First, targeting depends on the degree of tumor vascularization and angiogenesis (Allen & Cullis, 2004). Thus, the impact might not be accomplished all solid tumors due to variations in porosity and pore size of the blood vessels (Bae, 2009). Second, is the elevation of interstitial fluid pressure that is witnessed in tumor tissues hinders the penetration of therapeutic agents (Netti et al., 1999). Third, is the tissue penetration could be a vital barrier to the effectiveness of a nanomedicine. The presence of extracellular matrix and dense population of cells around blood vessels limits the power of nanomedicine to penetrate. As a result, the anti-tumor effectiveness of the nanocarriers is commonly impaired. Additionally, PEGylation itself is a hindrance since it not solely prevents the interaction between nanocarriers and opsonin however; additionally, between the nanocarriers and cell surface (Romberg et al., 2008). Fourth, is the non-uniformity or heterogeneity of tumor blood flow interferes with the consistent distribution of a drug inside the tumor (Hobbs et al., 1998). Therefore, so as to enhance EPR, researchers have to come up with many strategies such as altering physiological conditions, physiological modifications of tumor vasculature, inducing morphological changes in perivascular cells, etc. (Kobayashi et al., 2014a).

There were additionally factors that influence the EPR impact in tumors; first is the nature of both the vascular bed and surrounding stroma, the presence or absence of functional lymphatic and interstitial hydraulic conduction impacting interstitial pressure in conjunction with mechanical stresses generated by cancer and stromal cells impacting the extracellular matrix. Second, factor is the tumor size, type, and location (including primary tumor versus metastasis lesions). Third, the extent of phagocyte tumor infiltration and also the activity of the mononuclear phagocytic system (MPS), which might vary between and inside tumor varieties and patient characteristics (e.g., age, gender, tumor type, body composition, treatment). These factors result in accumulation of nanoparticles in both normal tissues and in several sections of the tumor, for instance, in the periphery, viable tumor, and necrotic sections; and

co-medications, which may affect, stroma and blood pressure (hypertension increases tumor blood flow). Additionally, many vascular factors (Table 1), like nitric oxide generators and bradykinin potentiators, i.e. ACE inhibitors that lower blood pressure, were found to have an effect on EPR and are comparatively safe and cheap to combine with a nanoparticles drug (Maeda et al., 2013).

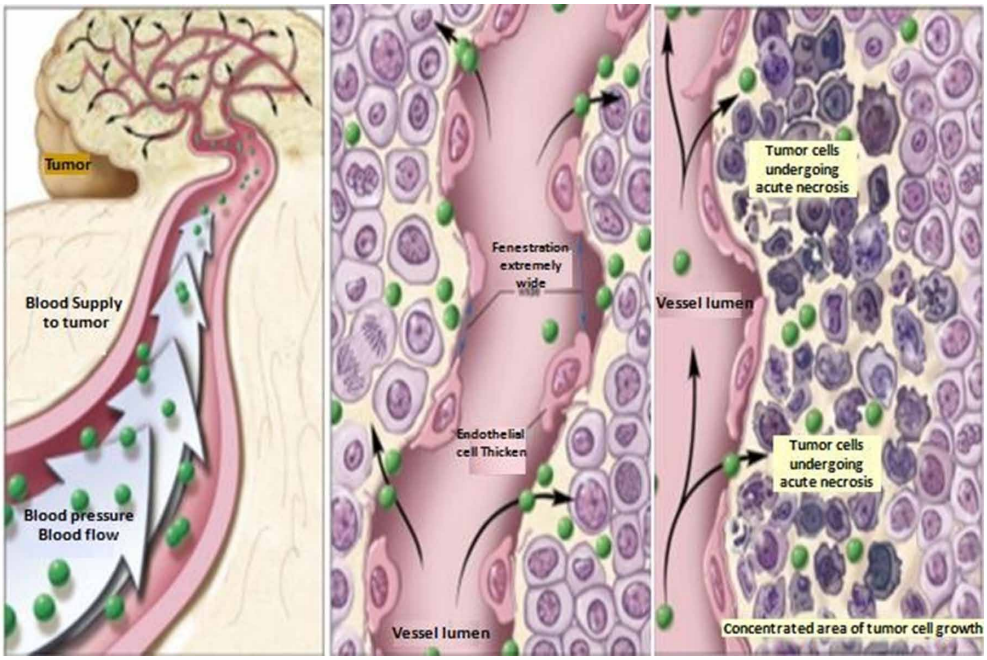
HOW TO IMPROVE THE EPR EFFECT

The extent of the EPR effect is dependent on a number of factors (Lammers et al., 2012). By achieving the manipulation of either local tumor or systemic conditions, EPR effects can be enhanced resulting in increased nano sized drug delivery. The three foremost modifiable factors that improve the tumor capillary wall's resistance were modulating the tumor blood flow; modulating the tumor vasculature and stroma; and killing the cancerous cells in order to reduce their barrier function (Figure 7 & 8) (Kobayashi et al., 2014b).

Table 1. Factors affecting the EPR effect of macromolecular drugs in solid tumors

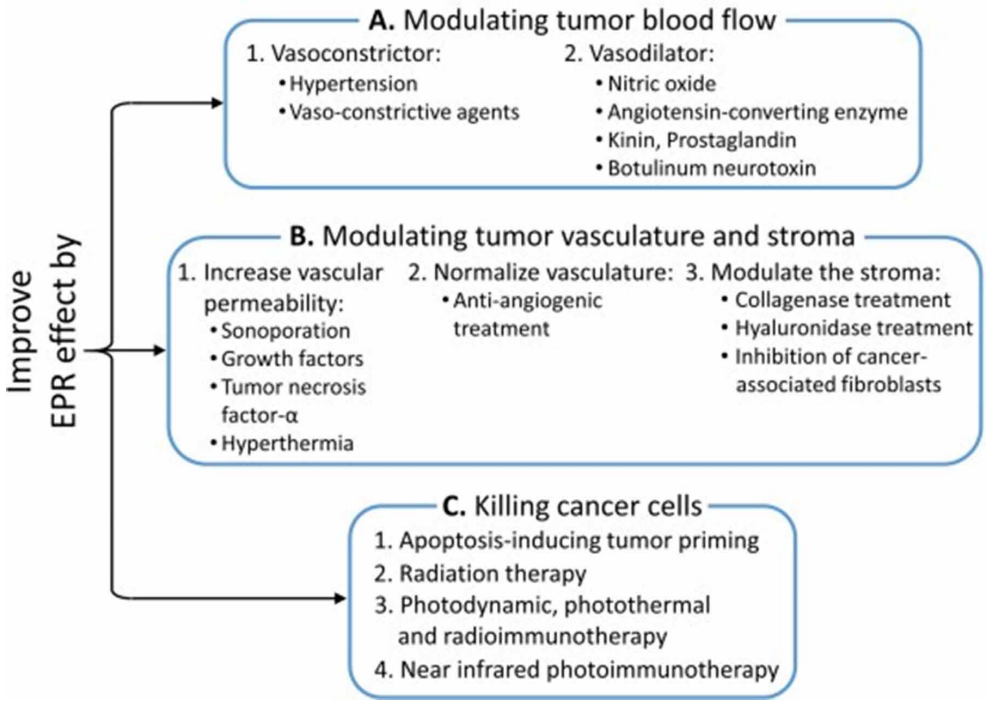
Mediators	Responsible enzymes and mechanisms	Possible application to therapeutic mechanism
Bradykinin	Kallikrein Protease	ACE inhibitors like Enalapril; blocking of kinin degradation elevates local kinin level and enhances EPR.
NO	iNOS	NO releasing agents such as nitroglycerin, ISDN, etc via denitrase and nitrite reductase to generate NO.
VPF/VEGF	Involved in NO generation	
Prostaglandins	Cyclooxygenase (COX I)	Beraprost sodium: PGI_2 agonist works Via vascular dilatation and extravasation.
Collagenase (MMPs)	Activated from pro- MMPs by peroxynitrite, or proteases	-
Carbon monoxide (CO)	Heme oxygenase (HO)-1	PEG-hemine via induction of HO-1 in tumor → CO generation
Induced hypertension	Using angiotensin II	Slow IV infusion → systemic hypertension, vascular extravasation selectively in tumor tissue.
Inflammatory cells and H_2O_2	Neutrophil/NADPH oxidase	-
Transforming growth factor (TGF)-β inhibitor	-	Inducing multiple inflammatory cytokines; NOS, COX, NO, PGs etc.
Tumor necrosis factor (TNF)-α	-	Inducing multiple inflammatory cytokines; NOS, COX, NO, PGs etc.
Anticancer agents	-	-
Heat	Vascular dilation	Gold nanoparticle or ferrite nanoparticle using electromagnetic, or laser, or microwave.

Figure 7. Figure showing increase blood flow and pressure, damaged vascular endothelial cells and destruction of tumor cells adjacent to tumor vessels
(Hisataka K. et al., 2014).



