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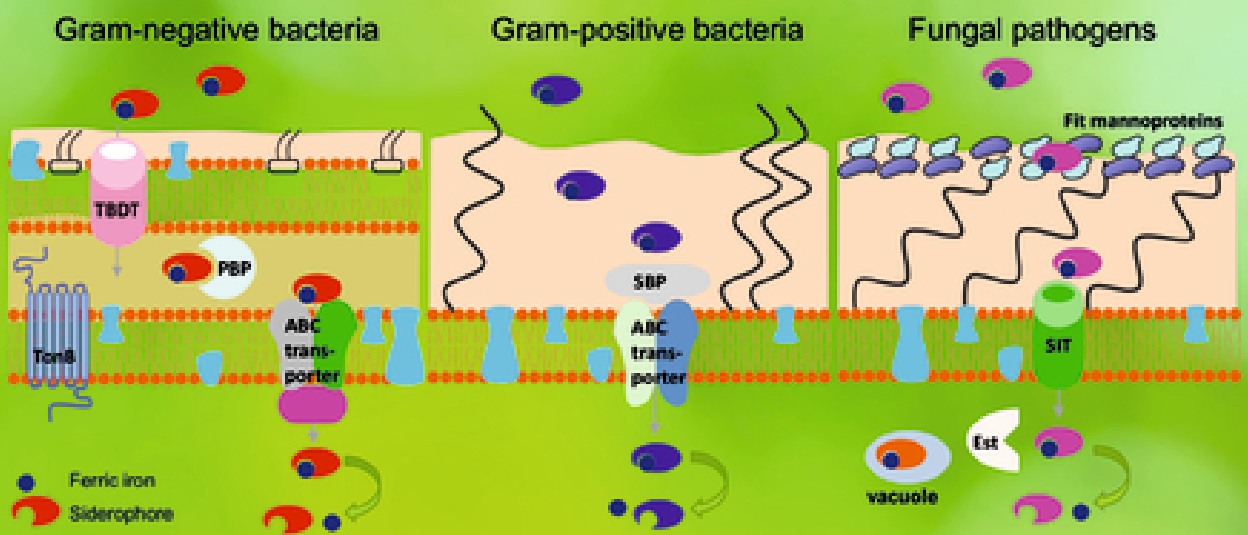
Peggy L. Carver (Ed.)

ESSENTIAL METALS IN MEDICINE

THERAPEUTIC USE AND TOXICITY OF METAL IONS IN THE CLINIC

Astrid Sigel, Eva Freisinger, Roland K. O. Sigel (Series Eds.)



METAL IONS IN LIFE SCIENCES 19

Astrid Sigel, Eva Freisinger, Roland K.O. Sigel
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Metal Ions in Life Sciences

Edited by
Astrid Sigel, Eva Freisinger and Roland K.O. Sigel

Volume 19

Guest Editor: Peggy L. Carver

Essential Metals in Medicine: Therapeutic Use and Toxicity of Metal Ions in the Clinic

DE GRUYTER

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Astrid Sigel has studied languages; she was an Editor of the *Metal Ions in Biological Systems* series (until Volume 44) and also of the "Handbook on Toxicity of Inorganic Compounds" (1988), the "Handbook on Metals in Clinical and Analytical Chemistry" (1994; both with H. G. Seiler), and on the "Handbook on Metalloproteins" (2001; with Ivano Bertini). She is also an Editor of the *MILS* series from Volume 1 on and she coauthored about 40 papers on topics in Bioinorganic Chemistry.

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Historical Development and Perspectives of the Series *Metal Ions in Life Sciences*[*]

It is an old wisdom that metals are indispensable for life. Indeed, several of them, like sodium, potassium, and calcium, are easily discovered in living matter. However, the role of metals and their impact on life remained largely hidden until inorganic chemistry and coordination chemistry experienced a pronounced revival in the 1950s. The experimental and theoretical tools created in this period and their application to biochemical problems led to the development of the field or discipline now known as *Bioinorganic Chemistry*, *Inorganic Biochemistry*, or more recently also often addressed as *Biological Inorganic Chemistry.*

By 1970 *Bioinorganic Chemistry* was established and further promoted by the book series *Metal Ions in Biological Systems* founded in 1973 (edited by H. S., who was soon joined by A. S.) and published by Marcel Dekker, Inc., New York, for more than 30 years. After this company ceased to be a family endeavor and its acquisition by another company, we decided, after having edited 44 volumes of the *MIBS* series (the last two together with R. K. O. S.) to launch a new and broader minded series to cover today's needs in the *Life Sciences.* Therefore, the Sigels new series is entitled

Metal Ions in Life Sciences.

After publication of 16 volumes (since 2006) with various publishers during the past 10 years, we are happy to join forces (from Volume 17 on) in this still growing endeavor with Walter de Gruyter GmbH, Berlin, Germany, a most experienced Publisher in the *Sciences.*

The development of *Biological Inorganic Chemistry* during the past 40 years was and still is driven by several factors; among these are (i) attempts to reveal the interplay between metal ions and hormones or vitamins, etc., (ii) efforts regarding the understanding of accumulation, transport, metabolism, and toxicity of metal ions, (iii) the development and application of metal-based drugs,

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(iv) biomimetic syntheses with the aim to understand biological processes as well as to create efficient catalysts, (v) the determination of high-resolution structures of proteins, nucleic acids, and other biomolecules, (vi) the utilization of powerful spectroscopic tools allowing studies of structures and dynamics, and (vii), more recently, the widespread use of macromolecular engineering to create new biologically relevant structures at will. All this and more is reflected in the volumes of the series *Metal Ions in Life Sciences.*

The importance of metal ions to the vital functions of living organisms, hence, to their health and well-being, is nowadays well accepted. However, in spite of all the progress made, we are still only at the brink of understanding these processes. Therefore, the series *Metal Ions in Life Sciences* links coordination chemistry and biochemistry in their widest sense. Despite the evident expectation that a great deal of future outstanding discoveries will be made in the interdisciplinary areas of science, there are still "language" barriers between the historically separate spheres of chemistry, biology, medicine, and physics. Thus, it is one of the aims of this series to catalyze mutual "understanding".

It is our hope that *Metal Ions in Life Sciences* continues to prove a stimulus for new activities in the fascinating "field" of *Biological Inorganic Chemistry.* If so, it will well serve its purpose and be a rewarding result for the efforts spent by the authors.

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Preface to Volume 19

Essential Metals in Medicine: Therapeutic Use and Toxicity of Metal Ions in the Clinic

The metal ions discussed in this volume play significant roles in medicine. Most are deemed 'essential' for life: iron, manganese, cobalt, zinc, copper, molybdenum, and the metalloid selenium. The essentiality of others, such as chromium, vanadium, and nickel, remains the subject of intense debate. As pointed out in *Chapter 1*, whether essential (or not), maintenance of metal ion homeostasis in humans is crucial, as perturbations often result in disease. In addition to the (possibly) essential metal ions, other metal ions play important roles in human health, often by causing harm (e.g., the metalloid arsenic) but also by their use in the diagnosis or treatment of human diseases (gadolinium, gallium, cobalt, molybdenum, lithium, gold, silver).

After going through a brief history of drug development in *Chapter 2*, readers may appreciate the continuing challenges faced in human medicine. While the rise of biopharmaceuticals has proven beneficial for numerous diseases, they are costly, and unlikely to provide 'cures' for all diseases. Thus, small molecules continue to play an important role in the treatment of human diseases, as outlined in the discussion of attempts to solve two therapeutic challenges: malaria and Alzheimer's disease.

Iron, while arguably the most intensely studied metal ion in human medicine, remains poorly understood. Iron is essential for life, yet toxic, as elegantly presented in *Chapters 3* to *7*. The essentiality of iron for both humans and pathogens means that the administration of iron is a double-edged sword. As discussed in *Chapter 3*, excess iron can prove toxic, and removal, via chelation therapy, remains a widely underappreciated problem in many countries where thalassemia is common and the availability of safe, inexpensive, and orally available iron chelators is desperately needed. Disruptions in iron homeostasis are clinically

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problematic. In *Chapter 4*, the role of iron in several common neurodegenerative diseases is reviewed, as well as novel therapeutic measures aimed at amelioration of these conditions by removal of iron from the brain.

In *Chapter 5*, the controversy over whether iron administration increases the risk of infections, by providing 'food' for pathogens seeking to infect humans, outweighs its risk, is reviewed. In patients in whom iron administration is needed, controversy also exists regarding the optimal formulation of intravenous iron. As noted in *Chapter 6*, intravenous iron products may have a varying ability to release labile iron into plasma, leading to induction of the formation of highly reactive free radicals. On the other end of the spectrum, *Chapter 7* discusses progress towards a long-held desire of clinicians to hijack the siderophore methodology commonly utilized by pathogens to scavenge iron as a means of delivering antimicrobials to those same pathogens, in a 'Trojan horse' approach to drug delivery. Despite many decades of work, this as-yet elusive dream has proved much more difficult than initially believed. Nevertheless, the first agents approved for human use may soon be available, if any of several agents that are currently undergoing clinical trials in humans prove successful.

Vanadium has had a long and storied history in medicine, with numerous claims of its beneficial effects on human health. Indeed, for many years, a variety of vanadium compounds were vigorously pursued as potential antidiabetic agents. In *Chapter 8*, the history of vanadium is reviewed: its long-time use as a 'health supplement', as a potential antidiabetic agent, and finally, recent realization of its potential as an adjunctive immunomodulatory agent for the treatment of cancer.

Like vanadium, the 'essentiality' of chromium in humans remains controversial, since in fact, the definition of 'essential' also remains a subject of debate. *Chapter 9* provides a historical overview of research attempts to elucidate the nature and roles of chromium in human health. Importantly, it points out that an in-depth knowledge of differences in the actions of metal ion species is crucial to our understanding of their biological roles and potential toxicities in humans; indeed, chromate, Cr(VI), is toxic and a carcinogen. Finally, the chapter provides approaches for resolving controversies in chromium biology in humans.

Manganese, the subject of *Chapter 10*, plays a significant role in human health and disease, including its essentiality while also noting the potential for toxicity, including neurotoxicity, when present in excess. Exposure to manganese is due to many sources, including environmental ones. Finally, remediation methods for patients with excess levels is also indicated.

The role of cobalt in human health is mainly based upon its important role in cobalamin (vitamin B_{12}), as has been extensively reviewed in a recent *MILS* text (Volume 13, Chapter 9). To date, the role of cobalt in human health has mainly been as a cause of contact dermatitis or (more rarely) as a cause of dilated cardiomyopathy in patients exposed to high levels from metal hip prostheses or from consumption of beer containing cobalt sulfate as a foam stabilizer. The radioactive isotope ^{60}Co is used to deliver radiation for cancer chemotherapy. *Chapter 11* reviews the potential role of cobalt as antimicrobial, antiviral, and chemotherapeutic agent, and as a possible inhibitor of amyloid-β (Aβ) formation for the treatment of Alzheimer's disease.

In addition to the essential roles copper plays in life, as discussed in Chapter 1, copper may be utilized as a drug, with the lethality of copper excess being exploited as a means of killing pathogens. In *Chapter 12*, the potential role of copper as an adjunctive chemotherapeutic agent to reduce angiogenesis is explored.

Chagas' disease, human African trypanosomiasis and leishmaniosis comprise three common, but neglected tropical diseases in desperate need of effective, affordable therapeutic agents. While a number of metal-based antiparasitic agents have been developed, as yet, none has been approved for use in humans, due to the rigorous regulations guiding drug approvals worldwide. *Chapter 13* outlines ongoing approaches to the development of these desperately needed agents.

While the biological functions of cyanide are not completely understood, its life-threatening toxicities are well recognized. Cyanide toxicity can occur due to active poisoning but, more commonly, from accidental sources such as fires. Outlining of the clinical aspects of cyanide in *Chapter 14* provides an excellent overview of the mechanisms of human cyanide toxicity and the metal-containing antidotes (generally cobalt or molybdenum compounds) used in its treatment.

Whether or not they are 'essential' for life, metal ions play significant roles in medicine, as diagnostic and therapeutic agents. Disruptions in metal ion homeostasis, whether genetically induced, or from environmental exposure, are implicated in the pathogenesis of human diseases. Utilizing translational approaches to furthering our understanding of the chemical basis and human (biological) consequences of metal ion homeostasis provides an important foundation for improving human health and disease.

Peggy L. Carver

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Comments and suggestions with regard to contents, topics, and the like for future volumes of the series are welcome.

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Metals in Medicine: The Therapeutic Use of Metal Ions in the Clinic

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Abstract: Metal ions are indispensable for living organisms. However, the roles of metal ions in humans is complex, and remains poorly understood. Imbalances in metal ion levels, due to genetic or environmental sources, are associated with a number of significant health issues. However, in clinical medicine, the role of metal ions and metal-based drugs is notable in three major areas: as metal-related diseases; as metal-based medicines (including drugs, imaging agents, and metal chelators); and as agents of metal-based toxicity.

Keywords: copper · gallium · gadolinium · iron · selenium · Wilson's disease · zinc

1. INTRODUCTION

1.1. Role of Metal Ions in Humans

Metal ions are indispensable for living organisms. Iron and zinc are the predominant metal ions found in humans and are arguably the best studied. However, the importance of other metal ions is increasingly recognized; some, such as manganese and nickel, though present in only 'trace' amounts, play vital roles [1, 2]. Molybdenum is found only in low levels in humans and rarely causes toxicity; however, it is found in key enzymes including xanthine dehydrogenase and oxidase. As discussed below, molybdenum's ability to bind copper has resulted in its use for Cu overload in patients with Wilson's disease [3]. Sodium, potassium, magnesium, and calcium play important physiological roles in humans and in human health and disease, as recently reviewed in detail [4–6]. Further, as alterations in the levels of one metal may affect the homeostasis of metals that share the same transporters (e.g., cadmium, copper, zinc, manganese, and iron) or signaling pathways, the importance of the interrelationships between metal ions must be kept always in mind [7].

Cytosolic, mitochondrial, and plasma antioxidants protect tissues from end organ dysfunction from reactive oxygen species (ROS) and reactive nitrogen species (RNS). Metal ion-containing enzymatic defense mechanisms, including catalase (Cu, Fe), copper-zinc superoxide dismutase (CuZn-SOD), manganese superoxide dismutase (Mn-SOD), and glutathione peroxidase, neutralize ROS and RNS [8, 9]. Activation of the nuclear transcription factor NF kappa beta by ROS is modulated by Se, Zn, and vitamins C and E. Cu serves an important role as an enzymatic cofactor, and is integral to a number of Cu-dependent enzymes, including ceruloplasmin, CuZn-SOD, hephaestin (which regulates intestinal transport of iron), and cytochrome *c* oxidase. Ceruloplasmin, like ferritin, is an acute phase reactant whose levels are increased in infections and in inflammatory processes such as coronary artery disease, diabetes, cancer, and renal failure [10, 11].

1.2. Overview of Metals in Medicine

Metals are increasingly utilized in humans in the diagnosis or treatment of disease. While trace amounts of metals are crucial, metal ion excess can also be similarly life-threatening to humans; thus, the role of metal ion chelators continues to be

a topic of intense research. The balance of metal ions in humans is complex, and still poorly understood. Excess, or deficiencies in metal ions, whether due to genetic or environmental causes, can result in clinical diseases.

While the use of metal ions as drugs or diagnostic agents has been employed since ancient civilizations, it remains less common than one would expect, given the major role metal ions play in human physiology [12]. Although recently, the role of metal-*based* drugs has been explored, most metal ions were used therapeutically in the chemical state found in nature. To cite just one example, the use of mercury as a treatment for syphilis was a widespread, but highly toxic practice until the discovery of one of the earliest antimicrobials, the arsenic-based compound arsphenamine, also known as 'salvarsan', which was used in the treatment of syphilis and trypanosomiasis [13]. Precious metals have also been utilized for a variety of medical indications. For example, silver has long been known to have antibacterial, antiinflammatory, and antineoplastic activity [14, 15]. Similarly, gold has long been utilized as a therapy for rheumatoid arthritis, and more recently, is being explored for its potential roles as in the therapy of viral and *Mycobacterium tuberculosis* infections and cancer chemotherapy [14, 16]. One of the earliest treatments for psychiatric disease was the use of lithium compounds for bipolar disease [17, 18].

Given the need for metal ions by both humans and pathogens, metal ions play a prominent role in infectious diseases. During infection, the host often appears to 'sequester' (hide) Fe, Zn, Se, and Mn from pathogens, in an attempt to starve pathogens, and limit their growth and virulence. For example, during the systemic inflammatory response syndrome (SIRS), decreased serum levels of Fe, Se, and Zn are observed, while Cu levels are increased [10, 19].

Metal sequestration by the host, termed 'nutritional immunity' results in the 'starvation' of invading pathogens [20, 21]. Microbes have evolved multiple strategies to control their access to divalent metals and circumvent nutritional immunity, while in turn, the hosts have presented countermeasures to these strategies [22, 23]. Several reviews of the roles of Fe, Se, Zn, Mn, and Cu in infectious diseases, and the 'tug of war' between pathogens and hosts have been recently published [24–26].

Medical strategies aimed at correcting acute or chronic imbalances in metal ion homeostasis must be approached with caution, since these imbalances often arise from defense strategies of the body to limit nutrients to microbes [24, 27, 28]. As discussed below (in the section on selenium), attempts to restore deficiencies may not prove beneficial. This may be due to compensatory mechanisms employed by pathogens, the host, or other as-yet unknown interactions with other metal ion systems of homeostasis.

In human health, metal ions play a number of important roles. Imbalances in metal ion levels, due to genetic or environmental sources, can lead to significant health issues. However, they also serve as major therapeutic agents in the clinic, for both the diagnosis and treatment of major human diseases. While this chapter cannot cover all of these areas, it will provide an overview of major concepts, in particular, those not covered in recent texts or in other chapters of this book. The information will be presented in three major categories: metal-related dis-

eases; metals as medicines (to include drugs, imaging agents, and metal chelators); and metals that are not used therapeutically but can be important clinically (generally in terms of toxicity to humans).

2. METAL-RELATED DISEASES

Wilson's disease and Menkes disease are the best known examples illustrating the significant role of Cu in human medicine. Both of these heritable diseases are caused by inborn errors of Cu metabolism [29, 30].

Wilson's disease is an autosomal recessive disorder caused by a mutation in a Cu-transporting enzyme ATPase 2 (Cu-ATPase ATP7B). Disease results from the release of 'free' (nonceruloplasmin-bound) copper into the bloodstream, rather than into the bile where it would be excreted via the gut. Select sites in human hosts normally serve as areas of low (brain and kidney) or high (serum, lung, liver, spleen) local availability of Cu. Tissue accumulation of Cu in the liver, cornea, and brain results in the development of cirrhosis of the liver, as well as neurological, cardiac and pancreatic abnormalities in affected patients [31]. Treatment for Wilson's disease consists of lifelong avoidance of high Cu foods and administration of potent Cu chelating agents and zinc salts. Therapy is usually initiated with the Cu chelators D-penicillamine or trientine, followed by maintenance therapy with trientine or Zn salts. Zn may be effective in delaying the onset of symptomatic disease if utilized early in the course of disease, and as therapy in pregnant women and children. However, while efficacious as initial therapy, it acts more slowly than penicillamine or trientine, requiring up to six months to alleviate symptoms of Cu toxicity [29].

Penicillamine, the first efficacious, orally available Cu chelating agent for the treatment of Wilson's disease, mobilizes Cu from tissues, allowing its excretion via urine. While very efficacious, penicillamine causes hypersensitivity reactions in >25 % of patients, in addition to proteinuria, myelosuppression, autoimmune reactions, and an initial worsening of neurological symptoms. The high rate of adverse effects has led to the more recent preference of clinicians in using trientine, a Cu chelator with a mechanism similar to that of penicillamine, as first line therapy. While trientine (often used in combination with Zn, if hepatic failure is present at the time of diagnosis) causes side effects similar to those observed with penicillamine, they are less common [29].

Unlike penicillamine and trientine, Zn is not a Cu chelator. Rather, Zn acts by reducing the systemic absorption of Cu from the gastrointestinal tract, by increasing enterocyte (and perhaps liver cell) levels of metallothionein, an endogenous Cu chelator that sequesters Cu in the enterocyte. As enterocytes are sloughed off every few days, the Cu held in the cell is eliminated in feces. In order to be efficacious, Zn must be taken on an empty stomach (i.e., separated by at least 1–2 hours from food or beverages other than the water taken to swallow the capsules). The rapid dissolution of Zn capsules in the stomach results in gastrointestinal discomfort, the main adverse effect of Zn, which is observed in ~25 % patients and results in non-adherence in ~10 % of patients [29].

The recent development of tetrathiomolybdate (TM), a Cu chelator and inhibitor of Cu absorption, has offered an alternative agent that appears to avoid the neurological worsening observed with penicillamine and trientine upon initiation of therapy. Although initially evaluated as an ammonium salt, recent studies have utilized the more stable bis-choline form [32]. TM is also being evaluated as adjunctive therapy in the treatment of cancer, to block tumor angiogenesis, as is discussed in more detail in Chapter 8 of this volume. TM acts in two ways to reduce Cu in patients with Wilson's disease: first, if administered without food, it is well absorbed and in the blood, forms a tripartite complex with albumin and free Cu. The complex is slowly metabolized by the liver and excreted, resulting in a significant reduction of free copper levels within 2 weeks. By contrast, when administered with food, TM binds to food proteins, and the Cu found in food, preventing the absorption of Cu [29]. Unfortunately, TM is not yet US Food and Drug Administration (FDA)-approved, hindering availability to patients.

Menkes disease is a congenital x-linked genetic disorder caused by a mutation of the transport protein Cu-ATPase ATP7A that mediates Cu transport from the enterocyte to the bloodstream [33]. The resulting severe Cu deficiency causes progressive neurologic deterioration and death during early childhood. Treatment consists of parenteral administration of a Cu-histidine complex, which is not consistently effective.

Several excellent reviews cover these two disorders in detail [57–59].

3. METALS AS MEDICINES

3.1. Metallodrugs Used in Medicine

Arguably, one of the most widely used metal-based therapeutic agents in current medical practice is the platinum-based agent cisplatin. The remarkable anticancer effects of cisplatin, discovered in 1965, revolutionized the modern treatment of cancer and have led to continued research in the search for additional anticancer metallodrugs. Cisplatin, and the development of the related platinum agents oxaliplatin and carboplatin, were reviewed along with the role of other metal ion developments (including ruthenium, gold, titanium, vanadium, and gallium as well as the essential metal ions Fe, Cu, and Zn) in a recent MILS volume devoted to this topic [34]. In addition, as comprehensively reviewed in a series of recent publications, a number of diagnostic metalloradiopharmaceuticals are in use; while the majority are technetium-based, gallium, indium, rubidium, gadolinium, and lanthanide derivatives have been widely investigated [12, 35]. 'Theranostic' metalloradiopharmaceuticals, which employ a diagnostic pharmaceutical, followed by the use of a therapeutic radiopharmaceutical (for example, ^{68}Ga diagnostic imaging with ^{90}Y treatment), are also under clinical investigation [36, 37]. As discussed in [38] and in Chapter 9 of this volume, vanadium-based compounds have been evaluated for their insulin-mimetic properties, and more recently, for their anticancer properties. Fe and Zn are the predominant metal ions found in humans. Fe, as a key component of host metabolic processes, is

tightly regulated, as reviewed in depth in Chapters 3 to 6 of this book. Pathogens have similar requirements, and the quest for Fe has led pathogens to develop complex mechanisms for gaining access to the tightly controlled levels of unbound Fe, as described in Chapter 4. The role of Zn in biological systems has been comprehensively reviewed [39, 40], including its role in the susceptibility and outcome of infectious diseases [24, 25].

3.1.1. Selenium

Se is an essential micronutrient metalloid which plays an important role in human antioxidant defense, mainly in superoxide dismutases (SODs) and as a component of the >30 identified selenoproteins, including the antioxidant glutathione peroxidase. The exact mechanisms of Se absorption from the gut in humans remain unclear and depend on the form of Se and region of the gut. Selenite is believed to be passively absorbed, while selenomethionine appears to be absorbed via an energy-dependent process [41]. Excretion appears to be via feces and urine. Deficiencies in Se have been linked to Keshan cardiomyopathy and Kashin-Beck disease, and cretinism, while Se excess is associated with selenosis. Clinical manifestations of selenosis include nausea, vomiting, diarrhea, hair and nail loss, mental status changes, and peripheral neuropathy [42].

Se deficiency (levels <85 μg/L) have been associated with heart failure, skeletal muscle dysfunction, and coronary artery disease, while severe deficiencies can lead to cardiomyopathy and sudden death. In many, but not all studies, human immunodeficiency virus (HIV)-infected patients demonstrate decreased serum levels of Se, which is linearly associated with decreases in the immune-based helper T cell (CD4+) counts, rapid progression of HIV, an increased viral load and risk of tuberculosis, and higher mortality [43–52].

The role of Se supplementation (alone or in combination with other micronutrients) has proved controversial. While Se supplementation may slow the decline of CD4+ cell counts and reduce infection-related hospitalizations in HIV-infected patients, most studies have failed to document improvements in viral loads or CD4+ counts [53–58]. Similarly, Se supplementation has been evaluated as a 'natural health' product for the prevention of lung cancer, with conflicting results: it reduces the risk of lung cancer in patients with lower (<106 ng/mL) baseline levels of Se, but increases its risk in patients with higher (≥121.6 ng/mL) baseline Se levels. In a small study, Se supplementation (in the form of kappa-selenocarageenan) reduced cisplatin-associated neutropenia and nephrotoxicity. In patients undergoing radiation therapy following cancer surgery, in whom Se plasma levels decrease, Se supplementation with sodium selenite can correct Se levels and improve antioxidant status [59].

Critically ill patients with septic shock demonstrate acute decreases of ~40 % in plasma Se, glutathione peroxidase, and selenoprotein-P levels [60, 61]. To replenish antioxidant levels, supplemental Se therapy, administered alone or in combination with other antioxidants, vitamins or trace elements, has been intensively studied, with conflicting outcomes. In these studies, a variety of Se dosages, formulations (generally sodium selenite, although ebselen has also been con-

sidered), routes of administration, and durations of therapy have been evaluated [62, 63]. Several small trials and early meta-analyses reported beneficial effects of Se on the rates of infections and mortality, and the duration of mechanical ventilation. However, following the addition of data from several larger trials, the conclusions of more recent meta-analyses are that Se supplementation, alone or as combined therapy, appears to provide little or no mortality benefit [8, 64–67].

3.1.2. Copper

Cu is an essential nutrient required by humans for proper organ function and a variety of metabolic processes [24]. Cu homeostasis is closely linked to Fe metabolism, given the critical roles of Cu-containing ferroxidase on transmembrane Fe transfer, which in turn, impacts Fe availability for host cells and microbes [68]. Cu is a redox-active metal, which exerts antimicrobial activities by radical and non-radical mediated processes [69].

Cu deficiency, while rare, can arise from malabsorption due to Crohn's disease, celiac disease, following gastric bypass, or removal of the small intestine [29]. High levels of Zn can upregulate metallothionein levels in enterocytes. As metallothionein has a greater affinity for copper than zinc, high doses of Zn (generally, >150 mg/day for long periods of time) can result in Cu-deficiency anemia in healthy individuals [33, 70], although (as discussed above) in Wilson's disease patients, it is utilized for its Cu-depleting effects. Excessive doses of zinc can be obtained by the consumption of large quantities of denture adhesive [29, 70].

Clinical manifestations of Cu deficiency include micro-, normo-, or macrocytic anemia, and myo- and optic neuropathies. The mechanisms by which these adverse effects occur is still unclear. While copper supplementation (over 4–12 weeks) can reverse the hematological effects of Cu deficiency, neurological and optical manifestations are incompletely reversible [33], as well as making patients more susceptible to infection. During sepsis, when serum levels of Fe, Se, and Zn decrease due to sequestration by host nutritional immunity, Cu levels increase as a means of 'Cu poisoning' [10, 19]. Excess Cu is toxic to bacteria and yeast, and they actively export virtually all that is taken up by the cell [71]. Cu-induced bactericidal activity has been exploited since the time of the Egyptians, and is currently being explored as a means to prevent hospital-acquired infections [72, 73]. For example, some hospitals are outfitting bed rails, toilet flush levers, grab bars, countertops, light switches, and other "high touch" hard surfaces with surfaces made of Cu alloy (90 % Cu, 10 % Ni, by weight), and patients are given Cu-laced linens and gowns.

Some Gram-negative bacteria counter this poisoning by producing Cu-sequestering siderophores [73, 74]. Yersiniabactin, a siderophore produced by many pathogenic Enterobacteriaceae, can sequester Cu extracellularly, in order to protect the bacteria from Cu intoxication. Conversely, methanobactins, the recently discovered Cu-chelating small molecules produced by methanotrophic Gram-negative bacteria, scavenge Cu to import it into methanotrophs, where it is used for the biosynthesis of the Cu-containing methane monooxygenase [73, 75]. However, while high Cu levels can prove toxic to pathogens, Cu limitation can

stimulate stress responses. For example, invasion of the kidney by *C. albicans* stimulates a decrease in kidney tissue levels of Cu, which is countered by a change in yeast expression of CuZn-SOD to Mn-SOD [72].

3.2 Metal-Based Imaging Agents

3.2.1. Gallium

Although gallium has no known physiologic function in the human body, it can interact with cellular processes and biologically important proteins, especially those associated with Fe metabolism. Gallium has a long history of use in the clinical setting as a diagnostic and therapeutic agent in medical imaging, oncology, and infectious diseases [76–80].

Ga is trivalent, and has a similar ionic radius, ionization potential, electronegativity, electron and ligand affinities, and coordination geometries as Fe(III) [81]; thus, Ga(III) can effectively mimic Fe(III) and display biological activity in many biological systems, including siderophores, transferrin, and lactoferrin, with stabilities only few orders of magnitude less than the corresponding Fe(III) species [81, 82]. However, unlike Fe(III), Ga(III) cannot be reduced under physiological conditions, and when taken up by cells, inactivates Fe(III)-dependent reduction and oxidation and alters protein conformation [83]. Ga also mimics Fe in mammalian storage and transport systems [84–86]. In human circulation, ^{67}Ga citrate is exclusively bound to the Fe transport protein transferrin, where it binds tightly, although about 300-fold more weakly than Fe(III) [81, 87, 88].

While the antimicrobial properties of Ga were known as long as a century ago, its use was largely ignored until recently [80]. In the early 1930s, Ga compounds were explored as treatments for syphilis and trypanosomiasis. More recently, interest has been piqued in 'repurposing' Ga compounds as antimicrobial agents, as they have been found to have antibacterial activity against several pathogenic bacteria known to possess extensive intrinsic and acquired resistance, including *Acinetobacter baumannii* [83, 89–93], *Neisseria gonorrhoea* [94], *M. tuberculosis and M. avium* [95–99] and *Staphylococcus aureus* [100]. Ga nitrate and transferrin-Ga can block the Fe-dependent growth of these pathogens, both extracellularly and within macrophages, and have proved successful in the treatment of murine models of *P. aeruginosa* pneumonia and thermal injury-induced infection [83, 93]. Several excellent reviews have discussed the use of gallium-based drugs as antimicrobial agents [77, 78, 101].

A. baumannii is a Gram-negative bacterium that has evolved numerous strategies for the acquisition of Fe, including high affinity siderophores and heme uptake systems [102]. Further, *Acinetobacter* is intrinsically resistant to most antimicrobials, and is often associated with biofilms (mixtures of proteins, polysaccharides and extracellular DNA, produced by bacteria on a surface). Bacteria within biofilms often develop to many-fold greater resistance than in a non-biofilm (planktonic) environment [89]. Ga nitrate, an FDA-approved drug for the treatment of degenerative bone diseases, can inhibit the planktonic growth

of *Acinetobacter*. However, *A. baumannii* quickly develops tolerance, due to its ability to utilize heme Fe. In an effort to avoid this resistance, gallium metal heme conjugates meso- and protoporphyrin IX were developed, which proved active against both planktonic and biofilm bacteria [92]. Use of Ga nitrate is limited by its toxicities, which include nephrotoxicity, with renal acidosis, and microcytic anemia [80].

In a 'dual drug' Trojan horse approach, Miller and colleagues [103] developed siderophore-conjugate drugs constructed from Ga-coordinated deferoxamine B linked to various thiol-containing fluoroquinolone and cephalosporin analogues. While limited in spectrum to Gram-positive pathogens, these compounds illustrate 'proof of principle' that the methodology could be expanded to include a spectrum including Gram-negatives. In particular, the high penetration of Ga(III) to the lung and blood is important, since multi-drug resistant *S. aureus* and *Acinetobacter* are common causes of bacterial pneumonia and secondary bacteremia.

^{67}Ga is widely used as an imaging agent to localize malignant cells, and sites of inflammation and infection. ^{67}Ga localizes in tumors, and the antineoplastic activity of gallium nitrate was explored in clinical trials for the treatment of non-Hodgkin's lymphoma and bladder cancer [104]. Unlike most chemotherapeutic agents, gallium nitrate does not produce myelosuppression [9]. During clinical trials for cancer, it was noted that decreases in calcium were observed in a number of patients, leading to further studies, and the eventual FDA approval of gallium nitrate ($(Ga(NO_3)_3)$; Ganite™) for the treatment of cancer-associated hypercalcemia. Recent novel applications related to its use as an imaging agent in the diagnosis of infections are of interest [105–107].

Recently, investigators have exploited the 'Trojan horse' approach to conjugation of drugs to the Fe siderophore system, whereby drugs are 'smuggled' into cells and tissues. Siderophores can bind Ga(III) at only slightly lower affinities than Fe(III) [108, 109]. Emery and Hoffer [110] first demonstrated that uptake of a ^{67}Ga analog of ferrichrome (^{67}Ga-deferriferrichrome) could mimic that of Fe(III) in *Ustilago sphaerogena,* and that Ga(III) effectively competes with Fe(III) for three other hydroxamate siderophores. More recently, the use of ^{68}Ga-siderophore complexes has been explored for the diagnostic imaging of *A. fumigatus* infection in a rat model of invasive pulmonary aspergillosis [107]. Lung uptake of the ^{68}Ga-N2-acetylated derivative triacetylfusarinine C correlated with the severity of infection, demonstrating 'proof of principle' for this technique. While ^{68}Ga-siderophores were taken up by *A. flavus*, *A. terreus*, *R. oryzae*, and *F. solani*, uptake was significantly lower in *A. fumigatus* and there was virtually no uptake in yeast (*C. albicans*) [111–114].

3.2.2. Gadolinium

Gadolinium-based contrast agents (GBCAs), which are composed of Gd^{3+} bound to a chelating ligand, were widely utilized in magnetic resonance imaging studies until recent reports of the development of nephrogenic systemic fibrosis sparked controversy regarding the safety of select GBCAs in patients with pre-

existing renal insufficiency [115, 116]. Structurally, GBCAs can be classified as linear or macrocyclic (based upon the structure of the chelating molecule) and further, as neutral or charged (ionic). Dechelation can result in the accumulation of the highly toxic free Gd^{3+} in bone, skin, liver, and brain tissue. While still debated, current opinion suggests the risk of dechelation of Gd^{3+} may be attributed to transmetallation, which is more likely with non-ionic, linear GBCAs than with ionic linear or macrocyclic chelates due to differences in the thermodynamic and kinetic stability of the chelating ligand. Transmetallation refers to the ability of soluble endogenous metal cations (most likely Zn^{2+}, Fe^{3+}, or Ca^{2+}) to attract the ligand, resulting in the release and tissue deposition of Gd salts [115]. Despite a lack of reports of adverse effects related to administration of GBCAs in patients with normal renal function, their use is known to result in the deposition and retention of Gd in brain tissue, raising additional concerns regarding its safety, and prompting a 2017 recommendation by the European Medicines Agency restricting the use of some commonly used GBCAs and the suspension of others [115, 117].

4. METAL ION TOXICOLOGY

4.1. Metal Ion Toxicity

While essential, many metal ions pose significant risks of toxicity when present in excess in humans as the result of exposure or ingestion. Worldwide, metal toxicity, particularly of cadmium, lead, manganese, mercury, aluminum, and arsenic, and (less commonly) thallium, tungsten, rhodium, ruthenium, iridium, palladium, and platinum and osmium are clinically important problems in human health. Several excellent reviews, texts (or chapters therein) are devoted to these topics and the interested reader is referred to them for more in-depth review [7, 118–124]. Despite its ubiquitous presence as a material for canning foods, tin, which has no known biological role in humans, is not easily absorbed and remains of low toxicity [125]. However, triorganotins, used as bactericidal and fungicidal agents, can prove quite toxic [126].

4.2. Metal Ion Chelators for the Treatment of Diseases

In patients with metal ion excess, chelation may provide a therapeutic option. As discussed in Chapters 3 and 6 of this volume, Fe chelation therapy is utilized in patients with Fe overload due to thalassemia, and has been utilized in the therapy of Alzheimer's disease. Fe overload is common in patients who have undergone multiple blood transfusions and in patients with renal failure [127]. In renal failure patients with iron overload, chelation therapy with deferoxamine was associated with an increased risk of infections caused by mucorales, a mold which is highly dependent on iron [128]. Deferoxamine was found to be acting

as a siderophore, resulting in the delivery of iron to the pathogen [129]. More recently, deferasirox, a non-siderophore iron chelator, was evaluated unsuccessfully as an adjunctive therapy of mucorales infections, in an attempt to 'starve' the pathogen of needed iron [130].

As noted in Section 2, Zn is used in the adjunctive therapy of Wilson's disease. More recently, chelation of Cu, with the use of TM for cancer chemotherapy, has been explored, as discussed in Chapter 8. The use of Zn chelators has been explored as a means to combat carbapenem resistance due to Zn metallo-beta lactamase, and as adjunctive therapy for difficult to treat pathogens such as *A. fumigatus* [65–69].

5. CONCLUDING REMARKS

The role of metal ions in medicine, whether as causative agents, or the treatment of human diseases, continues to be an exciting area of translational research. A solid understanding of the chemistry of metal ions is crucial to furthering knowledge in this important area, as is the continued collaboration of clinicians and basic scientists in applying the basic concepts learned in the laboratory to the complex environment of human health.

ABBREVIATIONS

CD4+	immune-based helper T cell
FDA	US Food and Drug Administration
GBCAs	gadolinium-based contrast agents
HIV	human immunodeficiency virus
RNS	reactive nitrogen species
ROS	reactive oxygen species
SIRS	systemic inflammatory response syndrome
SOD	superoxide dismutase
TM	tetrathiomolybdate

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2

Small Molecules: The Past or the Future in Drug Innovation?

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Abstract: With the impressive development of molecular life sciences, one may have the feeling that biopharmaceuticals will dominate the world of drug design and production. This is partly due to the evolution of pharmaceutical industry, especially since the 1980s. As a matter of fact, small molecules are still dominating the field of drug innovation, in contradiction with claims predicting their downfall and the exponential raise of biopharmaceuticals. The strong association of chemistry with biochemistry and pharmacology has been the scientific base of the establishment and the success of strong powerful pharmaceutical companies throughout the twentieth century. To meet the needs of new therapeutic agents, it is necessary to assess the role and future position of medicinal chemistry. In fact, the reasonable balance between small molecules and biopharmaceuticals will depend on scientific and economic factors, including the goal of having highly efficient drugs to cure the largest possible number of patients, at a cost that is compatible with the limits of national health budgets.

In the present chapter, we would like to emphasize the future important role of small molecules based on new chemicals, to build a new portfolio of efficient, safe and affordable drugs to solve major therapeutic challenges. Two examples are then given. In the blood parasitic diseases such as malaria and schistosomiasis, the iron of heme is an "old" and relevant therapeutic target to kill the parasite. Investigations on the mechanism of action of the antimalarial endoperoxide sesquiterpene artemisinin, have paved the way to the design of new efficient synthetic endoperoxide drugs. In the case of Alzheimer's disease, the loss of copper homeostasis in patient brain is one of the key features of neurodegeneration. The development of small copper specific ligands able to retrieve copper from its pathological sinks to reintroduce it into physiological circulation is a challenging but promising approach to effective therapy.

Keywords: small molecules · drug innovation · dual molecules · hybrid molecules · malaria · Alzheimer's disease

1. INTRODUCTION

With the impressive development of molecular life sciences (genetics, antibody production, gene therapy, cell therapy …) many policy makers have the feeling that biopharmaceuticals will dominate the world of drug design and production,

reducing the place of small molecules (the new name for drugs based on chemicals) to a very limited area in the arsenal of therapeutic agents.

The success of humanized monoclonal antibodies in cancer therapy has biased the judgment of many executive officers in large pharmaceutical companies. They too quickly considered that monoclonal antibodies and other biopharmaceuticals would provide the answer for all different types of diseases, including aging diseases located in the brain. This reductive vision of drug discovery, that can be accepted by managers that are not fully aware of the complexity of drug discovery and pharmacology, is misleading for drug discovery in several therapeutic domains. Antibodies are perfect for cancer therapy, since there are able to distinguish between tumor cells and normal cells. But because of their size, antibodies cannot be used for masked therapeutic targets and they are not able to cross the blood-brain barrier (BBB) to act as drugs in an aging disease located in the brain, like Alzheimer's disease. The other crucial aspect in favor of small molecules as drugs is the cost of therapy. Who is going to pay for a treatment based on biopharmaceuticals when the full cost is largely over 100,000 US dollars (US$), or (for a recent example) above 800,000 US$? The patient, a national health insurance, or private insurance? While the cost of small molecule treatment is ranging from few dollars to one or two thousands of US$, it is obvious that "small drugs" based on chemicals have a real future [1].

As mentioned in the article cited above [1], the real challenge is that "It is important to assess the role and future position of medicinal chemistry in the design of new therapeutic agents, in order to reach a reasonable balance between small molecules and biopharmaceuticals. The equilibrium between these two types of drugs will in fact depend on several factors, including the necessity of having highly efficient drugs for the treatment of diseases and health disorders at a minimal cost. The challenge is to cure the largest possible number of patients at a cost that is compatible with the foreseen limits of national health budgets in 2030".

In the present chapter, we would like to emphasize the future important role of small molecules based on chemicals, to build new portfolios of efficient, safe and affordable drugs to solve therapeutic challenges, taking as examples two domains: parasitic diseases (malaria and schistosomiasis) and a neurodegenerative disease (Alzheimer's disease).

2. BRIEF HISTORICAL OVERVIEW OF CHEMICAL DRUG DEVELOPMENT

At the beginning of the 19th century, improved command of extraction methods and analytical tools resulted in isolation and identification of "active principles" from plants identified by traditional medicines as curative of particular diseases. The first milestone of the pharmacology of alkaloids is the extraction and the purification of quinine from the bark of cinchona used in South America by Quechua people to reduce fevers, in particular the fever due to the endemic parasitic disease that will later be defined as malaria (the fever of swamps). The next step in quinine manufacturing has been the idea of making its total synthesis

to escape from the importation of cinchona from tropical countries. Perkin tried to prepare quinine from aniline without success. Instead of quinine, he obtained a nice dye that became famous under the name of Perkin's mauve [2]. Serendipity is always a significant route in chemical discoveries and also for making new drugs as we will see below!

The development of chemical syntheses at the beginning of the 20th century and the emergence of biochemistry are both at the origin of modern pharmacology, with the lock-and-key concept introduced by H. E. Fischer in 1894 [3] and the concept of receptors proposed by P. Ehrlich in 1906 [4]. The strong association of chemistry with biochemistry-pharmacology has been the scientific base of the establishment and the success of strong powerful pharmaceutical companies for nearly a century, e.g., Merck, Eli-Lilly, Bristol-Myers, Bayer, ICI, Hoechst, Rhône-Poulenc, Roussel-Uclaf … to name some of them. All these successful companies had "two legs": chemistry and pharmacology. In the 1980s, this industrial concept was changed under the pressure of the economic parameters, the way to manage companies and also the fact that biology and genetics became really molecular by using all the concepts of molecular chemistry. In a way, the triumph of chemistry, making biology more and more molecular, is mainly at the origin of the decline of chemistry in the pharmaceutical industry.

In the 1950s, the drug market was not controlled by "Big Pharma" companies. None of the different pharmaceutical companies had more than 2 or 3 % of the world drug market. Their development was mainly driven by innovation since the time from "patent to market" was rather short (ranging from 6 to 8 years, compared to 10 to 12 nowadays), many scientific researchers being in the executive committees of these "old-fashion" companies. Some tragedies created by bad drugs (the teratogenic thalidomide and the toxic stalinon are some examples) marketed after insufficient toxicological studies accelerated the inflation in regulations. The International Conference on Harmonization of Technical Requirements for Pharmaceutical Uses was created in 1990 (ICH guidelines). If we add on the costs of these regulations to those associated with much more detailed mechanistic studies on drugs, one cannot be surprised by the fast increase in the costs to get a new drug approved by national authorities.

2.1. The "Merging Period" of Pharmaceutical Industries

With the increase in costs for innovation and development of drugs, the trend in pharmaceutical industry was to move from innovation to merging operations. Within two decades [1980–2000], the pharmaceutical industry changed with the emergence of international companies, the so-called "Big Pharma". As an example, Sanofi, a French company created at the beginning of the 1970s by the fusion-acquisition of more than 40 small companies, that should be considered as the "start-up" companies of the middle of the 20th century, merged with Synthélabo, and then with Aventis. This latter company was the result of the merge of Rhône-Poulenc with Roussel-Uclaf, Marrion-Merrel-Dow, and finally with Hoechst-Pharma. Nobody can ignore that all these merging steps are not

the best way to create a friendly atmosphere for drug discovery within these companies. A large part of the energy of researchers is consumed by considerations related to reduction of research centers and staff. Since discovery is associated to risk acceptance, one can easily understand that nobody is going to take risk in restructuration periods.

These merging operations had also a strong impact on the composition of the headquarters of the Big Pharma. Very quickly, the management of these companies shifted to the hands of business and financial people. Most of the persons with scientific or medical expertise were progressively excluded from the executive committees and relegated to the so-called technical positions [1]. Recently, some of these Big Pharma re-introduced few of these "scientific experts" within the top-level circles to have their direct opinions, and to avoid of being too strongly influenced by external consulting companies that are usually experts to present complicated organization charts. Another consequence of the growth of the size of pharmaceutical companies (which, in fact, is also the case for all very large industrial or administrative structures) is the explosive number of internal reports circulating within the different complicated layers of these companies. Such proliferation of "reporting methods" has also been accelerated by the numerical revolution; it is so easy to produce long indigestible documents and to diffuse them to hundreds of people with "one click" through the Internet. The increasing storage capacity of computers allows to get these reports within "data graveyards". The "more you get, the less you read" is killing the diffusion of really important information and, in addition, the time allocated to think on research projects or to scientific discussions with co-workers is going decreasing dramatically. It is urgent to control the proliferation of low value electronic data and/or information to protect the researchers of universities or industrial companies from the waste of time and energy created by "mail and social networks" systems. Creativity and time for thinking should be the priority, not the production of reports or the compliance to time-consuming paper works.

2.2 Is Data Mining the Future for Drug Discovery?

The electronic revolution is now the alpha and omega of industrial managers. On one side, nobody can argue against the development of robots in manufacturing industries to avoid repetitive and painful movements of workers, and to accelerate the productivity of car production, as an example. To be sure that the pharmaceutical industry was following this trend, it was trendy in the 1990s to install high-throughput screening (HTS) of chemical libraries to search, in association with biological targets, for hits in order to identify new drug candidates. Despite huge financial investments in this strategy, one has to confess that the outcomes have not been at the level of the amount of money that has been dedicated to these methods. Among the different reasons of the failure of HTS methods, one can note that the used libraries of chemicals were mainly based on automated production of chemicals with limited structural diversity [5] and also because of a limited number of new targets despite the considerable work that has been

done on genetics to find druggable targets. There is no fast track from "gene to drug". An illustration of these difficulties has been provided by a very open-mind opinion by researchers of GlaxoSmithKline. After seven years (1995–2001) of evaluation of more than 300 gene products as potential targets for novel antibiotics with 70 HTS campaigns using large libraries of synthetic chemicals, no new drug was found [6]. One of the wrong sayings that is running within corridors of decision-makers, including within important pharmaceutical companies, is "we have enough molecules on the shelves, we don't need to make new ones with chemists". This is wrong, since we know now that the chemical space is just at the beginning of its exploration. Reymond and his group have identified 166 billion of feasible molecules that can contain up to 17 atoms of carbon, nitrogen, oxygen, sulfur and halogen atoms [7]. The "drug-like" chemical space obeying Lipinski's rules [8] for bioavailability is as high as 10^{20} for all molecules up to 30 atoms [7]. The Chemical Abstract Service just passed the 100-millionth registered chemical substance in 2015. So, we are far from having all drug-like molecules in hands. Presently, the "à la mode" idea is big data mining. With more and more powerful computers and algorithms, it will be possible to find new drugs and to boost drug discovery. It just feels that such saying is one of the good ideas that will go wrong within the next decade. All data bases are containing yesterday's, not tomorrow's, information. How can we get new drugs that have not been discovered and reported yet? Before the work of Youyou Tu (Nobel Prize 2015) on the extraction of *Artemisia annua* and structural characterization of artemisinin, who was able to imagine that the structure of this natural product with antimalarial activity would have been a sesquiterpene lactone containing a trioxane motif [9]?

2.3. The Rising of Biopharmaceuticals

Since the 1980s, the impressive development of molecular genetics (with the discovery of restriction enzymes and ligases making possible the manipulation of genes) strongly influenced the evolution of pharmaceutical companies. They moved towards more biological applications. The deep understanding of gene functions allowed the production of recombinant proteins and, consequently, the use of monoclonal antibodies as therapeutic agents, in particular, in cancer therapy. All the chemicals used in cancer therapy have a rather limited therapeutic window: they are more toxic on cancer cells than on normal cells, but the low difference is at the origin of side-effects, always present. The tuning in cell differentiation is easier with monoclonal antibodies than with simple chemicals. In addition, such fine tuning is applicable to the different tumor cells that have been identified for each category or sub-category of cancer lines. These antibodies are at the origin of the biopharmaceutical drugs that can be used for "personalized medicine". For obvious reasons, every patient is expecting a drug which should be good for himself, so it is easy to understand the success of these monoclonal antibodies in cancer therapy. In addition, the cost of the analysis of your own DNA decreased very quickly: many analytical companies now provide this ser-

vice for about one thousand US$. Sophisticated analyses of genes, proteins or metabolites became possible, opening the field of "omics" methods (genomics, proteomics, pharmacogenomics, metabolomics, ionomics …). These evolutions made possible the treatment of orphan genetic diseases. Blinded by the success of monoclonal antibodies in cancer, many decision makers in Big Pharma consider that the time of chemical drugs is over and that they will vanish in front of all these new biopharmaceuticals. In addition, the rather facile synthesis of many chemical drugs is not making a barrier against production of generic versions in countries with low-cost workforces.

Are we sure that the drug needed to cure all different diseases will be covered by biopharmaceuticals? Can we imagine a therapeutic arsenal without chemically based drugs? The answer is "no". Based on facts, it is obvious that the drug arsenal will be shared between chemical drugs and biopharmaceuticals. Before going to an estimate of what will be the ratio between these two complementary tools, we must consider the limits for the use of biopharmaceuticals.

One of the drawbacks of biopharmaceuticals is their size. Monoclonal antibodies are large protein structures, able to circulate in blood, but unable (i) to reach targets located inside another protein (how to inhibit a specific enzyme?) or (ii) to cross barriers such as the blood-brain barrier. In addition, the *per os* use of antibodies is not possible due to the capacity of the stomach to digest proteins. Such limitations leave open large numbers of opportunities for the so-called small molecules.

On top of the arguments, there is the cost of biopharmaceuticals. Details on this particular point have been reported in [1]. Since 2012, the costs are more and more becoming a real concern, due to the limits that national or private insurances can put to support expensive treatments. In August 2017 the US FDA approved a new treatment of acute lymphoblastic leukemia in children and young adults (tisagenlecleucel; trademarked Kymriak™) for a cost of 475,000 US$, one of the most expensive cancer treatments if one excludes the treatment of some very rare genetic diseases [10]. We have to keep in mind that the total health budget reached 17.4 % of the gross domestic product in the USA, at a plateau level for the last decade. In France and Germany, the health budgets were 10.1 and 10.0 % in 2014, respectively. With the aging of the population worldwide, one can anticipate an increase of health costs within the constraints of a slow GDP evolution, making difficult the use of highly expensive drugs. In such context, it is obvious that low-cost and efficient drugs based on chemicals have a real future.

2.4. Chemical Drugs Are Still Dominating Therapeutic Innovations

As a matter of fact, small molecules still dominate the field of drug innovation for the last two decades, in contradiction with claims predicting their downfall and the exponential raise of biopharmaceuticals. Each year, the FDA provides the list of the new drugs that have been approved by its Center for Drug Evaluation

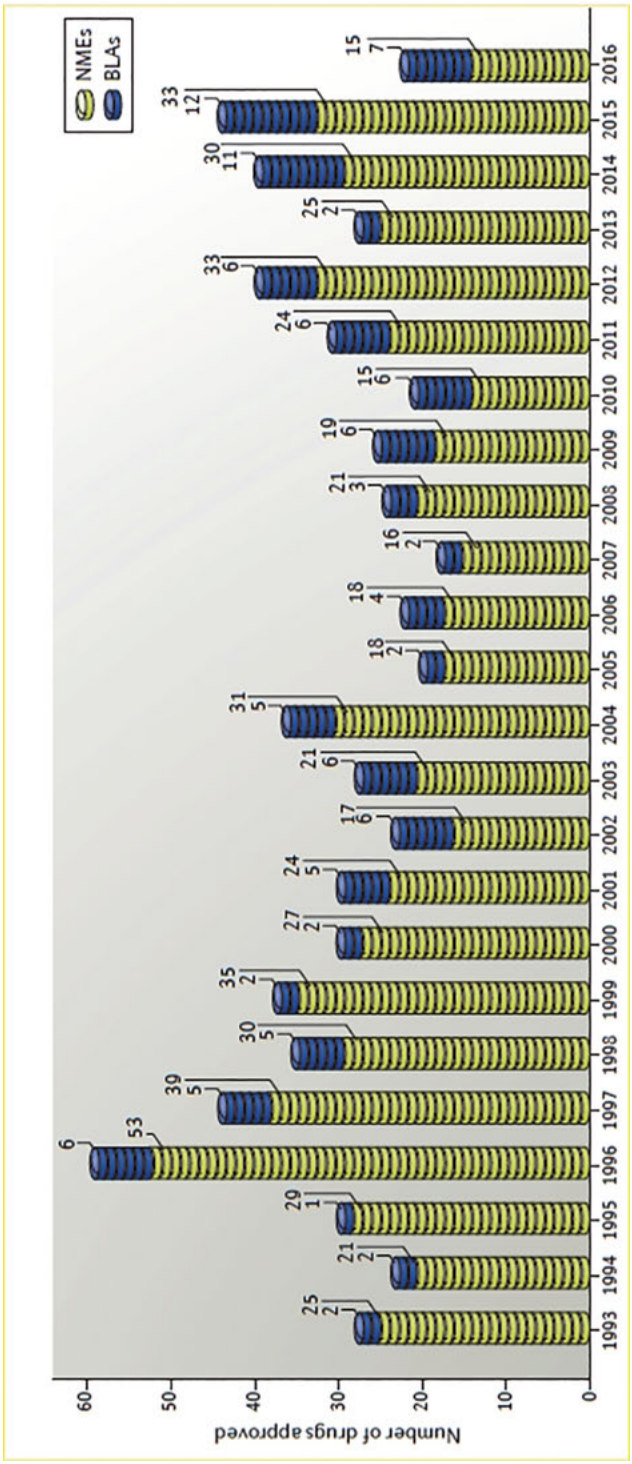

Figure 1. New drugs approved by FDA since 1993. New molecular entities (NMEs) and biologics licence applications (BLAs) approuved by the Center for Biologics Evaluation and Research (CBER) since 1993. Approvals by the Center for Drug Evaluation and Research (CDER) are not included in this count. Data are from Drugs@FDA. Reproduced from [11] with permission of Springer Nature; copyright 2016.

and Research, with details of the number of small molecules (chemical drugs), named "New molecular entities" (NMEs), and the number of biopharmaceuticals, named "Biologics license applications" (BLAs) (Figure 1) [11].

For this 24-year period, the numbers of chemicals and biologics were 566 and 114, respectively. On this large period, the "small molecules" represent 83 % of the total number of drugs that have been approved by the FDA. Facing this impressive fact, it is surprising to read comments by investors or opinion leaders of pharmaceutical companies on the decline of chemicals drugs in drug innovation, placing medicinal chemistry in an unfavorable position in Big Pharma companies. Most of them have closed their chemical facilities and expect to get new molecular entities from start-up companies or universities [1, 12]. However, many of large size "biotech companies" that have raised money from stock markets are not afraid by chemical drugs that are on the way to be approved after a successful proof of concept in clinical trials. Dimethylfumarate is a recent illustration of that particular point [13]. In March 2013, this very simple chemical molecule was approved by the FDA for the oral treatment of multiple sclerosis [14]. In April 2014, within one year after its entry on the market, the sales of this drug, commercialized by Biogen-Idec under the name of tecfidera, reached 1.3 billion of US$. The treatment cost (54,700 US$ per year) of this cheap chemical can be compared to the cost of fingolimod, that was already on the market for a cost of 60,000 US$/year. Dimethylfumarate has a long history, far from the usual biotech saga. In 1959, a German chemist decided to treat his own sporiasis with different fumaric esters, since this disease was associated to a low level of fumaric acid in the body [15]. His success led to fumaderm, a monoester of fumaric acid, as drug against psoriasis, marketed by the German company Fumapharma. This small company decided to initiate, in collaboration with Biogen-Idec, the first clinical trial to evaluate the activity of dimethylfumarate *per os* to treat multiple sclerosis. Just after the preliminary positive results, Biogen-Idec bought Fumapharma in 2006 and achieved all late-stage clinical trials, up to the final FDA approval [13]. This success story clearly illustrates the essential value, even with a simple entity, of "small molecules" as efficient drugs. In fact, we need "chemtech companies" as we need "biotech companies"! Last point, the cost of the treatment in the case of dimethylfumarate has nothing to do with the cost of the chemical itself, but has to do with many different financial aspects that are far beyond the scope of the present chapter dedicated to drug innovation.

2.5. Don't Forget Serendipity!

Medicinal chemistry has changed since the 1970s, with the emergence of new sophisticated techniques and equipment (high-field NMR spectroscopy, high-resolution mass spectrometry for small and large molecules, fast crystallography for chemicals or macromolecules) and computing facilities allowing virtual drug screening for example. All these new tools accelerate all the processes of medicinal chemistry, from the identification of hits to the efficient large scale synthesis of approved molecules. Based on the knowledge acquired by the understanding

at the molecular level of biochemical and biological mechanisms, we all have the feeling that we are on the way of "rational drug design". In fact we are just "on the way". A living system is complicated. The number of proteins from direct gene expression in a human body is close to 20,000, but much more if modified proteins are included (e.g., by glycation), and all these proteins are organized with glycoside-containing polymers and nucleic acids in about 10^{13} cells. Then, a full "*in silico*" study of a drug candidate, with all cellular and sub-cellular aspects at a molecular level, should involve complicated calculations that are not available. Parameters of chemical objects that can be successfully calculated with density functional theory methods are far below the ones of the universe created by exogenous molecules circulating with a human body.

Despite of all our wishes for rational drug design, it must be underlined that serendipity has played and will play a role in drug discovery. Serendipity has nothing to do with hazard games; no, the information that you get by serendipity is only accessible to prepared minds as previously said by Pasteur. Serendipity is at the origin of some very useful and efficient drugs, such as paracetamol and cisplatin to give only two examples. The discovery of the analgesic and antipyretic properties of paracetamol (4-acetylaminophenol) is due to a mistake [2]! This drug has been on the market since 1955, but its discovery goes back to the year 1880 at the hospital of Strasbourg. A senior medical doctor, Adolf Kussmaul, asked two young students, Arnold Cahn and Paul Hepp, to treat the fever of few patients with naphthalene (yes!). The successful reduction of fever with the first set of patients encouraged them to repeat the experiment. Unfortunately, the second attempt was a failure. A discussion with Kopp, the pharmacist who provided the drug, allowed to solve this problem: by mistake the first compound was not naphthalene but acetanilide (acetylaminobenzene), a compound that generates *in vivo* paracetamol by enzymatic hydroxylation in the *para* position. For a while, acetanilide was commercialized under the name of Antifebrin with severe side effects (formation of methemoglobin). In 1948, it was established that the hydroxylated metabolite of acetaniline, i.e., paracetamol, was a much better analgesic and antipyretic drug than its precursor, and had a lower toxicity. This second discovery is at the origin of the extensive use of paracetamol. The current production of this drug is reaching 147,000 metric tons per year [16].

Cisplatin, one of the major drugs used in cancer therapy, is another example of discovery due to serendipity. In a first article, Rosenberg, Van Camp, and Krigas published the observed role of an electric field on the growth of *E. coli* bacteria when using platinum electrodes [17]. The bacteria formed unusual long filaments, up to 300 times the normal length. The authors clearly indicated that such phenomenon was caused by the presence of transition metal ions in concentrations of about 1–10 parts per million. This key experimental observation led Rosenberg to explore the effect of different platinum complexes on the growth of bacteria or tumor cells: *cis*-diamminedichloroplatinum(II) (cisplatin or cisplatinum, for short) was one of them [18]. Being a physicist, Rosenberg engaged fruitful exchanges with Robert J. P. Williams, the well-known inorganic chemist of Oxford University, on the inorganic chemistry of platinum compounds [19]. Andrew Thomson, a PhD student of Williams spent two years in the Rosenberg's

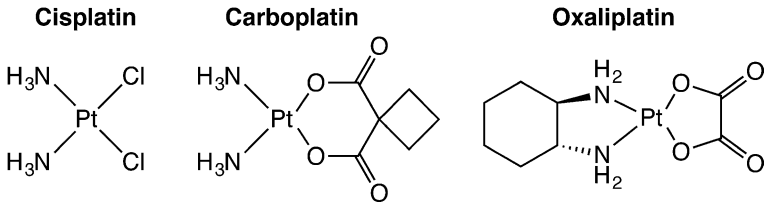

Figure 2. Structures of three successful anticancer platinum derivatives, cisplatin, carboplatin, and oxaliplatin.

laboratory to prepare a series of platinum compounds, including cisplatin, that have been evaluated as potential anticancer drugs [20].

Based on data obtained *in vitro* and *in vivo* with leukemia L1210 cells, cisplatin entered in the pipeline of the National Cancer Institute in the early 1970s, and in the clinic in 1972 with initial trials on patients at the Memorial Sloan Kettering hospital in New York City. Facing renal toxicity as major adverse effect, Esteban Cvitkovic decided to perform a chloride diuresis to maintain the excess of platinum, which was not reaching the drug target, in the inactive dichloride state, thus preventing the formation of the more toxic diaqua form [21]. Mannitol diuresis was also a way to reduce renal failure. Finally, in 1978, thirteen years after the initial observation of long filaments of *E. coli* within an electrolysis cell, cisplatin was approved as drug to treat testicular cancers and then its use was expanded to different cancers (bladder, ovarian, lung, neck and head) [22, 23]. Cisplatin has been followed by two other platinum derivatives, carboplatin and oxaliplatin, that are also used worldwide (see Figure 2 for structures). Other platinum complexes received only regional approvals [24].

The research on platinum derivatives is still very active and many efforts have been made to obtain new compounds active on cisplatin-resistant cancer cells, one of the limitations of chemotherapies with chemical drugs [25]. A last and important point on cisplatin: the approval of this efficient anticancer drug was based on two key parameters, the pharmacological activity and the methods to control its toxicity. In 1978, the DNA as potential target was only mentioned and was considered as questionable by many researchers. Fortunately, in the late 1970s, regulatory agencies were more interested by the clinical benefit for the patients than by a full documentation on the mechanism of action (MOA for short in all current guidelines) of the drug. According to the PubMed data base, between 1965 and December 1978, from the initial observation to the drug approval of cisplatin, only 272 articles containing the word cisplatin were published. In February 2018, the word cisplatin is mentioned in more than 66,900 articles, indicating that this anticancer drug, discovered by serendipity is still a leading subject of research. For the patients suffering from diseases for which there is an urgent need for new drugs, it is important not to forget to focus the research efforts on discovery, with a balanced effort on mechanistic aspects on drugs that have been discovered. This is one of the lessons that can be drawn from the history of cisplatin.

3. TRIOXAQUINES: ANTIMALARIAL HYBRID MOLECULES WITH A DUAL MODE OF ACTION

Malaria and schistosomiasis, caused by *Plasmodium* and *Schistosoma* sp. parasites, respectively, are the main blood parasitic diseases in the tropical regions. Malaria is a major public health issue for more than 3.2 billion people living in endemic areas, but also a significant threat for millions of travellers. It remains one of the three main infectious causes of death (with tuberculosis and HIV) despite the fact that, according to the World Health Organization (WHO), the estimated cases of malaria worldwide decreased from 233 million in 2000 to 216 million in 2016. The mortality rates were reduced by approximately half, from 985,000 to 445,000 deaths during the same period [26]. Artemisinin-based combination therapies (ACT) have been integral to this success. The 2015 Nobel Prize in Physiology or Medicine was awarded to Youyou Tu, from the China Academy of Chinese Medical Sciences in Beijing, for her key contributions to the discovery of artemisinin [9]. In the 1960s, the main treatments for malaria were chloroquine and quinine (Figure 3), but they were proving increasingly ineffective. The research of antimalarial drugs was prompted in China by the Vietnam War.

China was involved with North Vietnam, and malaria greatly reduced the combat strength. So in 1967, China established a national project, identified as "project 523", to discover new therapies against malaria. Tu and her team screened more than 2,000 Chinese herbal remedies, seeking drugs with antimalarial activity. From the wormwood plant *Artemisia annua*, that has been used for several centuries in traditional Chinese pharmacopeia, the researchers isolated chemically pure arte-

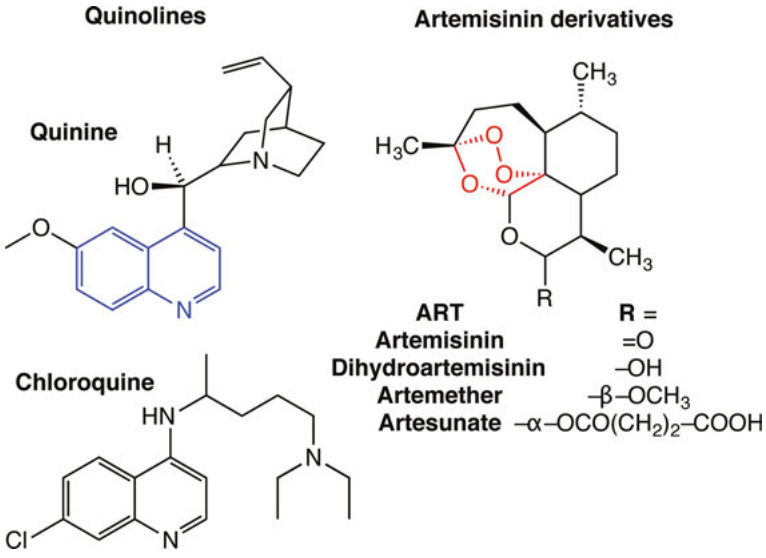

Figure 3. Structures of the antimalarial drugs quinolines (quinine and chloroquine), and artemisinin derivatives.

misinin (qinghaosu), a sesquiterpene lactone containing a peroxide function (Figure 3), that has now been proven to be efficient for decades on multidrug resistant *Plasmodium falciparum* parasites.

Artemisinin, and its hemisynthetic derivatives dihydroartemisinin, artemether, and artesunate (artemisinins refers to artemisinin and its derivatives, and is noted ART for short), clear parasitemia more quickly than any other antimalarial drug, including quinine [27]. As a unique feature among antimalarials, they are active against the early intraerythrocytic stages (ring stages) [28, 29]. They also reduce the number of gametocytes (sexual-stage parasites), thus resulting in reduced transmission to the mosquito vector, and limited spreading of the disease [28, 30].

Since 1994, artemisinins have been used to treat uncomplicated malaria, in combination with other effective antimalarial drugs (= ACT for "artemisinin-based combination therapy") having different mechanisms of action, and longer plasma half-lives than artemisinin. In fact, when the artemisinins are eliminated (1–2 hrs), the remaining parasites are exposed to the associated long-lasting drugs well after the end of the usual 3-day ACT course (up to a week). ACTs are the most effective antimalarial treatments available nowadays, and were recommended as first-line treatment for uncomplicated malaria by the WHO in 2001. They have replaced quinolines and antifolates as first-line drugs in the main part of endemic countries, with 409 million doses in 2016 [26].

Following the WHO recommendation, the availability and price of artemisinin fluctuated greatly from year to year, ranging from shortfall to oversupply. To alleviate these supply issues, production of artemisinic acid, a late-stage precursor of artemisinin, by yeast fermentation, followed by chemical conversion to artemisinin, was developed and entered commercial production by Amyris Inc. in 2013 [31].

3.1. Artemisinin: An Antimalarial Trioxane with Alkylating Properties Toward Heme

From the early studies on artemisinin, its antimalarial activity was found to be strongly dependent on the peroxide moiety, since an artemisinin derivative lacking the endoperoxide bridge (deoxyartemisinin) is devoid of antimalarial activity [32]. During the infection of erythrocytes, *P. falciparum* is able to digest 60–80 % of the host hemoglobin to collect amino acids for its own protein production. The released Fe(II)-heme is potentially a damaging catalytic source of reactive oxygen species (ROS). To avoid poisoning by this redox active species, the parasite efficiently polymerizes heme to hemozoin, the "malaria pigment" that is a crystalline, redox-inactive polymer of Fe(III)-heme [33].

In the presence of ART, the reductive cleavage of the peroxide bond of artemisinin is initiated by an electron transfer from the low-valent iron(II)-heme to the antibonding σ^* LUMO of the peroxide. This reductive activation generates a short lived alkoxy radical which quickly rearranges, *via* β-fragmentation, to a C4-centered primary radical thermodynamically facilitated by concomitant formation of an ester functionality. Intramolecular addition of this alkylating species

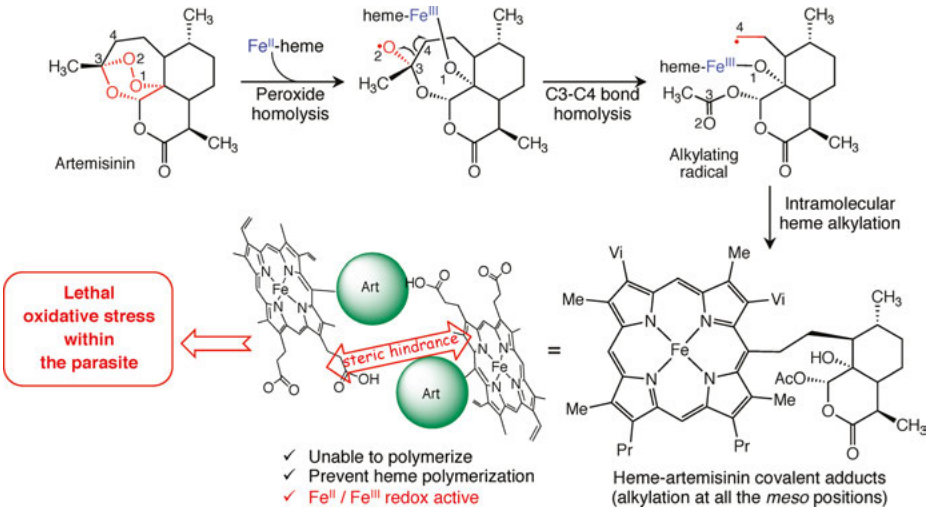

Figure 4. Reductive activation of artemisinin by iron(II)-heme, leading to potentially toxic heme-drug adducts.

occurs without regioselectivity on the four *meso* carbons of the porphyrin ligand, leading to the formation of covalent heme-drug adducts (Figure 4) [34–37]. After treatment with artemisinin at pharmacologically relevant doses, these heme-artemisinin adducts are detected in the spleen and urine of malaria-infected mice indicating that, also *in vivo*, heme can act as trigger and target of artemisinin [38]. Heme-artemisinin adducts are unable to polymerize, and are able to completely inhibit the polymerization of heme *in vitro*, suggesting that free heme derivatives should accumulate in parasites treated by artemisinin, thus increasing oxidative stress within the parasite [28].

When mice infected with an artemisinin-resistant strain *P. yoelii nigeriensis* (obtained after long-term drug pressure) were treated with artemisinin, significantly lower amounts of heme-artemisinin adducts were detected, compared to mice infected with a *P. yoelii* artemisinin-sensitive strain. After release of the artemisinin drug pressure, the parasite regains both susceptibility to artemisinin, and increased ability to produce heme-drug adducts, thus confirming that heme and/or hemozoin metabolism are key targets for artemisinin [39].

When incubated in *Plasmodium* cultures, radiolabeled artemisinin alkylates not only heme, but also some specific parasitic proteins [40–43]. *In vitro*, the C4 centered radical of artemisinin can react with the thiol function of cysteine or glutathione to alkylate the peptide through a thioether linkage [44], indicating that a covalent heme-protein coupling may occur by this way. However, no artemisinin-protein adduct has been characterized up to now, and *in vitro* these reactions are much less efficient than heme alkylation [45].

Iron metabolism is also likely to play a central role in the anticancer activity of artemisinin, associated to indiscriminate generation of oxidative stress as a consequence of heme-mediated endoperoxide cleavage, activation of caspases and consequent apoptosis [46].

3.2. From Artemisinin to Antimalarial Trioxaquines: Heme as Drug Target

Since heme released in the *Plasmodium* food vacuole is an old but specific pharmacologic target (hemoglobin digestion occurs only in infected erythrocytes), many semi-synthetic derivatives of artemisinin, or synthetic drugs containing the crucial endoperoxide bond of artemisinin have been developed, including trioxanes, trioxolanes, and tetraoxanes (for a recent review, see [47]). Among them, the trioxolane OZ439 (Figure 5) is, to date, the most promising drug in the pipeline [48–50]. Unfortunately, the single dose treatment of OZ439 (artefenomel) associated with piperaquine did not meet the target efficacy in a recent Phase II clinical trial [51]. A Phase II clinical trial of a combination of OZ439 and ferroquine [52] is still under way. These peroxide derivatives were able to alkylate heme or a synthetic heme model, and their alkylating ability correlates well with their antimalarial efficiency [53–55].

In Toulouse, we prepared hybrid molecules, named trioxaquines, containing a trioxane moiety with heme-alkylating property, like in artemisinin, covalently linked to a 4-aminoquinoline, having heme-stacking ability, like in chloroquine (Figure 5). Several trioxaquines were found highly active on chloroquine-resistant *P. falciparum*, and no cross resistance was observed with chloroquine and pyrimethamine [28, 56–59].

PA1103 was selected among 120 other active trioxaquines for pre-clinical development. PA1103 is highly active *in vitro* on several sensitive and resistant strains of *P. falciparum* (e.g., $IC_{50} \leq 10$ nM on the chloroquine-resistant strain FcM29-Cameroon) and also on multidrug-resistant strains obtained from fresh

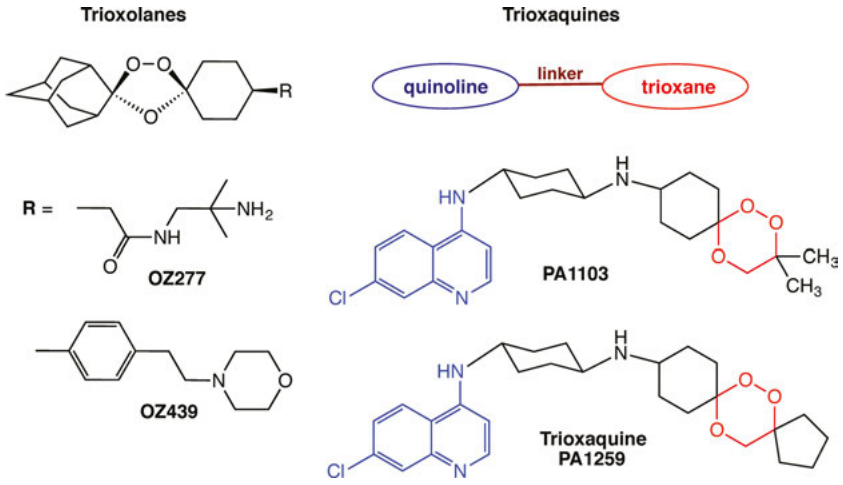

Figure 5. Structures of synthetic endoperoxides developed as antimalarial drugs: trioxolanes OZ277 (arterolane) and OZ439 (artefenomel), and trioxaquines PA1103 and PA1259.

patient isolates in Gabon. *In vivo*, PA1103 is efficient by the oral route, providing complete cure of mice infected with chloroquine-sensitive or chloroquine-resistant strains, when treated at 26–32 mg/kg. PA1103 is also highly effective in humanized mice infected with *P. falciparum*, and has a good drug profile (preliminary absorption, metabolism, and safety parameters) [60]. Heme-trioxaquines adducts were detected in the spleen and urine of *P. vinckei* infected mice orally treated with trioxaquines, thus confirming that heme is a target of trioxaquines *in vivo* [61]. The development of PA1103 was stopped in 2010 due to a lack of financial support.

3.3. Trioxaquines against Schistosomiasis

Schistosomiasis (or bilharzia), caused by the flat worm *Schistosoma* sp., is currently the second most prevalent parasitic disease after malaria, and is occurring in over 75 countries in tropical and sub-tropical regions. It is estimated that 600 million people are at risk of infection; 200 million people are infected, and at least 200,000 deaths per year are associated with the severe consequences of infection [62]. Schistosomiasis and malaria share most of the same geographic areas, and co-infection is usual [63]. The treatment and control of schistosomiasis is currently based for the last forty years on a single drug, praziquantel (PZQ). However, schistosome strains with lower sensitivity/resistance have been identified in African countries, leading to treatment failures [64, 65].

Despite their high phylogenetic difference, the two parasites *Plasmodium* and *Schistosoma* have a similar hemoglobin metabolism leading to the formation of hemozoin. For this reason, antimalarial drugs targeting heme have been evaluated as potential antischistosomals. However, artemisinin was evaluated, first in China, against *S. japonicum* endemic infections, long before investigations on its mechanism of action. In these conditions, targeting free heme released by hemoglobin metabolism, we evaluated the activity of trioxaquines against *S. mansoni*. Several of these molecules were found highly active on both larval and mature stages of *S. mansoni*, among them PA1259 (Figure 5 [66, 67]) alkylates heme in female adult *S. mansoni*, and heme-drug adducts were identified from treated worms [68]. PA1259 was found active *in vitro* on all the human-relevant development stages of *S. mansoni* (cercariae, schistosomules and adult worms). *In vitro*, treatment of female *S. mansoni* with PA1259 results in a drastic decrease of their hemozoin content, associated with nitric oxide production, suggesting that iron metabolism should be a valuable target for anti-schistosomal chemotherapy [67].

In infected mice, PA1259 is less active than praziquantel on adult worms (after a 4×50 mg/kg oral treatment, the reduction of worm burden was 40 % and 86 % for PA1259 and PZQ, respectively). PA1259 is slightly more effective than PZQ on schistosomules (53 % and 41 % for PA1259 and PZQ, respectively), thus opening the way to an efficient combination therapy of this neglected disease.

3.4. Resistance of *Plasmodium falciparum* to Artemisinin and Synthetic Peroxides

Parasite resistance after artesunate monotherapy was evidenced in 2008 on the Cambodia-Thailand border [69, 70]. The decline of ACTs efficiency was suspected for the first time in 2006 [71], and has now spread in the Greater Mekong subregion [72, 73]. Artemisinins resistance is characterized by a delayed parasitic clearance, and higher rate of clinical failures due to the increased parasite recrudescence [69, 70], meaning that a significant number of parasites survive artemisinin treatment, and continue their cell cycle after elimination of artemisinin. A delayed parasite clearance does not necessarily induce treatment failure, as long as the partner drug used in ACT remains effective.

Artemisinin resistance is characterized by an unconventional mechanism based on quiescence of a portion of *Plasmodium* at the young ring stage, leading to parasite recrudescence as soon as drug pressure is removed. The quiescence mechanism involves an almost complete arrest of the cell cycle, and an increased ability to manage oxidative damage [74]. This mechanism was first established on an artemisinin-resistant parasite line F32-ART5 obtained *in vitro*, after 5 years of exposure to sequential and increasing concentrations of artemisinin [75], and then confirmed on Cambodian isolates [76–78]. Quiescence due to a drug treatment also exists in bacteriology and oncology. In fact, the ability of human cancer cells to stop their cell cycle at the G1 phase make them able to survive anticancer chemotherapy [79]. At a molecular level, *Plasmodium* quiescence is induced by a mutation in the propeller domain of the gene encoding for the Kelch protein 13 (K13) [76–77]. A number of mutations of the gene *pfk13* have been characterized; at least one of these mutations is necessary, albeit not sufficient, for resistance [80].

The experimental *P. falciparum* artemisinin-resistant line F32-ART5 also enters a quiescent stage when treated with other antimalarial drugs such as pyrimethamine or quinolines, including quinine and mefloquine. Only atovaquone overcomes this multiresistance *in vitro* [81]. Unfortunately, resistance to atovaquone is easily and quickly selected when used in the field, regardless of the use of artemisinin [82].

A strong cross resistance was evidenced between trioxaquines and artemisinin, despite the hybrid structure of trioxaquines, that included both trioxane and quinoline moieties. In fact, the F32-ART5 parasite line recrudesced significantly faster than the wild-type F32-TEM line, after exposure to trioxaquines, as well as the artemisinin-resistant Pf K13 mutant field parasites. Moreover, the F32-Tanzania clone, cultured for 4 years under a dose-escalating regimen of trioxaquine DU1302, led to a trioxaquine- and artemisinin-resistant parasite line (F32-DU) containing the same K13 mutation that was previously found in the F32-ART5 line [83]. So, the hybrid structure of trioxaquines could not avoid the *in vitro* selection of resistant parasites. It was recently reported that K13 mutations also compromise the activity of the trioxolane OZ277. However, OZ439 remains effective against most artemisinin-resistant parasite cell lines, especially those containing the C580Y mutation of the *pfk13* gene, a mutation that is by far the

most frequent in Cambodia and Vietnam [84, 85]. These data indicate that specific peroxide antimalarial drugs can prove valuable to fight artemisinin-resistant *P. falciparum* infections.