Modulating Tumor Blood Flow: one of the hallmarks of solid tumors is inefficient blood flow, that limits drug transport within the neoplasm and contributes to a reduced or absent transcapillary pressure gradient. Both effects reduce the uptake and distribution of anti-neoplastic medicine. So, in order to restore an effective tumor blood flow, both vasoconstrictors and vasodilators has been used as promoter drugs that modulate tumor blood flow. This seems incomprehensible; however, both approaches aim to expand the trans-capillary pressure gradient via different mechanisms like increasing blood pressure with vasoconstrictors or decreasing flow resistance with vasodilators. Hence, in order to boost the nano-drug delivery into cancer tissue and not in normal tissue, one may increase the input function of the nano-sized agent. Traditional vessels retain their ability to respond to foreign vasoconstrictors, whereas neoplasm vessels lose their responsiveness to such agents. Muscular fibers within the vessel wall can contract, limiting blood flow in traditional tissues. Therefore, once vasoconstrictor medicines are administered, normal vessels are constricted and blood pressure is increased. In contrast, tumor vessels don't react to vasoconstrictors owing to deficient muscular system. This leads to a relative increase in the input performance in the neoplasm tissues (Maeda, 2013). This phenomenon was recognized in 1970"s during diagnostic angiography for tumor localization and was termed "pharmaco-angiography" (Novak & Weber, 1976). Throughout the diagnostic angiography, vasoconstricting agents including alpha-receptor agonists were injected to constrict normal vessels whereas accentuating neoplasm vessels (Georgi & Freitag, 1980). Later, pharmaco-angiography was used to constrict vessels; once the delivery of nano-drug therapy to prolong the exposure of the neoplasm to the therapy (Li et al., 1983). Diagnostic pharmaco- angiography was powered by additional sensitive techniques like computerized tomography and magnetic resonance imaging for increased drug delivery.

Figure 8. Methods for improving cancer nano sized drug delivery based on EPR effects by manipulating intrinsic physiological barriers



To enhance the EPR impact, varieties of vascular mediators were utilized (LaRocque et al., 2009; Maeda et al., 2016). Nitric oxide (NO) is an endogenous mediator that causes vessels to dilate and thereby lowers blood pressure. Several solid tumors manifest vascular embolism or vascular clogging. If nitroglycerin is injected to restore vascular blood flow of such tumors, drug delivery is enhanced and thus a greater EPR impact happens. It is reported about the application of nitroglycerin and angiotensin-converting protein (ACE) inhibitors, such as enalapril, in which they magnified delivery of Evans blue-albumin to tumors (Maeda,2014). Another vascular mediator concerned in the EPR impact, Kinin, could be a major mediator of inflammation that induces extravasation and accumulation of body fluids in inflammatory tissues (edema) (Del Rosso et al., 2008). Kinin is understood to activate endothelial cell derived NO synthase (Kou et al., 2002), that ultimately results in a rise in NO, an important mediator of tumor vascular permeability. Prostaglandins (PGs) are lipid compounds that are derived enzymatically from arachidonic acid by mean of cyclooxygenases (COXs) (Wu et al., 1998). Similar to bradykinin, PGs are vital mediators in inflammation and may be upregulated by inflammatory cytokines as well as kinin (Furuta et al., 2000). In a research it was shown that native administration of Clostridium Botulinum neurotoxin type A increase tumor oxygenation and perfusion, resulting in improvement to the tumor response to radiotherapy and chemotherapy. This is often the results of interference with neurotransmitter release at the perivascular sympathetic varicosities, resulting in the inhibition of the neurogenic contractions of tumor vessels and improvement of tumor perfusion and oxygenation (Baudele et al., 2006).

Targeting tumor vasculature or stroma: Another approach for improving nano-drug delivery into cancer tissue is to physiologically modify the tumor vasculature. Many anti-angiogenic medicines have

been approved and commonly used. Among them, the anti-vascular epithelial growth factor (VEGF) monoclonal antibody, Bevacizumab, has been used to block the impact of VEGF thereby, inhibiting neoplasm maturation and suppressing tumor growth (Jordan et al., 2005) by decreasing blood flow and vascular porosity. In contrast, VEGF itself could temporally increase leakiness and perfusion in tumor tissue as a possible way to physiologically augment the EPR impact (Cyran et al., 2012). It has been additionally argued that anti-angiogenic treatment leads to vascular standardization that improves the distribution of blood in the center of the neoplasm and hence, improves delivery of certain medications (Chauhan et al., 2012). In recent work, researchers describe targeting the endothelial cells of the neoplasm vasculature by targeting the αvβ3 integrin utilizing an RGD- peptide conjugated to a gold nano-particle. When light is applied, photo-thermal harm will increase anti-tumor and EPR effects (Xie et al., 2011). Similar effects were seen with ultrasound micro bubbles (Kiessling et al., 2012). Damaging endothelial cells of the neoplasm vasculature would possibly remove a barrier to drug delivery, however, it carries the chance of decreasing or perhaps shutting down the tumor blood flow because of thrombosis hence, reducing the input performance of medicine into tumors. There are many different approaches in targeting the vasculature or stroma to promote vascular supply and permeability in tumors such as hyperthermia (Kong et al., 2001), irradiation (Lammers et al., 2007), high intensity targeted ultrasound (Ranjan et al., 2012) and numerous mediators together with bradykinin (Fang et al., 2011), nitric oxide-releasing agent (Seki et al., 2009), angiotensin- converting enzyme inhibitors (Fang et al., 2011), tumor necrosis factor α (Seki et al., 2011), heme oxygenase-1 (Fang et al., 2012) and proteases together with collagenase (Eikenes et al., 2004) or hyaluronidase (Eikenes et al., 2005). Most of these mediators are low relative molecular mass and hence, once injected systemically, can have an effect on normal blood vessels in the vicinity of neoplasm, thus, facilitating extravasation not solely within however additionally around tumors. A theoretical concern is that compromising the integrity of cancer stroma could promote metastasis (Marcucci & Corti, 2012).

Tumor necrosis factor-α (TNF) is an inflammatory protein/cytokine that causes hemorrhagic tumor necrosis in mice. Studies showed high response rates wherever high-dose TNF together with chemotherapeutical medicine were administered by isolated-limb perfusion to patients with malignant melanoma or sarcoma of the extremities (Eggermont et al., 2003). Clear proof was obtained in these studies for TNF as a promoter drugs that increases tumor uptake and penetration of chemo therapeutically effector medicine. However, in humans the drug is incredibly toxic and functions solely in a very slender range of doses leading to severe systemic side-effects. High IFP (Interstitial Fluid Pressure) in tumors could be a direct consequence of angiogenesis and limits nano-sized drug extravasation. Targeting angiogenesis could be an easy approach to avoid this barrier (Sriraman et al., 2014). Paclitaxel treatment has been shown to be effective in reducing IFP values in the clinical studies (Taghian et al., 2005). A VEGF blockade to inhibit angiogenesis is another promising strategy to assist in drug penetration against the pressure gradient (Tong et al., 2004). Treatment with Imatinib, a PDGF receptor-β matter, leads to decreased VEGF expression and eventually decreased IFP (Vlahovic et al., 2006).Similarly, Dickson et al., have shown that pre-treatment with Bevacizumab, an anti-VEGF monoclonal antibody, helped to boost the antineoplastic efficacy of systemically administered topotecan in a murine malignant neuroblastoma model (Dickson et al., 2007). Vascular disrupting agents like combretastatin and ZD6126, a tubulin-binding agent, have additionally been accustomed with successful reduction in IFP (Ley et al., 2007). Abnormal ECM composition and structure in solid tumors are the main obstacles for penetration of metastatic tumor medicine, particularly in desmoplastic tumors. In solid tumors, penetration of macromolecular therapeutic agents is especially affected by interstitial stromal barriers like collagen networks (Brown et

al., 2003). Various studies have shown that the ECM-degrading enzyme collagenase may improve the distribution of macromolecules in solid tumors (Goodman et al., 2007).

Killing tumor cells: The use of nano-drug delivery procedures has been reportedly increased in several cancer therapies. The doubtless clarification for this is that, neoplasm cells themselves act as a barrier to deeper penetration of nanodrugs. The heterogeneity of the blood supply within the neoplasm microenvironment results in marked heterogeneity in the rate of cell proliferation. The cancer cells close to the vessels proliferate rapidly, whereas cancer cells far off from the vessels suffer nutrient deprivation and proliferate more slowly (Kizaka-Kondoh et al., 2003). Microscopy reveals that neoplasm cells grow as sleeves or sheaths concentric with the tumor vessels (Divan et al., 2001). Such extremely cellular layers could interfere with drug penetration to the inner layers of the tumors (Grantab et al., 2006). Radiation therapy has additionally been reported to extend perfusion and porosity of nanoparticles (Lammers et al., 2007). Radiation therapy primarily damages cancer cells with a less pronounced impact on the vasculature. Nano-sized molecules will enter the radiation treated tumors at a rate 2.2-fold more than non-irradiated tissue. Radiation killed well-oxygenated cancer cells close to neoplasm vessels; thus, radiation briefly magnified vascular porosity by reducing the barrier function of the cancer cells. The extreme cell harm occurs red in perivascular cancer cells, which eventually underwent programmed cell death. However, excessive radiation broken the vessels sufficiently to shut down blood flow, that negatively affected nanodrug delivery (Kobayashi et al., 2014).