Until now, ACTs remain effective in Africa [80, 86]. However, the risk of spreading ACT resistance from its actual epicenter to Africa should not be under-estimated. Beside genetic factors of *Plasmodium*, poor access to drugs in many countries may contribute to emergence of ACT resistance. Until December 2014, seven African countries still offered artemisinin as monotherapy, despite monotherapy is a main factor contributing to the development of resistance, and it was banned by the WHO [87]. In addition, the use of fake or counterfeit drugs may lead to spreading resistance, by using treatments at sub-therapeutic doses. Recent estimates from Southeast Asia suggest that up to 50 % of the artesunate sold are fake drugs, and the situation might be worse in other malaria-endemic countries. ACTs sold without any quality control are ubiquitous in Sub-Saharan Africa, both in the private and the public sectors, accounting for 32–89 % of the total ACT doses used [88, 89].

Despite development of resistance, ACTs are still the cornerstone of antimalarial therapy. However, no efficient medicine against a common pathogen can be regarded as definitive. In the more or less long term, drug pressure is expected to select parasite-resistant lines able to regain the lost ground. To avoid dramatic spreading of a disease due to the progressive failure of the actual treatments, research for new drugs is a race against the development of resistance of the previous drugs. Actually, the inevitable time lag to develop new anti-infective drugs is 12–17 years [90], a delay that makes the situation critical in particular for the domain of parasitic diseases that are not directly touching developed countries. The influence and efficiency of strong private foundations is a positive point, when they accept the idea to support drug discovery approaches based on medicinal chemistry. One has to keep in mind that the current control of HIV disease is mainly due to "small molecules" (from medicinal chemistry) and, despite important funds targeted on vaccine-candidates, one should recognize that no efficient vaccine is available to stop the spreading of this retrovirus.

4. REGULATION OF COPPER HOMEOSTASIS IN ALZHEIMER'S DISEASE

With increasing life expectancy in most countries, aging diseases are becoming the frontline health problems, especially the various kinds of dementia that occur in the elderly. Currently, about 35 millions of patients in the World are suffering from Alzheimer's disease (AD), a neurodegenerative condition related to aging, that accounts for about 70 % of cases of dementia, and this number is expected to reach 100 millions in 2050 [91, 92]. Since 99 % of cases have no genetic cause, but are sporadic, AD can occur in anyone, in any family, in any country. The irreversible progressive cognitive dysfunction, impaired language skill, and personality changes, together with the duration of AD pathology (5 to 8 years)

place a considerable burden on patients, families, caregivers and on public health budgets. Therefore, the research for efficient anti-AD therapies is currently one of the major challenges in drug discovery. In fact, current AD therapies based on four acetylcholine esterase inhibitors and memantine, a weak antagonist of the *N*-methyl-D-aspartate receptor [93], are not curative, offering only a short-term symptomatic relief. Moreover, these drugs have potentially serious side effects and their efficiency/cost ratios are questionable. Despite intensive efforts over the last two decades on genetics, biochemistry, and cell biology on AD, the pipeline of new drugs is rather unproductive. Since the approval of memantine in 2003, no new AD drug has been approved.

Clinical trials undertaken from 2002 to 2012 for AD involved 244 new compounds, but all of them, except one, failed at different stages, corresponding to an attrition of 99.6 % [94]. In fact, trials for AD drugs, while showing promise in animal studies, were discontinued at later stages. Drug screening for AD is actually exclusively done using transgenic mouse models. However, AD is not a simple monogenic disease, but a multi-parameter pathology involving epigenetics, far beyond the simple concept "one disease/one target-protein/one drug" that has been used to develop mono- or double-transgenic mice models. The use of transgenic animal models that do not accurately reflect human pathogenesis and, consequently, do not reliably predict drug efficacy, is probably one of the reasons of the research failures [95]. "Drug-industry scientists are failing themselves if their animal studies are poorly done or use the wrong model, and their companies are failing academics who do their phase 3 trials with them, trial participants, and shareholders. Perhaps the problem is "translational research" itself: a phrase much bandied around, but does anyone know what it really means, let alone how to do it?" [95]. So, to allow significant progress, one must think out of the box, and consider different targets to find new drugs able to stop the degenerative process as early as possible.

AD is characterized by abnormal deposition of the amyloid protein (Aβ) and the hyperphosphorylated tau protein, two proteins that have been considered as the main drug targets up to now. However, the post-mortem analyses of amyloid plaques indicate an excessive accumulation of copper, iron, and zinc by 5.7, 2.9, and 2.8 times the levels observed in normal brains, respectively [96]. There are extensive evidences linking tau pathology, Aβ-aggregation, and metal deregulation. In addition, *in vitro* incubation of metal ions with amyloid peptide Aβ promotes its aggregation. Conversely, incubation of plaques with metal chelators results in partial dissolution of the plaques. So, there are evidences of mis-metabolism of metal ions in AD (Figure 6). In addition, the distributions of Cu, Fe, and Zn are tightly linked together. For example, Zn(II) competes with Cu(II) for coordination to β-amyloids [97]. The amyloid precursor protein (APP) interacts with ferroportin, oxidizes Fe(II), and loads Fe(III) into transferrin, thus taking part in iron regulation [98]. This ferroxidase activity of APP is inhibited by Zn(II) supplied by extracellular Zn-Aβ deposits [98], resulting in cortical Fe(II) accumulation that characterizes AD pathology. As an important feature, Cu(II)- or Fe(III)-Aβ can be readily reduced by endogenous reductants such as glutathione, thus inducing the reduction of dioxygen by Cu(I)- or Fe(II)-Aβ and producing

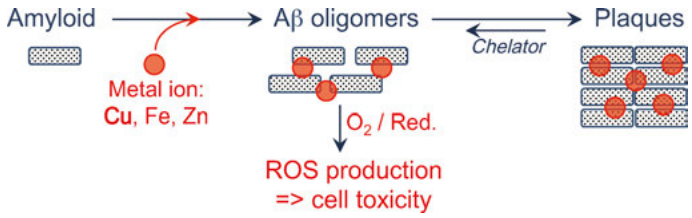

Figure 6. Aggregation of amyloids promoted by metal ions, and partial dissolution of plaques in the presence of metal chelators.

ROS responsible for neuron death. Such a catalytic process causes overconsumption of endogenous antioxidants, leading to their depletion in neurons.

In addition, copper sequestered in amyloid plaques may generate a deficit in other compartments of the brain, or a reduced superoxide dismutase-1 activity [99]. For these reasons, restoration of copper homeostasis in the brain is a kind of hub target, and copper chelators are considered for AD chemotherapy [100–105]. The difficulty for large copper-carrier proteins to directly extract copper ions from Cu(II)-Aβ may be at the origin of the rupture of the copper homeostasis in AD brains. An efficient copper chelator should therefore be able to extract copper ions from pathological sinks (amyloids being the major one) and to transfer these redox-active metal ions to copper-carrier proteins or copper-containing enzymes, in order to recycle copper in its physiological role. Under physiological conditions, glutathione is one of the providers of copper to proteins. The copper complex of glutathione is indeed able to release the metal ion in favor of carriers or enzymes [106, 107]. Unfortunately, *in vitro*, glutathione was found unable to fully extract copper from Aβ [108]. Obviously, the affinity of chelators for copper should not be a sufficient criterion, but the metal specificity, the ability of the chelators to release copper under specific biological conditions, and their ability to inhibit the ROS production induced by Cu-Aβ should also be needed specifications.

4.1. From Hydroxyquinolines to Bis(8-Amino)quinolines as Regulators of Copper Homeostasis

The historical prototypes of copper chelators that have been considered for AD therapy are clioquinol (CQ) and PBT2, based on a mono(8-hydroxy)quinoline scaffold (Figure 7) [109]. CQ was found able to decrease Aβ deposits and to improve learning and memory capacities of APP transgenic mice [110]. Unfortunately, clioquinol, formerly used as antifungal and antiprotozoal, was withdrawn from the market in 1983 [111], due to its neurotoxicity attributed to zinc chelation [112, 113]. In fact, 8-hydroxyquinolines are non-specific metal chelators [114, 115], and the affinity constant of CQ for Cu(II) is only one order of magnitude higher than that for Zn(II) (log K = 10 and 9, respectively) [102, 116]. In this series, PBT2 [102] was less toxic [117]. However, clinical trial of PBT2 as anti-AD drug was stopped due to its lack of efficacy. CQ and PBT2 were proposed to promote the

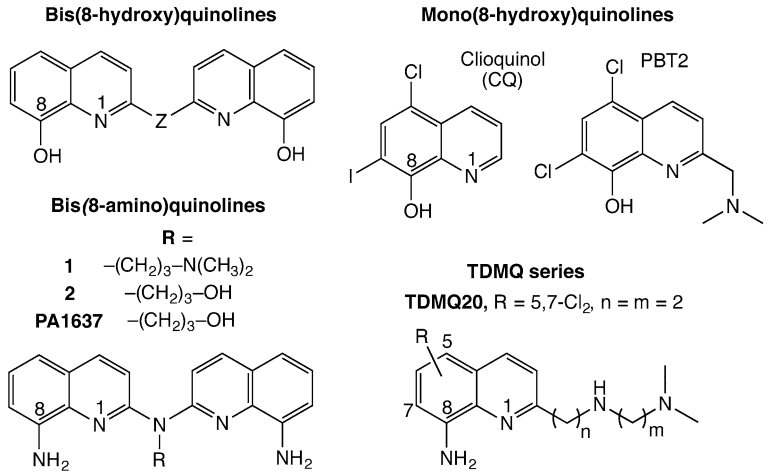

Figure 7. Structures of 8-aminoquinolines and 8-hydroxyquinoline copper chelators. Z stands for different types of linkers (alkyl, amine, ester …).

translocation of Cu(II) and Zn(II) from the extracellular environment to the inside of the cell, thus rectifying the misbalance of metal ions [102, 118].

However, CQ and PBT2 are bi- or tridentate ligands, while the stable coordination of Cu(II) requires (at least) 4 coordinating functions. The Cu(II) complexes of CQ and PBT2 have a 1/2 metal/ligand stoichiometry [102, 119] and, therefore, in the presence of Cu^{II}–Aβ *in vitro*, stoichiometric amounts of CQ or PBT2 result in stable ternary CQ- or PBT2-Cu^{II}-Aβ complexes [119, 120]. As a consequence, 8-hydroxyquinolines are unable to extract copper(II) from soluble amyloids, indicating that these bidentate ligands are not really adapted as mediators between Cu-Aβ and Cu-carrier-proteins for regulation of copper ions in AD brains [120].

Contrary to mono(8-hydroxy)quinolines, bis(8-hydroxy)quinolines (Figure 7) offer four binding sites N_2O_2 within a single molecule, giving rise to complexes with a ligand/metal ratio = 1/1 at low concentration [121–123]. Such tetradentate ligands have an affinity for copper(II) that is by 4 to 6 orders of magnitude higher than that of mono(8-hydroxy)quinolines, with log K_{app} of 15.5–16.6 at physiological pH, and their selectivity for copper with respect to zinc is 100 to 1000 (log K_{app} for Zn(II) = 12.5–14.2). These ligands are also highly efficient at solubilizing Aβ peptides, and as inhibitors of H_2O_2 production by the system Cu-Aβ/ascorbic acid. However, their affinity for Zn(II) is still higher than that of Aβ [124] and, for that reason, they do not fulfill the criteria recently proposed for biologically pertinent copper chelators [125].

To assist the transfer of copper from Aβ to glutathione and, therefore, restore homeostasis of copper in AD brains, we designed a series of blood-brain barrier-permeable tetradentate ligands L, based on a bis(8-aminoquinoline) scaffold, which are specific for chelation of copper (Figure 7, **PA1637**, **1**, **2**) [100, 126].

4.1.1. Bis(8-amino)quinolines Transfer Copper from Cu-Amyloid to Glutathione

These ligands L have a very high affinity for Cu(II) ions, with log K_{app} in the range 14–16 at pH 7.4, and their selectivity for Cu(II) with respect to Zn(II) is very high, with log [K_{app} Cu-L/K_{app} Zn-L] >12 [126]. *In vitro*, at micromolar concentrations, they efficiently and readily extract Cu(II) from Cu^{II}-Aβ, to provide the Cu(II)-L complex (Figure 8) [119].

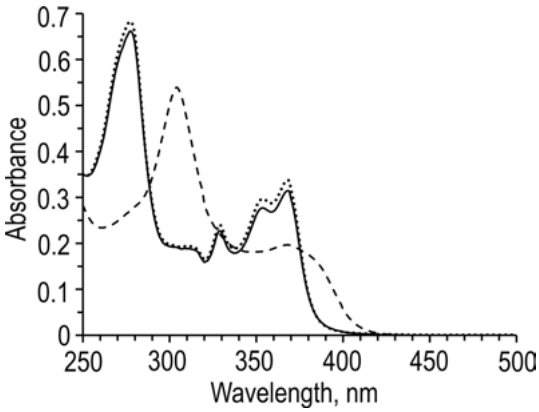

Figure 8. Extraction of Cu^{2+} from $A\beta_{1-28}$ upon addition of **2** (see Figure 7), evidenced by UV-visible spectroscopy. Spectrum of Cu-$A\beta_{1-28}$ + ligand **2** ($A\beta_{1-28}$/**2** mol ratio = 1/1, ——), compared with spectrum of Cu-**2** (·····), and spectrum of ligand **2** (- - - -).

Importantly, this series of chelators offers a square planar coordination that is suitable for Cu(II) but not for Cu(I). So, in the presence of glutathione (GSH), playing both the roles of reducing agent and competitive ligand for copper, the Cu(II)-L complex readily gives its copper ion to glutathione which will, in turn, give it to proteins (Figure 9) [127].

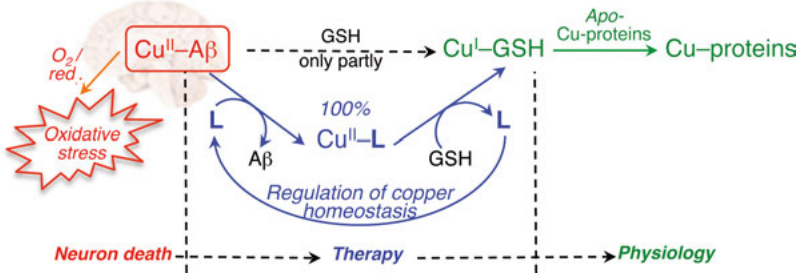

Figure 9. Transfer of copper ions from Cu-Aβ to Cu-proteins, using bis(8-amino)quinolines as mediating chelators.

In this process, the bis(8-amino)quinoline ligand is released and should be able to work in a catalytic way (Figure 9). Bis(8-amino)quinolines may therefore promote *in vivo* the transfer of Cu from Cu(II)-Aβ, to proteins, thus re-introducing the copper trapped in amyloids into a physiological circulation.

4.1.2. Bis(8-amino)quinolines Inhibit the Oxidative Stress Induced by Cu-Amyloid

In order to evaluate these copper chelators as drug candidates, a major issue is the potential ability of these ligands to inhibit the production of ROS induced by Cu^{II}-Aβ in the presence of a biological reducing agent. Therefore, we measured the ascorbate oxidation by Cu^{2+} or Cu^{II}-Aβ, in the presence of a chelator, as an indirect evaluation of its ability to reduce dioxygen and consequently to induce an oxidative stress [128]. As expected, the ascorbate was extensively oxidized in the presence of $CuCl_2$ (> 96 % in 30 min, Figure 10, trace b), or of Cu-$A\beta_{1-16}$ (92 % in 30 min, trace c). Addition of Cu-Aβ to ascorbate in presence of PBT2 also resulted in an extensive catalytic oxidation of ascorbate (80 %, trace d). This result indicated that PBT2 did not efficiently inhibit the production of ROS by Cu-Aβ in a reductive medium, and is consistent with the fact that the geometry of PBT2 is expected to chelate Cu(I) as well as Cu(II). Interestingly, the oxidation of ascorbate in the presence of Cu-Aβ and **2** (see Figure 7) was negligible (< 5 % in 30 min, trace e), confirming that Cu^{II}-**2** is unable both to reduce dioxygen and to release catalytically active copper ions under these reductive conditions.

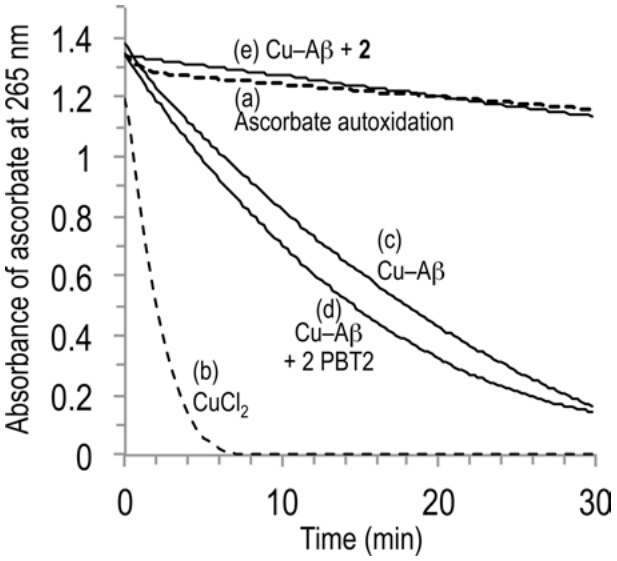

Figure 10. Measure of ascorbate oxidation under air in the presence of (a) no additive, (b) $Cu^{II}Cl_2$, (c) Cu^{II}-$A\beta_{1-16}$, (d) Cu^{II}-$A\beta_{1-16}$ + PBT2 (2 mol equiv), (e) Cu^{II}-$A\beta_{1-16}$ + **2** (1 mol equiv).

4.1.3. PA1637 Is a Bis(8-amino)quinoline Copper Chelator Able to Restore Episodic Memory in Alzheimer Mice

For the reasons outlined above, we developed a non-transgenic, reliable, and cheap mouse model of AD that has been validated with clioquinol and memantine. In a regular mouse, the memory deficit mimicking the early stage of AD is created by a single intracerebroventricular (icv) injection of $A\beta_{1-42}$ oligomers, while injection of the control anti-sense $A\beta_{42-1}$ has no effect on mice [129]. The ability of PA1637 (see Figure 7, for the structure) to inhibit the episodic memory loss was evaluated on this non-transgenic murine model. After oral treatment (8 doses of PA1637, 25 mg/kg each, over a 3-weeks period), the episodic memory of AD mice (Figure 11, grey bar) was similar to that of healthy animals (white bar), while that of AD mice that did not receive treatment was significantly impaired (black bars). Treatment with PA1637 at the lower dose of 12.5 mg/kg provided the same result (data not shown). These results clearly indicate that oral administration of PA1637 was able to fully inhibit the cognitive impairment induced by icv injection of amyloid oligomers.

Then, as a remarkable feature, the ability of bis(8-amino)quinoline ligands to transfer copper from amyloids to proteins, and to inhibit the oxidative stress produced by copper-loaded amyloids, is correlated to their capacity to restore episodic memory in non-transgenic amyloid-impaired mice. This result strongly supports the targeting of copper for AD chemotherapy.

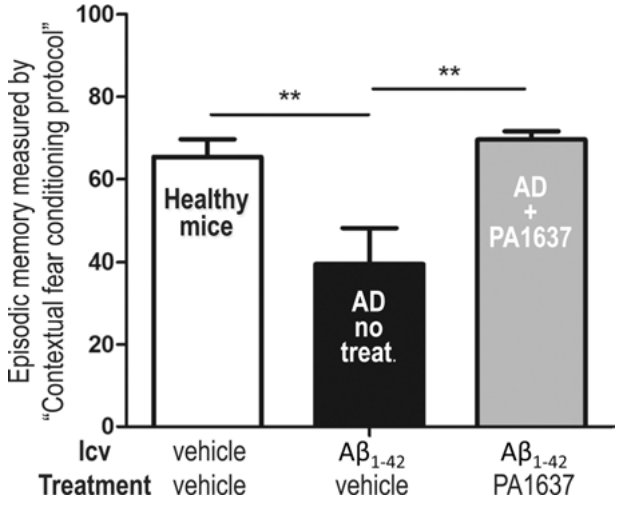

Figure 11. Episodic memory of non-transgenic AD mice treated with PA1637 at 25 mg/kg by oral route (grey bar), compared to healthy control mice (white bar) and untreated AD mice (black bar). Healthy control mice receiving icv injection of vehicle exhibited basal scores of episodic memory. Comparatively, the icv injection of $A\beta_{1-42}$ resulted in a significant decrease of the memory (black bar, $p = 0.008$). Treatment by PA1637 significantly improved the memory score of mice having received $A\beta_{42-1}$ (grey bar, $p = 0.003$). P values are given for the Fisher's LSD test. ** stands for p values in the range 0.01–0.001.

4.2. The Tetradentate Monoquinoline Series as Regulators of Copper Homeostasis

To enhance the efficiency and druggability of copper chelators, we designed a new series of tetradentate amino (TDMQ) copper ligands, based on a mono(8-amino)quinoline motif and a coordinating side chain, able to offer a N_4-tetradentate square planar coordination site for Cu(II) complexes, similar to that of bis(8-amino)quinolines (Figure 7) [130, 131]. Structural modulation of the polyamine chain allowed to tune the geometry of their coordination site and, consequently, their copper selectivity and their ability to inhibit oxidative stress. One of these ligands, TDMQ20 (Figure 7), is specific for copper compared to zinc, with 11–12 log units difference between its affinities for Cu(II) and Zn(II), thus supporting the capacity of this series to extract the copper ions linked to amyloids without disturbing homeostasis of zinc in the brain. *In vitro*, extraction of copper from Cu-Aβ, and complete inhibition of the ROS production triggered by Cu-Aβ associated with ascorbate, were fully achieved by TDMQ20, as well as the release of copper from Cu-TDMQ20 in the presence of glutathione. These features are clearly related to the association of a 8-aminoquinoline scaffold bearing a side chain suitable to chelate Cu(II) in a 1/1 square planar complex. In this TDMQ series, the most appropriate side chain meeting this requirement was the chain having n = m = 2 (2 + 2 methylene groups). This result supports the development of such copper chelators, which are very different from the former 8-hydroxyquinoline derivatives clioquinol and PBT2, in terms of coordination chemistry behavior [127]. The pharmacology of these TDMQ ligands is currently under investigation.

4.3. Rationale of the Copper Chelator Design: N_4-Tetradentate Ligands

The copper specific N_4-tetradendate ligands TDMQ20 and bis(8-amino)quinolines are able to transfer copper from Cu-Aβ to glutathione, and to fully inhibit *in vitro* the aerobic oxidation of ascorbate induced by Cu-Aβ, while other ligands with N_2O_2 or N_3O_2 coordination spheres failed to do so. In fact, the N_4-scaffold of these two aminoquinoline-based ligands, suitable for a square-planar coordination of copper(II), allowed them to enhance both the selectivity for Cu(II) and also the ability to reduce the oxidative stress induced by copper-amyloids.

The design of copper ligands able to counteract metal deregulation and deleterious consequences in AD brain requires a good understanding of their coordination properties. In this respect, the results obtained with 8-aminoquinoline chelators pave the way to the development of efficient regulators of copper homeostasis to inhibit and/or reverse the loss of memory in the early stages of human AD, when small cognitive impairments are detectable, but amyloid plaques and irreversible brain damage are not yet established.

5. GENERAL CONCLUSIONS

In the future, medicinal chemistry will continue to provide a large number of new, efficient, and low cost drugs to treat all types of different diseases. It will be a mistake to consider that biopharmaceuticals will provide an answer for all therapeutic areas. The success of antibodies in cancer therapy should not blind us and lead us to forget that many drug targets are not accessible for large biomolecules. Medicinal chemistry will continue to have a bright future in drug discovery.

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ABBREVIATIONS AND DEFINITIONS

Aβ	amyloid β
ACT	artemisinin-based combination therapy
AD	Alzheimer's disease
APP	amyloid precursor protein
ART	artemisinin or semisynthetic derivatives of artemisinin
BBB	blood-brain barrier
CQ	clioquinol
FDA	Food and Drug Administration
GSH	glutathione
HTS	high throughput screening
NMR	nuclear magnetic resonance
Icv	intracerebroventricular
PZQ	praziquantel
ROS	reactive oxygen species
TDMQ	tetradentate monoquinoline
WHO	World Health Organization

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3

Iron Chelation for Iron Overload in Thalassemia

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Abstract: This chapter is devoted to the chelation treatment of transfusion-dependent thalassemia patients. After a brief overview on the pathophysiology of iron overload and on the methods to quantify it in different organs, the chelation therapy is discussed, giving particular attention to the chemical and biomedical requisites. The main tasks of an iron chelator should be the scavenging of excess iron, allowing an equilibrium between iron supplied by transfusions and that removed with chelation, and protection of the individual from the poisonous effects of circulating iron. The chelating agents in clinical use are presented, illustrating the main chemical and pharmacological features, together with a comparative cost analysis of their treatments. As a final section, an overview is provided on chelators undergoing clinical trials, and on research in progress.

Keywords: chelation therapy · deferasirox · deferiprone · desferal · iron · iron chelating agents · β-thalassemia

1. INTRODUCTION

Iron chelation is a necessary clinical treatment for patients suffering from β-thalassemia to protect them from the toxicity of iron overload that occurs due to periodic blood transfusions. β-thalassemias are a group of hereditary blood disorders characterized by anomalies in the synthesis of β-chains of hemoglobin. The total annual incidence is estimated at 1 in 100,000 throughout the world and 1 in 10,000 people in the European Union, with the highest frequency in Cyprus (14 %) and Sardinia (10.3 %). However, population migrations and interethnic marriages have introduced thalassemia in almost every country of the world [1]. Individuals with thalassemia major usually present within the first two years of life with severe anemia, and require regular red blood transfusions for their survival. These transfusions lead to a number of complications related to iron

overload that, if untreated, account for most of the morbidity and mortality associated with this pathology [2, 3]. As humans have no effective mechanism for removing iron, the only way to remove an excess is with the use of iron chelating agents, which allow iron excretion through the urine and/or stool.

The first advances in iron chelation entered into clinical practice in the seventies of the last century [4–7]. Nowadays, with the availability of innovative techniques to measure the iron content in different organs and tissues [8] (such as magnetic resonance imaging [9] or magnetic susceptibility [10]) extraordinary advances have been made in iron chelation therapy (ICT), leading to a focused therapeutic approach that can be adapted to the requirements of each individual patient [11]. Thanks to the combined research efforts of clinicians, chemists, biologists, and biochemists, a second advancement was possible with the introduction of the two new oral chelators, deferiprone (DFP) and deferasirox (DFX). Prior to their development, desferrioxamine (DFO) was the only available drug to treat transfusional siderosis, and despite all its limits and the non-optimal compliance, it radically changed both the life expectancy and quality of life of thalassemia patients.

In this chapter, after a brief description of the pathophysiology of iron overload, we will present:

1. The available methods of evaluating and quantifying transfusional iron overload.
2. The iron chelation therapy, pointing out the main chemical and biomedical requirements of chelating agents.
3. A detailed presentation of the three currently approved chelating agents, focusing their chemical and clinical-pharmacological profile. Specific attention will be paid to the role of combination therapy and to the economic aspects of therapy.

This chapter also includes an overview on the present research on iron chelators, and on the chelators currently in trials.

2. OVERVIEW ON THALASSEMIAS

2.1. Pathophysiology of Iron Overload

Anomalies of β-thalassemias result in variable phenotypes ranging from severe anemia to clinically asymptomatic individuals. An excessive production of free α-globin chains causes ineffective erythropoiesis that results in profound anemia with associated clinical problems, such as erythroid marrow expansion, extramedullary hematopoiesis, splenomegaly, and increased gastrointestinal iron absorption. The most salient and ultimately fatal complication of this disease is the progressive iron overload in various organs, especially in the myocardium [12]. Three main forms, thalassemia major, thalassemia intermedia, and thalassemia

minor have been described. Individuals with thalassemia major, which usually appears within the first two years of life with severe anemia, require regular red blood cell transfusions for their survival. Untreated individuals present growth retardation, pallor, jaundice, poor musculature, hepatosplenomegaly, leg ulcers, development of masses from extramedullary hematopoiesis, and skeletal changes that result from expansion of the bone marrow. In β-thalassemia major, the repeated blood transfusions are the major cause of iron overload that, if untreated, account for most of the morbidity and mortality. Regular transfusions lead to a number of iron overload-related complications. Consequently, iron chelation and management of secondary complications of iron overload are necessary. The prognosis for patients affected by β-thalassemia has substantially improved in the last 20–30 years, as a result of advances in transfusion and in iron chelation. Nevertheless, cardiac complications remain the main cause of death in patients with iron overload.

Several different transfusional regimens have been proposed, but the most widely used aims are to achieve a pre-transfusional hemoglobin level of 9–10 g/dL and a post-transfusion level of 13–14 g/dL. In this way growth impairment, organ damage, and bone deformities are prevented, and a normal activity and quality of life can be reached [13]. The frequency of transfusion is typically every two to four weeks. The amount of blood to be transfused depends on several issues, the main ones being the weight of patient, and the target increases in hemoglobin level and hematocrit. Patients who submit to a regular transfusion regimen gradually develop the clinical manifestations of iron overload: hypogonadism (35–55 % of the patients), hypothyroidism (9–11 %), hypoparathyroidism (4 %), diabetes (6–10 %), liver fibrosis, and heart dysfunction (33 %) [1, 14, 15]. Iron status should be accurately and periodically monitored for evaluating its clinical relevance, the necessity of treatments, and the progression of chelation therapy [16–18].

The total body iron content in normal human adults is about 40–50 mg/kg body weight, corresponding to 2.8–3.5 g in a 70 kg man (male: weight 70 kg, total blood 5200 mL/5500 g, total plasma 3000 mL/3100 g; female: weight 58 kg, total blood 3900 mL/4100 g, total plasma 2500 mL/2600 g) [19]. About 80 % of the total iron content is found in hemoglobin in circulating red blood cells and in myoglobin in the muscles; the remaining 20 % are distributed between storage proteins, ferritin, and hemosiderin, a small amount in various iron-containing enzymes, and about 3–4 mg circulate in the plasma bound to transferrin. Iron in plasma is turned over about 10 times a day. Iron metabolism in the human body is essentially conservative: The average absorption is 1–3 mg/day, and almost the same amount is excreted by cell desquamation from gut and skin, and in women of fertile age, through menstruation or pregnancy.

In patients who do not receive transfusions, abnormal iron absorption produces an increase in the body iron burden estimated to be 2–5 g per year [4]; regular blood transfusions double this iron accumulation. The annual iron load from blood transfusions can be estimated from the number of red cell units given [20]. A unit (420 mL of donor blood) contains about 200 mg of iron. Excess iron is stored in hepatocytes and macrophages, utilizing an active cycle of iron use and recycling. Iron accumulation introduces progressive damage in liver, heart,

and in the endocrine system if a chelating therapy is not introduced. As the iron load increases, the iron-binding capacity of serum transferrin is exceeded and a non-transferrin-bound fraction of plasma iron (NTBI) appears which generates free hydroxyl radicals and induces dangerous tissue damage [21]. Iron accumulates at different rates in various organs, each of which reacts in characteristic ways to the damage induced by NTBI and by the intracellular labile iron pool (LIP). Based on these observations, chelators that remove iron from specific target organs would be desirable, above all in the heart, cardiac disease being the life-limiting consequence of iron overload. Chelators can act on different iron pools: (i) serum iron bound to transferrin; (ii) iron in the form of NTBI; (iii) when the transferrin is saturated, the iron stored in ferritin and in hemosiderin; (iv) the labile iron pool that is found in the cytoplasm.

Iron chelators should reduce tissue iron levels, allowing efficient transport and excretion without iron redistribution, prevent excessive organ iron accumulation and neutralize toxic labile iron pools. Absorption from the gastrointestinal tract and cell penetration, both of which depend on diffusion through the biological membranes, are governed by molecular size, lipophilicity, and net molecular charge, as will be discussed in Section 3.2. Iron status should be accurately assessed in order to evaluate its clinical relevance, the need for treatment, and the timing and monitoring of chelation therapy [22, 23].

2.2. Methods for Quantifying Iron Overload

The main aim of chelation therapy in transfusion-dependent thalassemia patients is to keep body iron content in the organism within safe limits. It is possible to calculate the annual iron intake from transfusions, which largely exceed dietary contributions, directly from the volume of transfusions. A correct chelation therapy should lead to the excretion of an equivalent or greater amount of iron in order to reach a negative or equilibrium balance between iron intake and iron removal. In this respect, the kind and the amount of administered chelating agent should be tailored to the requirements of the patient, requiring a periodic control of his own iron status. A variety of techniques are used for estimating iron overload, spanning from the measurement of serum ferritin levels (SF), to the determination of liver iron concentration (LIC), to the use of the "superconducting quantum interference device" (SQUID) and of magnetic resonance imaging (MRI). The determination of SF and LIC still maintain important roles in many situations and in many countries, despite the increasing availability of instrumental resources for performing MRIs. The pros and cons of these techniques will be briefly outlined to better understand their use in the following. For a detailed presentation of these methods we refer to the recent Guidelines of Wood [24], and to the Guidelines for the Clinical Management of Thalassaemia [25].

Determination of serum ferritin is surely the simplest and least expensive method for quantifying iron overload. SF values usually provide a reliable estimate of total iron stores in the body and are correlated with clinical observations

[26]. In particular, ferritin levels greater than 2,500 ng/mL are associated with an increased risk of morbidity and mortality and should trigger intensification of chelation therapy [27]. According to Wood [24], serum ferritin is best checked frequently (every 3–6 weeks) so that running averages can be calculated; this corrects for many of transient fluctuations related to inflammation and liver damages. The same author recommends to anchor the trend of serum ferritin, which provides a rapid indication of patient iron status, to gold standard assessments of iron burden. Further, a significantly lower risk of cardiac disease and death in at least two-thirds of cases is identified where serum ferritin levels have been maintained below 2,500 μg/L (with desferrioxamine) over a period of a decade [28]. Additional advantages are shown to be associated with an even lower serum ferritin of 1,000 μg/L [29]. It has to be noticed that the prediction of iron overload by SF can be unreliable, due to its fluctuations in response to inflammatory stimuli, hepatotoxic processes, and vitamin C deficiency.

Since the liver is the main storage organ for the iron excess, liver iron concentration is considered the reference standard for evaluating total body iron stores [23]. LIC is determined by chemical measurement of iron content obtained by a needle biopsy of the liver. Normal LIC values are up to 1.8 mg/g dry weight. High LIC values (15–20 mg/g dry weight) are normally associated with increases of the chelatable iron pool [30], labile plasma iron levels, liver fibrosis, cardiac iron deposition [31], and mortality [32]. Annual LIC determination was proposed as a standard procedure for the control of iron chelation therapy, since it provides a quantitative and specific measurement of non-heme storage iron, information on liver histology/pathology, and presents a positive correlation with morbidity and mortality. However, significant cardiac iron burden can be present (initially asymptomatically) despite the presence of a low LIC. In addition, LIC suffers from poor patient acceptance and unreliability of results (for a multiplicity of causes), all of which have resulted in this procedure becoming outdated, with a current preference towards the use of non-invasive instrumental techniques.

The urinary iron excretion, measured by the chemical determination of iron content in the daily excreted urine, permits the evaluation of the effect of iron chelating agents (with DFO about half of chelated iron is excreted in urine, with DFP over 80 %). Repeated determinations are necessary to counteract the variability in daily iron excretion.

Measurement of NTBI has proved a useful instrument for examining how chelators interact with plasma iron pools. Liver susceptometry biomagnetic technique uses a superconducting quantum interference device to measure the magnetic susceptibility of the liver, which is strongly related to its total iron content [33, 34]. This technique is generally accurate and reproducible, but the current limited availability of the necessary devices to only five centers in the world strongly limits the application of this technique in clinical practice.

At present, MRI can be considered the standard technique for non-invasive quantification of liver iron content. It permits evaluation of both iron distribution and the mean iron concentration. MRI is based on the effects that paramagnetic Fe^{3+} ions, bound by different endogenous ligands (proteins) in the tissues, exert on the signals of water protons, altering their NMR relaxation times, a

fundamental parameter of the ^{1}H NMR signal, in a way proportional to iron concentration. The technological progress in this field, instrumental and data treatment, has permitted the introduction and the rapid development of different MRI methods, above all the spin echo (SE) and the gradient recalled echo (GRE). Without entering into the details of these methods, widely discussed in the papers by Wood and Ghugre [35], Fischer and Harmatz [9], and Wood [24], and references therein, we report a bird's eye view of the fundamental parameters related to these last two techniques, and of the kind of results that each allows one to reach. The relaxation phenomena are described by using both the relaxation time, reported as T2 when the spin echo technique is used, or as T2* when the gradient echo is used, and the relaxation rates, expressed as R2 = 1000/T2 or R2* = 1000/T2*. The factor 1000 depends on the fact that the relaxation times are generally expressed in ms, while the relaxation rates are expressed in Hertz (s^{-1}). R2 and R2* are directly rather than inversely proportional to iron. Historically, R2 is used in spin echo measurements and T2* in gradient echo, similarly to what happens in spectroscopy where wavelengths are used in UV-vis and frequency in IR spectroscopy. MRI results in the liver are typically reported as R2 and R2* values [36, 37] and LIC units, whereas the use of T2 and T2* is more common in the heart [38, 39].

Gradient echo methods are easier and faster, and the latest generation instruments allow clinicians to gain all the R2* information in a single breath-hold, although they are prone to distortion makers such as air-tissue boundaries or metal implants.

Since the removal of iron from different organs by chelation takes place at different rates, generally hepatic iron load improves more quickly than cardiac iron load. Therefore, both hepatic and cardiac iron should be measured to optimize chelation therapy [40]. MRI of the heart offers valuable practical information of myocardial iron through cardiac T2*, the optimal range being above 20 ms. T2* values lower than 10 ms are indicative of high risk of rhythm disturbances and of contractile dysfunction [41]. Systematic monitoring of the left ventricular ejection fraction permits to identify patients with poor prognosis at high risk of heart failure and death [33].

3. IRON CHELATION THERAPY

3.1 Aims of Iron Chelation Therapy

The chelation therapy of thalassemia patients has three main goals: reaching an equilibrium between the amount of iron acquired via blood transfusions and that excreted through chelation; protecting patients from the adverse effects of the circulating NTBI (mainly from the production of reactive oxygen species (ROS) via Fenton reaction); and scavenging iron previously stored in different organs.

In the following sections, the main requirements of an iron chelator will be outlined as chemical and biomedical requirements, even if this subdivision appears to some extent artificial and forced because of the strong interplay be-

tween these definitions. Nonetheless, these requirements will be the reference points for the evaluation of the degree at which the single drugs adhere to them in the exploitation of their chelating action.

3.2. Chemical Requirements of an Iron Chelator

The chemical requirements that an iron chelator must reach have been better defined over the years, as sketched in the following.

(i) The stability of the formed complexes with iron must be higher than with endogenous ligands. This stability is a necessary requisite so that the metal ion can be completely transformed into a chelated species that can be excreted. In addition to the stability constants, other factors including solubility of the formed complex, stoichiometry of the complex, and proton competition contribute to the binding efficiency of a ligand for a given metal ion. The methods for quantifying the ligand effectiveness toward a target metal ion have been recently reviewed by Bazzicalupi et al. [42]. In the present chapter, we will make use of the pM parameter, proposed in 1981 by the group of Raymond [43] as $-\log[M_f]$ at $[M_T] = 1\times10^{-6}$ M and $[L_T] = 1\times10^{-5}$ M at pH 7.4, where $[M_f]$ is the concentration of free metal ion and $[M_T]$ and $[L_T]$ are the total concentrations of metal and ligand, respectively. The higher the stability of the formed complex the less metal ion remains un-chelated in solution (free metal ion), determining a higher pM value.

(ii) The kinetic exchange of iron between endogenous ligands and chelator must be fast.

(iii) The chelator must be selective for iron. For a full understanding of the selectivity of an iron chelator, it is important to evaluate both the influence of various essential metal ions, such as Cu^{2+} and Zn^{2+}, on iron complexation, and the perturbations induced by the chelator on the homeostatic equilibria of the essential metal ions. These two effects depend on the thermodynamic and kinetic properties of the interaction between the ligand and the involved metal ions. The interactions of chelators with metal ions other than iron can be crucial for the drug bioavailability and for possible side effects. Furthermore, any disturbance of the homeostasis of essential metal ions induced by chelating agents can have serious health consequences: the depletion of essential metal ions during iron chelation therapy is widely reported [20, 44–50].

(iv) The redox potential of complexes must be appropriate. The aptitude of an iron complex to catalyze the Fenton reaction depends on its redox potential, which should be in the proper range to satisfy two constraints: The first is that the Fe^{3+} complex must be reducible by the reductants in physiological environment (-0.16 $V_{/NHE}$ $O_2/O_2^{\cdot-}$, -0.28 $V_{/NHE}$ ascorbate/ascorbyl, -0.324 $V_{/NHE}$ $NADP^+/NADPH$). The second requires that the redox potential of the Fe^{3+} complex must be smaller than $+0.46$ $V_{/NHE}$ ($H_2O_2/OH^{\cdot}$, $OH^{\cdot}/OH^-$), for the electron transfer from the Fe^{2+} complex

to H_2O_2 to be possible. To prevent iron participation in a catalytic cycle, with production of dangerous ROS, its redox potential must be controlled by proper chelation. In this way, the redox potential of iron can be removed from the region in which it undergoes redox cycling.

(v) The chelator should not be transformed, once in the organism, into non-chelating metabolites. The chelating agent generally reaches its maximum concentration in plasma in a time determined by the kind of delivery, by the absorption, and by other minor factors. It then disappears from plasma because of metabolism, excretion, and transfer to the tissues; which of these predominates varies according to the chelating agent. Many chelating agents are metabolized in the body to species that lose the chelating properties of the parent molecule. These reactions can be very different, as the glucuronidation of hydroxypyridinones, the acetylation of triethylenetetramine (trien), or the formation of –S–S bonds between 2,3-dimercaptopropan-1-ol (BAL) and SH-containing ligands. The correct choice of drug administration becomes of vital importance when this kind of metabolic transformation is rapid as, for example, the subcutaneous infusion of desferal. All these processes, by reducing the amount of chelating agent in plasma, reduce the efficacy of its action. Therefore, when comparing the efficacy of two chelating agents, in addition to the thermodynamic properties, the kinetic behavior must also be considered.

3.3. Biomedical Requirements of an Iron Chelator

The main biomedical requisites of an iron-chelating agent should include:

(i) Low toxicity of both the chelating agent and the formed complexes.

(ii) Good intestinal absorption and good bioavailability of the chelator in entering the target cells. The chemical requisites regarding the absorption and the bioavailability have been pointed out by Ma et al. [5]. Three key parameters regulate diffusion through biological membranes: molecular size, lipophilicity, and net charge [51]. Specifically, the cut-off molecular weight for drug absorption in human intestine is ~500 g/mol. Lipophilicity is generally estimated by the water–octanol partition coefficient (P). These general properties are imbedded in Lipinski's rule of five (RO5) [52], adopting a four parameter analysis characterized by the number 5. Their guidelines state that a poor absorption is likely when:

- molecular weight > 500 g/mol
- log P > 5
- more than 10 hydrogen bond donors are present in the molecule (expressed as a sum of OH and NH groups)
- more than 10 hydrogen bond acceptors are present in the molecule (expressed as a sum of O and N atoms).

The same parameters also determine the ability of the chelating agent to enter a cell, and the excretion of the formed metal complex. In this latter case, the formation of a neutral complex is of paramount importance. In the case of Fe^{3+} complexes, it is more appropriate to use a chelating agent bearing coordinating groups such as ─CO─COH or ─CO─NOH (like in hydroxypyridinones or in hydroxamates) rather than ─COH─COH or COH─COOH (as in catechol or in salicylate), since the first ones lead to easily extractable uncharged complexes, and the second ones to negatively charged complexes.

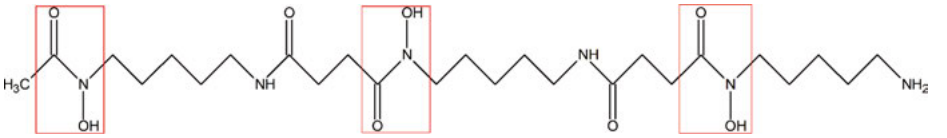
To conclude, the patient must comply with the mode of administration, and the chelating agents must have a low cost to allow clinical access to the largest number of patients.

4. CHELATORS IN USE

4.1. Desferal

Desferal, the mesylate salt of desferrioxamine B (DFO), is a drug approved in the US, Canada, Europe, and other countries for the treatment of transfusional iron overload. For about 30 years, it was the only available drug. In spite of all its limitations, including non-optimal patient compliance, it has led to extraordinary progresses in the quality of life and overall survival of patients with transfusion-dependent blood disorders [28]. According to Bernhardt [53] "*β-thalassemia patients now in their 50s who have undergone DFO chelation therapy since childhood are living proof of the value of this drug. Those afflicted with this disease prior to the emergence of DFO, or who have been unable to cope with the demands or cost of DFO therapy, have typically died in their teens*". DFO is included in the Model List of Essential Medicines by the World Health Organization (20th ed., March 2017, amended August 2017) in the section "Antidotes used in poisoning" [54].

Some historical notes can be useful to focus the discovery of desferrioxamine, and the early chemical and clinical studies. "*Its discovery was a fruitful chance occurrence. The story of its discovery is most unusual because the failure of research in one direction led to success in another*" [55]. Desferrioxamine is derived from ferrioxamine B (FOB), an iron-bearing metabolite produced by *Streptomyces pilosus*. Seven ferrioxamines (A–G) were discovered in 1959 as impurities in the course of research by Ciba on the iron-containing antibiotics, ferrimycines. Ferrioxamines are iron-containing antibiotic antagonists. Although the Ciba project was discontinued at the end of 1959, Bickel et al. produced a few grams of these antagonists, including FOB, in May 1960 [56]. Prelog and his co-workers soon elucidated the structure of FOB [57]. FOB was thought to be a potential iron donor for patients with iron-deficiency anemia, so it was passed to Prof. Wohler, at the University of Freiburg (Germany), for clinical trials. After animal toxicity studies, it was delivered to patients, who excreted it unchanged and in the same quantity in urine, without passing iron to the body. This behavior permitted



Scheme 1. Molecular formula of desferal, with highlighted iron chelating hydroxamic groups.

Wohler to realize that the iron in FOB is bound with exceptional strength, suggesting that if iron-free FOB could be produced, it should be capable of removing iron accumulated in the body in different pathologies. Therefore, in the same year Bickel obtained desferrioxamine [58]. Before testing DFO on animals, its protein targets were determined, in order to assess if it could remove iron from hemoglobin. As it was found to remove iron only from ferritin and hemosiderin, and not from hemoglobin and transferrin, DFO passed to clinical studies. After favorable results from animal studies, in mid-1961 Wohler treated the first patient with severe hemochromatosis and was able to achieve massive iron excretion in urine. In the following year, 22 patients were treated with good results [59]. DFO was registered in Switzerland in June 1963, only 2.5 years after its first production, and introduced on the market [60]. In speaking with one of the authors of these first clinical studies, he explained to us that the lack of rigid constraints in the 1960s in clinical trials permitted such a rapid approval of DFO for the treatment of transfusion-dependent thalassemia patients.

4.1.1. Chemistry

Desferal is a trihydroxamic acid, $C_{25}H_{48}N_6O_8$ of molecular weight 560.68 g/mol, whose solubility at 20 °C in water is greater than 20 % [61]. It is characterized by the four protonation constants log K_1 = 10.84, log K_2 = 9.46, log K_3 = 9.00, and log K_4 = 8.30, the first attributed to the terminal amino group, the other ones to the hydroxamic groups. At pH 7.4 it is mainly in the fully protonated, positively charged LH_4^+ form, and for about 10 % in the neutral form LH_3, as can be seen from the distribution curves presented in Figure 1. DFO forms with Fe^{3+} two differently protonated 1:1 complexes, $[FeLH]^+$ and FeL, characterized by complex formation constants log β_{111} 41.01 and log β_{110} 30.4 [62]. The speciation plot in Figure 2 shows that the protonated complex is completely formed at pH 2, and loses the proton on the terminal amino group with pK 10.61, very close to the value of 10.84 in the free ligand. The protonation and complex formation constants allow estimating a p[Fe] value of 26.5.

The crystal structure of DFO has never been determined. The structure of the complex formed between DFO and iron, reported by the group of Crumbliss in 2001 [63] is presented in Figure 3.

The structure of the complex is described by two closed loops, and an open chain with the protonated amine, which points out from the chelate rings. It was

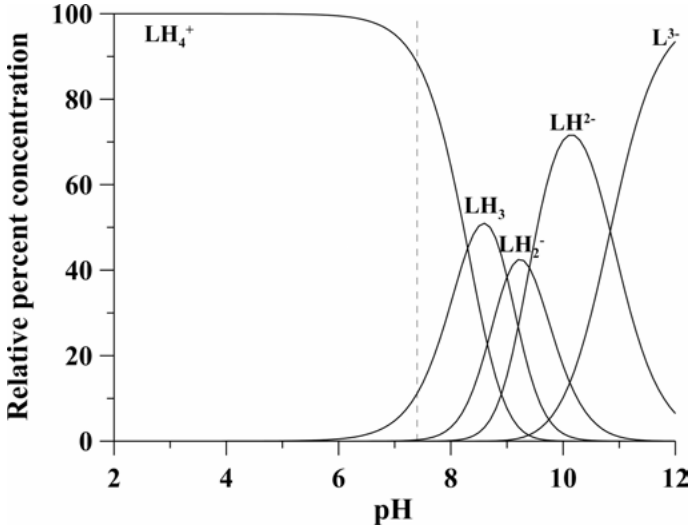


Figure 1. Distribution curves of the various protonated forms of desferal.

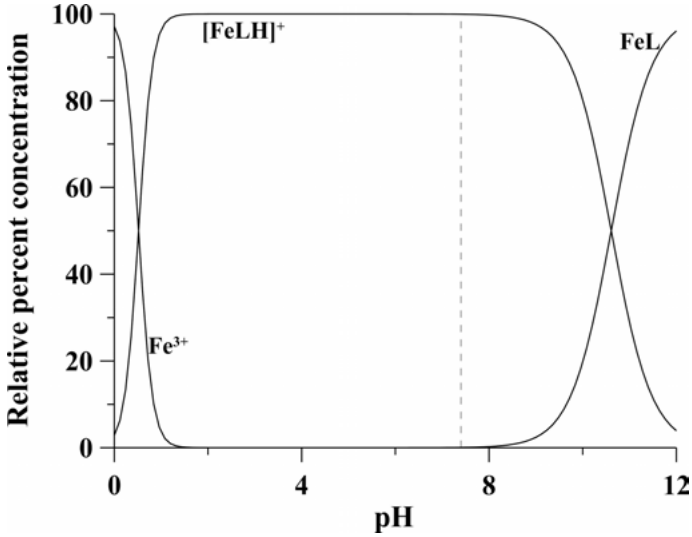


Figure 2. Distribution curves of the different Fe^{3+}-desferal complexes, calculated with the Fe^{3+} concentration of 1 μM and the DFO concentration of 10 μM.

proposed that this pendant protonated amine could play an important role in the recognition and membrane transport processes.

The three 5-membered chelate rings are very similar, with mean distances N–O 1.376(5) Å, C–O 1.284(8) Å, Fe–O_{oxime} 1.979(7) Å and Fe–$O_{carbonyl}$ 2.036(7) Å. The shorter Fe–O distance for the oxime oxygen depends on the greater negative

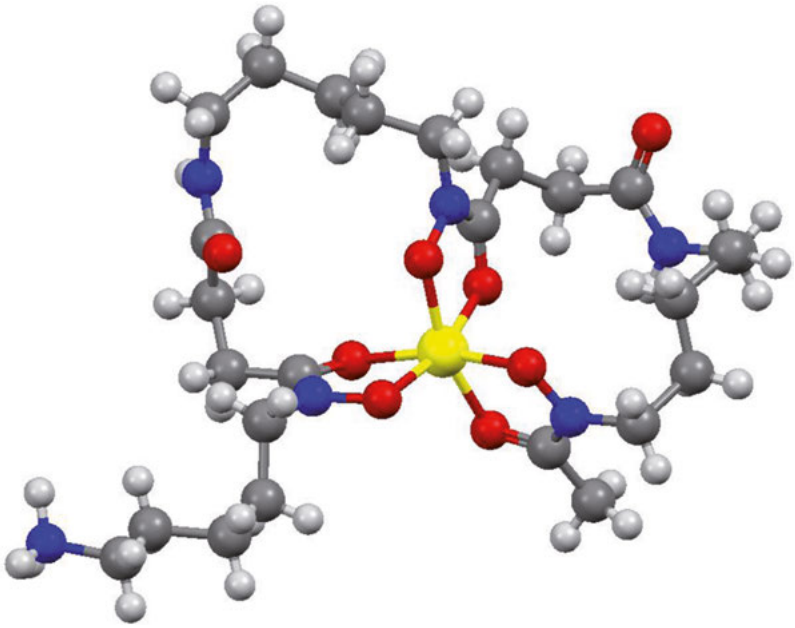


Figure 3. Crystal structure of the Fe^{3+}-desferal complex [63], (H: white, O: red, N: blue, C: grey, Fe: yellow). Coordinates obtained from the Cambridge Structural Database, CSD entry code OFUYET, images created with Mercury 3.5.

charge on this oxygen atom with respect to the carbonyl oxygen atoms. This uneven charge distribution provokes a small deviation from a perfect octahedral configuration around the iron atom [63].

The value of the octanol/water partition coefficient (log $P = 0.614$) determines the high hydrophilic character of DFO. The compact structure and the high stability lead to the extremely low redox potential of the Fe^{3+}-DFO complex, -475 mV at pH 7.5 [64], inaccessible to biological reductants [6]. DFO complexation completely protects the coordinated Fe^{3+} cation from reduction, avoiding ROS formation via Fenton reaction.

4.1.2. Formulation, Doses, and Administration

DFO is indicated for chronic iron overload due to transfusion-dependent anemias. It is furnished in two different dosage forms, 500 mg vials or 2 g vials containing the sterile lyophilized powder of desferrioxamine mesylate without non-medicinal ingredients.

The chemical features of DFO do not allow its oral absorption, so it must be administered by intravenous (recommended in cases of acute iron intoxication) or subcutaneous infusions in patients with chronic iron overload. Having a short plasma half-life of 20–30 minutes, DFO should be administered over an extent of 8–10 hours a day, on 5–7 days a week [65].

The daily dose ranges between 20 and 60 mg/kg/day, depending upon the severity of the iron load. The total daily dose should not exceed 6.0 g.

4.1.3. Pharmacodynamics and Pharmacokinetics

Following parenteral administration of DFO, tissue iron is mobilized resulting in a sharp rise in serum iron concentration. DFO chelates Fe^{3+}, either free or bound in ferritin and hemosiderin, forming the extremely stable complex ferrioxamine. Since ferrioxamine is completely excreted, DFO promotes iron excretion in the urine and feces, so reducing pathological iron deposits in the organs. The affinity for divalent metal ions such as copper, zinc, and calcium being substantially lower, DFO treatment has little influence on the excretion of these essential trace elements.

DFO is poorly absorbed orally, while it is well absorbed following intramuscular or subcutaneous routes of administration. The binding to serum protein is less than 10 %. It is distributed throughout all body fluids, and is excreted by the kidneys via glomerular filtration and tubular secretion. Different metabolites have been isolated and identified from the urine of patients being treated for iron overload. These metabolites derive from transamination and oxidation reactions yielding an acid metabolite, from beta-oxidation yielding an acid metabolite, and from decarboxylation and N-hydroxylation yielding natural metabolites.

Plasma concentrations ranging from 80 and 130 μmol/L can be measured 3 minutes following an intravenous injection of DFO (10 mg/kg) in healthy subjects and in patients with transfusion-dependent iron overload. These concentrations halve within 5–10 minutes, and then decline more slowly. This rapid fall in concentrations is due in part to distribution and excretion of the active substance, and in part to the formation of the iron complex ferrioxamine (which starts within a few minutes, and depends on the iron grade of each individual) and to metabolic transformations.

During continuous intravenous infusion of DFO (100 mg/kg in 24 mL sterile water, at a rate of 1 mL per hour), plasma concentrations of DFO and ferrioxamine in healthy subjects increase, reaching a plateau after 6 h or, more frequently, after 12 h, always depending on the individual iron status, reaching maximum concentrations of 20 μmol/L for DFO and 2.75 μmol/L for ferrioxamine. The corresponding concentrations in patients are 8.3 μmol/L for DFO and 12.9 μmol/L for ferrioxamine. The 48-hour urinary excretion averages 118 μmol in healthy subjects and 836 μmol in patients.

4.1.4. Mechanisms of Action

DFO is an iron-chelating agent that binds free iron in a stable complex, preventing it from engaging in chemical reactions. Desferrioxamine chelates iron from intra-lysosomal ferritin and siderin forming ferrioxamine, the water-soluble chelate excreted by the kidneys and in the feces via the bile. DFO does not readily bind iron from transferrin, hemoglobin, myoglobin or cytochrome.

4.1.5. Adverse Effects

The main adverse effects of DFO consist of growth delay (it is recommended to monitor pediatric patients by measuring body weight and growth every 3 months), skeletal alterations, ocular and auditory disturbances (vision and auditory function should be assessed every year), respiratory distress syndrome that can take place during the treatment of acute iron intoxication, and in irritation at the site of the infusion.

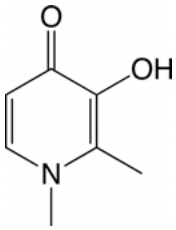
Since desferrioxamine given orally is poorly absorbed, to be effective it must be administered by intravenous infusion with a small portable syringe pump, ideally for 9–12 h each day. This administration route easily explains why only part of thalassemia patients comply with DFO chelation therapy. This limited compliance, which decreases the efficacy of iron chelation, can be considered one of the most influent limitations of DFO treatment.

4.2. Deferiprone

Deferiprone (Ferriprox®; Apotex Inc., Toronto, Canada) or DFP was the first orally available iron chelating agent. It was synthesized and characterized at Essex University in 1981 [66], and its biological characteristics were assessed at the University College Hospital in London [67]. DFP was patented for the treatment of iron overload in 1982 [68] and authorized for clinical use in India in 1995 and in European, South American and Asian countries in 1999. The US Food and Drug Administration (FDA) approved DFP in the US in 2011, limiting it to 'the treatment of patients with transfusional iron overload due to thalassemia syndromes when current chelation therapy is inadequate' [69]. Now DFP is available in more than 50 countries.

4.2.1. Chemistry

Deferiprone (Scheme 2), 3-hydroxy-1,2-dimethylpiridin-4(1H)-one, $C_7H_9N_2O_2$, MW 139.152 g/mol, is characterized by a log *P* 0.61 and by a water solubility 0.1075 M (14.95 g/L) at 25 °C and 0.1 M ionic strength, and 0.15015 M (20.94 g/L) at 37 °C and the same ionic strength [70]. Two dissociation constants, pK_1 3.66



Scheme 2. Molecular formula of deferiprone.

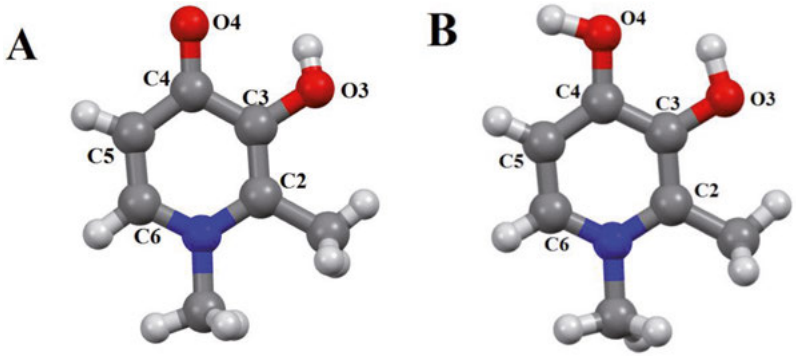


Figure 4. Crystal structures of deferiprone (**A**) and DFP·HBr (**B**) [72], (H: white, O: red; N: blue, C: grey). Coordinates obtained from the Cambridge Structural Database, CSD entry codes GALDEC01 (A) and SIHJIC (B), images created with Mercury3.5.

and pK_2 9.82 can be calculated [71], the first relative to the dissociation of the positively charged H_2L^+ form, protonated on the carbonyl group, and the second to the dissociation of the 3-hydroxyl group.

The structures of DFP and DFP HBr [72] reported by Hider (and shown in Figure 4) show that a further proton in the H_2L^+ form is located on the carbonyl oxygen atom O4. This leads to a lengthening of the C4–O4 bond from 1.271(1) to 1.329(5) Å, with the contemporary decrease of the distances C3–O3 (from 1.364(1) to 1.343(6) Å), C3–C4 (from 1.438(2) to 1.384(7) Å), and C4–C5 (from 1.407(2) to 1.391(6) Å).

The speciation plot in Figure 5 shows that, at physiological pH, DFP exists in the neutral form LH. Thanks to the molecular weight, the lack of charge, and the log *P* value of 0.61, DFP is intestinally adsorbed, and penetrates most cell membranes.

DFP forms 5-membered chelate rings with iron, where the metal ion is coordinated by the oxygen atoms of the carbonyl and of the hydroxylate groups [73]. DFP forms with Fe^{3+} the three complexes $[FeL]^{2+}$, $[FeL_2]^+$ and FeL_3, characterized by the formation constants log β_{110} 15.01, log β_{120} 27.30 and log β_{130} 37.43. The resulting p[Fe] value of 20.70 explains the good chelating properties of this molecule [71]. The related speciation plots (Figure 6) give evidence that, in the ratio of DFP/Fe^{3+} 3:1 or more, the only species that exists at pH 7.4 is the neutral complex FeL_3.

The redox potential of FeL_3, variable from −535 to −620 mV depending on the experimental conditions [74], renders it inaccessible to biological reductants. On the contrary, the $[FeL_2]^+$ complex formed at high Fe^{3+}/DFP ratios, cannot safeguard the coordinated Fe^{3+} ion from reduction, allowing the dangerous formation of ROS via Fenton reaction.

Iron is coordinated in the FeL_3 complex by the two oxygen atoms of the three DFP molecules in an almost perfect octahedral coordination $Fe^{3+}O_6$, as can be observed in the crystal structure presented in Figure 7.

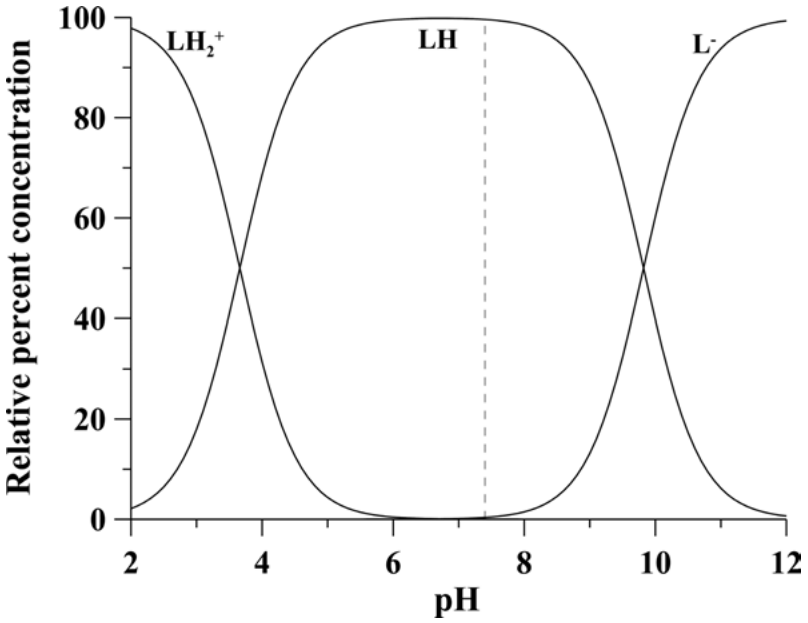


Figure 5. Distribution curves of the various protonated forms of deferiprone.

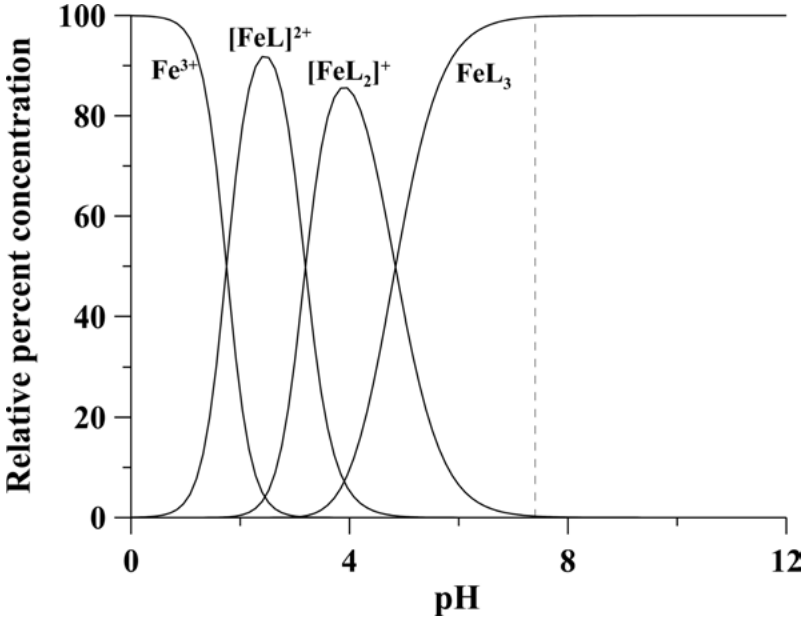


Figure 6. Distribution curves of the different Fe^{3+}-deferiprone complexes, calculated with the Fe^{3+} concentration of 1 μM and the DFP concentration of 10 μM.

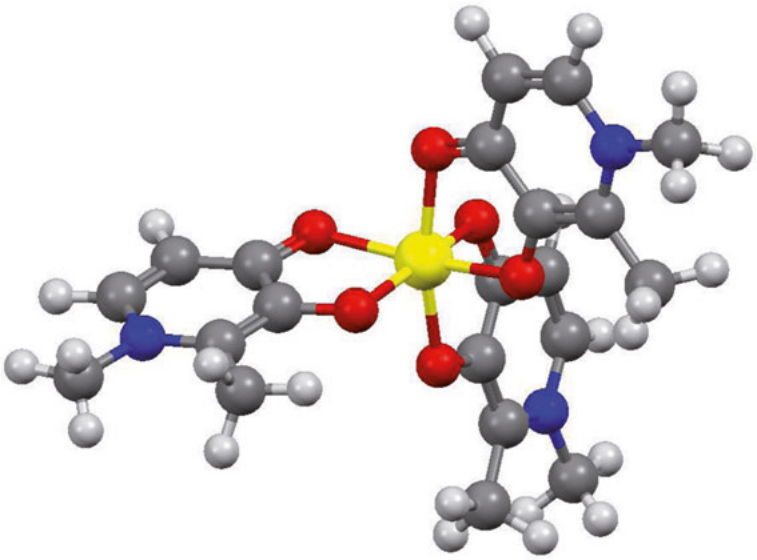


Figure 7. Crystal structure of the deferiprone-iron complex FeL_3 [75], (O: red; N: blue, C: grey, Fe: yellow). Coordinates obtained from the Cambridge Structural Database, CSD entry code JAWSEF01, images created with Mercury3.5.

4.2.2. Formulation, Doses, and Administration

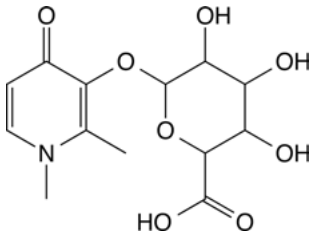
Ferriprox™ is available commercially as tablets and as oral solution. DFP is also produced by CIPLA Ltd. in India as Kelfer™, and by GPO (Government Pharmaceutical Organisation) in Thailand.

The recommended dose of DFO is 25 to 33 mg/kg body weight three times a day, corresponding to a total daily dose of 75–100 mg/kg. The tablets or volume of solution to be administered can be easily calculated according to the patient's weight.

4.2.3. Pharmacodynamics and Pharmacokinetics

The amount of iron excretion from the body induced by DFP depends on its dose, and is influenced by the entity of the existing iron load.

DFP is efficiently absorbed by the intestinal tract [76, 77], thanks to its low molecular weight 139.152 g mol^{-1} and lipophilicity (log P = 0.61), appearing in the blood within 5 to 10 minutes of oral administration. Following a single 1500 mg (~21.6 mg/kg) oral dose of DFP under fasting conditions in healthy subjects, the mean maximum concentration (C_{max}) of deferiprone in serum is 20 μg/mL. Peak serum concentrations occur ~1 h after administration of a single dose to fasting subjects, and up to 2 h after a single dose in the fed state. Administration with food decreases the C_{max} of DFP by 38 % and the absolute bioavailability (AUC)



Scheme 3. Molecular formula of the glucuronide metabolite of deferiprone.

by 10 %. Following a single dose of 33 mg/kg in healthy subjects under fasting conditions, the mean C_{max} is 35 μg/mL. Mean maximum serum DFP concentrations are reached at ~0.8 hours and then decline in a multi-exponential manner. Exposures to DFP are dose-proportional over the dose range of 22–50 mg/kg.

The main metabolite in man is the glucuronide form, which renders the glucuronide derivative unable to bind iron, blocking the 3-hydroxyl function [78, 79] (Scheme 3). To overcome the transformation in the non-chelating o-glucuronide form, DFP needs to be administered two or three times per day.

The metabolic transformation of DFP to an inactive glucuronide species determines its pharmacokinetic profile. The maximum concentration of DFP in plasma is reached two hours after administration, thereafter it completely declines over six hours. This behavior entails three separate administrations during the day. The majority of the DFP-iron complex formed is excreted by the kidneys (70 %).

4.2.4. Mechanisms of Action

DFP is a chelating agent with a high affinity for ferric ions, binding them in neutral 1:3 Fe^{3+}:DFO complexes. It removes Fe^{3+} from ferritin, and even from hemosiderin. DFP has a lower binding affinity for essential bivalent metal ions, such as copper and zinc, than for ferric ions. Nevertheless, zinc and copper depletion during DFP therapy has been reported by different authors [49], and a quantitative evaluation of this depletion has been recently proposed based on a speciation study [20]. The lipophilicity of DFP, and its low molecular weight, enable this small molecule to gain access into myocytes. DFP has the clear advantage over DFO of being orally active, and, at doses of 75–100 mg/kg/day, it may be as effective as DFO in removing iron [49]. DFP therapy is significantly more effective than DFO in decreasing myocardial siderosis [80–82], giving a significantly greater cardiac protection than DFO to transfused thalassemia patients [83].

4.2.5. Adverse Effects

Agranulocytosis/severe neutropenia are the most serious side effects associated with DFP, occurring in about 1.7 % of the patients [49]. The mechanism by which DFP induces agranulocytosis is unknown. Agranulocytosis and neutropenia com-

monly resolve by suspending the DFP treatment; however, post-marketing observations report cases of agranulocytosis leading to death. Thus, patients on DFP treatment must be closely monitored for agranulocytosis and neutropenia by obtaining a complete blood count weekly [84].

Less severe common side effects are gastrointestinal symptoms, musculoskeletal and joint pains, zinc deficiency, and fluctuating liver enzymes.

4.3. Deferasirox

Deferasirox (DFX) was synthesized by the Nick team at Novartis. According to Nick et al. [85], DFX emerged, among more than 700 screened ligands, as the chelating agent with the best combination of high oral efficacy and tolerability in animals. This study is indicative of the importance of a well conducted preliminary chemical research in the selection of proper chelating agents.

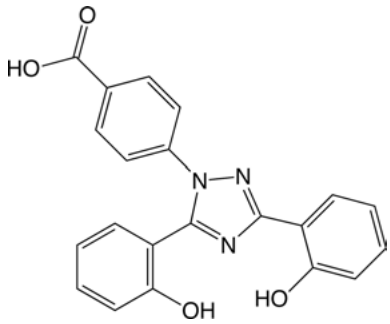
DFX was the second oral iron chelator entered in clinical use: it received EU authorization in 2002 and in most countries in 2006. At the moment it is the only oral iron chelator whose daily dosage can be given in a single administration [86].

4.3.1. Chemistry

Deferasirox (Exjade®), 4-[3,5-bis(2-hydroxyphenyl)-1H-1,2,4-triazol-1-yl]-benzoic acid, $C_{21}H_{15}N_3O_4$, molecular weight 373.36 g/mol, is characterized by two hydroxyphenyl groups bonded to a triazole unit, at which a benzoic acid is also attached which determines the solubility of the molecule (Scheme 4).

DFX's solubility in water at 25 °C is 0.4 g/L at pH 7.40, and log P = 6.30 at pH 7.4 [87]. It is characterized by three protonation constants (log K_1 = 10.6, log K_2 = 9.0, and log K_3 = 3.7, the last ascribed to the carboxylic group [88]), and at pH 7.4 is found as the negatively charged form LH_2^- (Figure 8).

The solid state structure presented in Figure 9 [88] shows the formation of a stabilizing intramolecular hydrogen bond between the N2 nitrogen atom in the triazole ring and the OH group on the corresponding phenolic ring; the OH



Scheme 4. Molecular formula of deferasirox.

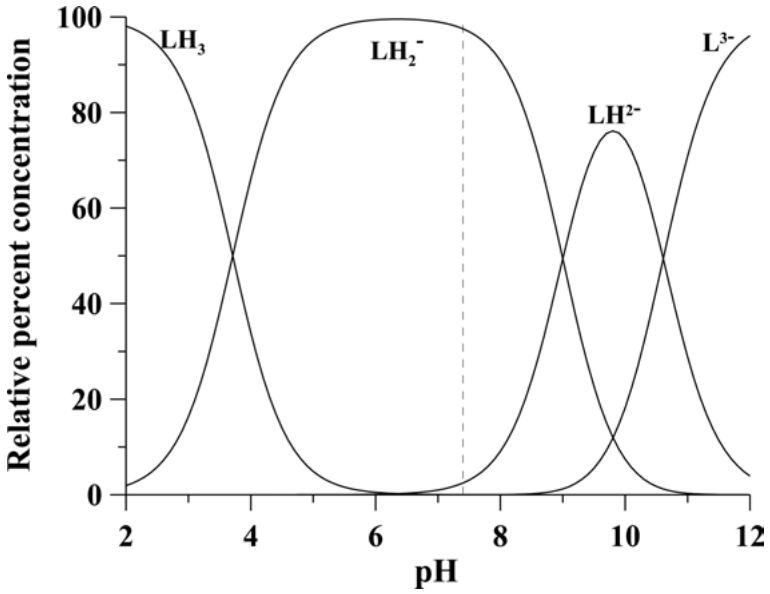


Figure 8. Distribution curves of the various protonated forms of deferasirox.

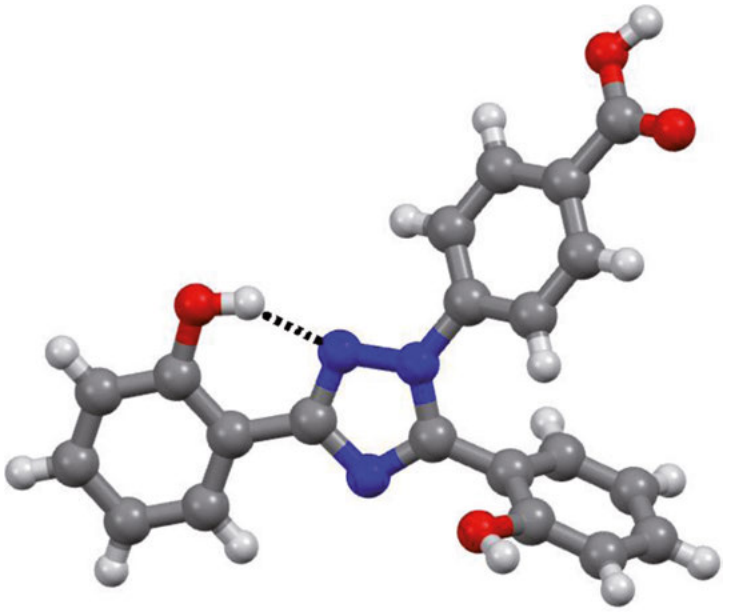


Figure 9. Crystal structure of deferasirox [88], (H: white, O: red; N: blue, C: grey). Coordinates obtained from the Cambridge Structural Database, CSD entry code SAJFIT, images created with Mercury3.5.

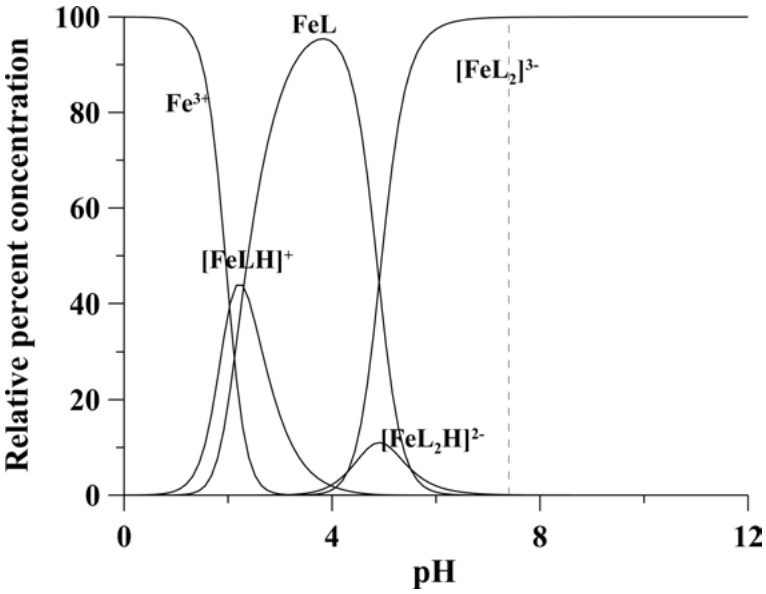


Figure 10. Distribution curves of the different Fe^{3+}-deferasirox complexes, calculated with the Fe^{3+} concentration of 1 μM and the DFX concentration of 10 μM.

group on the second phenolic group is nevertheless involved in intermolecular O–H···N bonding. The phenolic ring involved in the intramolecular hydrogen bonding is roughly coplanar with the triazole ring with a torsional angle of 2.3(3)°, while the second stands almost perpendicular to the triazole moiety with a torsional angle of 77.7(3)°. The benzoic acid unit is tilted out of the triazole plane by an angle of 13.9(3)°.

At pH >1 this tridentate molecule forms two 1:1 iron complexes, $[FeLH]^{+}$ and FeL, and three 1:2 complexes at pH >4, $[FeL_2H_2]^{-}$, $[FeL_2H]^{2-}$, and $[FeL_2]^{3-}$, in which iron is coordinated by the two phenolate moieties and by one nitrogen atom. The complex formation constants are log β_{111} 24.3, log β_{110} 22.0, log β_{122} 43.4, log β_{121} 41.2, and log β_{120} 36.9, which determine a p[Fe] value of 23.18 [89]. The speciation plot in Figure 10 shows that at pH 7.4 only the tri-negative anionic complex $[FeL_2]^{3-}$ exists. Its redox potential (−600 mV at pH 7.2) renders Fe^{3+} completely inaccessible to biological reductants.

According to Steinhauser et al. [88], there have been several unsuccessful attempts to crystallize Fe^{3+} complexes with deferasirox. However, a crystalline complex of the same FeL_2 stoichiometry was obtained with the corresponding decarboxylated ligand (Figure 11). This complex adopts the expected bis-structure with a meridional coordination mode of the two ligands, a distorted octahedral coordination environment, and Fe–O distances at the short end of the range expected for high-spin Fe^{3+}. The Fe–N distance of 2.092(5) Å is also remarkably short. The ligand in this complex is again not planar, the torsional angles being between the phenolate rings and the triazole unit 14.1° and 23.5° respectively.

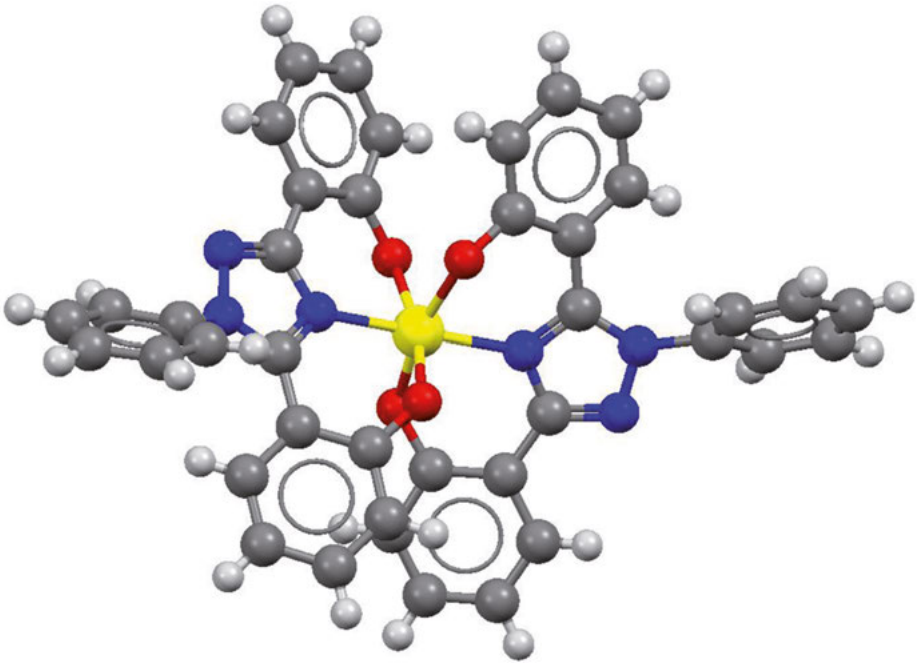


Figure 11. Crystal structure of the 1:2 complex between Fe^{3+} and the decarboxylated deferasirox ligand [88], (H: white, O: red; N: blue, C: grey, Fe: yellow). Coordinates obtained from the Cambridge Structural Database, CSD entry code SAJFAL, images created with Mercury3.5.