Light medical care or therapy with conventional photodynamic therapy (PDT) may enhance the EPR impact up to 3-fold compared with control tumors, although this impact is prescribed to be within0 and 12 h after PDT (Gil et al., 2011). But, similarly like radiation therapy, PDT causes harm to both neoplasm vasculature and the tumor vascularity, there's a danger that PDT may also reduce vascularity, thus negatively affecting drug delivery (Dubreta et al., 2009). In recent work, the cancer cells were targeted via the GRP78 receptor employing a GRP78-targeting amide conjugated to a PEGylated gold nano-rod. When light was applied, photothermal harm leads to neoplasm cell killing that magnified EPR effects up to approximately 2-fold compared with untreated controls (Gormley et al., 2012). Moreover, general radio-immuno conjugates ideally killed perivascular neoplasm cells leading to improved drug delivery (Clarke et al., 2000). But, these strategies might additionally harm tumor vasculatures leading to thrombotic occlusion from the bystander impact. Near- infrared photo immunotherapy (NIR-PIT) is a recently developed cancer treatment that employs a targeted monoclonal antibody conjugated to a photo absorber, IRDye700DX (IR700, silicon phthalocyanine dye) (Mitsunaga et al., 2011). The first-in-human section 1 trial of NIR-PIT in patients with inoperable head and neck cancer targeting epidermal protein receptor was approved by the United States FDA, and is underway as of June 2015 (https://clinicaltrials.gov/ct2/show/NCT02422979). During this trial, a patient is injected with an antibody-photo absorber conjugate (APC) that binds to target molecules on the tumor's cell membrane. After 24 h, the neoplasm is exposed to NIR light at a wavelength of 690 nm that is absorbed by the dye. This induces nearly immediate death necrobiosis instead of apoptotic cell death. Within minutes, cells treated with NIRPIT rapidly increase in volume, resulting in rupture of the cytomembrane and extrusion of cell contents into the extracellular space (Sato et al., 2014). In distinction, cell death by programmed cell death tends to shrink tumor size without membrane disruption and needs a period of several days (Ziegler & Groscurth, 2004).Moreover, since the APC tends to preferentially bind to the layers of cells in the immediate perivascular space, succeeding NIR- PIT treatment results in perivascular neoplasm necrobiosis, thereby promoting increase in vascular permeability and allowing even nano-sized particles to enter the treated tumor beds. The dramatic increase in porosity for nanoparticles, followed by their retention in NIR-PIT

treated tumors, has been termed as super-enhanced permeability and retention (SUPR). SUPR impacts induced by NIR-PIT have been reported to permit a rise in nanodrug delivery up to 24-fold compared with untreated tumors during which solely the EPR effect is present (Hanaoka et al., 2015). In contrast to radiation therapy and PDT, NIR-PIT will solely induce SUPR effects because of the particular killing of cancer cells adjacent to tumor vasculature that removes the "solid stress" while not decreasing the blood flow in tumors. It has been additionally reported that cell killing after NIR- PIT was mainly on the surface; but APCs administered immediately after NIR-PIT penetrated deeper into tissue compared to the primary NIR-PIT session, leading to improved cell killing after a second NIR-PIT session (Nagaya et al., 2016). However, these changes occur within 20 min of NIR light exposure, but yet gross neoplasm size and form don't modify for many days after NIR-PIT (Kobayashi & Choyke, 2016). The effects were however, short-lived, lasting a period of hours after NIR-PIT, and sufficient to administer nano-sized medicine in combination. Thus, NIR-PIT might have an on-spot impact on the therapeutic effects of nano-sized cancer medicine.

Recently, another additional selective methodology of killing tumor cells to enhance drug delivery, named photo-immunotherapy (PIT), has been reported (Mitsunaga et al., 2011). PIT will specifically kill cancer cells exposed to near infrared light emission by inducing immediate necrosis, while not damaging normal cells (including vascular endothelial cells). Since most of the initial cell killing happens within the perivascular neoplasm sheaths, increase in nano-drug delivery up to 24-fold compared with untreated control tumors, can be observed (Sano et al., 2013). This magnified permeability was induced directly after exposure to near infrared emission. Dynamic fluorescence imaging showed that intravenously injected, non-targeted polyethylene glycol coated quantum dots (PEG- QD) quickly accumulated in the PIT-treated tumor bed compared with the non-treated controls. Microscopic anatomy after PIT showed a markedly expanded neoplasm vasculature in the widened tumor interstitium together with neoplastic cell debris. In addition, intravenously injected PEG-QD leaked throughout the cancerous tissue. Thus, PIT induces immediate necrosis particularly within the layers of cancer cells encompassing the neoplasm vasculature, while not damaging vascular cells themselves. This initially results in decreased interstitial pressure and a coextensive rise in perfusion. Therefore, PIT induces selective harm to perivascular cancerous tissues hence, markedly augmenting the EPR impact and dramatically increasing the delivery of drug.

CONCLUSION

Size plays an important role in the delivery of nanoparticles to solid tumors; however, it's not the sole parameter that affects transport. Nanoparticles form and charge may additionally be of equal importance and further studies are needed to elucidate the impact of these two physical properties. Elongated particles (e.g., nanorods) have shown superior trans-vascular flux compared with spherical particles of equal hydrodynamic radius. It's additionally been shown that cationic nanoparticles will additional effectively cross the neoplasm vessel wall compared with their neutral or anionic counterparts. Therefore, it'd be additional useful if we have a tendency to utilize not solely the size-dependence, however additionally the shape and charge- dependence of nanoparticles accumulation in solid tumors. Recently, period of time nanoparticles formulations is developed whose size modification in response to the properties of the microenvironment. PH-responsive nanoparticles aim to utilize the acidic microenvironment of the many tumors. However, the decrease in hydrogen ion concentration is comparatively small and happens at a distance of many micrometers from the blood vessels. Therefore, effective opening transport is a very

important parameter that should be thought-about. Currently, clinically approved nanoparticles formulations exploit the EPR impact and passive delivery. Active delivery of nanoparticles with targeting ligands on their surface is another approach that allows specific binding to cancer cells. Although addition of targeting ligands has usually did not improve intratumoral penetration, there were cases wherever targeted nanoparticles has been shown to considerably increase delivery and therapeutic outcome. Therefore, the sphere of active delivery of cancer nanomedicine is promising and further studies are useful. Intracellular delivery of drug-loaded organic compound conjugates and pharmaceutical nanocarriers accumulated in tumors via the EPR impact will be expedited by numerous means. Consequently, the opportunities for the neoplasm drug delivery look simply. However, no matter complicated schemes are being developed to effectively bring metastatic tumor medicine and genes into tumors, the EPR effect-mediated neoplasm accumulation remains the primary crucial step. Nano-sized cancer medicines were promising as they will be extremely loaded with anti-cancer agents and desirable neoplasm delivery supported the unmodified EPR impact. Many strategies to boost nano-drug delivery into cancer tissue have been discovered. Those strategies improved delivery by the maximum amount, about 2-fold compared with non-treated tumors. However, increased EPR effects that occur once induces harm within the layers of cancer cells directly adjacent to the neoplasm vasculature and have dramatic effects on introduction with enhancements in the delivery of nano-particles of up to 24-fold compared with untreated tumors. The magnitude of the nano-delivery improvement might have an on the spot impact on the therapeutic effects of nano-sized cancer medicine presumably leading to dose reductions. Overall, additional selective targeting of tumor vasculature that doesn't because occlusion would be a key factor for winning improvement of nano-drug delivery supported changed EPR effects.

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Chapter 27
Monocytes as Targets for Cancer Therapies

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ABSTRACT

The importance of monocytes in modulating the lymphocyte dependent tumor necrosis is a target for cancer therapeutics. Monocytes produce a plethora of chemokine receptors. Lymphocyte to monocyte ratio is one of the negative factors in cancer patients. It is being targeted for treatment of abnormal lymphocytopenia and monocytosis in untreatable metastatic cancer patients. The aim of the chapter is to throw light on the circadian and psychological factors that modulate the progression of cancer and identify novel targets for controlling transformation of preneoplasms to neoplasms, invasiveness, and metastasis.