4.3.2. Formulation, Doses, and Administration

Novartis supplies Deferasirox in two different formulations, as Exjade® since 2006 and as Jadenu® since 2016. According to the Exjade® Product Monograph (Date of Preparation October 18, 2006, revised October 23, 2017), Exjade® is furnished as dispersible tablets for oral suspension of 125 mg, 250 mg, and 500 mg, containing lactose monohydrate as a clinically relevant non-medicinal ingredient. According to the Jadenu® Product Monograph (Date of Preparation February 19, 2016, revised October 23, 2017), Jadenu® is furnished as film-coated tablets of 90 mg, 180 mg, and 360 mg, containing a number of inactive ingredients in the core tablet, and a composite film-coating material.

DFX, formulated as Exjade® dispersible tablets, are a formulation of deferasirox with lower oral bioavailability as compared to Jadenu® film-coated tablets; thus, Exjade® requires a different dosing regimen and method of administration as compared to Jadenu®, and the recommended dose of Jadenu® should be about 30 % lower than that of Exjade®. To avoid dosing errors, it is important that prescriptions of deferasirox specify both the type of formulation (dispersible tablet or film-coated tablet) and the prescribed dose in mg/kg/day.

4.3.3. Pharmacodynamics and Pharmacokinetics

Pharmacodynamic effects tested in an iron balance metabolic study showed that deferasirox (10, 20, and 40 mg/kg/day) was able to induce net iron excretion (0.119, 0.329, and 0.445 mg Fe/kg body weight/d, respectively) within the clinically relevant range (0.1–0.5 mg Fe/kg/day). Iron excretion was predominantly fecal.

Daily treatment with Exjade® at doses of 20 and 30 mg/kg for one year in frequently transfused adult and pediatric patients with β-thalassemia leads to reductions in indicators of total body iron. Liver iron concentration is reduced by about 0.4 and 8.9 mg Fe/g liver (biopsy dry weight) and serum ferritin by about 36 and 926 μg/L on average, at the two different doses, respectively. At these doses the ratios (iron excretion/iron intake) were 1.02 (indicating net iron balance) and 1.67 (indicating net iron removal), respectively [90].

In patients with cardiac iron deposition (MRI T2* <20 ms), treatment with Exjade® removes cardiac iron, as demonstrated by progressive improvements in T2* values over 3 years of observation, and it can mobilize iron from the heart [91]. Exjade® is absorbed following oral administration with a median time to maximum plasma concentration (t_{max}) of about 1.5 to 4 hours. The C_{max} and AUC of DFX increase approximately linearly with dose after both single and multiple dose administration, with an accumulation factor of 1.3 to 2.3 following administration of multiple doses. The AUC of DFX tablets for oral suspension is 70 % compared to an intravenous dose. The plasma half-life after a single oral dose in humans is about 10 h allowing once daily dosing [92].

In humans, DFX is highly (~99 %) protein-bound, almost exclusively to serum albumin, with a mean elimination half-life ($t_{1/2}$) ranging from 8 to 16 hours. The percentage of deferasirox confined to the blood cells is 5 %. The volume of distribution at steady state (V_{ss}) of DFX is 14 ± 3 L in adults. Glucuronidation is the main (84 % of the dose) metabolic pathway for DFX, with subsequent excretion of drug and its chelates via bile into feces, while renal excretion is minimal (8 % of the dose) [93].

4.3.4. Mechanisms of Action

DFX is an orally active chelator that is highly selective for Fe^{3+}. This tridentate ligand binds iron with high affinity in a 2:1 ratio. Although its highest affinity is for iron, DFX has a significant affinity for aluminum and a very low affinity for zinc and copper, and there are variable decreases in the serum concentration of these trace metals after its administration. The clinical significance of these decreases is uncertain. A detailed description of trials and of clinical results of DFX treatments can be found in the recent review by Saliba, Harb, and Taher [94].

4.3.5. Adverse Effects

DFX is contraindicated in patients with moderate and severe renal impairment and has not been studied in patients with severe hepatic impairment. Acute renal failure, hepatic failure, and gastrointestinal hemorrhage and perforations are

clinically significant adverse events. At the recommended dose 20–40 mg/kg/day, its most frequent adverse events include transient gastrointestinal disturbances and skin rash. Mild, usually non-progressive increases of serum creatinine, observed in approximately one third of patients, spontaneously returns to baseline in the majority of cases [95]. Renal failure, reported following post-marketing use, is an important complication [96]. According to Hider [47], the formation of zinc polymeric complexes plays a possible role in the origin of this complication.

4.4. Comparative Cost Analysis

Different studies have evaluated costs related to iron chelation therapy (ICT), and compared the relative costs of the chelating agents in clinical use (DFO, DFP, and DFX). A number of factors contribute to the total cost of ICT either as single or combined chelating agent therapy. The first factor is the drug cost for unitary weight that has to be weighted by daily dose in mg/kg/day and weight of patient, and by the days of treatment per week. These parameters were taken from two studies evaluating the ICT in transfusion-dependent thalassemia populations in two different local situations, Thailand [97] and Great Britain [98].

The pure drug costs per gram of the three drugs are quite different, and varies in Thailand from $ 58.56 per gram for DFX, to $ 10.77 per gram for DFO to $ 2.09 for DFP (Kelfer) or $ 0.20 for DFP (GPO-L-One), while in Great Britain varies from £ 33.6 ($ ≃ 47.7) per gram for DFX, to £ 8.5 ($ ≃ 12.1) per gram for DFO to £ 3.2 (about $ 4.5) for DFP. Taking into account the daily dose in mg/kg, the days of treatment per week and the mean weight of patient of 63 kg, the cost/year of each treatment can be evaluated, which amounts to £ 23,179 ($ ≃ 32,914) for DFX, £ 5,584 ($ ≃ 7,929) for DFO and £ 5,519 ($ ≃ 7,837) for DFP in Great Britain. For the population in Thailand, this cost/year is calculated taking into account the mean weight for different age ranges; at any rate, using the same mean weight used in Great Britain and an identical dosage, the cost year amounts to $ 40,397 for DFX, $ 7,075 for DFO, and $ 6,605 for DFP (Kelfer) or $ 345 for DFP (GPO-L-One).

Besides the direct cost of drugs, further costs have to be taken into account, related to the treatment (the necessary infusional equipment for DFO), to the monitoring therapy for the major side effects (audiological and ophthalmological controls in patients treated with DFO, treatments for neutropenia and agranulocytosis in patients treated with DFP, etc.), to the compliance of patients (economic penalties of a poor compliance), and finally, to the quality of life.

As concluding remarks, Bentley et al. [98], taking into account four ICT treatments (DFO, DFP, DFX, and combined DFO-DFP) and assuming that all the treatments had a comparable effect, reached the conclusion that DFP appears as the most effective treatment. An analogous conclusion was reached by Luangasanatip et al. [97]. The work of McLeod et al. [99] to assess the cost-effectiveness of deferasirox concluded that "*DFX may be cost-effective compared with DFO in patients with β-thalassemia, but it is unlikely to be cost-effective compared with DFP*". Analogously, Delea et al. [100], who compared the cost effectiveness

of DFX *versus* that of DFO, reached the conclusion that DFX is a cost-effective chelating agent from a US healthcare perspective.

5. COMBINATION THERAPY

5.1. Aims of Treatment with More than One Chelator

Based on the observation that some transfusion-dependent thalassemia patients do not reach a negative iron balance with DFP at dosages of 75 mg/kg/day, Wonke et al. [101] proposed in 1998 the use of the combined administration of DFO and DFP. Besides the greater compliance by patients for this administration regimen, this combined therapy presents a number of potential advantages. First, the use of the two chelators allows the access to different iron pools and a better control of the toxic effects of NTBI. Further, the doses of both chelating agents can be decreased, decreasing their toxic effects, safeguarding at the same time a high chelation efficacy. The application of combined chelation therapy has shown in its application marked benefits in myocardial iron deposition [102] and iron balance [103]. Galanello et al. [104] have suggested restricting the term "combination therapy" to the chelation regimens that utilize co-administration of the two chelating agents in the same day (for example, DFP during the day, and DFO at night). The term "alternate chelation" should be instead reserved to administration of the two chelating agents on different days. The amount of scavenged iron in combination therapy has shown additive effects in all patients (the quantity of excreted iron equal to the sum of that excreted individually by each chelating agent). In a relevant number of patients, synergistic effects were observed, i.e., that the amount of removed iron was greater than the sum of removed individually by each chelating agent. The "shuttle hypothesis" has been invoked [105–107] to explain the efficacy of combined therapy. According to this hypothesis the smaller DFP molecule accesses iron pools not available to DFO, and then "shuttles" the chelated iron in plasma, where it is exchanged with the stronger DFO; the newly formed complex is then rapidly excreted and the released DFP molecule is free to repeat its scavenging action. Even if extremely attractive, the shuttle hypothesis cannot be true for the large part of regimens because a direct interaction between the two chelating agents is not possible for their limited co-existence in plasma before their metabolic deactivation.

5.2. Desferal with Deferiprone

DFO with DFP is the best-studied combination of iron chelating agents, starting with the early work of Wonke et al. in 1998 [101]. It has been experimented in a large variety of combined regimens, for the time of administration and for the dose levels, which have been the object of two extensive reviews by Saliba et al. [94] and Kwiatkowski [108]. Here we only mention the principal advantages offered by this combined DFO-DFP therapy, the main one being a significant

contribution to reducing cardiac morbidity and mortality in patients with transfusion-dependent thalassemia [109]. DFO and DFP together have shown to produce a rapid and effective decreases in liver and cardiac iron stores [99, 110, 111]. In particular, this DFO-DFP combination improves left ventricular ejection fraction and decreases cardiac and liver iron content in patients with severe myocardial siderosis [108]. Based on the wide experience with this combination therapy and on its distinct benefits, it can be recommended whenever possible for patients with important cardiac iron loading [112].

5.3. Desferal with Deferasirox

The first reported combined treatment with DFO and DFX dates to 2011, by Voskaridou et al. [113]. These authors treated successfully a patient with transfusion-dependent thalassemia with a combination of DFX and DFO, obtaining significant improvements in markers of iron overload. Without reviewing the numerous studies which appeared since 2011, the obtained experience has shown that the combined therapy with DFO and DFX is particularly effective at rapidly reducing high liver iron content, and to a lesser extent cardiac iron. Thus, this treatment appears of particular efficacy for patients with severe hepatic iron loading but without important cardiac iron loading and cardiac dysfunctions [108].

5.4. Deferiprone with Deferasirox

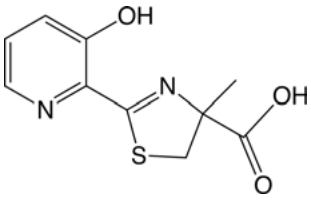
Some cases of successful use of a DFP and DFX combination have been reported, and at the moment it is the subject of several ongoing studies. This combination appears particularly attractive since it does not require parenteral administration.

In a major study by Elalfy and coworkers in 2015 [114], a randomized controlled trial of DFP combined with DFO or DFX in pediatric patients with mild cardiac siderosis, over a 1-year period the ferritin levels and LIC improved with both combinations of chelating agents. Cardiac T2* also improved with both combinations, but the improvement was more effective and rapid in the DFP-DFX group. This more rapid improvement in cardiac T2* merits additional studies, particularly in patients with important cardiac iron loading and cardiac dysfunctions. If the combination DFP-DFX is confirmed to be effective and well tolerated, it would offer an easier alternative treatment, removing the problem of non-compliance with DFO administration.

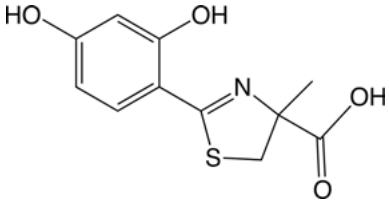
6. NEW CHELATORS

6.1. New Chelators in Trials

Desferrithiocin, a natural siderophore, is a three-dentate chelator (Scheme 5) that forms with Fe^{3+} a single $[FeL_2]^-$ complex with a p[Fe] value of 20.4. The trivalent metal ion is completely coordinated in an octahedral fashion through



Scheme 5. Molecular formula of desferrithiocin.



Scheme 6. Molecular formula of deferitrin (GT-56–252).

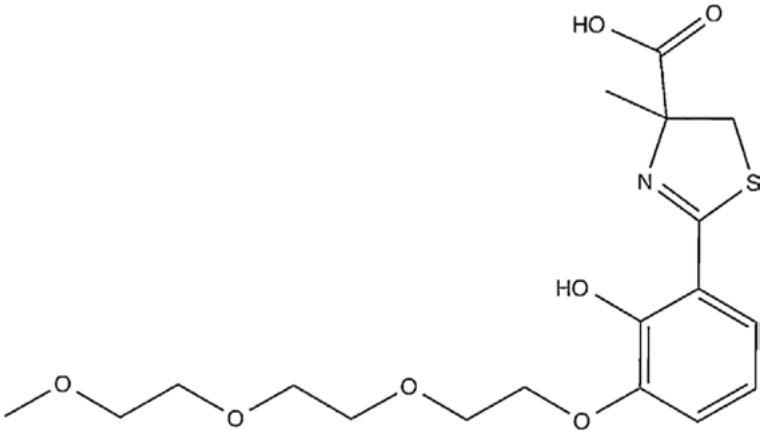
the two oxygen atoms of phenolate and carboxylate groups, and the heterocyclic thiazole nitrogen atom. Desferrithiocin presented remarkable oral activity and scavenging properties both in rodents [115] and in primates [116], but resulted in renal toxicity in rats [117].

In the following years, the group of Bergeron [118], starting from the scaffold of desferrithiocin, designed, synthesized, and analyzed different ligands in order to overcome toxicological problems preserving iron scavenging properties. These studies led first to deferitrin and then to deferitazole. Deferitrin (GT-56–252) (Scheme 6) was very promising in phase I human clinical trials that evaluated its safety and pharmacokinetic parameters [119], but in the following studies at higher doses (12.5 mg kg^{-1} twice a day), it presented an unacceptable renal toxicity in three patients that led to the termination of the study [118].

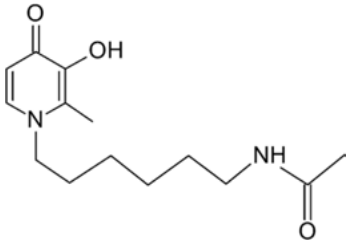
In the following, a series of polyether derivatives of desferrithiocin were examined, and deferitazole (FBS0701, or SSP-004184, or SPD602) resulted as the most promising one (Scheme 7).

Deferitazole is an oral chelator with a low toxicity, and its half-life (16.2–21.3 h) allows once daily administration. Hider et al. [120] have characterized the chemical features of this molecule which forms with Fe^{3+} two different complexes, a 1:1 complex with $\log \beta_{110} = 17.70$ and a 1:2 complex with $\log \beta_{120} = 33.14$, with a p[Fe] of 22.3. The assessment of deferitazole in clinical trials in thalassemia and sickle cell anemia patients in several centers worldwide [121–123] ended in 2014 due to the treatment being stopped, because of non-clinical safety results [124].

Hider et al. have studied different hydroxypyridinones with the aim of improving the properties of deferiprone, and reaching a log *P* that can give a greater lipophilicity and greater bioavailability to the molecule. Preliminary log *P* calculations of a series of 1-(ω-acetamido-alkyl)-3-hydroxy-2-methylpyridin-4-ones predicted that 1-(N-acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one or



Scheme 7. Molecular formula of deferitazole.



Scheme 8. Molecular formula of CM1.

CM1 (or LINAII) would possess a log *P* value slightly higher than that of DFP. This ligand, CM1, has been synthesized and characterized [125]. Its partition coefficient is suitable to allow membrane permeation, rendering it capable of entering mammalian cells to scavenge excess intracellular iron and to efflux it from cells as the neutral 3:1 complex.

CM1 has shown promising results in an animal model of thalassemia [126]. It is orally active, possesses a higher efficiency than DFP in rats, and displays no toxicity to a range of cell types. These promising studies are going to be extended to monitor its pharmacokinetics and metabolism, and render it a potential candidate for phase 1 clinical trials.

6.2. Overview on Recent Research

In this section, a selective survey of the most recent progresses in the study of iron chelators is presented. Particular attention has been paid to the 3-hydroxy-4-pyridinone (3,4-HP) ligand family. The 3,4-HPs bind Fe^{3+} with high affinity via the two oxygen atoms (bidentate {O,O} chelators) in the same fashion as

DFP. These ligands can be easily extra-functionalized to improve both the chelating ability and the bioavailability. The group of Santos has been proposing a number of tetradentate 3,4-HP chelators [127, 128]. This research presented a ligand formed by two 3,4-HP chelating moieties connected by an iminodiacetic acid (IDA) scaffold, with a 1,4-disubstituted arylpiperazine on the nitrogen atom in the linker. Successively the same authors presented:

(a) Two hexadentate ligands, based on the KEMP acid scaffold to which three 3-hydroxy-4-pyridinone chelating moieties are attached by two spacers of different size, the tris-hydroxypyridinone compounds KEMPPr(3,4-HP)$_3$ and MPBu(3,4-HP)$_3$ [129]. These ligands are effective chelating agents towards iron, and for the group III metal ions.

(b) Two tris(3,4-HP) hexadentate chelators, NTA(BuHP)$_3$ and NTP(PrHP)$_3$, in which the three hydroxypyridinone units are joined by nitrilotriacetic acid (NTA) and nitrilotripropionic acid (NTP). Their iron complex formation ability was studied in solution, and their scavenging capacity was assayed *in vivo* on iron-overloaded animals. The remarkable stability of iron complexes should be pointed out (p[Fe] = 27.9 for NTA(BuHP)$_3$ and p[Fe] = 29.4 for NTP(PrHP)$_3$) [130].

(c) A tripodal hexadentate ligand, NTP(PrHPM)$_3$, with three hydroxypyrimidinone (HPM) chelating units attached to a nitrilotripropionic acid [131]. The thermodynamic stability of the complexes with iron, aluminum, and gallium was studied, and subsequently the capacity to remove hard metal ions from an animal model overloaded with Ga was assayed *in vivo*. The new tris-HPM ligand, an interesting alternative to the reported hexadentate analogue NTP(PrHP)$_3$, still keeps high chelating capacity for hard metal ions and presents better water solubility (log $P = -1.51$). The *in vivo* studies show that it induces a faster clearance from main organs and an enhancement of overall excretion, as compared with DFP, slightly lower than the tris-hydroxypyridinone analogue, NTP(PrHP)$_3$.

For improving the properties of DFP, reaching a better stability (p[Fe] >20) or a better log *P*, the group of Hider [132] has synthesized different hydroxypyridinones. An extensive series of the so-called "high pM" hydroxypyridinones have been investigated, consisting in a variety of different 2- and 6-amido-3-hydroxypyridin-4-ones; all these ligands exhibit lower pK_a values than DFP due to the inductive effect of the amido group. These lower pK_a values led to a lower proton competition, and therefore to better chelating properties in comparison with those of the parent DFP [133]. None of these chelators has been identified with superior efficacy/toxicity ratios than those of DFP [132, 134].

Our group studied the chelating ability of a number of bisphosphonate ligands showing their high efficiency, with p[Fe] values greater than that of DFP [135]. Two different groups proposed the conjugation of bisphosphonates with other strong coordinating groups to further improve their chelating properties. Ding et al. [136] proposed catechol-bisphosphonate conjugates and Bailly et al. [137]

proposed bisphosphonate-hydroxypyridinonate compounds. We studied the iron-chelating properties of these compounds, finding that in both cases the short linker prevents simultaneous tetradentate coordination [138, 139]. In the last years, we presented a series of tetradentate derivatives of kojic acid (KA). This research started from a paper by Fox and Taylor [140], who experimented with an interesting iron chelator, formed by two kojic acid units linked by a methylene group, for the *in vivo* mobilization of ferritin-bound iron, and proved its high efficiency. Following the work of Fox and Taylor, we characterized this ligand and its iron complexes. It forms stable iron binuclear complexes, characterized by the extremely high p[Fe] value 20.5 [141]. Encouraged by the interesting result we designed and synthesized a variety of chelators based on two KA units joined by different linkers [142–144]. These ligands act as tetradentate chelators, by the formation of binuclear high stability complexes Fe_2L_2 and Fe_2L_3, the first stoichiometry being favored by the shorter linkers. The presence of two linked KA units leads to the significant increase of the stability of complexes compared with that of KA. The p[Fe] value 13.3 for KA increases up to 9.8 units passing to these derivatives. The ligands containing a -CH_2-NR-CH_2- linker form Fe_2L_3 complexes in which the three ligands completely satisfy the coordination sphere of two iron ions. The structures for the iron complexes reported in reference [7] show that the coordination of each iron atom with the bis-kojic acid perfectly resembles the one of KA, in which the three ligands were free to get the best placement around the metal ion. This fact indicates that the linker has the correct length and flexibility to allow the best iron coordination without strain. Consequently, the enthalpic contribution to the formation of the bis-kojic acid-iron complex should be similar to that of KA, not disturbed by strain effects. Consequently, the entropic contribution depending on the preorganization of the tetradentate ligand strongly favors the formation of a Fe_2L_3 complex, determining the remarkable increase of p[Fe]. Their relatively low molecular weights (340–450 g mol^{-1}) and many opportunities to modulate their binding ability by employing proper substituents in the KA units and in the linker suggest their possible roles as oral chelators.

7. CONCLUDING REMARKS

In a review of 1999 entitled "Oral iron chelators for clinical use" [145] we reported some information on DFX: "*In a recent meeting a new potential tridentate oral chelator (4-(3,5-bis(2-hydroxyphenyl)-1,2,4-triazol-1-yl)-benzoic acid) has been proposed* [146] *which emerged from a selection process as the compound which best combines high oral potency and tolerability. It forms 1:1 and 1:2 differently protonated iron complexes characterized by extremely high formation constants* (K. Hegetschweiler, personal communication)", and on the clinical trials on DFP – at that time under an important scientific/legal dispute on its clinical safety – "… *3-hydroxy-4-pyridinone presents the most interesting properties and its 1,2-dimethyl derivative (L1) has been tested clinically since 1989 by different thalassaemia hospitals all over the world*". Finally, we concluded: "*There is an*

obvious need for alternative, orally effective iron-chelating drugs, which would be more convenient to use than Desferal, and therefore could be available to a larger number of patients who, at the present, are unable to afford the cost or to comply with the need for long-term subcutaneous infusion."

Since then, a massive progress has been made, and the two oral chelators DFP and DFX have now reached well-established clinical use. The development and availability of new instrumental techniques for a precise and accurate evaluation of iron overload in different organs, and the possibility of administering chelating agents through different options, have nowadays made it possible to adjust the therapy (number of transfusion, kind and amount of chelating agents) according to the needs and the compliance of each individual patient.

Besides these ongoing progresses in transfusion therapy, a series of new treatments for thalassemia patients is now on the horizon.

Nevertheless, at the expense of this optimistic picture of continuous progress, some realistic considerations have to be done on the world distribution of thalassemia and on the percentage of patients that can access to the correct necessary treatments. In her recent review, Deborah Rund [147] reports: "*It is well known that the world's distribution of thalassemia patients is predominantly in developing countries which do not have the resources (financial and technological) to adopt the use of most of these modalities. Tragically, there are many countries in which the most basic routine care of transfusion and chelation are either non-existent or woefully underutilized due to financial constraints. To those in the industrialized world, it is shocking to read that only about 12 % of children born with thalassemia worldwide are transfused and of these, only about 40 % are adequately chelated. Therefore, the survival of thalassemia patients in low income countries at present is similar to that in the Europe and the United States 50 years ago. Annually about 1.33 million pregnancies worldwide are at risk for thalassemia but only a small fraction has access to genetic counseling and prenatal diagnosis*."

From this dramatic picture, it clearly appears that the health problems of thalassemia have to be faced not only by a scientific but mainly by a political approach. Policy makers and public health organizations must implement all the necessary actions to prevent the diffusion of this disease, and to provide to patients the best medical treatments.

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ABBREVIATIONS AND DEFINITIONS

AUC	absolute bioavailability
BAL	2,3-dimercaptopropan-1-ol

CM1	1-(N-acetyl-6-aminohexyl)-3-hydroxy-2-methylpyridin-4-one
DFO	desferal, desferrioxamine
DFP	deferiprone
DFX	deferasirox
FDA	US Food and Drug Administration
FOB	ferrioxamine B
GRE	gradient recalled echo in MRI
HPM	hydroxypyrimidinone
3,4-HPs	3-hydroxy-4-pyridinone ligands
ICT	iron chelation therapy
IDA	iminodiacetic acid
KA	kojic acid
KEMP	1,3,5-trimethyl-1,3,5-cyclohexanetricarboxylic acid
L1	old acronym of DFP
LIC	liver iron concentration
LIP	intracellular labile iron pool
log *P*	log of the partition coefficient octanol/water
MRI	magnetic resonance imaging
NADP	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
NTA	nitrilotriacetic acid
NTBI	non-transferrin-bound fraction of plasma iron
NTP	nitrilotripropionic acid.
R2	relaxation rate, when spin echo is used in MRI
R2*	relaxation rate, when the gradient echo is used in MRI
ROS	reactive oxygen species
SE	spin echo in MRI
SF	level of serum ferritin
SQUID	superconducting quantum interference device
T2	relaxation time, when spin echo is used in MRI
T2*	relaxation time, when the gradient echo is used in MRI
trien	triethylenetetramine

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4

Ironing out the Brain

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Abstract: Our understanding of the broad principles of cellular and systemic iron homeostasis in man are well established with the exception of the brain. Most of the proteins involved in mammalian iron metabolism are present in the brain, although their distribution and precise roles in iron uptake, intracellular metabolism and export are still uncertain, as is the way in which systemic iron is transferred across the blood-brain barrier. We briefly review current concepts concerning the uptake and distribution of iron in the brain, before turning to the ways in which brain iron homeostasis might be regulated. The distribution of iron between different brain regions is then discussed as is the increase in brain iron with normal aging, and the different forms in which iron is present.

The increased levels of iron found in specific brain regions and their potential contribution to neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, Huntington's disease and other polyglutamine expansion diseases, amyotrophic lateral sclerosis, Friedreich's ataxia, as well as a number of neurodegenerative diseases with iron accumulation, are discussed.

The interactions between neuroinflammation and iron are presented, and the chapter concludes with a review of current clinical studies and discussion of the potential and efficacy of iron chelation therapy in the treatment of neurodegenerative diseases.

Keywords: brain · chelators · homeostasis · iron · neurodegeneration

1. IRON HOMEOSTASIS IN THE BRAIN

1.1. Systems Involved in Uptake and Distribution of Iron in the Brain

While our understanding of human iron metabolism and homeostasis has advanced greatly over the past few decades [1, 2], the ways by which iron is handled within the brain remain an enigma [3]. We begin by briefly outlining the essential characteristics of the brain, its interface with the peripheral tissues and fluids, often designated as the blood-brain barrier, the types of specialized cells of which it is constituted, and the components of general iron metabolism which are reported to be present in brain cells. Based on this data, we then present an overview of iron uptake and distribution in the brain.

The brain is the most complex organ of the human body, responsible for all of our thoughts and actions, our memory, and our feelings. Although it constitutes only 2 % of body mass, it receives 15 % of cardiac output, consumes 20 % of total O_2 consumption, and accounts for 25 % of total body glucose utilization. Enclosed within the confines of the skull, suspended in the cerebrospinal fluid with the consistency of soft gelatine, the human brain contains about 10^{11} specialized nerve cells, neurons. Each neuron can form tens of thousands of connections with other neurons. There are three types of neurons (Figure 1): (i) Multipolar neurons, with profusely branched dendrites, receive synaptic signals from several hundred of other neurons and transmit them to many other neurons, motor neurons, and sensory neurons. (ii) Motor neurons transmit nerve impulses to muscle cells, and their single, often very long axons, extend from the cell body of the neuron to the effector muscle cell. Mammalian motor neurons have an insulating sheath of myelin covering all parts of the axon except for the nodes

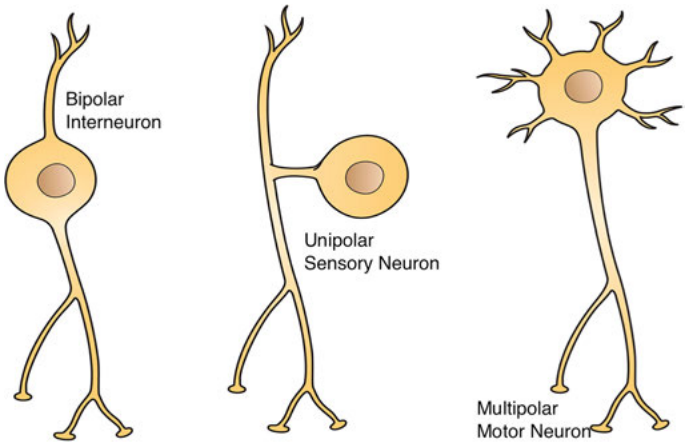

Figure 1. The three types of neurons found in the CNS (Quia). Artwork by Holly Fischer – http://open.umich.edu/education/med/resources/second-look-series/materials – CNS Slide 12; PNS Slide 18, CC BY 3.0, https://commons.wikimedia.org/w/index.php?curid=24367144.

of Ranvier and the axon terminals at the neuromuscular synapse. (iii) Sensory neurons collect all sorts of information, concerning light, smell, sound, pressure, touch, etc. through specialized receptors, and transform this information into electrical signals. In sensory neurons, the axon branches when it leaves the cell body. The peripheral branch carries the nerve impulse from the receptor cell to the cell body. The central branch then carries the impulse from the cell body, located in the dorsal root ganglion close to the spinal cord, either to the spinal cord or to the brain.

The human brain also contains three different types of cells, called glial cells, namely astrocytes, oligodendrocytes, and microglia (Figure 2). Together, they form the matrix within which the gigantic neuronal switchboard is embedded. Astrocytes are the brain cells which have direct contact with the endothelial blood-brain barrier (BBB), and therefore represent the potential point of entry for iron from the peripheral circulation. They perform many functions, including biochemical support of endothelial cells that form the BBB, provision of nutrients to the nervous tissue, maintenance of extracellular ion balance, and a role in the repair of the brain and spinal cord. Oligodendrocytes are involved in the electrical insulation of the axons of nerve fibres. Morphologically they resemble cellular octopuses, wrapping up to 150 layers of myelin sheath approximately 1μm thick around the axons of neurons, rather like electrical insulating tape. One oligodendrocyte can extend its processes to up to 50 neuronal axons. In the peripheral nervous system the function of the oligodendrocytes is replaced by Schwann cells, which, however, can wrap around only one axon. Microglia are the resident macrophage cells, acting as the active immune defense in the central nervous system (CNS). They can communicate with astrocytes and neurons and with cells of the immune system by a large number of signalling pathways. In normal circumstances they function

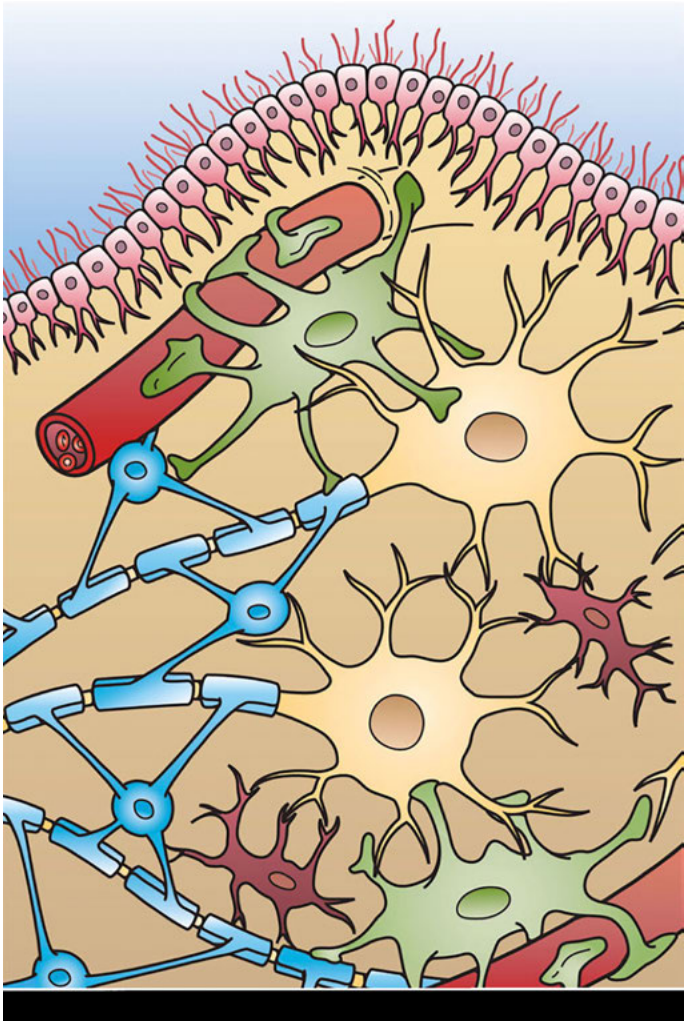

Figure 2. The four different types of glial cells found in the central nervous system: Artwork by Holly Fischer – http://open.umich.edu/education/med/resources/second-look-series/materials – CNS Slide 4, CC BY 3.0, https://commons.wikimedia.org/w/index.php?curid=24367125.

as surveillance cells, regulating the removal of cell debris after neuronal death, after which they return to their quiescent state.

We now address the way in which iron crosses from the peripheral circulation into the brain. The brain is hidden behind a relatively poorly permeable vascular barrier, which limits its access to plasma nutrients, such as metal ions. There are three principal barrier sites between blood and brain [4]: (i) the blood-brain barrier proper, formed by the endothelial cells of cerebral blood vessels, which form the walls of the capillaries, (ii) the blood-CSF (cerebrospinal fluid) barrier,

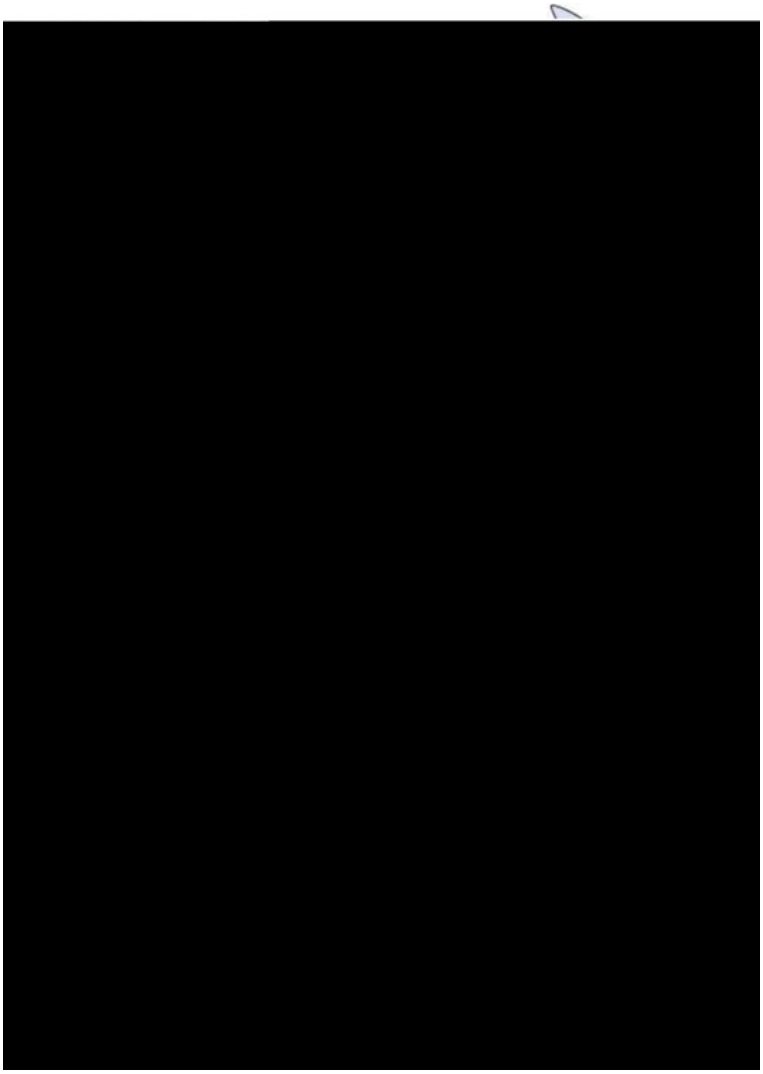

Figure 3. Schematic of neurovascular unit components. Schematic drawing of the neurovascular unit, indicating the close spatial relationship and the complex physiologic interactions between endothelial cells and pericytes, astrocytes, microglia, and neurons. Reproduced by permission from [8]; copyright 2017 Elsevier.

located at the choroid plexus in the lateral, third and fourth ventricles of the brain, and (iii) the arachnoid barrier, provided by the arachnoid epithelium, lying underneath the dura and completely enclosing the CNS. At the BBB, brain capillaries bring blood close to neurons, and as the brain endothelium forms the largest interface for blood-CNS exchange, the activities of the BBB are key to brain homoeostasis [4].

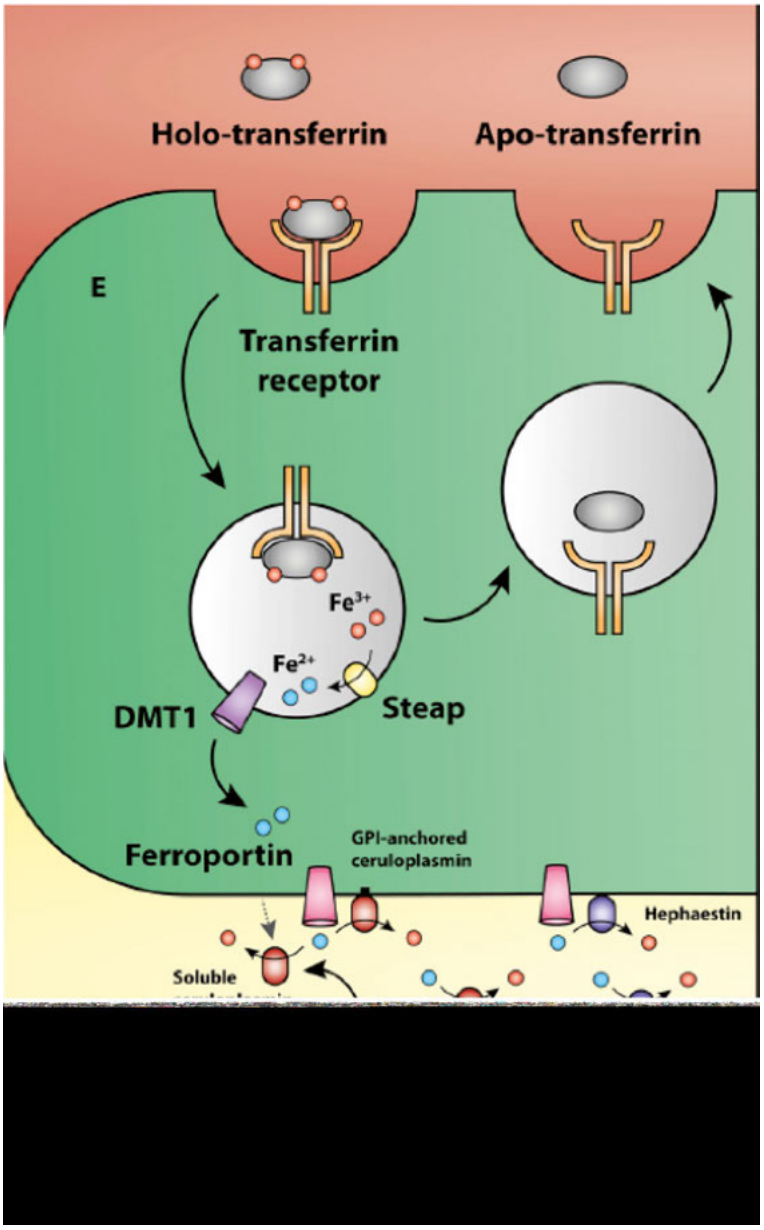

Figure 4. A proposed model for iron transport across the blood-brain barrier. Reproduced by permission from [10]; copyright 2016 Springer Science+Business.

The endothelium of the BBB acts within a coordinated cellular complex [5, 6], known as the modular neurovascular unit (NVU), composed of a capillary segment with its associated pericytes, basement membranes, perivascular astrocytes,

and microglial cells, which serve the needs of the small number (typically less than 8) of local client neurons (Figure 3) [4, 5–8].The components of the neurovascular unit all contribute to BBB function. Disturbance of neural function can result from dysfunction of any of these elements and of the wider network of neuronal connections [9].

A model for iron transport across the BBB is presented in Figure 4 [10]. The first step in iron transport into the brain involves transferrin uptake through the brain capillary endothelial cells (BCECs) at the BBB [11, 12]. The way in which iron is transported across the BCEC to the abluminal membrane has been the subject of some discussion, but recently it has been reported that rat brain endothelial cells express all of the relevant proteins required for iron uptake by the classic transferrin to cell cycle, notably DMT1 (divalent metal transporter 1) [10]. This implies that the transcytosis model [12], in which diferric transferrin (Fe_2Tf, or holo-transferrin in Figure 4) crosses the BCEC and is released intact at the abluminal surface, which was not supported by quantitative transport studies [12, 13], is probably not involved. Fe_2Tf binds to transferrin receptors on the luminal surface of the brain endothelial cell, and the complex is internalized within endosomes. The action of a proton pump decreases the pH within the endosome to pH 5.6, causing dissociation of Fe^{3+} from transferrin, whereupon ferrireductases of the STEAP (six-transmembrane epithelial antigen of prostate) family of proteins reduce the Fe^{3+} to Fe^{2+}, which is then transported across the endosomal membrane to the cytosol by DMT1 to join the intracellular labile iron pool (LIP). Apo-transferrin bound to the transferrin receptor is recycled back to the luminal membrane, where it is released. Fe^{2+} is transported out of the brain endothelial cell at the abluminal membrane into the brain interstitial fluid (ISF) by the transmembrane Fe^{2+} export protein, ferroportin (FPN). Fe^{2+} can then be oxidized to Fe^{3+} by the ferroxidase activity of either glycosylphosphatidylinositol (GPI)-anchored ceruloplasmin or GPI-anchored hephaestin, both of which are expressed on rat brain endothelial cells, and by the soluble form of ceruloplasmin secreted from pericytes and astrocytes [10].

The perivascular end feet of astrocytes (Figure 3) ensheath the abluminal membrane of the BBB [14]. Astrocytes do not seem to have transferrin receptors, and are thought to take up Fe^{3+} in the form of low molecular weight complexes (Figure 5) with citrate, ATP or ascorbate, which the astrocytes themselves secrete [3, 12]. Iron, within the LIP of the astrocyte, can be stored in ferritins (*vide infra*), cytosolic heteropolymers made up of 24 subunits composed of variable amounts of H- and L-chains, utilized for incorporation into iron-containing proteins, or else be exported via FPN. The location of astrocytes in such close proximity to brain endothelial cells implies that they play a key role in distributing iron to other cells within the NVUs [15], which are bathed by the ISF [16].

Transferrin in the brain is principally secreted by the choroid plexus into the CSF [17], which is in extensive communication with the ISF. Fe^{2+} exported from astrocytes by FPN can be oxidized by ceruloplasmin and inserted into ISF transferrin (Figure 5). Neurons take up iron, essentially from transferrin through the classic TfR1-mediated endocytosis pathway described above, and export excess

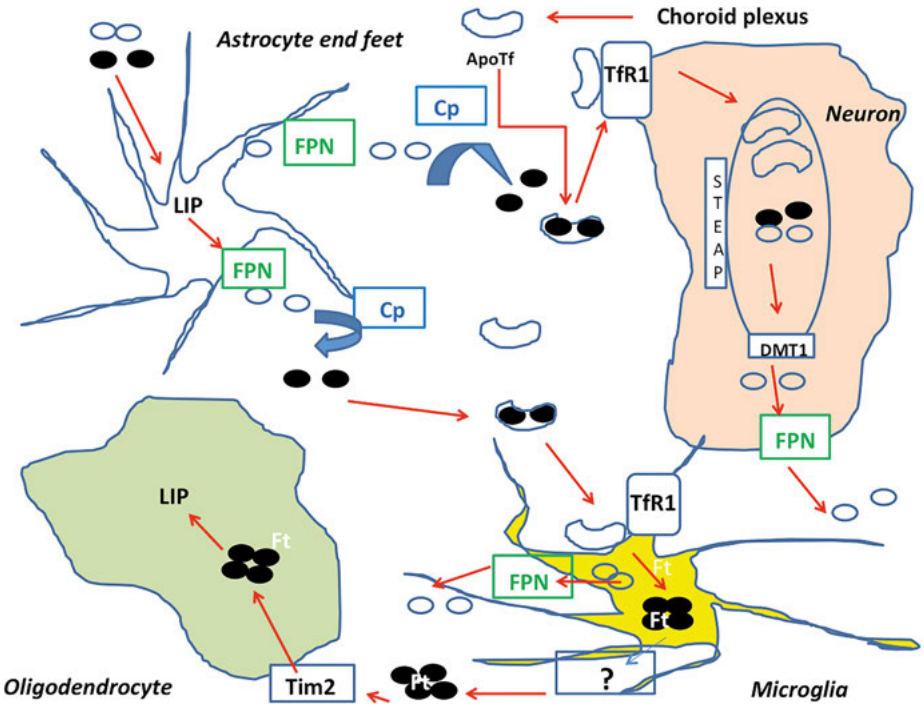

Figure 5. Iron transport in brain. Once iron (Fe^{2+} indicated by open circles and Fe^{3+} as closed circles) has been released from brain capillary endothelial cells, it is taken up by the perivascular end feet of astrocytes. The astrocytes then release Fe^{2+} via FPN, which can be oxidized by ceruloplasmin (Cp), and incorporated into apotransferrin (apoTf), which can then be taken up by neurons and microglia. This involves the classic Tf to cell cycle involving STEAP reductases and DMT1. Oligodendrocytes may take up iron from ferritin (Ft) released into the interstitial fluid from microglia, via TIM2.

iron via FPN. In common with the neuroglial cells, neuronal iron from the cytosolic LIP can be stored in ferritin or used for the metallation of Fe-containing proteins. As indicated above, astrocytes take up low-molecular-weight iron from the BBB. Microglia have been reported to have both transferrin receptors and ferroportin [18, 19]. The main iron-containing cells in adult brain, reflecting their involvement in myelin formation are the oligodendrocytes, although in the course of brain development, microglia are the first to stain for iron as ferritin, with iron staining shifting subsequently to oligodendrocytes [20].

Mature oligodendrocytes do not express transferrin receptors, and it has been proposed that they take up iron as H-chain ferritin via a specific receptor, the membrane protein TIM2 (Tcell immunoglobulin domain 2 protein) [21, 22]. However, this poses several questions – whether microglia secrete H-chain ferritin oligomers, how one can explain iron uptake by oligodendrocytes in man if the TIM2 gene is absent from the human genome [23], and how can a physiologically coherent mechanism for delivery of an essential cellular nutrient be carried out by a protein with a variable yet important amount of a toxic transition metal?

1.2. Iron Homeostasis

Having outlined the way in which iron is taken up, stored, and exported from the different cell types within the brain, we now turn to the way in which brain iron homeostasis is regulated. In most cells of the body iron homeostasis is regulated at the post-transcriptional level [24, 25] by the iron regulatory proteins (IRPs), IRP1 and IRP2 (Figure 6) which, in conditions of iron insufficiency, bind to *cis*-regulatory RNA hairpin structures, known as iron regulatory elements (IREs). The IREs are located within the 5′-untranslated region (UTR) of the mRNAs of H- and L-chains of iron storage ferritins, the iron exporter FPN, δ-aminolevulinic acid synthase, the first and key regulatory enzyme of the heme biosynthetic pathway, the transcription factor hypoxia inducible factor 2α, and mitochondrial aconitase. They are also found in the 3′-UTRs of transferrin receptor 1 and one of two transcripts of DMT1. Binding of the IRPs to the

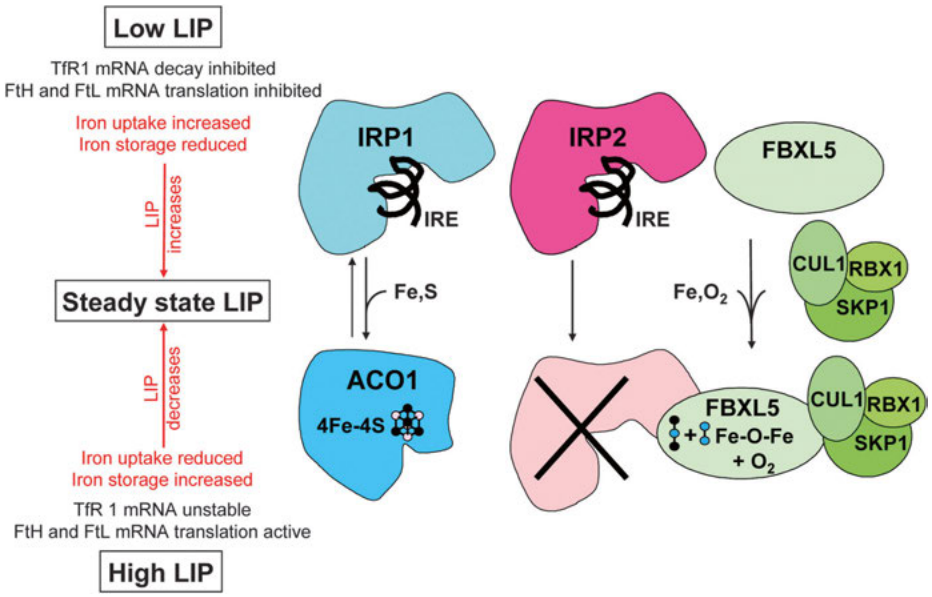

Figure 6. Feedback mechanisms that control cellular iron homeostasis. The scheme depicts IRP1 and IRP2, which are active as RNA-binding proteins at low LIP levels. By binding to IREs, they inhibit the translation or degradation of mRNAs encoding proteins required for cellular iron storage and import, thereby increasing the LIP. Once it has reached a sufficiently high concentration, labile free iron then contributes to the assembly of the [4Fe–4S] cluster that inactivates RNA binding of IRP1. Concomitantly, insertion of a diiron center into a hemerythrin-like domain of FBXL5 renders this protein more stable such that it combines with additional subunits to form an E3 ubiquitin ligase complex, which then binds IRP2 and induces its degradation by the proteasomal pathway. The assembly of these two iron centers corresponds to an iron-sensing mechanism, in which free iron acts on its own level through these elaborate feedback loops. Reproduced by permission from [25]; copyright 2015 Royal Society of Chemistry.

5′-UTRs prevents translation of the mRNAs, thereby preventing iron storage, export, and utilization. In contrast, binding to the 3′-UTRs prevents nuclease degradation of the mRNAs and allows the cells to take up iron from circulating transferrin and to release it from the endosomes, thereby increasing the intracellular iron level within the LIP. Once this level reaches a tipping point, the IREs lose their capacity to bind to the IREs, and as a consequence, the mRNAs with IREs in the 5′-UTR can now be translated, increasing iron storage, export, and utilization. Inversely, the mRNAs with IREs in the 3′-UTRs are degraded by nucleases, and iron uptake is prevented. Thus, 'iron controls iron' [25] – the iron content of the LIP acts as a trigger, switching the IRE/IRP system between its two states, thereby ensuring the fine tuning of intracellular iron metabolism. IRPs are ubiquitously expressed [25], and have probably evolved to maintain the cytoplasmic LIP at an appropriate level. IRP2 predominates in the CNS [26], and gene knock-out studies show that IRP2, encoded by the IREB-2 gene, is the master regulator of brain iron levels [27]. IRP2 deficiency causes iron dysregulation in the CNS, and in motor neurons of spinal cord, characterized by increased expression of ferritin, decreased expression of TfR1, and significant iron accumulation in neurons throughout the brain.

Systemic iron homeostasis involves the hepcidin/FPN axis which coordinates iron export into the bloodstream from duodenal enterocytes, macrophages involved in iron recycling from effete red blood cells and periportal hepatocytes, through the interaction between FPN and the peptide hepcidin (Figure 7) [28]. Hepcidin is synthesized and secreted by the liver, and its expression is regulated by a number of factors, including iron status, erythopoietic demand, and inflammation [1, 2]. Binding of hepcidin to FPN triggers ubiquitination of multiple lysine residues in FPN, accompanied by its endocytosis, internalization, and degradation, thereby abolishing iron export. When hepcidin synthesis stops, FPN can once again resume iron exportation.

Does this concept of hepcidin as the key element of systemic iron homeostasis, together with FPN, still hold within the confines of the brain? Firstly, is hepcidin able to cross the BBB, or is it synthesized within the brain, and if so, where is it synthesized and in which type(s) of cell? The answer is probably that it does not cross the BBB and that it may well be synthesized by astrocytes, as we will see later. Secondly, when we introduce hepcidin into brain cells (usually in cell culture), do they respond in the same way as in the periphery? The answer is unquestionably yes – iron exportation stops and iron assimilation in certain cells increases, notably as ferritin. And so to our final questions – what are the mechanisms and what are the extracellular parameters (transferrin saturation and/or concentration, inflammation …) which regulate hepcidin expression from the cells in which it is synthesized? For these, we still await the reply.

Another factor which increases the difficulty in understanding brain iron homeostasis is that as a function of their localization within different brain regions, the neurons, and neuroglial cells can find themselves in quite different environments. For example, dopaminergic neurons of the substantia nigra (SN) and locus coeruleus (LC) deposit iron in neuromelanin (giving them their characteristic dark color), and the way in which this is regulated is unknown.

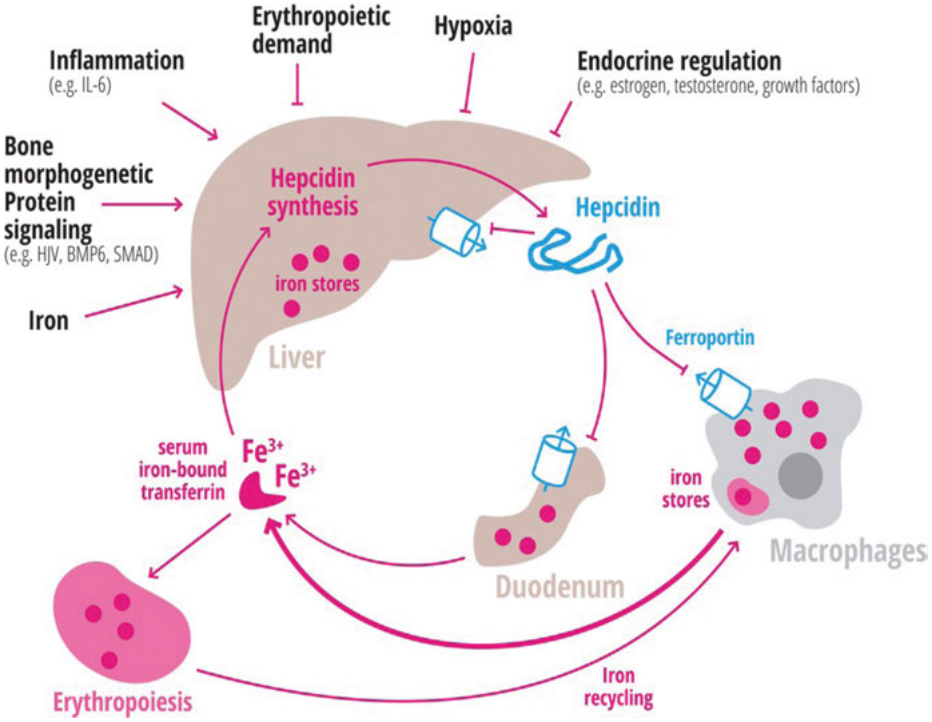

Figure 7. Regulation of hepatic hepcidin production. Hepatic hepcidin synthesis is regulated by iron, bone morphogenetic protein signaling, inflammation, erythropoiesis, hypoxia or endocrine stimuli. FPN1, which is expressed predominantly in hepatocytes, macrophages, and enterocytes is internalized and degraded following hepcidin binding. Iron is transported in the blood bound to transferrin. Most iron is required for erythropoiesis. Aging erythrocytes that exceed a life-span of approximately 120 days are recycled in macrophages. Transferrin-iron is a critical indicator for systemic iron homeostasis and regulator of hepcidin expression. Reproduced from [28]; this is an open access article distributed under the Creative Commons Attribution License (CC BY).

1.3. Iron Content of Different Brain Regions

The brain iron content is less than 2 % of total body iron content. In early studies *post mortem* material was used for the quantitation of iron in a number of brain regions. In 1958 Hallgren and Sourander [29] quantitated iron in several brain regions after acid digestion and quantitation of iron by colorimetry with orthophenanthroline. The highest iron content was in the globus pallidus (GP) followed by the red nucleus, SN, putamen, and dentate nucleus. The lowest iron content was in different regions of the cortex. Later studies [30] used Perl's and Turnbull's staining methodology to make a detailed quantitative regional analysis of non-heme iron in the human brain. The highest levels of stainable iron were in the GP and SN with moderate staining intensity in the forebrain, midbrain, and cerebellar structures. The brain-stem and spinal cord generally only

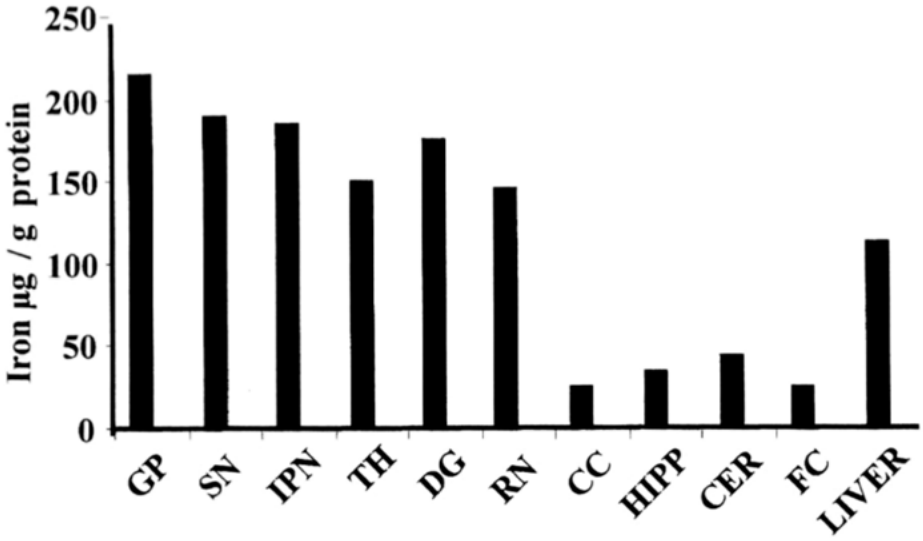

Figure 8. Distribution of iron in human brains. Terms: GP, globus pallidus; SN, substantia nigra; IPN, interpeduncular nucleus; TH, thalamus; DG, dentate gyrus; CC, cerebral cortex; HIPP, hippocampus; CER, cerebellum; FC, frontal cortex. Note that a similar type of distribution is also observed for the monkey, rat, and dog brains. Reproduced by permission from [31]; copyright 2004 John Wiley & Sons.

showed staining with the intensification procedure and even this was of low intensity. Microscopically, non-heme iron was present predominantly in glial cells as fine cytoplasmic granules. Iron-positive granules appeared to be free in the neuropil as well as around blood vessels in the GP, striatum, and SN. The neuropil shows a fibrous impregnation when stained for iron which is, in part, derived from glial processes, myelinated fibers, and fiber bundles. Neurons showed low reactivity for iron. In the GP and SN zona reticulata, neurons with highly stainable iron content are found with granular cytoplasmic iron reactivity similar to that seen in the local glial cells [30]. Later studies by Götz et al. [31] reported high iron concentrations in GP (21.3 mg/100 g fresh tissue), with high iron amounts also in dentate gyrus, interpeduncular nucleus, thalamus, ventral palladium, nucleus basalis, and red nucleus (Figure 8). The regions of the brain associated with motor functions tend to have higher iron content that non-motor-related regions.

With the advent of magnetic resonance imaging (MRI) techniques such studies of brain iron concentrations in different brain regions can now be performed *in vivo*. Magnetic susceptibility can provide a direct and reliable quantitative measurement of iron content and can be used clinically, at least in regions with high iron content. Wharton and Bowtell [32a] showed that high quality whole-brain susceptibility mapping at 7 T was feasible, and confirmed that the highest amounts of iron was present in the GP and SN and lowest in thalamus, and upper grey matter (Figure 9).

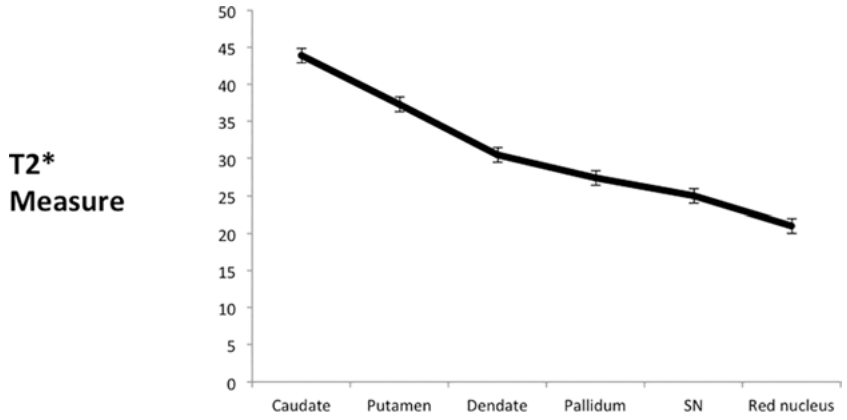

Figure 9. MRI measurements of control brains using a 3T Siemens Verio (Siemens Healthcare, Erlangen, Germany) equipped with a 32-channel phased array head coil. T2* measurement are inversely related to the iron concentrations in the different brain regions.

1.4. The Aging Brain: Significance of Increasing Iron Content

One of the major achievements of the modern era is the extension of the human lifespan through improvements in medical care, such as the use of antibiotics and screening programs for various diseases, nutrition, sanitation, and access to clean water. Over the last 100 years, life expectancy at birth in the UK has risen by almost 30 years so that life expectancy in both men and women is over 80 years. In a shifting population demographic, almost 1 in 5 of the UK's total population is aged 65 or over and this is expected to rise to 1 in 4 by 2050. Therefore there is an urgent need to ensure that there is healthy aging well into the 80s [32b].

With aging, cells may enter a senescent state which will have a profound effect on both molecular and biochemical processes [33]. For example, there may be a decline in the maintenance of repressive heterochromatin, an increased mutation burden as DNA repair processes decline, changes in epigenetic markers and decreases in stem cell renewal. In response to genomic and epigenomic damage, there is upregulation of micro-RNA-29, which will target IREB-2, the gene encoding the iron regulatory protein IRP-2 [34]. Biochemically impaired mitochondrial and metabolic function may occur, there could be accumulation of misfolded proteins, as well as a decline in cell communication and signalling. Upregulation of genes which promote inflammation may occur, which will result in an increased production of proinflammatory cytokines and chemokines, chronic inflammation (inflammaging), leading to upregulation of hepcidin [35] thereby promoting intracellular iron storage. Increases in circulating levels of IL-6 and the C-reactive protein have been reported in the elderly [36, 37]. Indeed, higher IL-6 is an independent predictor of mortality and morbidity in older adults [36]. IL-6 plays an important role in iron homeostasis as it has been demonstrated that IL-6 upregulates both IRP1 and DMT1 expression and down-regulates FPN1 expression through c-Jun N-terminal kinase signaling pathways in BV2 microglial cells [38].

1.5. Iron Accumulation in the Brain with Aging

With aging, the iron content of various brain regions increases, notably in the SN, putamen, globus pallidus, caudate nucleus, and cortex, while other regions such as the LC retain low iron concentrations throughout life [3]. A variety of explanations have been presented for this, which include increased BB permeability, inflammation, inflammaging, and, as discussed later, redistribution of iron within the brain and changes in iron homoeostasis [3]. Why there should be a selective increase of iron in only some brain areas is unknown but importantly such regions with an increased iron burden with age are often involved in neurodegenerative diseases.

In normal circumstances, the oligodendrocytes within the brain contain the highest iron content while astrocytes show low cellular iron [39].With aging microglia and astrocytes accumulate iron [40] which could be a result of inflammatory processes, both cell types being able to respond to inflammatory stimuli. No changes in the iron content of aged oligodendrocytes have been identified despite their higher concentrations of cellular iron [41]. Iron is mostly contained within ferritin, a mixture of H- or L-ferritin oligomers (heavy or light chain); neurons express predominantly H-ferritin while microglia express mostly L-ferritin [42, 43].

Dopaminergic neurons of the SN and the noradrenergic neurons of the LC show the presence of the pigment neuromelanin (NM), a black insoluble molecule composed of different components of melanin, proteins, lipids, and metal ions, particularly iron. NM is synthesized from L-dopa in dopaminergic cells and forms stable complexes with Fe(III). NM is located within cytosolic organelles, its size varying between 0.5 and 3.0 μm, surrounded by a double membrane. Early histological studies showed contrasting results; NM pigment concentration increased with age [40] or was a linear fallout of pigmented neurons with advancing age in the pars compacta of the caudal SN [44]. More recently, MRI has been utilized to identify NM in the brains of elderly subjects. In one study where *in vivo* high-resolution T1-weighted FLASH imaging at 3 T was used to localize the human LC in young and older adults, the topographical distribution of LC signal intensity, equivalent to neuromelanin, changed across the entire rostrocaudal length of the LC revealing age-related differences that were non-uniform [45]. In another study, neuromelanin-sensitive weighted MRI was used to visualize and measure the mean signal intensity of the LC in healthy younger and older adults (LC being a source of noradrenaline in the cortex and thought to modulate attention and memory) [46] and showed that LC signal intensity was significantly higher in older than younger adults and significantly lower in women than in men. In addition, both verbal intelligence and a composite reserve score were positively associated with LC signal intensity in older adults. Such results concluded that *in vivo* estimates of LC neuromelanin signal intensity were associated with cognitive reserve in normal aging. Fast spin-echo T1-weighted MRI at 3T, which was optimized to detect neuromelanin-related contrast showed significant positive correlation with normal aging and was slightly but significantly higher in women than in men [47].

2. NEURODEGENERATIVE DISEASES, DISRUPTION OF IRON HOMEOSTASIS, AND IRON SPECIATION

2.1. Parkinson's Disease

Parkinson's disease (PD) is the second most common form of motor system degeneration, and is characterized by a progressive loss of dopaminergic neurons in the SN pars compacta in the ventral midbrain. There is an increased accumulation of iron in the SN, with smaller accumulation of iron in other brain regions such as red nuclei, GP, and cortex of PD patients. As the severity of the disease increases the total iron content increases in the SN which correlates with motor disability [48]. The reason for such accumulation of iron in the SN of patients with PD is unclear, but various factors have been suggested: increased permeability or dysfunction of the BBB; increased pro-inflammatory state; increase of lactoferrin receptors in neurons and microvessels; increased expression of DMT1 in dopamine neurons; altered iron transport by transferrin-TFR type 2; and mutations in genes relevant to iron transport and binding (reviewed in [3]).

Semi-quantitative histochemical studies of the SN show that iron deposits are present within the neurons and glia of the SN, putamen, and GP, with an increase of ferritin-loaded microglia cells in the SN [49]. One of the characteristic histopathological signs of PD is the presence of Lewy bodies, which are composed of aggregated α-synuclein, inside the neurons. The olfactory bulb is typically the first region in the body to accumulate α-synuclein aggregates [50]. Early studies indicated that iron was present in Lewy bodies and was redox-active. A method that detects redox-active iron *in situ* was able to demonstrate strong labeling of Lewy bodies in SN pars compacta neurons in PD [51], but suggested that such sequestration of iron was a protective rather than degenerative mechanism. The increase of iron in the SN of PD patients is associated with increased ferritin and neuromelanin iron loading [49, 52] as well as increased expression of divalent metal transporter 1 that may contribute to PD pathogenesis via its capacity to transport ferrous iron [53]. Studies of the temporal cortex of PD brains identified lower iron when compared with age-matched healthy controls as well as decreased levels of DMT1+IRE, TfR1, FPN1, and IRP1 [54]. The authors concluded that iron redistribution may occur between the temporal cortex and the SN of patients with PD. Intense microgliosis occurs around extraneuronal neuromelanin (released by dying neurons) in the SN of patients with PD [55, 56], which could be an important factor in inducing iron accumulation within cells.

Preliminary reports suggested that identification of neuromelanin (rather than iron) by MRI might be feasible in patients with PD [57, 58]. Indeed, the loss of NM in the LC and SN, together with the associated loss in the ability to sequester iron, might be a characteristic sign of PD [59]. There are many studies investigating neuromelanin in PD brains. For example, using three dimensional neuromelanin-sensitive ^{31}P MRI, for discrimination of patients with PD from healthy controls [60], showed that signal densities and contrast ratios were significantly lower in the SN of PD patients compared to controls. Another study of 13 late stage PD patients and 12 *de novo* PD patients (2–5 years duration) showed that

the NM signal was significantly decreased in late stage PD patients compared to *de novo* PD while in the lateral SN region, a decrease in the contrast ratios was detected in all PD groups compared to controls. Interestingly, the NM signal area significantly correlated with Hoehn Yahr Stage and Movement Disorder Society Unified Parkinson's Disease Rating Scale part II, while a weak correlation was found with part III [61]. Such results may indicate that measurement of the NM content in the brain by MRI techniques could be an important diagnostic approach.

2.2. Alzheimer's Disease

Alzheimer's disease (AD) is a fatal age-related neurodegenerative disease which results in cognitive decline, memory loss, and psychosis. AD clinically is characterized by progressive dementia. Initial symptoms are short term memory loss which is followed by extensive neuronal loss in the hippocampus and selected cortical and subcortical area. There is abnormal protein processing with the accumulation of the peptide β-amyloid (Aβ), which is deposited extracellularly and manifests as amyloid plaques, and the protein tau in the form of neurofibrillary tangles. Both of these abnormal protein aggregates are definitive markers of the disease in *post mortem* material.

There is considerable evidence that there is defective homeostasis of iron in the brain. Increases in the iron content of AD have been in the literature for over 50 years. In a recent paper which made a comprehensive systematic meta-analysis and review of over 2556 publications, 43 eligible studies were identified where the iron content in AD serum, CSF or brain tissue had been studied. In nineteen studies, the iron content in twelve selected brain regions was analyzed and it was concluded that eight specific brain regions (the temporal, parietal, and frontal lobes) had higher iron concentrations which correlated with the clinical diagnosis of AD [62]. A recent laser ablation inductively coupled mass spectroscopy study also identified increases of iron in the frontal cortex [63].

It is of interest to know in which form the increased iron is present – whether as the redox-inactive iron ferrihydrite within ferritin or in a more active redox state. As early as 1992, Kirschvink and his team [64] reported the presence of magnetite in AD brains. Magnetite (Fe_3O_4) is a ferromagnetic iron oxide, with alternating lattices of Fe^{3+} and Fe^{2+} which are antiferromagnetically coupled. More recent studies of AD tissues have confirmed that there are increased proportions of iron containing these Fe^{2+} biomineralization products [65–68]. Such results suggest that there is abnormal iron biomineralization in the AD brain, a process which occurs in plaques or the surrounding diseased tissue. Furthermore, the size distribution of the magnetite cores implies formation from a ferritin precursor, possibly implicating a malfunction of the primary iron storage protein in the brain [66]. Interestingly, it was reported that *in vitro* the amyloid peptide, Aβ (1–42), is capable of reducing ferrihydrite to a pure Fe^{2+} mineral where antiferromagnetically ordered Fe^{2+} cations occupy two non-equivalent crystal symmetry sites [69]. Overall these results suggest that Aβ is involved in the

formation of wüstite (FeO) via reduction of ferrihydrite. The formation of such redox active ions may play an important role in AD pathogenesis since such a sustained source of ROS would be capable of inducing widespread neuronal damage.

Pathologically, iron is present primarily within ferritin, both in amyloid plaques and neurofibrillary tangles. Whether this might induce a deficiency of iron in adjacent areas is unknown [70].

It remains unclear whether iron within the ferrihydrite core of the ferritin, or as unligated iron, could drive the translation of several mRNA species, e.g., APP, as well as being involved in iron toxicity to generate hydroxyl radicals. *In vitro*, Aβ (1–42) has been reported to convert the redox inactive ferric iron (Fe^{3+}) (present as ferrihydrite) to the redox-active ferrous (Fe^{2+}) iron, which can act as a catalyst of the Fenton reaction to generate toxic free radicals, which could drive neuroinflammation [69, 71]. Furthermore, the presence of magnetite and wüstite in the AD brain seems to indicate that Aβ (1–42) can oxidize Fe^{3+} to Fe^{2+} [66, 68, 72]. Both iron mineralized forms, magnetite/maghemite, are found inside and outside of ferritin. Neuroinflammation plays an important role in AD, activated microglia increasing their intracellular ferritin content, mainly as L-ferritin. Weakly-bound iron is also associated with microglia over-activation, which can promote neurodegeneration in AD.

2.3. Huntington's Disease and Friedreich's Ataxia

Huntington's disease (HD) is the most common of nine neurological disorders which result from expansion of CAG repeats coding for a poly-Q tract in the corresponding proteins, which are particularly prone to aggregation [73]. HD has a frequency of 10.6–13.7 individuals per 100 000 in Western populations [74] and is characterized by a progressive degeneration of large sensory neurons and cardiomyopathies associated with defects in both motor and cognitive functions. The Huntington protein, huntingtin (HTT), is one of the longest proteins known (3144 residues long) and is expressed ubiquitously in the body with the highest levels in CNS neurons. While the function of HTT is not clear, it interacts with several proteins involved in transcription, cell signalling and intracellular transport. Alterations in brain iron metabolism have been reported, resulting in increased iron deposition in caudate, putamen, and cortex [75].

Friedreich's ataxia (FA) is one of a number of neurological disorders caused by the anomalous expansion of unstable nucleotide repeats in which, unlike in HD, the nucleotide expansion occurs in the non-coding part of the gene (the introns). FA is characterized by a progressive degeneration of large sensory neurons and cardiomyopathies [76]. Although rare, FA is the most frequent inherited ataxia, with an estimated prevalence of two to four people in 100 000 individuals. Most patients carry homozygous GAA expansions in the first intron of the frataxin gene on chromosome 9. Whereas the critical pathologic triplet repeat threshold is 66 repeats, the average expansion can be as many as 890 GAA repeats. This results in the partial silencing of frataxin, a small mitochondrial

protein which plays an essential role in iron-sulfur cluster (ISC) biogenesis, an essential metabolic pathway found in all organisms [77, 78]. Frataxin interacts directly with the two central components of the ISC biogenesis machine, the NFS1/IscU complex [79]. Correct ISC synthesis in mitochondria is closely linked to cellular iron homeostasis [80] and lack of frataxin therefore causes dysregulation of iron metabolism. As a consequence, failure to assemble mitochondrial Fe-S proteins results in increased cellular iron acquisition, mitochondrial iron overload [81], and mitochondrial iron deposits in some FA patients [82].

2.4. Neurodegeneration with Brain Iron Accumulation

Syndromes of neurodegeneration with brain iron accumulation (NBIA) are a group of inherited neurodegenerative disorders characterized by abnormalities in brain iron metabolism with excess iron accumulation, particularly in the basal ganglia, and are frequently associated with cerebral and cerebellar atrophy [83–85]. Patients present with a progressive hypo- and/or hyperkinetic movement disorder, parkinsonism, and neuropsychiatric disturbances. NBIA are relatively rare disorders (Table 1), with a cumulative prevalence of less than 1 in 10^5. The two most common conditions are pantothenate kinase-associated neurodegeneration (PKAN) (35–50 % of NBIA patients), and phospholipase A2 group VI (*PLA2G6*)-associated neurodegeneration (PLAN) (around 20 % of NBIA patients). Mutations in genes such as *Pank2, Pla2G6, COASY*, *FA2H, ATP13A2,* and *WDR45* induce iron loading in the brain although their protein functions are not related to iron metabolism. However, of these, the first four encode for proteins localized in mitochondria, which are directly or indirectly implicated in lipid metabolism and mitochondrial membrane remodelling (Figure 10). We will briefly discuss PKAN and PLAN, before turning to two less common NBIAs, aceruloplasminemia and neuroferritinopathy, which clearly involve genes coding for proteins of iron metabolism.

PKAN (formerly known as Hallervorden-Spatz syndrome) typically presents in early childhood and is caused by mutations in pantothenate kinase, the first enzyme in the biosynthesis of coenzyme A from pantothenate. A pathophysiologically closely related NBIA is COASY-associated neurodegeneration (COPAN), due to mutations in the CoA synthase (COASY) gene. Both PKAN and COPAN map into CoA synthesis, a key molecule in the metabolism of fatty acids, carbohydrates, amino acids, and ketone bodies (Figure 10). A characteristic diagnostic feature of PKAN in T_2-weighted MRI scans is “the eye of the tiger” (Figure 11), which shows diffuse bilateral low signal intensity of the GP (due to iron deposition) with a region of hyperintensity in the internal segment due to axonal vacuolization [86].

PLAN typically occurs because of mutations in the phospholipase A2 group VI gene (*PLA2G6*) [87], encoding a calcium-independent phospholipase A2 enzyme involved in phospholipid metabolism, signal transduction, cell proliferation, and apoptosis. Cerebellar atrophy is an early sign in MRI scans, while evidence of iron accumulation appears later [88].

Table 1. List of neurodegeneration with brain iron accumulation (NBIA).

Human disorder	Brain regions interested by iron deposition	Symptomatology
Aceruloplasminemia	Dentate nucleus, globus pallidus, putamen, caudate	Diabetes, anemia, dementia, dystonia, dysarthria
Neuroferritinopathy; hereditary ferritinopathy; NBIA type III	Dense ferritin-Fe spheroid inclusions in dentate nuclei, globus pallidus, putamen, caudate, thalamus, and red nuclei	Dementia, dystonia, dysarthria in some cases cognitive decline
Pantothenate kinase-associated neurodegeneration (PKAN, NBIA type 1, Hallervorden-Spatz syndrome)	Globus pallidus (eye of the tiger)	Dystonia, with predominant oro-lingual-mandibular involvement, and spasticity
Phospholipase 2, group VI-associated neurodegeneration (PLAN, NBIA type II, INAD1; Karak syndrome)	Globus pallidus and substantia nigra (in < 50 % of patients)	Infantile neuroaxonal dystrophy: hypotonia, visual disturbance, motor and mental retardation. Atypical neuroaxonal dystrophy (late onset): dystonia, dementia, and parkinsonism
Mitochondrial membrane protein-associated neurodegeneration (MPAN)	Globus pallidus and substantia nigra	Dysarthria, gait abnormalities, dystonia, and parkinsonism
Fatty acid hydroxylase-associated neurodegeneration (FHAN)	Globus pallidus and substantia nigra in some patients	Dysarthria, gait abnormalities, dystonia, and parkinsonism
COASY protein-associated neurodegeneration (CoPAN)	Globus pallidus and substantia nigra	Oro-mandibular dystonia with dysarthria and parkinsonism, cognitive impairment
β-Propeller protein-associated neurodegeneration (BPAN)	Globus pallidus and substantia nigra	Parkinsonism, dystonia, dementia, and global development delay
Kufor-Rakeb syndrome	Globus pallidus and substantia nigra in few cases	Dystonia and parkinsonism
Woodhouse-Sakati syndrome	Globus pallidus and substantia nigra in few cases	Dystonia and deafness

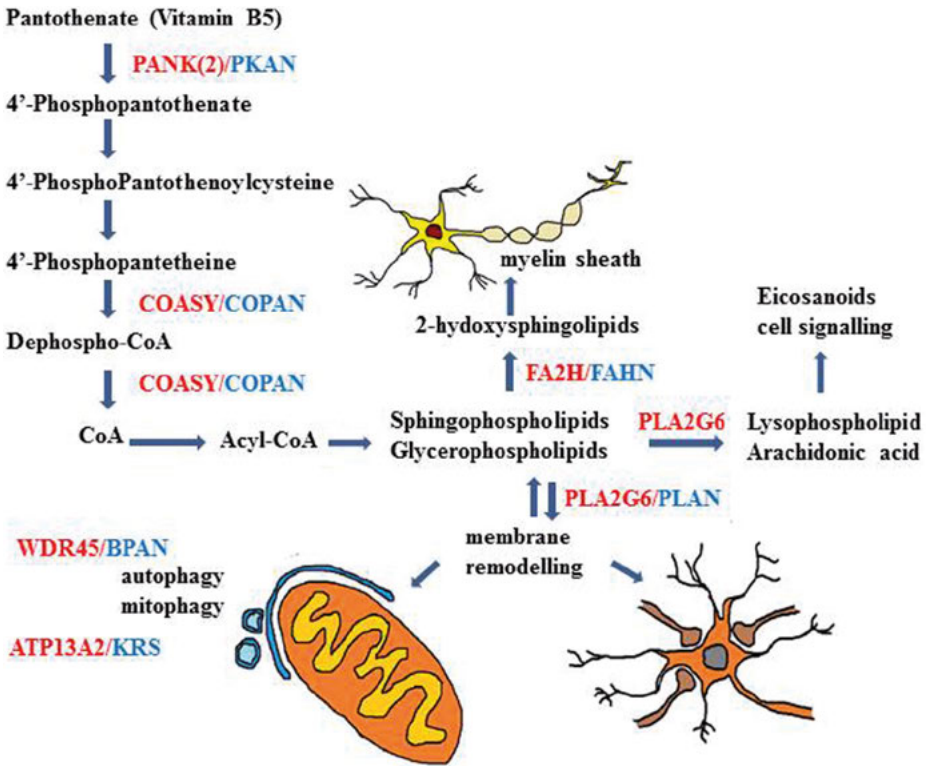

Figure 10. Schematic description of genes and biochemical pathways involved in different types of NBIA disorders. The biochemical pathways of lipid metabolism and membrane/organelles (mitochondria) remodeling seem to play an important mechanistic role in many of the genetic NBIA disorders so far identified. Reproduced from [85]; this is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY).