INTRODUCTION

They are the largest type of cells in the peripheral blood under normal conditions. Their cytoplasm has azurophillic granules which stain purple to dark blue. They originate from the myeloid or progenitor cells in the bone marrow both during homeostasis as well as inflammation and migrate into blood. The works by (Dunay et al., 2008) and Serbina et al(2006) have thrown some light on the mechanism by which the monocytes leave bone marrow. They have shown that these cells egress from the bone marrow under the influence from CCR2 expressed by Gr-1hi monocytes in mouse (Dunay et al., 2008 and Serbina et al., 2006). The circulating TLR –Ligands can induce the production major monocyte chemoattractant MCP-1 by the bone marrow mesenchymal and progenitor cells (Shi et al., 2011). MCP-1 binds to CCR2 and promotes their egress from the bone marrow. In humans the exact mechanism is still not known. Further the exact mechanism of functioning of Gr1 low (mouse) monocyte subsets is yet to be ascertained. Apart from the bone marrow the spleen has a large reservoir of these cells.

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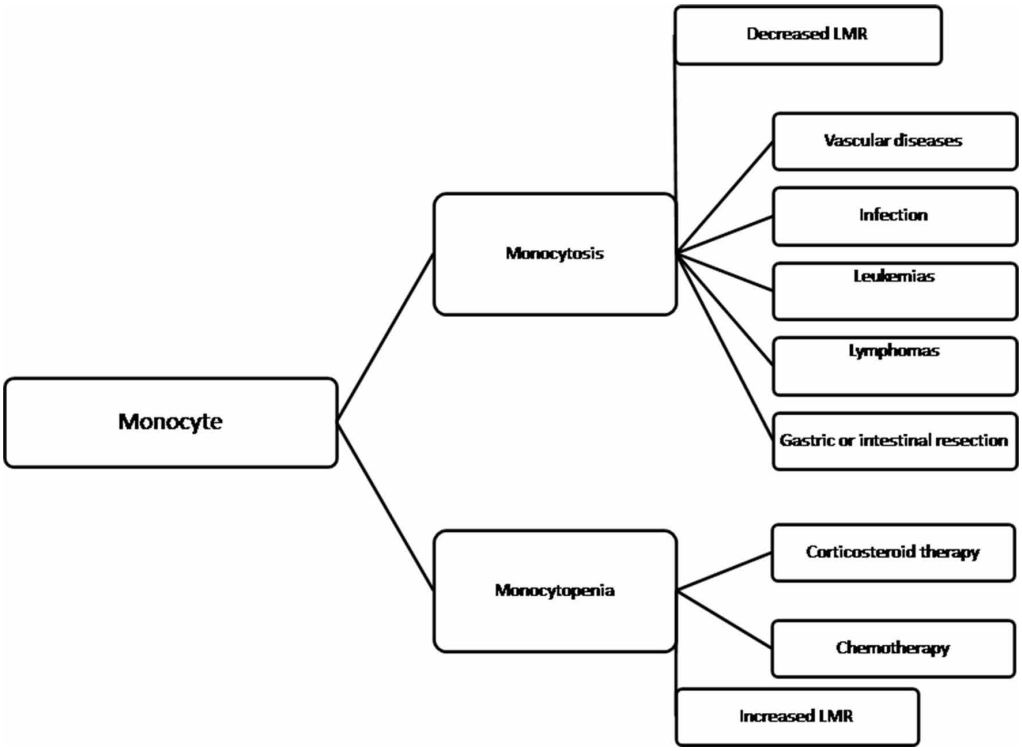
ROLE

The monocytes exit spleen and migrate to the site of injury (Reiss 1999), infection (Dunay et al., 2008, Dunay 2010, Voisine et al., 2010; Shi et al., 2011; Kim et al., 2011) or inflammation (Karlmark et al., 2012). It is in these sites of injury or inflammation that these monocytes will differentiate into various types of macrophages and dendritic cells. There are three types in humans based on the cell surface marker expressed i.e., classical $CD14^{++}$ $CD16^{-}$, non classical $CD14^{+}$ $CD16^{++}$and intermediate $CD14^{++}$ $CD16^{+}$. The mechanisms involved by the macrophages deploy to kill pathogens is phagocyting them to etosis. Monocytes produce a plethora of chemokine receptors. The interaction of chemokine receptors with the corresponding ligand governs the fate and function of monocytes. Upon infection or injury first line of defense comes from neutrophils followed by the monocytes which take over by producing the cytokines like IL-1Beta, IL6, TNF-ALPHA to modulate inflammation The Monocyte chemoattractant protein 1 (MCP1) is the major ligand for CCR2. The cytokine receptors SDF1-CXCR4 interactions are important for the homing of leukocytes in the bone marrow. Hence the importance of monocytes in modulating the lymphocyte dependent tumor necrosis is a target for cancer therapeutics. Mutations in the CXCR4 may cause WHIM (Swirski et al., 2009; Hernandez, et al., 2003; Gorlin, et al., 2000). Monocyte subsets with chemokine receptors with their corresponding ligands are as follows: Classical monocytes Mouse: Gr-1hi Human: CD14++CD16– CCR1 CCR2 CXCR2 MCP-2, MIP-1α (CCL3), RANTES (CCL5) MCP-1 (CCL2), MCP-3 (CCL7), MCP-5 CXCL1 (GROα), CXCL2 (GROβ), CXCL3 (GROγ) Non-classical monocytes Mouse: Gr-1low Human: CD14+ CD16+ CX3CR1 CCR5 CCR6 CX3CL1 (Fractalkine) CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) CCL20 (MIP-3α). Mouse models have been extensively used to discover the molecular mechanisms involved in monocyte mediated control of infections. Using mouse models, it has been shown that the monocytes Gr-1hi CCR2+ differentiate into TipDCs under certain microbial infection and produce TNF-a and iNOS as a strategy to control the infection (Gorlin, et al., 2000; Geissmann, Jung, & Littman, 2003 Serbina et al. 2003 and Serbina et al. 2009). Further these cells promote adaptive immune response by acting as antigen presenting cells to lymphocytes. It has also been shown in case of experimental muscle injury that these cells phagocytize the apoptotic cells followed by a phenotype switching where these cells become anti-inflammatory from proinflammatory and start producing IL10 and TGF-B1, for regeneration of muscles (Aldridge et. al., 2009). It has also been shown that during the inflammation of the skin and lungs they differentiate into Langerhans cells and CD103+ pulmonary dendritic cells. In a hepatic injury model, the monocytes have been shown to modulate disease progression by either producing iNOS or Arginase in response to the TH1/TH2 environment (Shi et al., 2011). Immunosuppression caused by monocyte macrophage system can be assessed by PB monocyte count. The degree of monocyte macrophage system regulates T lymphocytes via cytokines (Dunay et al., 2016). Leukocytosis has also been found to be correlated with depression. Further, both monocytosis and neutrophilia are associated with depression. The effects of depression may be caused by an underlying inflammatory process. Such effects are most profound in the cases of major depression whereas those suffering from minor depression have intermediate effects as compared to the normal controls (Serbina et.al., 2012). Hence the additional effects of depression on progression of disease are evident. Monocytosis may be induced by several factors from infections to neoplasms as well as therapies for treatment. Infections caused by viruses like HIV, Epstein Barr virus etc and bacteria like mycobacterium tuberculosis, Treponema pallidum, etc., protozoans such as Leishmania, Trypanosoma, Typhus etc., Neoplastic disorders like hairy cell leukemia, acute myeloid leukemiA, lymphomas etc., Monocytosis in patients with vascular disorders has been correlated with increased risk

of heart attack (Arnold et al., 2007) Monocytosis may occur in combination with lymphopenia and neutropenia in primary immune deficiencies like GATA 2 involved in the hematopoetic cells (Maes et al., 2009). Apart from these gastric or intestinal resection patients show monocytosis. The opposite of condition arises when monocytes in blood are less than $< 0.5 \times 109/L$ (MSD Manuals). It is caused under certain conditions like Chemotherapy induced myelosuppression in single and combinational therapies for treatment of malignant cancer has been seen. This results in monocytopenia along with deficiencies of other blood cells. (Mary 2020). Corticosteroid therapy induced monocytopenia was evident in the initial response to therapy (Nazir et al. 2016). Monocytopenia is evident in peripheral blood but the tissue macrophages are retained as specific types in different tissues hence PB monocyte count is not indicative of tissue monocytopenia. It increases the chances of infection and has been associated with chronic infections and MonoMAC disease (Rinehart et al. 2016). Monocytes are integral part of innate immune response and is also involved in a cross talk between innate and adaptive immune systems as they can differentiate into macrophages and dendritic cell types. The later are involved in antigen presentation for cell mediated immune response. Macrophages can be free or fixed and differentiate into kuffer cells in liver, histocytes in lungs alveolar macrophage and mesangial cells in kidney and microglial cells in brain. There are three subsets of monocytes based on the pattern of CD 14 and CD16 cell surface proteins. They have distinct functional properties with differential gene expression. Monocytes secrete many cytokines including factor (TNF), IL-1a, IL-1b, IL-6, IL-8, IL-10 and IL-12, IL-18 etc,.24 Monocytes are stimulated by kinin system to produce a number of cytokines and bk2 induced pig rat and human macrophages increased arachidonic acid and prostaglandins. (Sampath et al.,