To date, only two genes coding for iron proteins have been identified as responsible for NBIA subtypes: mutations in the ceruloplasmin gene (*CP*) causing aceruloplasminemia [89] and the L-ferritin gene (*FTL*) altered in neuroferritinopathy [90]. Defects in these genes lead to early deposits of iron in the striatum, thalamus, GP, dentate nuclei, cortex, and retina.

Ceruloplasmin, the principal copper-binding protein in serum, is a ferroxidase and it has been proposed that ceruloplasmin has a custodial role *in vivo*, ensuring that Fe^{2+} released from cells via ferroportin is oxidized to the potentially less toxic Fe^{3+} prior to its incorporation into apotransferrin. The central role that ceruloplasmin plays in cellular iron efflux is underlined by aceruloplasminemia, a neurodegenerative disease associated with the absence of functional ceruloplasmin due to the presence of inherited mutations within the ceruloplasmin gene. This condition results in disruption of iron homeostasis, with extensive iron accumulation in both parenchymal tissues and in the basal ganglia in the brain, with iron accumulation in both neurons and microglia. However, in these pa-

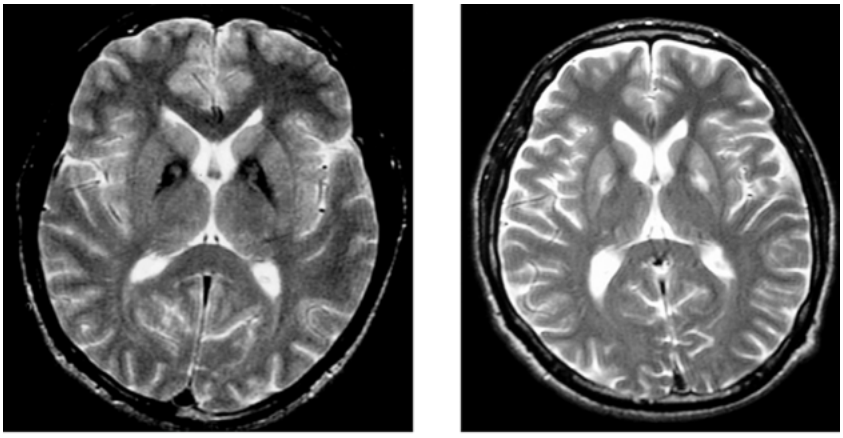

Figure 11. (**a**) Pantothenate kinase-associated neurodegeneration (PKAN) 'the eye of the tiger' sign. This characteristic T2-weighted image shows the diffuse bilateral low signal intensity of the globus pallidus (due to iron deposition), with a region of hyperintensity in the internal segment due to axonal vaculization. Contrast this with (**b**): Neuroferritinopathy, where the T2-weighted image shows a high signal bilaterally in the globus pallidus. Reproduced by permission from [129]: copyright 2004 Elsevier).

tients, as in aceruloplasminemic mice, both copper transport and metabolism are normal, providing strong evidence against the role of ceruloplasmin as a major copper transporter. In the brain a GPI-anchored form of ceruloplasmin is expressed on the surface of astrocytes [91]. It is thought that in its absence, iron will initially accumulate in a non-toxic form within ferritin, which gradually becomes filled to capacity because of the reduced ability of the cell to efflux iron. The cascade of events leading to neuronal death are not fully elucidated but oxidative stress, exacerbated by heavy metal accumulation is the primary cellular toxic event.

Neuroferritinopathy is a rare monogenic progressive movement disorder caused by mutations in the gene encoding the L chain of ferritin (FTL). Ferritins are tetracosameric proteins (24mers), made up of two types of subunits, H and L, which are assembled in different proportions [92]. The 24 subunits form a hollow protein shell within which an iron core is stored. The three dimensional structure of the two subunits is very similar, consisting of a bundle of four parallel helices, and a fifth smaller helix called E, at the C-terminus, which is directed toward the center of the cavity. Seven pathogenic mutations have been reported in exon 4 of the *FTL1* gene [93], and all are predicted to result in alterations of helix E, extending the ferritin light chain at the site of the four-fold channel (Figure 12). These mutant FTL subunits can readily undergo assembly with wild-type FTH or FTL chains, forming dysfunctional ferritin shells [94] which exhibit iron loading-induced ferritin aggregation and decreased iron incorporation. Although the structure of the spherical shell is maintained in the crystal structure

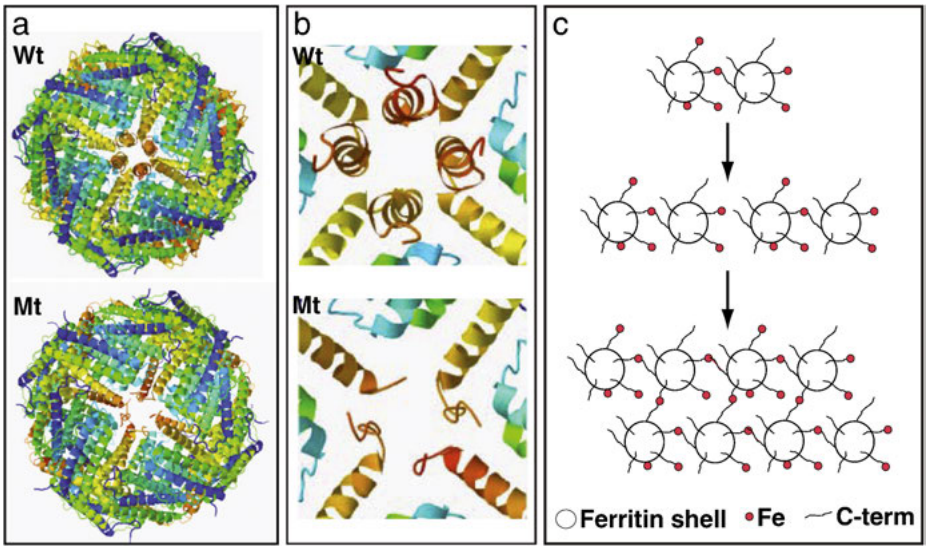

Figure 12. Ferritin structural disruption and aggregation caused by mutant FTL subunits. (**a**) The structure of the spherical shell is maintained in mutant ferritin as seen in the crystallographic structures of Wt- (wild type) and Mt- (mutant) FTL homopolymers viewed down one of their 4-fold axes. (**b**) Close up views of the 4-fold pore from the interior of the Wt- and Mt-FTL structures show remarkable disruption in Mt-FTL making the pores unstable and leaky. Note that since the last 26 amino acids of Mt-FTL remained unaccounted for crystallographically, mutant C-termini are substantially longer than represented in (b), and if extended could reach as far as the diameter of the ferritin shell. (**c**) Iron loading-induced aggregation of Mt-FTL homopolymers is consistent with a model in which iron binds to the unraveled and extended portion of the mutant C-termini on two different ferritin shells bridging them and initiating a gradual accumulation of ferritin and iron into a precipitate. Bridging is not necessarily restricted to C-termini and may become more general, e.g., between a C-terminal group and a surface amino acid which both have affinity for iron. Structures were taken from RCSB (code 2FG8 for Wt-FTL and 3HX2 for Mt-FTL). Reproduced by permission from [95]; copyright 2011 Elsevier.

of mutant (Mt-FTL) homopolymers, the 4-fold channels show remarkable disruption in Mt-FTL making the pores unstable and leaky. Iron loading-induced aggregation of Mt-FTL homopolymers is consistent with a model in which iron binds to the unraveled and extended portion of the mutant C-termini on two different ferritin shells bridging them and initiating a gradual accumulation of ferritin and iron into a precipitate [95].

3. IRON AND THE IMMUNE SYSTEM

Iron and the immune system are closely linked in that activation of cells will result in changes in iron fluxes across the cellular membrane. The immune system can be divided into two interactive systems, namely innate and adaptive immunity. Within the brain there are two glial cell types which predominantly

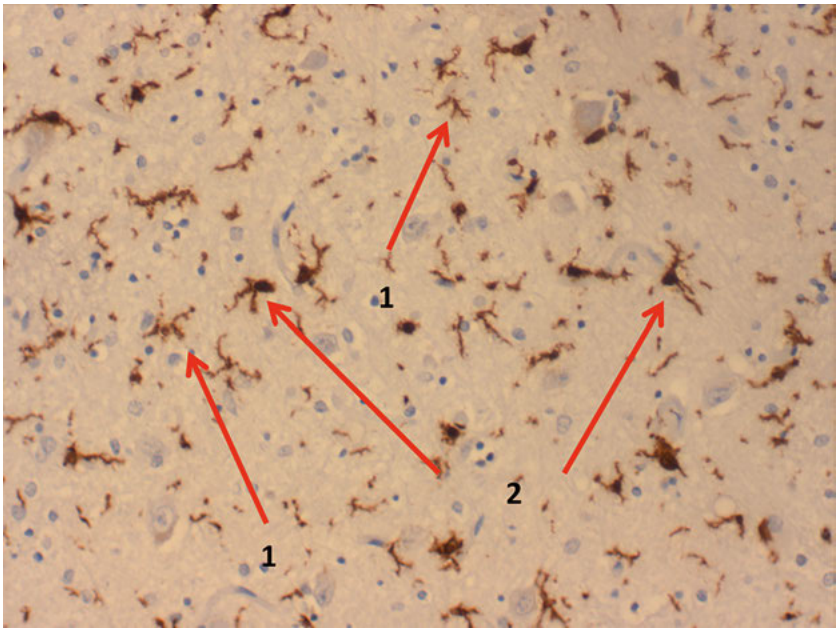

Figure 13. Immunohistochemical Iba staining for microglia in substania nigra region of a PD patient. Arrows indicate the two forms of microglia as well as the pigment, neuromelanin.

play an important response to any perceived abnormality, microglia, and astrocytes. Microglia are the resident macrophage population in the central nervous system and have many important functions. For example, they are involved in the maintenance of synapses, being able to interact with termini, spines, astrocytic processes, and the synaptic cleft (reviewed in [96]). Numerous neurotransmitter receptors are present on the microglia surface to augment such actions [97]. When challenged, microglia are capable of acquiring diverse and complex phenotypes which permit them to participate in (i) toxic response, (ii) immune regulation or (iii) injury resolution. Microglia are considered to be the primary mediators of neuroinflammation. In normal circumstances, in the healthy adult brain, microglia are in a non-activated state, displaying a ramified morphology (Figure 13). When abnormities are detected by a range of effector molecules, e.g., toll-like receptors, receptor for advanced glycation end products, nuclear factor kappa B, hypoxia factor 1, and heme oxygenase, a sequence of events is set in motion which results in the mobilization of cytokines, e.g., IL-6, neurotropic factors chemokines, reactive oxygen and reactive nitrogen species. Microglia convert to an amoeboid phenotype and release IL-6, IL-1β, and TNF-α. As a result of such an inflammatory response, there are changes in iron fluxes across the cell membrane which are thought to be mediated by hepcidin.

Astrocytes are the other major glial cell in the brain, and support metabolic functions of neurons and maintain CNS homeostasis as well as showing immune responses (reviewed in [98]). Furthermore, astrocyte responses to various pathological CNS insults, are characterized by variations in morphology and molecular

expression pattern and the release of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and CCL2 [99–102]. In addition iNOS and COX2 expressions may increase in activated astrocytes, resulting in neuroinflammation by the production of the cytokines NO and PGE2 [102, 103].

During an innate immune response, systems are rapidly mobilized to combat the perceived abnormality. A variety of effector molecules such as the pro-inflammatory cytokine IL-6 will bind to the IL-6 receptor to increase hepcidin production via two pathways. The IL-6 receptor has two major subunits, an 80-kDa subunit (IL-6-R) with ligand specificity, and a 130-kDa β subunit (gp130) with signal transduction capability. Binding of IL-6 to the IL-6-R subunit effects homodimerization of gp130, which recruits cytoplasmic JAK (Janus kinase) proteins to phosphorylate gp130. Two distinct downstream pathways are activated, the MAPK (mitogen-activated protein kinase) and the STAT (signal transducer and activator of transcription) pathways. Downstream events in the MAPK pathway culminate in the nuclear translocation of the extracellular signal-regulated kinase proteins, which in turn influence the activities of specific transcription factors. In the STAT pathway, phosphorylation of gp130 by JAK, STAT1 or STAT3 proteins bind to a distinct gp130 domain, which are then phosphorylated and released from gp130, dimerize, and translocate to the nucleus. In the nucleus the STAT dimers bind to specific *cis*-acting genomic elements, STATBS, in the hepcidin promotor to enhance hepcidin expression.

In recent years research on hepcidin in brain has focused on two questions – firstly, what is the source of hepcidin in brain and secondly, what is its role there? The first question effectively comes down to whether hepcidin is released from specific cells within the brain, or is derived from the liver, and appears to favor the former hypothesis. After inflammatory stimuli there is an increased expression of hepcidin in astrocytes and microglia, but not in neurons [104]. In addition, changes compatible with increases in hepcidin are evident, e.g., increased expression of DMT1 in neurons, astrocytes, and microglia, and a decreased expression of FPN1 in all three cell types [104]. The net result of such alterations is increased iron accumulation in neurons and microglia but not in astrocytes [104]. After administration of hepcidin to the lateral cerebral ventricle, FPN1 levels are decreased and iron overload can be identified in the cerebral cortex, hippocampus, and striatum [105] as well as in neurons. In other studies hepcidin expression has been detected in different brain areas, including the olfactory bulb, cortex, hippocampus, amygdala, thalamus, hypothalamus, mesencephalon, cerebellum pons, spinal cord, and dorsal root ganglia [105–107] but whether these are a source of hepcidin production in the brain awaits clarification.

Recent studies have identified the important role played by IL-6 in the production of hepcidin. It has been suggested that astrocytes are the source of hepcidin in the brain. You et al. [35] showed that when astrocyte hepcidin expression was disrupted, apoptosis of neurons was prevented, and FPN1 levels were maintained to prevent iron accumulation. Such data are consistent with a model whereby inflammation initiates an intercellular signalling cascade in which activated microglia, through IL-6 signalling, stimulate astrocytes to release hepcidin which, in turn, signals to neurons, via hepcidin, preventing their iron release (Figure 14), and resulting in neuronal cell death.

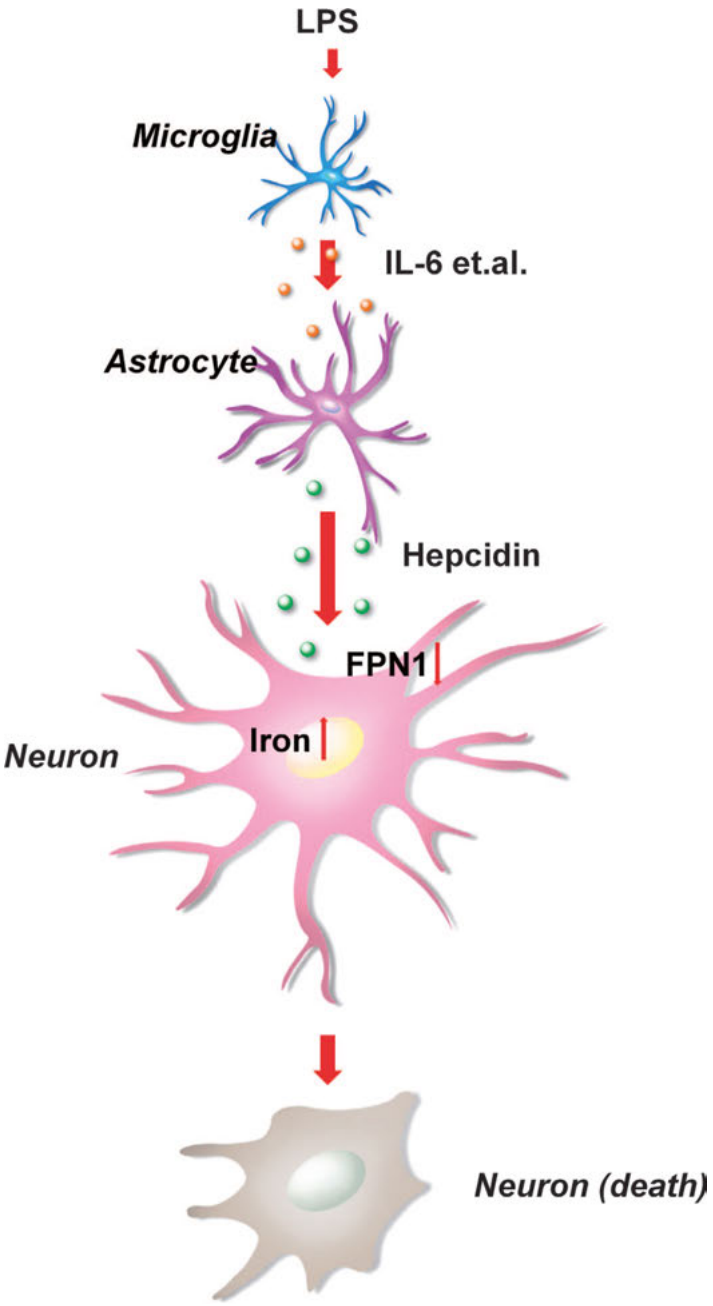

Figure 14. LPS-induced hepcidin expression in astrocytes is regulated by IL-6 from activated microglia. Astrocyte hepcidin is an effector in the pathway of LPS-mediated apoptosis of neurons by decreasing neuronal FPN1 protein levels, which leads to a concomitant increase in neuronal iron levels. Reproduced from [35]. This work is licensed under a Creative Commons Attribution 4.0 International License.

However, the effect of inflammation on hepcidin may not be entirely mediated by IL-6. For example, IL-6 knock-out mice maintain some hepcidin responsiveness to LPS [108]. In addition, a combination of both anti-IL-6 and anti-IL-1 neutralizing antibodies are necessary to abrogate completely the stimulatory effect of conditioned macrophage media on hepcidin in cultured mouse hepatocytes. Although IL-1 can induce IL-6 production in hepatocytes, studies indicate that IL-1 has a direct action in stimulating hepcidin expression in both mouse hepatocytes and human HuH7 cells [108, 109], but exactly what action IL-1 may have on hepcidin production in the brain awaits investigation. In addition, the signal-transduction pathway responsible for the effect of IL-1 on hepcidin expression remains to be elucidated.

Adaptive immunity is elicited by B and T lymphocytes which utilize immunoglobulins and T cell receptors, respectively, as antigen receptors to recognize non-self molecules. These receptors are generated via DNA rearrangement and respond to a wide range of antigens. T cells, B cells, macrophages, and natural killer cells appear to differ from one another in the ways in which they synthesize and utilize iron-binding proteins and in the amount of iron they take up and store [110]. The role played by iron in the function of adaptive immunity awaits clarification.

4. IRON CHELATION PRESENT AND FUTURE DIRECTIONS

4.1. Parkinson's Disease

Chelation of iron in the SN of PD brains was proposed many years ago. In our initial animal studies over 15 year ago [111, 112], we showed that two iron chelators, already in clinical use for the treatment of iron loading systemic diseases, i.e., deferrioxamine (DFO) and deferiprone (DFP), were able to cross the BBB and reduce the iron content in various brain regions. We subsequently were able to show that all three clinically approved iron chelators, DFO, DFP, and deferasirox could also induce neuroprotection in an animal model of PD [113].

One early study reported the beneficial use of deferiprone in one PD patient, such that after one year administration at 30 mg/kg/day there was an improvement in the UPDRS score and a decrease in iron accumulation in the bilateral dentate nuclei as well as the SN [114]. Subsequently, two clinical trials were initiated in 2011 and 2013 to investigate whether DFP would reduce iron content of the SN over a period of 6–9 months as well as induce changes in UPDRS. Both studies [115, 116] established the beneficial effects of the chelator. In the study of Devos et al. [115] R2* sequences were used to assess iron content in the SN, while UPDRS scores were acquired at various intervals to assess clinical parameters and serum ferritin was assayed as a marker of iron stores and inflammation. After 6–9 months decreases in SN iron content were present, there were improvements in UPDRS motor scores. In the second study [116] of 6 months duration, again decreases in SN iron content were assayed by T2* in patients receiving 30 mg/kg/

day and there were indications of an improvement in UPDRS. There was no evidence that the chelation therapy had an adverse effect on hematological parameters. The only disadvantage of the use of DFP was the incidence of neutropenia or agranulocytosis which occurred in 8 % of the patients, but which resolved rapidly upon cessation of the oral therapy. Such a side effect required that all PD patients entering into the clinical trial required a weekly blood test. Such positive results from the clinical trials have confirmed that iron chelation therapy could be a therapeutic option for the treatment of PD, although, if the side effects could be eradicated, it could become a more generally applicable treatment.

4.2. Alzheimer's Disease

Current therapies for Alzheimer's disease such as the acetylcholinesterase inhibitors and the NMDA receptor inhibitors may provide moderate symptomatic delay at various stages of the disease, but do not arrest the disease progression or effect meaningful remission. The confirmation of altered iron homeostasis in the brain of AD patients has opened the possibility of using iron chelators as a new therapeutic approach. Various animal models of AD have been utilized to investigate the therapeutic action of iron chelators. For example, the iron chelator (–) epigallocatechin-3-gallate and M-30 reduced APP expression in cultured cells [117, 118].

Although it is known that there is an increase of iron in various brain regions in AD patients, there has been no clinical evidence proven to support the use of chelating agents as an adjunctive treatment for AD. Tea polyphenols and curcumin have been advocated as metal chelators for the treatment of AD although the efficacy of such natural products awaits further investigation.

In 1991 McLachlan showed that DFO significantly reduced the behavioral and cognitive declines of AD patients [119]. However, since this ground-breaking study, it is only now that further studies are being undertaken to investigate the therapeutic efficacy of iron chelators in AD. Currently a phase 2, randomised, placebo-controlled, multicenter clinical trial (Neuroscience Trials Australia) to investigate the safety and efficacy of DFP has just started recruiting. Approximately 171 participants with mild cognitive impairment prodromal Alzheimer's disease and mild Alzheimer's disease will be recruited for the study. Delayed release of DFP will be used as this may prevent the unwanted side effects of neutropenia and agranulocytosis. The aim of this study is to ascertain whether DFP (15 mg/kg BID orally) slows cognitive decline in Alzheimer's patients. MRI will ascertain the iron content of brain regions during the period of the study (Clinical Trial Deferiprone to Delay Dementia).

4.3. Friedreich's Ataxia

In a clinical trial DFP (10–15 mg/kg, twice daily) was administered to Friedreich's ataxia (FA) patients in a small clinical trial over a 6 months period. In 9 adolescent patients with no overt cardiomyopathy, brain iron reduced significantly in the

dentate nuclei. The chelator treatment caused no apparent hematologic or neurologic side effects while reducing neuropathy and ataxic gait in the youngest patients [120]. In a second clinical trial DFP (10 mg/kg) was administered in combination with idebenone (a synthetic analog of coenzyme Q_{10}) (20 mg/kg) to 20 FA patients. No significant differences were observed in total ICARS scores when comparing baseline status and the end of the study in the whole group of patients. Echocardiography data showed a significant reduction of the interventricular septum thickness and in the left ventricular mass index after the start of the therapy. After 11 months of treatment, iron was reduced in the dentate nuclei, there was a worsening of posture and gait compared to baseline [121].

4.4. Pantothenate Kinase-Associated Neurodegeneration

Variable results were reported from the initial studies where the efficacy of deferiprone was investigated in pantothenate kinase-associated neurodegeneration (PKAN) patients. In the study of Abbruzzese et al. [122] four PKAN patients received DFP for one year but only two subjects showed mild-to-moderate improvement in motor symptoms, documented by changes in clinical rating scales and blinded assessment of video tapes. Decreases in the iron content of GP were identified although this did not necessarily correspond to an improvement in clinical symptoms. In the study by Zorzi et al. [123] nine PKAN patients received DFP for six months but showed no clinical improvement. It was concluded that six months of DFP treatment was too short and that the PKAN patients were severely affected and had been ill for a long period of time. In a later study five patients with PKAN (confirmed genetically) received DFP solution at 15 mg/kg orally twice daily for 48 months. At the end of this period, there was stabilization of motor symptoms, while MRI measurements of the brain identified a decrease in iron content of the GP [124]. A phase 2 randomized, double-blind, placebo-controlled clinical trial (NBIA Diseases Association) is now underway to investigate if DFP has an impact on the course of PKAN. The drug will be tested for 18 months in 90 patients. The study will be carried out at five centers: Munich (Germany), Warsaw (Poland), Milan (Italy), Newcastle (United Kingdom), and Oakland (California, United States).

5. CONCLUDING REMARKS

When we published the 1st edition of our book ‘Metal-based Neurodegeneration. From Molecular Mechanisms to Therapeutic Strategies’ [125] in 2006, our objective was to establish the basic principle that metal-derived oxidative stress played an important role in the development and progression of a number of neurodegenerative diseases. By the time that the 2nd edition appeared [126] in 2014 it was well established that transition metal ions, particularly iron, were present in increased levels in the affected brain regions in a great many neurode-

generative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, Friedreich's ataxia, and, of course, the neurodegenerative disorders with brain iron accumulation (NDIAs). However, it had also become apparent that neuroinflammation also contributed enormously to the progression and severity of virtually all neurodegenerative diseases.

Despite this, even today, the etiology of most neurodegenerative diseases which involve elevated iron in specific brain regions remains unknown. Exceptions are the monogenic disorders, HD, FA, and the NDIAs, where the primary cause is clearly mutations in the affected gene – huntingtin, frataxin, ceruloplasmin, and L-chain ferritin, for example, although other factors clearly affect the specific brain regions. For those neurodegenerative diseases in which multiple genes are involved, like AD, PD, and MS, causality remains an enigma, and defining their etiology will require a much more coherent understanding of brain iron homeostasis.

From a therapeutic point of view therefore, we are still only at the level of treatments targeted at symptoms of the disease rather than attacking the causes directly. Once again, exceptions are the monogenic disorders, where, for example CRISPR/Cas9-mediated gene editing has been shown to ameliorate neurotoxicity in a mouse model of HD [127]. However, the growing awareness of the important role of iron and of neuroinflammation has resulted in a timid, but thus far promising, use of iron chelation therapy in both PD and FA (*vide supra*). The results from the two clinical trials revealed positive results in that the iron content of the substania nigra decreased after 6–9 months of treatment and that the progression of the diseases was slowed. While there are still problems to be resolved, notably of neutropenia and granulocytosis observed in a very small number of patients ($<2\,\%$), the potential use of slow-release formulations of DFP may help to make the use of chelation therapy a more practical option. The potential to treat the neuroinflammatory aspects of many neurodegenerative diseases make antiinflammatory therapy an attractive therapeutic approach, and raises the possibility that combining chelators and antiinflammatories could prove an attractive approach. A recent epidemiological study showed a positive link between patients taking disease-modifying antirheumatic drugs particularly methotrexate to treat arthritis, and reducing their risk of developing dementia [128]. Clearly, further research is needed before any firm conclusions can be reached about arthritis drugs as a treatment for dementia.

Clearly, our long-term objective is to establish the definitive etiology of neurodegenerative diseases and to establish the role of iron therein. Meanwhile, symptomatomic approaches based on chelation and anti-inflammation therapy may represent our best current therapeutic strategies at present.

ABBREVIATIONS

Aβ(1–42)	amyloid β-peptide, residues 1–42
AD	Alzheimer's disease
ATP	adenosine 5′-triphosphate

BBB	blood-brain barrier
BCEC	brain capillary endothelial cells
BCSFB	blood-cerebrospinal fluid barrier
BID	twice a day
CNS	central nervous system
COASY	CoA synthase
COPAN	COASY-associated neurodegeneration
CP	ceruloplasmin
CRP	C-reactive protein
CSF	cerebrospinal fluid
DFO	deferrioxamine
DFP	deferriprone
DMT1	divalent metal transporter 1
ERK	extracellular signal-regulated kinase
FA	Friedreich's ataxia
FLASH	fast low angle shot (magnetic resonance imaging)
FPN	ferroportin
FSE	fast spin-echo
FTL	ferritin light chain
GM	grey matter
GPI	glycosylphosphatidylinositol
HD	Huntington's disease
HIF2α	hypoxia-inducible factor 2α
HTT	huntingtin
ICARS	International Cooperative Ataxia Rating Scale
IL	interleukin
IRE	iron regulatory element
IRP	iron regulatory protein
ISC	iron sulfur cluster
ISF	intertitial fluid
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
L-dopa	L-dopamine
LC	locus coeruleus
LIP	labile iron pool
LMW-Fe	low-molecular-weight iron
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MDS-UPDRS	Movement Disorder Society Unified Parkinson's Disease Rating Scale
MRI	magnetic resonance imaging
NDIA	neurodegenerative diseases with iron accumulation
NFκB	nuclear factor κB
NM	neuromelanin
NMDA	N-methyl-D-aspartate
NVU	neurovascular unit

PD	Parkinson's disease
PKAN	pantothenate kinase-associated neurodegeneration
PLAN	phospholipase A2 group VI -associated neurodegeneration
poly-Q	poly-glutamine
RNS	reactive nitrogen species
ROS	reactive oxygen species
SN	substantia nigra
STAT	signal transducer and activator of transcription
STEAP	six-transmembrane epithelial antigen of prostate
TfR1	transferrin receptor 1
TH	thalamus
TIM2	T cell immunoglobulin domain 2 protein
TLR	toll-like receptor
UPDRS	Unified Parkinson's Disease Rating Scale
3′-UTR	untranslated region of a mRNA located at the 3′-end, involved in mRNA stability
5′-UTR	untranslated region of a mRNA located at the 5′-end, involved in formation of the ribosomal initiation complex

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5

Infections Associated with Iron Administration

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Abstract: A dynamic interplay between the host and pathogen determines the course and outcome of infections. A central venue of this interplay is the struggle for iron, a micronutrient essential to both the mammalian host and virtually all microbes. The induction of the iron-regulatory hormone hepcidin is an integral part of the acute phase response. Hepcidin switches off cellular iron export via ferroportin-1 and sequesters the metal mainly within macrophages, which limits the transfer of iron to the serum to restrict its availability for extracellular microbes. When intracellular microbes are present within macrophages though, the opposite regulation is initiated because infected cells respond with increased ferroportin-1 expression and enhanced iron export as a strategy of iron withdrawal from engulfed bacteria.

Given these opposing regulations, it is not surprising that disturbances of mammalian iron homeostasis, be they attributable to genetic alterations, hematologic conditions, dietary iron deficiency or unconsidered iron supplementation, may affect the risk and course of infections. Therefore, acute, chronic or latent infections need to be adequately controlled by antimicrobial therapy before iron is administered to correct deficiency. Iron deficiency *per se* may negatively affect growth and development of children as well as cardiovascular performance and quality of life of patients. Of note, mild iron deficiency in regions with a high endemic burden of infections is associated with a reduced prevalence and a milder course of certain infections which may be traced back to effects of iron on innate and adaptive immune function as well as to restriction of iron for pathogens. Finally, absolute and functional causes of iron deficiency need to be differentiated, because in the latter form, oral iron supplementation is inefficient and intravenous application may adversely affect the course of the underlying disease such as a chronic infection.

This chapter summarizes our current knowledge on the regulation of iron metabolism and the interactions between iron and the immune response against microbes. Moreover, some of the unanswered questions on the association of iron administration and infections are addressed.

Keywords: anemia of chronic disease · anemia of inflammation · chronic kidney disease · ferritin index · ferroportin-1 · hepcidin · infection · iron administration · iron deficiency · macrophage · renal anemia · sepsis

1. INTRODUCTION

1.1. Iron in Biological Systems

In biological systems, iron exists as either ferrous (Fe^{2+}) or ferric (Fe^{3+}) ion. Due to its ability to switch between these two valences, iron is implicated in a broad range of redox reactions. Typically, these take place in a strictly regulated way catalyzed by enzymes carrying iron-containing functional groups such as heme or iron-sulfur clusters. Therefore, an adequate supply of iron is required for basic biochemical reactions including oxygen transport and storage, cellular respiration and DNA synthesis and repair [1–3]. In contrast, when surplus amounts of iron are present in an unbound labile form, iron can catalyze the

non-enzymatic generation of reactive oxygen species (ROS) via Fenton-/Haber-Weiss chemistry [4]. ROS in turn damage DNA, proteins, and lipids, resulting in cytotoxicity and tissue damage over time [5]. Therefore, iron plays a dual role in living cells because it has both vital and toxic qualities. Living cells have thus evolved sophisticated strategies to control iron fluxes and to maintain this labile iron pool (LIP) within a narrow range. Mechanisms of iron storage and cellular efflux are in place to counteract any potentially harmful expansion of the LIP. In addition, antioxidative mechanisms are induced to neutralize otherwise toxic ROS whenever they are produced in the presence of labile iron.

1.2. Regulation of Systemic and Cellular Iron Homeostasis

1.2.1. Intestinal Iron Absorption

In mammals, systemic iron homeostasis is maintained by the controlled transfer of iron to the circulation. Because no effective mechanism for the excretion of surplus iron exists, the two regulated processes to maintain systemic iron homeostasis are dietary iron absorption in the upper small intestine and iron recycling by macrophages [6].

Iron is present in food in diverse forms including complexes that contain heme or non-heme iron [7]. Heme-iron is taken up as intact molecule by the sequential action of transport proteins of the Slc48 (solute carrier-48) family at the apical surface of enterocytes and members of the Slc49 family basolaterally [8] and loaded onto hemopexin (Hpx), its carrier protein in serum [9].

The absorption of non-heme ionic iron is initiated by duodenal cytochrome B (DcytB), located at the apical surface of enterocytes, which reduces ferric iron to its ferrous form, and divalent metal transporter-1 (Dmt1), a H^+-coupled symporter [10, 11]. In the apical region of the enterocyte's cytoplasm, ferrous iron is thought to be bound by PCBP2 (poly(rC)-binding protein-2). PCBP2 is an iron chaperone that escorts ferrous iron from the site of cellular entry via Dmt1 to its intracellular destination such as the basolateral surface where ferroportin-1 (Fpn1) is present to mediate transmembraneous export [12, 13]. A certain amount of iron is also stored in enterocytes in ferritin (Ft) and this process is necessary to regulate intestinal iron absorption. Mice lacking Ft heavy chain (Fth) in enterocytes show increased iron absorption that results in tissue iron overload (IO) [14]. Also, absorptive enterocytes express iron regulatory protein (IRP)-1 and IRP2 which, in concert with transcriptional mechanisms, regulate Dmt1 and Fpn1 levels [15, 16]. When IRP1 and -2 are missing in enterocytes, a progressive and lethal enteropathy characterized by malabsorption of nutrients and water occurs [17]. This suggests that the cellular regulation of iron transporters in enterocytes is not only critical for systemic iron homeostasis but also for proper function of the absorptive epithelium itself.

The proportion of iron absorbed from dietary sources is low, varying between 5 and 15 %, possibly to limit its potential availability in the circulation for microbes that have invaded the blood stream [18]. In fact, intestinal iron absorption

typically provides only 1–2 mg of iron each day, which just compensates the obligatory daily losses that occur through desquamation of senescent epithelia from the skin and the gastrointestinal tract [19].

After export across the basolateral membrane, iron has to be converted to its ferric form by membrane-bound ferroxidase hephaestin or the soluble oxidase ceruloplasmin [20]. In serum, transferrin (Tf) constitutes the most important iron carrier protein, as its supplies most cell types with iron, including rapidly proliferating tissues such as the intestinal epithelium and the erythropoietic bone marrow. Tf has two binding sites for ferric iron and, at steady state, is only saturated to 15–45 %, ensuring adequate capacity for the acceptance of iron from enterocytes and macrophages. Mutations in the C-terminal iron binding site of Tf in humans reduce its binding affinity to the bacterial transferrin acceptor and provide protection from infection, because some pathogens, such as *Neisseria meningitidis*, use Tf-bound iron (TBI) as a major source for the metal [21].

The recycling of iron from aged red blood cells (RBC) constitutes the quantitatively most important pathway for iron turnover and transfer of the metal to the circulation. The uptake of aged RBC, the break-down of hemoglobin (Hb) and the recycling of iron are key functions of the mononuclear phagocyte system (MPS) and are mainly executed by macrophage populations in spleen, liver and bone marrow [22, 23]. This may also be relevant during the course of infection, because macrophages are in the first line of defense against invading microbes, which are recognized via pathogen-associated molecular patterns (PAMP), possibly enabling macrophages to autoregulate iron homeostasis and immune responses [24]. In steady state, recycling of Hb iron makes approximately 20–30 mg of iron available for reutilization [25, 26]. In both intestinal iron absorption and macrophage iron recycling, the rate-limiting step is mediated by the iron exporter Fpn1 which thus determines the relevant iron fluxes to the circulation [7, 27]. Therefore, Fpn1 constitutes the ideal target for hormonal negative feedback regulation of iron transfer by liver-derived hepcidin [28].

1.2.2. Hepatic Regulation of Iron Homeostasis

Hepcidin is the body's iron-regulatory hormone and is mainly produced by hepatocytes. Its mode of action is unique in that hepcidin physically binds to Fpn1 and induces its internalization from the cell surface so that iron transfer to the circulation is switched off [29]. Since absorptive enterocytes, iron-recycling macrophages, periportal hepatocytes, and the syncytiotrophoblast in the placenta are the cell types expressing the highest levels of Fpn1, the hepcidin-Fpn1 interaction controls the most important iron fluxes in the body [27, 30]. A raise in serum iron levels as well as increased iron storage are two of the key variables that stimulate hepcidin production. Inflammation does so too as detailed in a later paragraph.

Serum iron levels are sensed by hepatocytes themselves via a multimeric complex consisting of the hemochromatosis-protein Hfe, transferrin receptor-1 (Tfr1), Tfr2, and hemojuvelin (Hjv) on their surface [31, 32]. The sensing of tissue iron levels however, mainly relies on liver sinusoidal endothelial cells

(SEC) which secrete bone morphogenetic protein-2 (Bmp2) and Bmp6 as stimuli for hepcidin production in adjacent hepatocytes [33, 34].

During episodes of increased erythropoiesis or in cases of anemia and hypoxia, the need of iron for Hb synthesis rises, requiring unrestricted iron transfer to the circulation and the erythron, consequently. The key regulators to decrease hepcidin production, when iron is needed for erythropoiesis, are soluble mediators including erythropoietin (Epo), erythroferrone (Erfe), growth differentiation factor-15, and platelet-derived growth factor-BB (PDGF-BB) [35, 36]. For example, Erfe is produced by erythroid progenitors and transmits the local need of iron for Hb synthesis from the bone marrow to the liver [37], where hepatocytes downregulate hepcidin production allowing macrophages to transfer iron to the circulation. Hypoxia may exert an even faster inhibitory effect on hepcidin production that is mediated by PDGF-BB, an inhibitor of hepcidin transcription in hepatocytes [38].

1.2.3. Cellular Iron Homeostasis

TBI is the major transport form of iron in the serum and as such, is the predominant iron source for many cell types including erythroid progenitors. Binding of TBI to Tfr1 on their cell surfaces initiates receptor-mediated endocytosis [39] (Figure 1). Subsequently, acidification of the internalized endosome causes the complex to dissociate so that ferric iron becomes free and can be reduced to its ferrous form, which is then exported to the cytoplasm via an endosomal Dmt1 isoform [40]. Tfr1 itself is recycled to the cell surface for additional rounds of endocytotic TBI uptake. Tfr1 is also secreted during the process, primarily by erythroid progenitors, and therefore serum levels of soluble Tfr (sTfr) reflect the erythropoietic activity of the bone marrow and its need for iron [41].

Independent of TBI uptake, Dmt1 is also present on the cell surface of many cell types and cooperates with DcytB to mediate the acquisition of ionic non-transferrin-bound iron (NTBI) [42, 43] (Figure 1). Once ferrous iron has entered the cytoplasm, it can be channeled into metabolic pathways, transferred into organelles such as mitochondria for utilization, or inserted into Ft for storage [44–46].

Macrophages possess a number of additional mechanisms for iron uptake which are prerequisites for their ability to maintain tissue homeostasis. Red pulp macrophages in the spleen and Kupffer cells in the liver carry scavenger receptors (SR) such as subtype A (SR-A) and CD36 and specialize in the recognition and elimination of aged and damaged RBC and their degradation products [26, 47–51] (Figure 1).