Figure 1. Schematic representation showing overall monocyte count and associated conditions



2018; Bo¨ckmann et al., 1998; Lerner et al.,1989; Tiffany et al., 1989 and Burch et al., 1989). MLR can be a good biomarker for TB (Monif et al., 2018). Monocytosis has been associated with respiratory diseases. Lung function impairment fever and smoking has also been found to trigger it. It also associates with neural, cerebral and vascular diseases. Monocytosis has been linked with high mortality in cardiovascular diseases, atherosclerosis, rheumatoid arthritis, systemic lupus erythematosus and also negatively influences disease outcomes (Gary et al.,1979). The role of monocytosis in cancer was shown as early as 1979 including the suppression of T cells (Marc et al., 2019). The relative ratio of monocytes to lymphocytes is a prognostic factor in epithelial ovarian cancer (Feng et al., 2016). The relative values of tumor size and MLR, PLR MWR has shown promising result for predictiveness in gastro intestinal stromal tumor prognosis. Increased MLR and in gastric cancer has been linked to poor overall survival and treatment outcome in metastatic gastric cancer (Wan et al., 2018; Zhou et al.;2018 and Feng et al., 2016). Monocytosis has also been found an independent prognostic factor for disease free survival and overall survival in cervical cancer (Tamar 2016). In diffuse large B cell lymphoma, it has been associated with poor survival (Stakheyeva et al., 2019). Persistent monocytosis is one of the diagnostic factors for chronic myelomonocytic leukemia (Larionova et al., 2019). The use of monocytes as anti-cancer therapy agent is being widely acknowledged. Brief monocyte count related outline is shown in figure 1. A phase two trial study has shown that high MLR treatment can improve immune status of cancer patients for treatment of metastatic cancer patients. Systemic lupus erythematosus (SLE) is associated with age dependent pathogenesis promoted by *Nba2* locus stimulating FcγR, apart from deregulated activation of autoreactive B cells (Shuichi et al., 2006). Lymphocyte to monocyte ratio is one of the negative factors in cancer patients. It is being targeted for treatment of abnormal lymphocytopenia and monocytosis in untreatable metastatic cancer patients using pineal hormone Melatonin (Shuichi et al., 2006). Using melatonin therapy immunosuppression has been shown to reduce in cancer patients. Further research on melatonin therapy mechanism of action will throw light on the circadian and psychological factors that modulate the progression of cancer and will help identify novel targets for controlling transformation of preneoplasms to neoplasms, invasiveness and metastasis.

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About the Contributors

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Mohd Aleem studied at Jamia Millia Islamia New Delhi, India and completed here his master of science with distinction in Biochemistry in 2016. He did his six months internship on the topic of "Repeated dose toxicity and biochemical parameters in female wistar rats by dermal administration of Gold nano-particles containing gel" under the supervision of Dr. Himanshu Ojha Sc, D from Institute of Nuclear Medicine and Allied Sciences (INMAS-DRDO), New Delhi, India. He had done his graduation with the subjects Zoology, Botany, and Chemistry from CCS University Meerut, UP, India. He has qualified CSIR/UGC NET (Life Sciences) AIR 62 and GATE -XL (Life Sciences) AIR 1016. Presently, he is doing PhD in Neurobehavioural Sciences from Institute of Nuclear Medicine and Allied Sciences. Mr. Aleem has research interest on the applications of nanomaterials and their catalytic roles on various drugs and finding out a mechanism to cope up with traumatic injuries.

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Farhad Bano began her research in the lab of Prof. Banalata Mohanty at the University of Allahabad, Prayagraj, India, awarded PhD in March, 2020 with her thesis focused on the "Effect of Endocrine Disruptors on Immune System". She subsequently joined the lab of Dr Ayub Qadri at National Institute of Immunology, New Delhi, India and presently interested to elucidate the immunomodulatory effect of nanoparticles based vaccines on diseases.

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Muneesh Kumar Barman's areas of interest are virology and molecular biology. After completing Masters degree in Biotechnology, he taught as an Assistant Professor in Biotechnology at Bastar University (a state university in Chhattisgarh, India) for 2.5 years. Currently he is affiliated to National Centre for Cell Science, India and his research focus on HIV-Host protein interactions and their effects on HIV pathogenesis.

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Prudhvi lal Bhukya is a young molecular virologist currently serving ICMR- National Institute of Virology, Pune as a Project Scientist-B. Currently he is working on Prevention and control of diseases and development of tools to prevent out breaks. He has registered for Ph.D and about to submit his thesis on human hepatitis B virus. He proved his credentials by working on HBV and HEV with his publication record. He has presented his work in numerous national and international conferences. He has proved his accolades with several publications in International Journals like Journal of General Virology Journal of Virology; Science of Total Environment. He is a well experienced manuscript writer and has contributed to several book chapters as Author for publication bodies like Springer Nature CRC-Press, Elsevier & Intech open

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Khalid Umar Fakhri has completed his graduation in science subject and post-graduation in Biomedical Sciences from Dr. Ambedkar Center for Biomedical Research, New Delhi. Thereafter, he went on to acquire her Doctor of Philosophy from Jamia Millia Islamia in human colorectal cancer under the supervision of Dr. M. Moshahid Alam Rizvi. At Jamia Millia Islamia, he is also handling UGC–SAP DRS II project of Rs 1 Crore 15 Lakh entitled " Impact of defined flavonoids on colon cancer intervention: Targeting signaling cascade under in vitro and in vivo systems" independently. His key area of research includes cancer chemoprevention based on the use of crude traditional medicines, purified medicinal products, and synthetic drugs. His current research work is based on cancer therapeutics. It includes the impact of the anticancer effects of anthraquinone and various flavonoids on different colorectal cell lines and tumor-bearing mice model system.

Abhilash G. has completed his Ph.D in Computational Biology from CSIR-Institute of Genomics and Integrative Biology, New Delhi. He received his M.Tech in Computational Biology from Jawaharlal Nehru University, New Delhi in 2011. His research interests include studying the predictive ability of aggregated genetic interactions measures derived from high-throughput functional genomics studies in context of long term evolution experiments and characterization of transcriptional biomarkers in limbal epithelial stem cells used in corneal grafts. He is deeply invested in science communication especially through scientific visualizations and is the joint recipient of the second prize in the Nature India Essay Competition 2020.

Sarbani Giri is Principle investigator of Laboratory of Molecular and Cell Biology with more than 24 years of teaching experience and currently serving as Head of the Department of Life Science and Bioinformatics. Assam University, Silchar. She is recipient of DBT Long term Overseas Associateship and joined Laboratory of Metabolism, National Cancer Institute, National Institute of Health, Maryland USA (2005- 2006). She was awarded with Indo-US Science and Technology Forum fellowship (2008). She joined Department of Molecular and Cell Biology, University of California, Berkeley, USA, as long term visiting scholar. Her research focus is mainly Life style factors and its impact on human health, xenobiotic / drug metabolism, role of tight junction integrity in oral cancer, downstream signalling pathways involved and environmental health assessment.

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Zubair Bin Hafeez is working as a Research Associate at the Department of Biosciences, Jamia Millia Islamia. His research area is cancer cell biology and therapeutics with seven years of research experience to evaluate the molecular mechanism of tumor suppressor genes involved in the development of cancer. His research interest is to identify the multiple molecular targets that play a potential role in cancer progression, thereby inhibiting the use of natural inhibitors. He completed his Ph.D. degree from the Department of Biosciences, Jamia Milia Islamia, New Delhi. He has expertise in Cell culture techniques (Primary cell culture, in vitro transformation, Cell biology techniques (ImmunoCytogenetics and molecular biology cytochemistry, flow cytometry, Western Blotting, etc.) techniques and in vivo imaging. He has published several research articles and abstracts in international journals and presented his work in National/ International symposiums.

Mohammad Raghibul Hasan appointed as a faculty member (2018) in the Department of Biochemistry and medical laboratory Sciences at Shaqra University, Riyadh, Saudi Arabia. Currently, he heads the Scientific Research Unit at College of Applied Medical Sciences, Al- Quwayiyah, Shaqra University. His research interests include translational cancer research from benches to clinics. He has been working on biomarker development on gastrointestinal cancers. His interests are focused on next-generation sequencing to study the miRNA and its targets in human gastrointestinal cancers. He has developed and conducted numerous courses focused on biochemistry, cancer research, and various other topics that also serve on various academic committees. He has been published and presented his work both nationally and internationally. Hasan completed his BSc in Biochemistry at All India Institute of Medical Sciences (AIIMS) New Delhi (2002) and moved to Madurai Kamaraj University, Tamil Nadu, India, and was awarded his M.Sc. in Biotechnology in 2005. He earned his Ph.D. degree in Biochemistry from AIIMS, New Delhi, in 2012. His Ph.D. thesis is entitled "Clinical and Functional Characterization of Genes with Altered Expression in Esophageal cancer." He has an exemplary academic record comprising a national level fellowship from the Department of Biotechnology, Junior Research Fellowship (DBT-JRF), and Senior Research Fellowship (SRF) from India's Government. In recognition of his novel research contribution on RNA mediated down-regulation of TC21 sensitizes esophageal cancer cells to cisplatin, he received the Scholar in Training Awards for American Association for Cancer Research (AACR) for poster presentation in International Conference on "Advances in Cancer Research: From

Laboratory to Clinic" in Jordan 2010. After completion of his degree, he appointed as a Scientist Lab. Operations in Preventive Life Care Pvt LTD in Mumbai for the development of diagnostic kits for inborn errors of metabolism for one and a half years (2013). In November 2013, he joined a short postdoc in a molecular genetics laboratory at AIIMS to study RB genes. Hasan undertook postdoctoral studies at the Gastroenterology, AIIMS and studied the role EpCAM in tumorigenesis of esophageal squamous cell carcinoma (2014-2017). He also served as a Guest faculty of Biochemistry at Jamia Millia Islamia (2017-2018). His laboratory is currently working on understanding the role of miRNA in gastrointestinal cancers. He currently serves on the Editorial Board of Frontiers in Oncology, specific to gastric cancers.

Ziaul Hasan is an energetic and dedicated young researcher in the field of Biological Sciences. At present, he is in the mid-phase of his research career. Mr. Hasan did his Graduation and Masters in Biochemistry from Aligarh Muslim University. Currently, he is working as a Predoctoral Student in the Department of Biosciences at Jamia Millia Islamia Central University, New Delhi, India. His research interest includes protein biochemistry, mycology and molecular biology. Currently, he is associated with Novel Global Community Educational Foundation, Australia as an honorary student counsellor representing India. He has been awarded many prestigious scholarships and honours during college days. He is also Life Member of Indian Science Congress Association (ISCA) and Natural Product Science Task Force (NPST). He is recently appointed as Ambassador of Youth Combating Neglected Tropical Diseases (NTDs). He is now working to suggest natural products as an alternative to commercial synthetic antifungals by studying their biophysical aspects in combination with the computational approach.

Vishal Jain presently working as assistant professor at university Institute of Pharmacy, Pt. Ravishankar Shukla University, Raipu(CG) and has has nearly 16 yrs of teaching and research experience. He has over 50 publications to his credit published in national and international journals. He has presented 50 papers in various national and international conferences. He is an active member of IPA, APTI, and Indian Society of Pharmacognosy (IST). His research interest extends from Phytochemical screening and standardization of herbals. He has sucessfully guided two Ph.D. and several m. pharm student.

Zakia Kazim has done her PhD from Jamia Millia Islamia under the supervision of Prof. Moshahid A Rizvi on the topic of Molecular Analysis of PTEN Gene in Breast Cancer Patients. During PhD, she worked on different genetic and epigenetic studies of PTEN gene in breast cancer. In addition to her PhD thesis work, she was involved in other ongoing projects on PTEN in the Lab. She has been a recipient of Women Scientist Fellowship from DST (Department of Science and Technology), India and UGC fellowship under Maulana Azad National Fellowship scheme. She expertise with most of the modern molecular and biochemical techniques such as MS-PCR, SSCP-PCR, RT-PCR, Immunohistochemistry, western blotting, chromatographies, PAGE, DNA fingerprinting, spectrophotometry etc. She also took the lead in training the graduates and postgraduates for their dissertations. She has demonstrated her capability to perform well in diverse fields of research and can work very well both independently and in a team. She has independent ideas and high learning skills, which will make her a very effective and responsible researcher in future.

Raj Kumar has done PhD from Jawaharlal Nehru University in 2018 and is currently working as post-doctoral fellow in USA. He has extensively worked on cancer biology and chemo preventive molecules. His research interests include in Virology, Microbiology and Cancer Research. Their most recent publication is 'Piperazine clubbed with 2-Azetidinone derivatives suppresses proliferation, migration and induces apoptosis in human cervical cancer HeLa cells through oxidative stress mediated intrinsic mitochondrial pathway'.

Umesh Kumar has done his doctoral thesis entitled "Epigenetic Regulation in Breast Carcinogenesis" in Dr. B. R. Ambedkar Center for Biomedical Research (ACBR), Delhi University (North Campus) in 2013. From his thesis he was able to publish his interesting findings in peer reviewed international journals of repute. Dr Kumar joined Division of Molecular Oncology in Institute of Cytology & Preventive Oncology (ICMR), Noida which was also WHO collaborated South East Asia Referral Laboratory for the Diagnosis of HPV induced Cervical cancer and later he shifted to Dr. B. R. Ambedkar Center for Biomedical Research (ACBR), Delhi University (North Campus) in 2010. After his doctoral thesis He joined Stem Cells Biology Laboratory for his Post Doc in National Institute of Immunology, New Delhi in 2014 where His Scientific Group has published in the field of Epithelial ovarian cancer stem cell in Nature Oncogene. In 2016, he worked in Department of Biochemistry as Scientific Officer for DNA Sequencing and Microarray facility in South Campus, University of Delhi up to July 2017. In August 2017 he joined Molecular Oncology laboratory in Drug Discovery & Development Laboratory, Department of Chemistry, Delhi University. He has studied promoter hypermethylation of important tumor suppressor genes including BRCA1, p16, CDH1, GSTP1, HIC1, MGMT gene in sporadic breast cancer and their correlation with increasing severity of breast lesions. Study indicated pivotal role of epigenetic silencing of specific genes in breast cancer which may serve as potential therapeutic target for breast cancer.

Sai Tejaswi Lavuri is currently pursuing 3rd year of her MBBS degree and interested in the fields of oncology, immunology, and infectious diseases. She would like to pursue her masters and research in viral oncology for better treatment prospects in the future.

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Salonee Martins has graduated with a Bachelors degree in Microbiology from the University of Pune and is currently pursuing a Masters in Virology from the National Institute of Virology, Pune. Her research interests lie in the fields of immunology, evolutionary biology and space biology. She is passionate about scientific communication, especially where climate change and wildlife conservation is concerned. She aims to apply for a PhD programme in the near future.

Ankit Mathur is working as a post-doctoral research fellow at Dr. B.R. Ambedkar Center for Biomedical Research, University of Delhi. He is a cancer cell biology researcher with more than nine years research experience contributing to cell biology studies for biomedical interventions to improve radio/chemo therapeutic outcome. His general research interest as a postdoctoral fellow is to target cellular and molecular mechanisms of acquired or intrinsic resistance towards radio and chemotherapeutic agents. He completed his PhD from Institute of nuclear medicine and Allied Sciences (DRDO), Delhi. He have expertise in Cell culture techniques (Primary cell culture, in vitro transformation, 3D culture etc.), Cell biology techniques (Immuno-cytochemistry, flow cytometry, Western Blotting etc.),Cytogenetics and molecular biology techniques. He has excellence in fluorescence microscopy. He has published several research articles and abstracts in several reputed journals and also presented his work in several national/International conferences. He was nominated as top five young scientists by Society of Radiation Research.

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Sana Nafees has research experience in the field of carcinogenesis, cancer chemoprevention, molecular basis of chemical carcinogenesis, molecular biology, role of free radicals in tumor promotion, progression, prevention of chemically induced toxicity in in vivo as well as in vitro system. She has skills related to cell culture, cell handling, as well as animal handling. In addition, her expertise of handling techniques like Real-Time PCR (RT- PCR), Western blot and Immunofluorescence are an added advantage. Her post-doctoral experience at Jamia Millia University based on in vitro and her Ph.D. expertise on in vivo models. She proved her credentials with her publication record.

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doctoral research work at Genome Biology Laboratory in Jamia Millia Islamia under the broad area of Molecular Oncology and Therapeutics where he investigated the role and the interaction of novel Parkin (PARK2) with another tumor suppressor gene P53. His research has highlighted some important aspects of two tumor suppressor proteins (Parkin and p53) regulating different cell cycle regulators involved in colorectal cancer progression. He has several peer reviewed publications to his credit and has presented his doctoral research data in an international conference on Precision medicine at Keystone Symposia, Sweden. Broadly speaking, he has a deep interest in understanding the molecular mechanisms involved in the initiation and progression of colorectal cancer and developing strategies to intervene through novel small molecules or natural products.

Abid Qureshi has done PhD from Institute of Microbial Technology (IMTECH), Chandigarh. He has worked as a lecturer in Punjab university and then he joined Sher-i-Kashmir Institute of Medical Sciences (SKIMS), J&K as a research scientist. His areas of interest include:Antiviral molecules, HIV, RNA interference, siRNA, miRNA, Peptides, Drugs, Machine learning, Prediction, Web programming, Databases, Data visualization and QSAR.

Rashi Rai is a self-motivated biotechnology graduate having experience in several areas of research like cloning, expression studies, cell culture, and drug repurposing with a record of achievement providing outstanding research skills, comprehensive knowledge of all standards and regulations, and operational dexterity in fast-paced laboratory environments. Able to combine current academic knowledge and theoretical training with practical experience to provide solution-oriented service.

Garima Rathi is a Post Graduate Teacher in Delhi Public School Ghaziabad,Meerut Road Uttar Pradesh. She is having vast experience of 11 years in Biology teaching.She has published several articles in breast cancer research. She did her masters in Biotechnology from Chaudhary Charan Singh University Meerut and graduated in Education from University of Delhi, Delhi.Her research area is breast cancer epigenetics.

Nerethika Ravichandran is pursuing M.Sc., Biomedical Science in Bharathidasan University. trained in the techniques of molecular biology dissertation work on the title " PHLPP1 role in the macrophage polarisation on IL-4 treatment " in Dr. Reddy's Institute of Life Sciences (DRILS), Hyderabad.

Md. Rizwanullah is currently working as Senior Research - CSIR fellow at the Department of Pharmaceutics, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi. He received his M. Pharma in Pharmaceutical Sciences (Pharmaceutics) from the School of Pharmaceutical Science and Research, Jamia Hamdard, New Delhi. He has more than five years of research experience in the field of Pharmaceutics. His major areas of interest include drug delivery, development of controlled release nanoparticle systems and cancer nanotechnology. He has authored over 20 publications in various peer-reviewed journals and eight chapters. He has also participated and presented his research work at various national and international conferences. He is a member of the IPGA (Indian Pharmacy Graduates' Association) and reviewer of various international journals.

Shalini Sakthivel, pursuing Integrated M.Sc in Bio-medical Science, Bharathidasan University, Tiruchirappalli. She has skills related to cell culture, cell handling, and various molecular techniques like cloning, transformation, and transfection. Her dissertation project experience at ICMR-National Institute of Virology, Pune about Gene expression profiling studies on Hepatitis B virus (HBV). In addition to she expertise in the qPCR technique.

Purva Salvi has graduated with a Bachelors degree in Microbiology from the University of Pune and is currently, a post-graduate student at the National Institute of Virology, Pune. She is extremely passionate about helping further research in Life Sciences. Her interests lie in the fields of Virology, Genetics, Astrobiology and Quantum Biology. She would like to pursue a PhD in the near future.

Deepshikha Sharma has completed her graduation in Life Sciences from University of Delhi, India where she learnt molecular techniques, like DNA isolation,PCR, agarose gel electrophoresis, SDS-PAGE. She has completed her post-graduation in Bioinformatics from Jamia Millia Islamia, India where she learnt various bioinformatics techniques, such as Molecular Modelling, Molecular Docking, and programming languages like C, SQL, BioPerl. She is currently learning Python programming language.

Deepti Sharma is a Ph.D. student in the Division of CBRN Defence, INMAS, DRDO in India. She is currently working on the development of topical formulations for the healing of wounds. She also has an expertise in animal handling techniques and various spectroscopic techniques like UV-Vis spectroscopy, fluorescencespectroscopy, circular dichroism spectroscopy, isothermal titration calorimetry, etc. She has completed her post-graduation in Biosciences from Jamia Millia Islamia, India where she learnt the cell culture techniques. She is working on wound healing models in vivo and in vitro. The in vivo models include excision wounds, incision wounds and radiation burn on rat's skin and in vitro models include scratch wounds, radiation wounds on human skin cells (fibroblasts and epidermis) to assess the efficacy, toxicity and molecular mechanism of the prepared formulations behind the wound healing.

Lakshit Sharma is a post graduate in Biotechnology from IMS Ghaziabad. He did his master's dissertation from National Institute of Cancer Prevention or Research, Noida on examining the anti-cancer effects of a homeopathic medicine on HeLa cells. Prior to this, he did bachelor's in Zoology honors from the University of Delhi. He also did online courses like Introduction to Cancer Biology offered by John Hopkins University. He intends to pursue his PhD in Cancer biology.

Shruti Sharma is currently pursuing her PhD from Department of Experimental Medicine & Biotechnology, PGIMER, Chandigarh. Ms. Sharma has qualified the National level fellowship (ICMR-JRF) and scored top 5 position. She is the founding member of Integrated Association of Medical Basic and Social Scientists (IAMBSS). Ms. Sharma has presented her research at various scientific meetings and conferences.

Ujjawal Sharma is a Research Scientist at Department of Community Medicine & School of Public Health, PGIMER, Chandigarh. He has completed his PhD from Department of Biochemistry, PGIMER, Chandigarh in the area of cancer biology. Dr Sharma has nine years of post PhD teaching and research experience from various prestigious institutes of India like National Institute of Immunology, New Delhi, Government Medical College, Chandigarh and PGIMER, Chandigarh. Dr Sharma has more than

25 research publications in journal of national and international repute and three book chapters in his credit. He has been invited to present his research work at various national and international conferences. He has been the organizing Chair of National conference "IAMBSSCON 2009". Dr Sharma has been awarded with various national and international awards like Young Investigator Award by Malaysian Society of Interventional Radiology, Integrated Leadership Award-2019 by Integrated Association of Medical Basic and Social Scientists (IAMBSS), India and International Travel Award by various agencies. Dr Sharma is an active member of various scientific associations and is co-founder and President of Integrated Association of Medical, Basic and Social Scientist (IAMBSS).

Madhu Singh has completed her Master Degree in Biotechnology as a subject from Jamia Millia Islamia, New Delhi. Later she joined Jawaharlal Nehru University for pursing doctoral degree and further advance her interest in p53-SIRT1 crosstalk under normal physiological and different stress conditions. Thereafter, she again joined Jamia Millia Islamia as DSK postdoctoral fellow to further advance the knowledge in cancer and redox signaling.

Subash Sonkar working as Scientist-II at Maulana Azad Medical College, New Delhi. He worked as Consultant and Technical Advisor implementation in National Mental Health Program at Indian Council of Medical Research and he was an Ex-Principal Investigator on NIH funded project affiliated with Florida International University in collaboration with Public Health Research Institute of India. He is an internationally renowned transnational research professional and academician with more than 10 years of experience in project planning, review, evaluation and technology assessment. He is associated as Editor, Reviewer and Member of many online journals. His work focuses upon diverse themes crossing the interface between laboratory-based research and transnational research in valuating diagnostic tests and effective diseases managements.

Manjita Srivastava is a molecular virologist working as DST Women Scientist A at National Institute of Virology, Pune working as Principal Investigator for the project "Role of cellular autophagy and its modulation in the replication of Hepatitis E virus". She has been funded by Department of Science & Tech, India of Rs 32 lacs. She has skills related to cell culture, cell handling, various molecular techniques like cloning, transformation, transfection, in-vitro transcription. In addition, her expertise of handling techniques like Real-Time PCR (RT-PCR), Western blot, Immunofluorescence, Confocal Microscopy, Sequencing and FACS are an added advantage. Her post-doctoral experience at Brown University on oil based microfluidic chip for RNA isolation and her PhD expertise on handling viruses like Hepatitis B and Hepatitis E, along with clinical samples and working on proinflammatory cytokines and therapeutic outcome will be an extra credit for this project. She proved her credentials with her publication record.

Armiya Sultan is working as a Post-Doctoral Research Associate at Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia (A Central University) New Delhi. His area of research interest is cancer biology, cancer chronobiology and chronotherapy, gut-microbiota, structure assisted drug designing and targeted cancer therapy. His has more than eight years of research experience. He is well experienced in molecular biology, cell biology, cell culture and psycho-neuroendocrinology techniques. He has published several research articles in various peer reviewed reputed national and international journals. He has also presented his work in several national/International symposia/conferences and has been awarded with several awards.

Mohamad Taleuzzaman passed his B. Pharm. and M. Pharm. (Pharmaceutical Analysis) from Jamia Hamdard, Delhi in 2005 and 2007 respectively. Ph. D. From Glocal University Saharanpur, 2018. Qualified GATE examination, conducted by IIT Mumbai in 2005 with percentile score 92.49. Completed M. Pharm. research project from Fortis clinical research Ltd. (FCRL), Faridabad in 2007. Total professional experience more than twelve years in academia and industry. Contributed thirty five research / review article in international refereed journals Published scientific abstract in national and international conferences. Attended Conferences, Workshops and also completed one month of instrumental training. Editorial /Advisory Board Member- Austin Chromatography Journal, Asian Journal of Pharmaceutical Analysis, Medicinal Chemistry and Chronicles of Pharmaceutical Science. Research interest area- Method development, validation and characterization of drugs by an instrument like UV-Spectroscopy, FTIR, HPTLC, HPLC, and LC-MS/MS.

Asma Tariq is currently working as the Dental Surgeon practising at Government sector. She has received her masters in hospital administration and in sociology in 2014. She has been awarded bachelors degree from Rajiv Gandhi University in 2010. Over the last five years, she has contributed extensively in patient care and set up and has been engaged in publishing original articles and book publications from IGI global related to human disease. Her interest in clinical research will pave way for effective patient management and mental health.

Basharat Bashir Teli is currently pursuing PhD in Life Sciences at School Of Life Sciences Jawaharlal Nehru University New Delhi 110067, India. He has qualified various state and national-level examinations, including the JK SET, Graduate Aptitude Test in Engineering (GATE) in Biotechnology, ICMR JRF Examination as JRF in ICMR funded Research Projects/Scheme, Joint CSIR UGC NET JRF examination in Life Sciences. In addition to carry out his doctoral research studies he is receiving fellowship from CSIR UGC JRF from the University Grants Commission, New Delhi India. He has also qualified for Interview based on BARC TS Online Examination in Bioscience discipline conducted by the Bhabha Atomic Research Centre Training Schools. Currently, Basharat Bashir Teli is actively involved in Exploring the Functional Genome of Candida albicans. He has passed his postgraduation in Biotechnology at Department Of Biotechnology (DBT Funded M Sc Program), University Of Kashmir, Srinagar, Jammu and Kashmir, India. He has pursued his master's dissertation at Department Of Biotechnology (DBT Funded M Sc Program), University Of Kashmir, under the supervision of Dr. Altaf Bhat and worked on PCR-mediated direct gene disruption (SPBC16G5.16) in Schizosaccharomyces pombae for functional analysis of heterochromatin.

Bui Thanh Tung received his PhD in Pharmacology from the Seville University, Spain in 2014. Then he worked in School of Medicine and Pharmacy, Vietnam National University Ha Noi as lecturer. In 2018 he was pointed as Associate professor. His main research focuses on medicinal plants for the treatment of various diseases such as diabetes, Alzheimer, cancer… His special interest is on the development of new drugs from Vietnamese natural resources. He writes and presents widely on issues of natural products, bioactive compounds and their pharmacological activities.

Piyush Kumar Tiwari has completed his B.Sc. from university of Delhi in Life sciences and Completed his Master in Industrial Biotechnology from Sardar Patel University sponsored by DBT India. He had a worked in Genome informatics at Bionivid, Bangalore. Currently he is working on engineering Prospective of genome editing, genome sequencing and Proteomics. He is an active participant of National Training and Workshop of genomics and proteomics, and attended workshops at various reputed institutes like IIT Delhi, NCL Pune etc.

Gyanendra Tripathi is pursuing Ph.D. in Bioengineering from the department of bioengineering, Integral University.

Puja Upadhaya has done her Ph. D from the Department of Life Science and Bioinformatics, Assam University, Silchar under the supervision of Prof. Sarbani Giri.

Shekhar Verma working as a Professor & Principal at Pt Deendayal Upadhyay Memorial Health Sciences and Ayush University of Chhattisgarh. Raipur, India. He has done his graduation from University teaching department, Gurughasidas University Bilaspur (CG) and done post graduation from B.R.Nahata College of Pharmacy Mandsaur (MP). He has completed his PhD from Gurughasidas Central University Bilaspur (CG). Dr, Shekhar Verma has vast experience of academic, administrative and research. Dr. Shekhar Verma actively involved in a research in field of Novel and Controlled Drug Delivery System, Non aqueous dispersion for poorly aqueous soluble drugs, Herbal Nutraceuticals, Nutraceuticals etc. Presently he is working on few funded research project. Dr Shekhar Verma is also Nominee of committee for the purpose of control and supervision of experiments on animals (CPCSEA), New Delhi. Recently he has awarded best young teacher award by premier pharmacy publication company, Nirali Publication New Delhi. Dr. Shekhar Verma published his research work in many national and international journals having high impact factor and also presented his work in different national and international conferences and seminars. He also participated and presented his research paper in international conferences at Dubai (UAE) and Bangkok, Thialand and wuhan university of Technology, China Dr Shekhar Verma is a member of editorial board in different national and international journals. He has also chaired different scientific session in national and international seminar-conferences. Dr Shekhar Verma is a life member of Professional bodies like Association of Pharmaceutical Teachers of India (APTI), Indian Pharmaceutical Association (IPA) and Indian Chemical Society (ICS). He is chairman of board of studies of Pharmacy, Pt. Deendayal Upadhyay Memorial Health Sciences and Ayush University of Chhattisgarh, Raipur and previously he was member of board of studies at Chhattisgarh Swami Vivekanand Technical University, Bhilai. Presently four PhD students registered under his supervision. Dr. Shekhar Verma associated with different research and funding agencies such as Ayush research department, ICMR, CCOST, AICTE, DST and INSA. His major area of interest is Novel Drug Delivery Systems & Nutraceuticals and He loves to teach different subject offered by Department of Pharmaceutics.

Barre Vijay Prasad completed Master's Degree in Psychology from Andhra University, he later went to acquire his first M Phil in Rehabilitation Psychology from National Institute for the Mentally Handicapped (NIMH). Thereafter, he went on to acquire his second M Phil in Clinical Psychology from one of the most reputed institutes in India Institute of Human Behaviour and Allied Sciences (IHBAS), Delhi. He is the first psychologist to have accreditation as a dually trained professional psychologist with qualifications as a Clinical Psychologist and a Rehabilitation Psychologist from Rehabilitation Council

of India (RCI) - Delhi. He is a Profes-sional Life Member of Indian Association of Clinical Psychology (IACP). He has awarded Doctoral Degree in Psychology (Psychooncology) from Centre for Health Psychology, University of Hyderabad. He has been awarded: Centenary Merit award during beginning of his professional psychology career. Currently he is working as Assistant Professor of Clinical Psychology in department of Psychiatry at All India Institute of Medical Sciences (AIIMS)-New Delhi, prior to this Institute he worked as Associate Professor and Head of the Department of Clinical Psychology at Dhar-wad Institute of Mental Health and Neurosciences (DIMHANS-India). He is now working to produce ways of working manuals for reducing psychological distress amongst mentally ill and patients with cancer in the field of Psycho-oncology. He is trained at conducting various assessments such as Psycho-diagnostics, personal-ity assessments amongst other skills. Not only does he work on assessments he is proficient in providing interventions using multi-modal approaches. He is trained in providing support for various initiatives disability rehabilitation, promotion of mental health and wellbeing. He specializes in cognitive behavioral therapies and behavioural interventions for various clinical disorders. He has published many articles in national and international journals, book chapters and books as well, and presented several conference papers on mental health, disability rehabilitation and psycho-oncology.

Durdana Yasin has recently defended her PhD in the Department of Biosciences, Jamia Millia Islamia, New Delhi, India. She has worked on anticancer and pro-apoptotic efficacy of Cyanobacterial extracts on cancer cells in vitro. She has expertise in handling animal cell cultures and cyanobacterial cultures. She knows various biochemical and molecular assays and techniques like UV-Vis spectroscopy, Antioxidant assays, MTT assays, Florescent microscopy, Real-Time PCR, Western Blotting etc. She has also worked on biosynthesis of metal nanoparticles from cyanobacteria. She has completed her graduation and post-graduation in Biosciences from Jamia Millia Islamia, India where she learnt the cell culture techniques. She has 7 publications to her credit which include research articles and book chapters.

Md. Zafaryab is working as a Senior Research Fellow (SRF) at the University of Delhi, India, under the Department of Biotechnology (DBT) funded project " Small molecule functionalized bio-polymeric nanoparticles encapsulating siRNA for target delivery to osteoblast for managing bone disorders." As a research fellow of this project, he isolated the primary cells of mice and differentiated into osteoblast cells and established osteoporosis mice model. Besides this, they conjugated the sost gene siRNA to Cs-AuNP to target both in-vitro and in-vivo for managing the bone disorder. He completed his Bachelor's and Master's degree from Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi. During the Ph.D. his main research area was an in-vitro assessment of monoterpenes on the molecular interplay of apoptotic regulatory genes. He has the expertise to maintain various cancer and normal cell lines. In addition to that, he has good knowledge of different biochemical, molecular as well as microscopic techniques.

Bushra Zeya is pursuing her Ph.D. degree from Department of Biosciences, Jamia Millia Islamia, New Delhi, India. She has done her graduation from Science College, Patna and post graduation from Jamia Hamdard, New Delhi. She has experience in academic and research works. Her current research work is based on cancer therapeutics. It includes the study of anti-cancerous effects of flavonoid on different cancerous cell lines and its mechanism. She is also involved in taking Laboratory classes of B.Sc. and M.Sc. curriculum. Ms Bushra Zeya has also presented her research work in international conferences and attended workshops.

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