Alternative to its storage, surplus iron can also be exported through the cell membrane via Fpn1 which is considered the most important cellular iron exporter in mammals. It is thus not surprising that several types of regulatory mechanisms control Fpn1 levels. First, oxidative stress induces Fpn1 transcription via Nrf2 (nuclear factor erythroid 2-related factor-2), the central stress-responsive transcription factor [52] (Figure 1). Second, iron and heme stimulate Fpn1 transcription directly [15, 53]. Third, the 5′ untranslated region (UTR) of Fpn1's mRNA

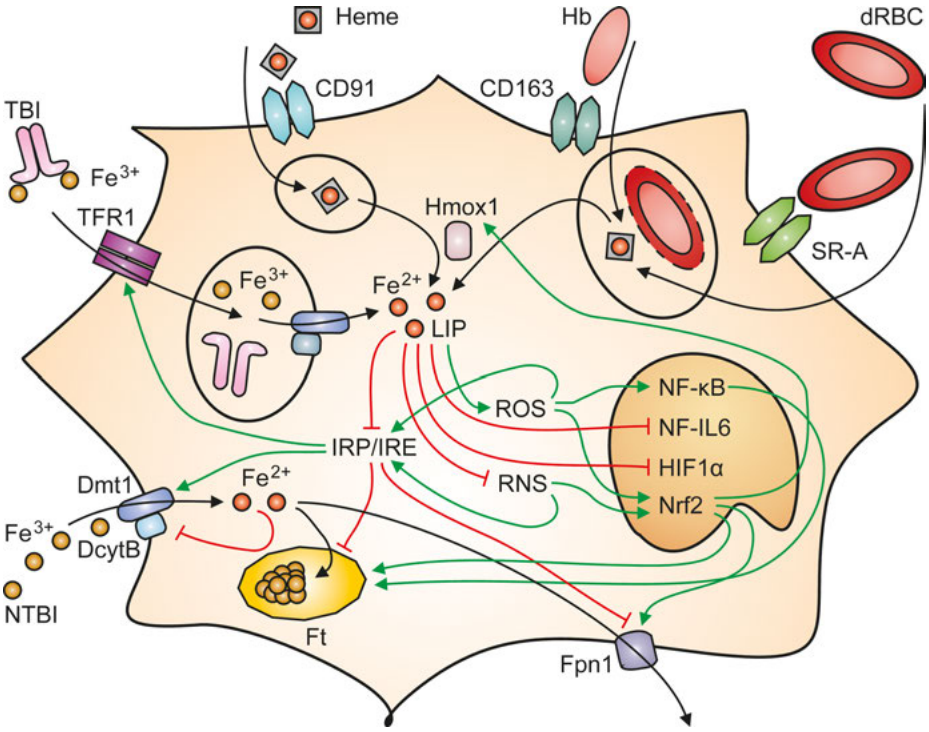

Figure 1. Cellular iron homeostasis – the macrophage as prototypical example. A macrophage possesses multiple mechanisms for the acquisition of iron. These include Dmt1 for the import of ionic non-transferrin-bound iron (NTBI), Tfr1 for the endocytosis of TBI and scavenger receptors CD91, CD163 and SR-A for the clearance of hemopexin-bound heme, haptoglobin-bound Hb, and damaged RBC (dRBC) from the extracellular space. Heme is degraded by Hmox1 and labile iron transported to the cytosol to enter the LIP. The LIP is kept small by IRPs which become activated by cellular ID to prolong Dmt1 and Tfr1 mRNA translation and repress Ft and Fpn1 formation. Excess labile iron generates ROS which in turn activate transcription factors NF-κB and Nrf2. Subsequently, Ft and Fpn1 transcription are induced to limit iron- and ROS-induced toxicity. Arrows denote iron fluxes (black), stimulatory effects (green) or inhibitory effects (red).

(messenger ribonucleic acid) contains an iron-responsive element (IRE), a specific RNA-structure allowing for iron-mediated translational control by IRP [54]. Concretely, the binding of IRP to Fpn1's IRE inhibits the initiation of mRNA translation (and thus ongoing iron export) when cytosolic iron levels are already low [55]. Whenever cells are iron-replete, their IRP are inactive and fulfill other functions such as cytosolic aconitase activity [56, 57]. In contrast, cellular iron deficiency (ID) – as well as radicals – activate IRP1 and -2 so that they can interact with IRE (Figure 1). An IRE is also present in the 5′ UTR of Fth's and Ftl's mRNA to inhibit iron storage [1, 58]. *Vice versa*, the mRNA coding for Tfr1 and Dmt1 carry 3′ IREs which mediate mRNA stabilization and prolong translation resulting in a compensatory increase of cellular iron uptake. Fourth,

Fpn1 acts as hepcidin receptor, which inhibits iron export both at the systemic and cellular levels [29]. Hepcidin is induced by IO, physically binds to Fpn1 and induces its internalization from the cell surface so that iron export is stopped [59]. Although several cell types including monocytes, macrophages, lymphocytes, and adipocytes produce hepcidin [24, 60], albeit in minute amounts, hepatocytes may be the only cell types in which hepcidin transcription is iron-sensitive, presumably because of their unique repertoire of iron-sensing surface proteins and the close proximity to SEC. However, inflammatory signals such as lipopolysaccharide (LPS), activin-B or interleukin-6 (IL-6) can trigger hepcidin secretion from immune cells such as macrophages giving them the opportunity to fine-tune iron content in an autocrine and paracrine fashion [24, 61–63].

2. IRON, IMMUNE FUNCTION, AND INFECTION

2.1. Regulation of Immune Function by Iron

The majority of studies on the immune-regulatory functions of iron have been conducted *in vitro*, although a considerable amount of experimental data from small animal models and clinical trials exists as well. Most of these reports have investigated the effects of iron on innate immunity and show that IO inhibits macrophage functions because the iron content of macrophages directly impacts the binding activity of pro-inflammatory transcription factors [64]. Specifically, the activities of nuclear factor (NF)-κB, NF-IL6, hypoxia-inducible factor (HIF)-1α, and Nrf2 are all regulated by iron, albeit not uniformly. By promoting the generation of ROS, iron stimulates NF-κB activity, one of the central transcription factors in the initiation and amplification of the immune response (Figure 1). NF-κB *trans*-activates a whole spectrum of pro-inflammatory cytokines, chemokines, and antimicrobial enzymes as well as antibacterial peptides and adhesion molecules [65–67]. Based on the activation of this pathway, labile iron has pronounced pro-inflammatory properties. However, at the same time, NF-κB activation seeks to limit iron- and ROS-induced cytotoxicity because it also induces transcription of the *Fth* and *Ftl* genes.

In sharp contrast to NF-κB, NF-IL6 and HIF-1α are typically inhibited by IO in myeloid cells (Figure 1). In macrophages stimulated with IFN-γ and LPS to mimic T helper (T_H1) immunity, the addition of iron reduces NF-IL6 activation thus impairing transcription of the *Nos2* (nitric oxide synthase-2) gene and NO (nitric oxide) production [68, 69]. Analogously, the addition of the iron chelator deferoxamine (DFO) activates HIF-1α resulting in enhanced *trans*-activation of the *Nos2* gene [70]. In addition, toll-like receptor (TLR)-4 signaling induces NF-κB dependent Ft transcription which reduces the cytoplasmatic LIP [71]. Since prolyl hydroxylases require iron as cofactor for continuous HIF degradation, this pathway stabilizes HIF-1α activation following stimulation with LPS. In macrophages infected with *Streptococcus pyogenes* or *Pseudomonas aeruginosa*, HIF-1α is essential to limit bacterial replication because it drives the expression of tumor necrosis factor (TNF), Nos2 and other antibacterial effectors. Corre-

spondingly, mice lacking HIF-1α in myeloid cells are more susceptible to skin infection by *S. pyogenes* [72]. HIF-1α is also essential for host defense against *Leishmania*, but to which extent HIF-1α or iron *per se* may affect the immune response of parasite-harboring macrophages is unknown [73]. NF-κB and HIF-1α also cross-regulate each other because NF-κB stimulates HIF-1α transcription [74, 75] and HIF-1α inhibits NF-κB induced gene expression [76]. In infections, both NF-κB and HIF-1α get activated and concertedly induce the expression of shared target genes essential for host defense, thus determining the outcome of prevalent infectious diseases.

In conclusion, macrophages regulate systemic iron homeostasis and iron affects macrophage effector functions at several levels including transcriptional and translation regulation of pro-inflammatory products. However, macrophages not only control systemic iron fluxes, they are also producers and targets of hepcidin, providing a potential mechanism of auto-regulation and a further link between iron homeostasis and immunity [61, 62, 77, 78]. In addition, iron has diverse effects on lymphocyte functions. Strikingly, a mutation in the gene coding for Tfr1, *TFRC*, results in a combined immunodeficiency characterized by functional defects of T and B cells because of impaired internalization of Tfr1 [79]. Iron also affects T_H cell responses by modulating the balance of T_H1 and T_H2 activity. Both ID and IO are thought to impair T_H1 responses because T_H1 cells are exquisitely sensitive to disturbances of iron homeostasis in either direction. On the one hand, T_H1 are highly dependent on the uptake of TBI via Tfr1 because they are only equipped with limited capacity of iron storage [80]. On the other hand, IO impairs T_H1 immunity because it negatively affects myeloid cell functions and interferon-γ (IFN-γ) signaling including the expression of major histocompatibility complex (MHC)-II, TNF, and IL-12 [81–83]. This results in an imbalance between T_H1 (such as IFN-γ) and T_H2 cytokines (such as IL-4 and IL-10) in favor of the latter and in impaired immunity against *Candida albicans* infections [84]. However, virtually nothing is known about the effects of iron on the function of T_H17 and T_{reg} cells.

2.2. Alteration of Iron Homeostasis by Inflammation

The sensing of PAMP or of danger-associated molecular patterns by the pathogen recognition receptors (PRR) repertoire elicits the acute phase response to protect the integrity of the human body. A key component of this response is the profound alteration of systemic iron homeostasis that is characterized by iron sequestration in the MPS and reflected by reduced serum iron levels. Serum iron itself, however, is a poor indicator of the body's iron status because it fluctuates after meals and (less so) due to intrinsic circadian rhythmicity [85, 86]. Upon immune activation, serum iron levels drop promptly (hypoferremia of the acute phase response). In parallel, the transferrin saturation and total iron binding capacity decrease. In contrast, the concentration of serum Ft, an iron-poor variant mainly derived from macrophages, increases (hyperferritinemia) [87]. This may be attributed to in-

creased transcription, translation, and secretion of Ft from macrophages and hepatocytes [88–91]. For example, ROS stimulate Fth transcription, while IL-1ß and IL-6 promote Ft translation [92, 93]. Whether Ft secretion is also regulated by either of these mediators is largely unknown, though.

In critically ill patients, excessive serum Ft levels are associated with increased disease severity and in part mortality from sepsis [94–96]. It is likely though, that serum Ft is a surrogate for the degree of macrophage activation and the severity of sepsis rather than a causative factor. Although serum Ft levels > 10,000 ng/mL suggest the presence of macrophage activation syndrome, the therapeutic consequences of this finding are uncertain [97–99].

As serum sTfr primarily reflects the erythropoietic activity of the bone marrow, its levels can decrease in the setting of infection, as a consequence of immune activation [41, 100]. However, after prosthetic joint revision, sTfr was higher in the presence of infection suggesting that sTfr may deviate in either direction dependent on the type, severity or duration of an underlying infection [101]. In any case, biochemical indicators of inflammation such as C-reactive protein (CRP), IL-6, and procalcitonin may help in the interpretation of iron indices. Moreover, it has been proposed that CRP and other markers of inflammation should be used to correct iron indices such as serum Ft for the degree of inflammation but no broadly applied algorithm has been established.

These alterations of iron homeostasis following immune activation are orchestrated, to a large extent, by IL-6 and hepcidin. In fact, IL-6 may be the single most important cytokine to divert iron fluxes into the MPS [61, 102, 103]. IL-6 is produced by many cell types including myeloid cells themselves, T_H1 cells, and parenchymal cells following their activation by PRR, by antigen presentation and co-stimulation through direct contact or other pro-inflammatory cytokines [104]. Hepatocytes are equipped with the IL-6 receptor which signals via the JAK-STAT3 (Janus kinase and signal transducer and activator of transcription, respectively) pathway, thus inducing hepcidin transcription [105, 106]. In infections, IL-6 levels rise and the subsequent increase in serum hepcidin levels executes the development of hypoferremia and hyperferritinemia that is required to contain the infection [107]. Basically, the same signaling cascade is turned on by the T_H17-type cytokine IL-22, which also stimulates hepcidin and Hpx transcription and has various other effects on epithelial cells [62, 108, 109]. Hepcidin produced during immune activation interacts with Fpn1 on iron-absorbing enterocytes and RBC-degrading macrophages, which results in reduced dietary iron assimilation and in iron sequestration within the MPS [110, 111]. The mechanism is further enhanced by Tfr1 and Dmt1 both of which are induced in the context of immune activation, for instance via IFN-γ [89]. Therefore, also the active uptake of TBI and NTBI into macrophages is enhanced in the context of a T_H1 response.

Other target genes of IL-6 have central functions in iron metabolism as well, including Tf, haptoglobin, and Hpx. Tf expression in hepatocytes is negatively regulated by IL-6 which further reduces the TBI pool in the serum [112]. Haptoglobin and Hpx, in contrast, are induced by IL-6 so that the serum's capacity to capture free Hb and heme, respectively, is increased [113]. This arm of the acute

phase response protects from the cytotoxic effects of Hb and heme and is relevant in many infections which are associated with damage to RBC. In this setting, the severity of RBC damage can range from a mild and compensated reduction of their lifetime to massive hemolytic anemia. Hemolysis during infections may have several causes such as bacterial hemolysins, pathogen proliferation inside RBC, the production of ROS, the activation of the complement cascade or pathogen-induced cross-reactive auto-antibodies against RBC as observed in *Mycoplasma pneumoniae* infections. In addition, the capacity of macrophages for erythrophagocytosis can be induced by pathogens themselves or by inflammatory mediators such as IFN-γ [114, 115]. Thus, immune-mediated iron sequestration in the MPS, hemolytic episodes, and enhanced erythrophagocytosis all may contribute to a reduction of Hb levels upon immune activation resulting in the anemia of inflammation [116]. Its degree is further aggravated by the fact that pro-inflammatory cytokines also inhibit the generation of Epo in the kidney and the response of the erythron to the Epo that is being produced [117, 118].

Other alterations of systemic iron homeostasis in the presence of microbes occur independently of IL-6 and solely require the ligation of PRR. For instance, Fpn1 transcription is inhibited by TLR2, -4 and -6, allowing for a hepcidin-independent downregulation of Fpn1 levels [78, 119, 120]. In addition, the antimicrobial peptide lipocalin-2 (Lcn2) is directly induced by TLR ligands such as LPS. First identified as granule content in neutrophils, Lcn2's primary function is to scavenge distinct groups of microbial siderophores [121]. Siderophores are small molecules of diverse chemical structure which bind ferric iron with extremely high affinity [122]. In addition, Lcn2 also has immune-modulatory effects [123]. For example, Lcn2 stimulates the chemotaxis of neutrophils and is more broadly required for proper neutrophil functions because Lcn2-deficient neutrophils also fail to phagocytose bacteria and fungi, which also impairs host defense against siderophore-independent pathogens. Lcn2 also controls macrophage differentiation and limits the production of IL-10, conferring a more pro-inflammatory macrophage phenotype and a further link between iron homeostasis and innate immunity [124–127].

2.3. The Importance of Iron for Microbes

Iron is a vital trace element to almost all pathogens. *Borrelia burgdorferi*, the causative agent of Lyme disease, is the only known notable exception in that its enzymes to not utilize iron as central cation [128, 129]. Given its role as essential micronutrient, pathogens have evolved strategies to acquire iron from host sources, and the expression of iron uptake genes is often linked to virulence. During host-pathogen interaction, the availability of iron thus modifies both, immune effector mechanisms and microbial proliferation. Therefore, the acquisition of sufficient amounts of iron is a major determinant of the outcome of infectious diseases [130–135]. It is thus not surprising that IO disorders increase the risk of bacterial infections and result in more severe disease courses and uncommon

manifestations. For example, in hematologic patients requiring repetitive transfusions of packed RBC, there is a strong positive association between IO and infections. In thalassemia syndromes, RBC transfusions, ineffective erythropoiesis, and increased iron absorption contribute to IO [136, 137]. The accumulation of surplus iron that results is not only toxic to parenchymal organs such as heart and liver, IO also predisposes to infections. In fact, invasive infections with *Klebsiella pneumoniae*, *Escherichia coli*, *P. aeruginosa*, *Staphylococcus* and *Salmonella* species have been reported in ß-thalassemia patients. The incidence of these infections directly correlates with the degree of IO and a delay in the therapeutic administration of an iron chelator [138]. In sickle cell disease patients, similar pathogens have been isolated during bacteremia episodes as have *S. pneumoniae* and *Haemophilus influenzae* for which splenic dysfunction was implicated [139]. In a similar fashion, IO is an independent negative predictor of survival in leukemia and myelodysplastic syndrome (MDS) patients after stem cell transplantation (SCT). Specifically, there is a positive association between iron stores as estimated by determination of Ft in serum and infection-related mortality in such patients. Therefore, invasive bacterial infections are a major contributing factor to poor outcome in such settings of IO [140].

The host iron sources potentially accessible to microbes are as diverse as the iron acquisition mechanisms employed by distinct pathogens. These are directly linked to microbial virulence because iron is a growth-promoting element [141, 142]. RBC may provide the richest source of iron because in the human adult, they make up more than 80 % of all cells and in total contain approximately 2,500 mg of iron. Iron in RBC is targeted by several strategies. *Babesia*, *Bartonella*, *Plasmodium* and *Toxoplasma* species directly invade RBC [143–145]. Other pathogens express hemolysins and receptors for Hb or Hpx to destroy RBC and harvest heme-iron. Hemolysins are bacterial toxins, which attack the RBC membrane and result in target cell destruction. Many pathogenic bacteria including *Staphylococcus aureus*, *Streptococcus* species, *Salmonella* species and *Escherichia coli* produce such hemolysins to increase their virulence and secure iron acquisition [146–148].

Both TBI and NTBI in serum are potentially accessible to microbial siderophores, because their affinity for iron outcompetes binding by Tf by far. Siderophores fall in several molecular classes [122, 149–152]. Widely distributed variants are enterobactin and yersiniabactin produced by Enterobacteriaceae, mycobactins, and carboxymycobactins from *Mycobacterium* species and *Pseudomonas*' pyoverdine [151–154]. Moreover, *Aspergillus* species produce siderophores, which are critical for their virulence [155, 156].

Other pathogens carry receptors for Tf, Ft or lactoferrin for the endocytotic uptake of these iron-containing proteins from the host [132, 157, 158]. Consequently, the reduction of the TBI during infections and the fact that serum Ft is iron-poor *per se* limit the availability of host iron sources to microbes. Based on these interconnections and on the *in vitro* effects of iron chelators on microbial proliferation, the withholding of iron from pathogens is considered an efficient antibacterial strategy termed 'nutritional immunity'.

2.4. Systemic and Cellular Iron Homeostasis During Infections

In the presence of infectious agents, iron homeostasis is modulated at both the systemic and cellular levels. The predominant mechanism of regulation, however, may depend on the type and localization of the pathogen. Specifically, the host needs to differentially respond to extracellular as opposed to intracellular microbes in order to limit the availability of iron in exactly the microenvironment a given pathogen inhabits to proliferate and persist. The presence of extracellular microbes is sensed by the cell surface repertoire of TLR present on myeloid cells and other types of immune as well as parenchymal cells and activates NF-κB and other signaling pathways [159, 160]. IL-6, generated upon NF-κB activation, and LPS itself act on hepatocytes and stimulate hepcidin expression so that Fpn1 is internalized from cell surfaces [161, 162]. In parallel, Tfr1, Dmt1, and Ft expression is induced in macrophages to stimulate iron uptake and storage [89]. As a result, serum iron levels drop, and iron is trapped in the MPS. Therefore, extracellular pathogens are fought off by systemic adaptation of iron homeostasis.

Infections with intracellular pathogens require a different type of host response [141]. In macrophages infected with *Chlamydia pneumoniae*, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Trypansosoma*, Fpn1 is induced to direct iron fluxes out of the cytoplasm and away from invaded pathogens [163–168]. Fpn1 induction has also been observed in MPS organs upon infection with *Mycobacterium bovis* BCG, *Salmonella* Typhimurium and *Trypansosoma brucei* suggesting the pathway is relevant to invasive infections with these pathogens as well [166, 169, 170]. Although the sensing mechanism that initiates this response remains unknown, it was found that T_H1 immunity promotes the mechanism by inducing Nos2 transcription. Nos2, in turn, produces high levels of NO which activates Nrf2, resulting in transcriptional induction of Fpn1 [52, 170]. The increased iron export depletes both the cytoplasmic LIP and the Ft-associated iron pool. Although Ft is supposed to render iron inaccessible for bacteria and other pathogens that reside either in the extracellular space or, after phagocytosis, in the phagosome (e.g., *E. coli*), professional intra-macrophage pathogens such as *Salmonella* Typhimurium, *Listeria monocytogenes* or *Mycobacterium tuberculosis* may actively seek access to Ft-encapsulated iron [82, 168, 171]. Therefore, in infections with intracellular microbes, iron homeostasis is mainly modulated at the cellular level, possibly only in infected cells in which a reduction of the Ft-iron pool may also serve to withhold iron. Consequently, the induction of hepcidin in the presence of intracellular microbes does not generally impact on the disease course [172–174].

Since some microbes such as *Salmonella* species can replicate both extra- and intracellularly, an even more differentiated immune response needs to be in place. This is also true of malaria, more so because *Plasmodium* has a complex life-cycle, too. In addition, co-infections with these two pathogens and others such as human immunodeficiency virus (HIV) are not uncommon, especially in sub-Saharan Africa. Moreover, polymicrobial infections are a concern when natural barriers are broken for instance during intraabdominal surgery. How these mechanisms of nutritional immunity are initiated, maintained and fine-tuned is subject to ongoing investigation.

3. IRON ADMINISTRATION AND INFECTION

3.1. Linkage of Iron Deficiency and Iron Supplementation to Infection in Animal Models

Data obtained in animal models show that ID and iron supplementation (IS) can affect the outcome of infections in either direction from the perspective of the host dependent on the specific type of pathogen present. For example, a large body of evidence suggests that IS increases the severity of diseases caused by mycobacteria [175, 176]. In line, genetic IO promotes *M. tuberculosis* infection and treatment with the iron chelator DFO ameliorates the disease course [177]. However, it was recently described that mild dietary IS facilitates the generation of ROS in mice infected with *M. bovis* BCG, thus reducing the mycobacterial load and improving disease outcome [169]. This contrasts with clinical studies, the majority of which suggests that increased iron content is a risk factor for the occurrence and progression of tuberculosis in human subjects [175].

Similarly, conflicting experimental results have been obtained in mice infected with *Salmonella* Typhimurium. On the one hand, dietary ID improves disease outcome because of impaired bacterial proliferation [178]. On the other hand, therapeutic application of DFO impairs the generation of ROS by the phagocyte oxidase Nox2, thus decreasing survival [179]. In contrast, dietary IO promotes *Salmonella* Typhimurium replication in spleen and liver despite appropriate induction of pro-inflammatory cytokines and antimicrobial enzymes such as TNF, IL-6, and Nos2 [180].

The reasons for such discrepancies are hard to grasp. Although both *Mycobacterium* and *Salmonella* species are facultative intracellular and macrophage-tropic, their relative distribution between intra- and extracellular compartments may be diverse. Also, the regulation of systemic iron fluxes may underlie different spatio-temporal dynamics during these types of infection. For example, the extent and timing of hepcidin induction may be different between *Mycobacterium* and *Salmonella* infections which may explain why hepcidin deficiency or antagonism does not affect outcome of experimental tuberculosis yet ameliorates the course of murine typhoid [172, 174]. In addition, iron not only promotes microbial proliferation, it also has immune-modulatory effects, for example on the generation of NO and ROS and the expression of TNF and MHC-II [81, 83, 163, 181]. Since NO generation by Nos2 has divergent effects on *Mycobacterium*, *Salmonella* and other intracellular infections, some of the differences observed may be due to iron's immune-modulatory properties [170, 182–184].

3.2. Dietary Iron Fortification and the Risk of Infection

ID is a global health concern affecting more than a billion children and women of reproductive age in developing countries. Because ID results in impaired cognitive development in pre-school children [185, 186], randomized controlled trials (RCT) investigated the potential beneficial effects of dietary iron fortifica-

tion in sub-Saharan Africa and South Asia. Initial studies suggested that IS is safe as it did not increase the risk of infections, even in countries in which malaria is endemic [187]. Rather, IS appeared to reduce the risk of severe malaria courses in a small trial [188]. In contrast, in a large study conducted in Eastern Africa, the supplementation of iron and folic acid without or with the addition of zinc was associated with increased risk of hospitalization and death from infections including malaria in comparison to control groups [189]. These results were confirmed in a large RCT in South Asia where children receiving micronutrient powder containing iron between the age of six and eighteen months experienced significantly more episodes of diarrhea and bloody diarrhea as compared to children without iron-fortified diet [190]. This appears to be directly related to the availability of iron for bacteria and parasites in serum and tissues [191–193].

Exactly in the countries in which large-scale micronutrient supplementation studies have been carried out, infections with *Plasmodium*, *Salmonella* species and other bacterial and viral microbes causing diarrhea or respiratory infections, *M. tuberculosis* or HIV and numerous other pathogens including helminths are a major health concern. This is relevant because iron affects the clinical course of either of these infections and may contribute to the risk of super-infection and poor clinical outcome [175, 194, 195]. Children with *Plasmodium falciparum* malaria are at increased risk of acquiring a subsequent bacterial blood stream infection, specifically with non-typhoidal *Salmonella* serovars, which are otherwise non-invasive, but are most commonly recovered from blood cultures in this setting [196–198]. Putative underlying mechanisms have been experimentally addressed. Both iron and the presence of damaged RBC impair innate immunity against non-typhoidal *Salmonella* and other Gram-negative pathogens [199]. It was also shown that *Plasmodium*-induced hemolysis, which results in iron deposition in myeloid cells, results in increased IL-10 production by macrophages, but also impaired ROS generation by neutrophils [200–203]. In both situations of dysregulation, the induction of Hmox1 appears to play a major causative role because inhibition of Hmox1 restores the killing of *Salmonella* Typhimurium in a mouse model [204]. However, the anti-inflammatory activities of Hmox1 also impact on host response to *Plasmodium* itself [205, 206]. While hepcidin induced during malaria counteracts super-infection by *Plasmodium*, it promotes intracellular replication of *Salmonella* Typhimurium and HIV [174, 207, 208]. In addition, monocyte dysfunction, complement defects and spleen dysfunction may contribute, especially when HIV-coinfection or a congenital hemolytic anemia is present [209, 210].

Therefore, the adaptation of host iron metabolism during immune defense against one pathogen may favor infection by another one. Upon oral supplementation, iron may have additional effects in the intestine which could be mediated via epithelial or immune cells or secondary by modulating the composition or the metabolism of the gut microbiota [211, 212]. The intestinal epithelium is one of the body's major barriers to infection. While engaged in the absorption of water and nutrients including iron in steady state, intestinal epithelial cells switch to a pro-inflammatory and antimicrobial program in the presence of pathogens which are sensed by PRR [213, 214]. IO may impair this phenotypic change and facilitate

pathogen invasion because high iron levels impair the production of pro-inflammatory mediators by enterocyte-like cells [215]. Furthermore, the mechanism of colonization resistance, by which the presence of commensals counteracts the proliferation and invasion of pathogens, is partly iron-based, too [216].

The role of iron availability for the risk and course of infection has been investigated in several observational trials. A prospective study carried out in Tanzania found that children with ID had fewer episodes with malaria and lower parasite counts when infected which translated into lower mortality rates over the first years of life [217]. Similar results were obtained in a prospective trial carried out with HIV-infected children in Malawi, demonstrating that IS ameliorated Hb levels but increased the risk for malaria [218]. This suggests that active infection should be ruled out or properly treated before IS therapy is initiated. This assumption was confirmed in a double-blinded RCT carried out in Ghana. Children received either iron micronutrient powder containing 12.5 mg of iron or no iron. Insecticide impregnated bednets and malaria treatment were available. Under these circumstances, IS did not increase the risk of malaria episodes [219]. Importantly, while a certain degree of ID exerts protection against malaria in infants and pregnant women [220], severe ID negatively impacts on the outcome of infections as shown in HIV-negative and -positive patients suffering from tuberculosis. Both, IO as well as ID were associated with an adverse outcome [221]. This may be attributed to the fact that ID negatively affects the proliferation and differentiation of lymphocytes and other immune cells whereas IO blocks antimicrobial immune response directed against mycobacteria and improves the availability of the nutrient iron for these bacteria [222, 223].

3.3. Clinics and Practice of Oral and Intravenous Iron Supplementation

Patient selection is critical to identify individuals who will most likely benefit from IS and least likely have adverse reactions or unintended side effects. The first step in this benefit-risk assessment is the appropriate interpretation of iron indices and the rational differentiation between absolute and functional ID as well as combined conditions. While Ft and sTfR are excellent markers to monitor the availability of iron in the serum or the needs of iron for erythropoiesis in subjects without inflammation, their diagnostic capacity in subjects with inflammatory diseases and consecutive diversion of iron traffic with subsequent anemia is massively restricted which is due to the fact that the concentrations of both biomarkers are affected by inflammatory cytokines [224–226]. Thus, the Ft index (sTfR/log Ft) was introduced as an aid for the differential diagnosis although it has not been investigated in prospective RCT [227, 228]. In patients with immune activation, an elevated Ft index suggests the concurrent presence of absolute ID warranting correction [87, 229]. However, the cut-offs required to make the differential diagnosis are largely dependent on the specific diagnostic test used for determination of sTfR precluding their broad clinical application [228, 230–232]. Several other markers have been used in patients with chronic disor-

ders to identify true ID including the percentage of hypochromic RBC, Hb levels in reticulocytes, reticulocyte numbers or simply erythrocyte indices such as mean corpuscular Hb or mean cellular volume. More recently, the determination of hepcidin in serum as well as combinations of several iron metabolism parameters together with markers of inflammation have been used to provide a more accurate differential diagnosis between true and functional ID in the setting of inflammatory diseases, however, no gold standard parameter has been identified thus far. This differential diagnosis is of major importance because true *versus* functional ID may warrant different therapies. In true ID, iron is lacking and needs to be substituted to meet the body's needs for iron for central metabolic pathways including Hb synthesis. In functional ID, iron is retained in the MPS due to the combined action of cytokines and hepcidin making the metal unavailable for erythroid progenitors. However, little information from prospective clinical trials is available toward the therapeutic efficacy of iron in advanced inflammation and potential off target effects specifically in relation to the interaction of iron with immune function and as a potential nutrient for microbes [233, 234]. Of note, new drugs have been developed in recent years to overcome inflammation and/or hepcidin-driven iron retention. Most of these drugs aim to block hepcidin expression or neutralize hepcidin in the circulation whereas others stabilize or increase Fpn1 expression. All these strategies attempt to increase macrophage iron egress and to enhance the availability of iron for erythropoiesis in order to reverse anemia [235–237].

In general, iron can be supplemented either orally, as iron salts, or intravenously (IV) as iron-carbohydrate complexes. For further details on dosing, pharmacology, indications, and benefits *versus* risks of different drugs the reader is referred to recent reviews [238–242].

Both, oral and IV iron, appear to be comparably effective in patients with ID anemia. However, in the setting of inflammation oral iron absorption is impaired due to hepcidin-mediated blockade of iron transfer from the diet [111]. This is specifically relevant in advanced inflammation and functional ID, and increased circulating hepcidin levels have been associated with a poor erythropoietic response to oral iron supplements or erythropoiesis stimulating factors in field studies and preclinical models of inflammatory anemia [243–245]. While IV iron has been shown to exert therapeutic efficacy in patients with chronic inflammatory disease and renal anemia, little information is available on whether or not IV applied iron will also become sufficiently available for erythropoiesis in subjects with more advanced inflammation. This notion is based on the fact that IV iron preparations are taken up by macrophages and iron is then utilized and redistributed to the circulation [246]. However, in inflammation iron egress via Fpn1 is blocked by circulating hepcidin and iron is retained within macrophages. Questions on therapeutic efficacies as a function of the patients' inflammatory status and underlying diseases can only be answered in prospective clinical trials. These may also address the different pharmacokinetics of oral and various IV iron preparations in these situations along with their potential off target effects specifically related to iron availability for microbes. As the host has developed different strategies to restrict iron availability for specific infections [247], differences

of iron distribution in serum, tissues and cells following oral *versus* IV iron application may be of interest to predict a specific risk for infections upon IS specifically in regions with a high endemic burden of infectious diseases.

Finally, many patients with inflammatory diseases and anemia suffer from true ID because of concomitant gastrointestinal or urogenital blood losses. This situation results in reduction of circulating hepcidin levels and slightly improved dietary iron absorption as compared to subjects suffering from classical anemia of chronic disease [111]. In such situations, oral and IV drugs appear to the comparably effective as evidenced by clinical studies, e.g., in patients with inflammatory bowel disease [248], however, we have to identify which mode of iron substitution is preferable to reduce the likelihood of certain infections in patients with specific environmental or individual risk factors.

3.4. Iron Supplementation and Risk of Infections in Chronic Kidney Disease

Patients with chronic kidney disease (CKD) and specifically those on dialysis are at a high risk of infection due to the dialysis procedure along with the risk of bacteremia. In addition, impaired kidney function with concomitant retention of potentially toxic metabolic products exerts negative effects toward immune function and immune cell proliferation [249]. Patients with CKD are often anemic which is due to multiple factors, including Epo deficiency and reduced iron availability as a consequence of repeated blood losses but also based on increased circulating hepcidin levels causing iron retention in the MPS [250–252]. Thus, treatment of renal anemia not only warrants Epo therapy but also iron substitution [253]. This leads to the question whether iron substitution may impact on the risk of infections in such patients and whether or not certain threshold levels of biomarkers such as Ft exist which are associated with a negligible or increased risk for infections.

In patients with end-stage CKD on intermittent hemodialysis, high serum Ft levels are associated with reduced production of TNF and IL-6 by peripheral blood monocytes following stimulation with IFN-γ and LPS. In contrast, intravenous IS restores iron and Ft within monocytes and results in increased phosphorylation of NF-κB and enhanced secretion of TNF and IL-6 [254]. Accordingly, dialysis patients receiving Epo and iron presented with a reduction of circulating TNF levels in blood over time as compared to dialysis patients receiving Epo alone [81]. This leads to the question whether or not such alterations of circulating cytokine levels impact on the risk for or the course of infections.

So far, there was inconsistency regarding the association of iron therapy and higher risk of infection in patients with end-stage CKD. A meta-analysis evaluated the link between iron and infection in hemodialysis patients [255]. Among the studies that examined the risk of infection according to serum Ft levels, nine reported an association while four did not. In general, studies comparing high (defined as >500 or 1000 ng/mL) *versus* low serum Ft levels reported higher rates of bacterial infection and more patients with infection in the high serum

Ft group. They also reported a 1.5- to 3.1-fold higher incidence of bacterial infection or infection-related mortality, which translated into an excess of 16 to 50 bacterial infections per 100 patient-years among patients with high serum Ft levels. Among the four negative studies, three were retrospective; of these, one reported a non-significant difference in bacteremia-free tunneled catheter survival among 89 patients with serum Ft levels > 500 versus < 500 ng/mL, and another one found a non-significant difference in the percentage of patients with infection, pneumonia or cellulitis/carbuncle between patients with serum Ft > 600 or < 600 ng/mL [249, 255].

One study prospectively examined risk factors for bacteremia among 985 patients on hemodialysis in France [256]. During a 6-months follow-up period, 51 episodes of bacteremia occurred, with an incidence of 0.93 episodes per 100 patient-months. In a multivariable Cox proportional hazards model, temporary catheters (particularly long-term indwelling catheters), history of two or more episodes of bacteremia, current immunosuppression, and lower Hb levels were independently associated with a higher risk of bacteremia. However, there was no significant difference in serum Ft levels between patients with (346 $\pm$ 502 ng/mL) and without bacteremia (353 $\pm$ 434 ng/mL; p = 0.44). It is important to note that serum Ft was an independent risk factor for bacterial infection in a previous study published by the same authors [257]. They attribute the discrepancy to a lower prevalence of IO (defined as serum Ft levels > 1,000 ng/mL) in the latter study (5 % *versus* 10 %), which most likely reflected concomitant differences in Epo use (16.1 % *versus* 51.5 %).

Interestingly, Epo impacts on innate immune function by blocking NF-κB mediated immune activation and antimicrobial host responses [258]. Once again, it has to be pointed out, that high Ft levels in the setting of a chronic inflammatory disease do not directly reflect massive iron loading but rather a more advanced inflammatory state which is likewise associated with a worse prognosis. In this respect a study is noteworthy which found that infection-related mortality is lowest in dialysis patients with Ft levels between 100–200 ng/mL whereas it increases in subjects with Ft levels below 100 and above 300 ng/mL [259, 260].

A recent meta-analysis of 72 studies conducted between 1966 and 2012 which included a total number of 10,602 patients found that the administration of IV iron was associated with a significantly higher risk of infection (OR 1.33; CI 1.10–1.64) compared with oral or no IS [261]. Of interest, a retrospective study identified profound differences in the risk for infection depending on the drug used. The authors found a significantly higher risk for bacteremia when using ferric sucrose instead of ferric gluconate (OR 2.9) and also described that a cumulative dosage of iron > 2,000 mg over a period of 10 month was associated with an increased risk for bacteremia as compared to cumulative dosages < 2,000 mg (OR between 1.5 to 2.4) [262]. The latter results were confirmed by another retrospective analysis indicating that the risk for infection increased in dialysis patients receiving > 1,050 mg in three months or > 2,100 mg of IV iron during six months [263]. Of note, a recently published retrospective study comparing the short-term effects of both IV iron drugs discussed above found a reduced incidence of infections in patients treated with iron sucrose as compared

to therapy with ferric gluconate during an observation period of 90 days but also when studying long term effects [264, 265]. These authors also found that patients receiving bolus iron application as compared to dialysis subjects receiving maintenance therapy were at a higher risk for infections and infection-related mortality [266]. Patients at highest risk were those with an implanted catheter or a history of a recent infection. A higher risk for infection-related hospitalization or mortality was also observed in patients receiving higher *versus* lower bolus dosages of iron which was largely independent of baseline iron status of these subjects [266]. In confirmation of that, a prospective observational study involving 1,086 patients in Japan found the highest hazard ratio for infection in patients receiving high dose IV iron, followed by subjects receiving low dose IV iron and finally subjects treated with oral iron [267]. This may also relate to the fact that patients receiving oral iron have poor iron absorption due to hepcidin-mediated blockage of iron transfer from enterocytes to the circulation. This conflicting data, more of which can be found in recent reviews [249, 268–270], on the use of iron in dialysis patients do not indicate that IS is a general hazard for subjects on dialysis. It rather points to the fact that we need to identify those patients who benefit from IS and those who will not. In addition, we need information to calculate the optimal individual single iron dose and cumulative dosages over time, an issue which can only be met by prospective RCT funded by governmental research bodies. Withholding iron from dialysis patients is also problematic because they will suffer from more severe anemia and negative effects of ID on cardiovascular performance, cellular respiration or quality of life. Of interest, a single center observational, prospective study involving 235 incident dialysis patients found that IS at any time resulted in a significant reduced mortality as compared to patients who never received iron [271].

3.5. Iron Supplementation and Infection Risk in Critically Ill Patients

In critically ill patients, hemolysis and immune activation stimulate erythrophagocytosis, which results in macrophage IO. In addition, the uptake of Hb-haptoglobin and heme-Hpx complexes as well as stimulation of TfR1- and Dmt1-mediated iron import further contribute to iron loading of the MPS [272]. This results in functional ID but also impacts on antimicrobial effector function of innate immune cells [223].

In a prospective study, functional ID (as estimated by a proportion of hypochromic RBC $>10\,\%$) was found in 35 % of intensive care unit (ICU) patients. Importantly, functional ID was an independent predictor of longer ICU stay and longer duration of the systemic inflammatory response but not of mortality [273]. In a retrospective analysis of an independent cohort, sTfR levels >280 mg/L were associated with higher mortality, possibly as a sign of increased inflammation. However, whether functional ID is an indicator of reduced nutritional status prior to or of increased disease severity during critical illness or an actual driver of the disease processes remains unknown. Of note, a liberal as compared

to a restrictive transfusion strategy in patients with upper gastrointestinal bleeding at the ICU was associated with a significantly increased mortality [274]. The underlying mechanisms were not entirely clear but appeared to be related to portal vein pressure, specifically in patients with impaired liver function but also to an increased risk of infection. The latter notion was confirmed by observations that peri-operative application of RBC transfusions was associated with a significantly increased risk for surgical site infections (OR, 3.21; 95 % CI, 1.41–7.31) [275]. However, ID may negatively impact on cardiovascular performance and physical activity. Studies have indicated that IV iron administration improved walking distance and left ventricular function in patients with congestive heart failure [276] which may relate to distinct effects of the metal on mitochondrial respiration [2, 3, 277]. Therefore, a prospective, multicenter, double blinded trial was initiated to study the effects of iron administration on transfusion dependence of anemic patients at ICU [278]. The immediate initiation of IV iron therapy during ICU stay did not result in a reduction of transfusion requirements, however, patients at discharge had higher Hb levels. In respect to infectious complications, there was a non-significant difference in the percentage of patients with nosocomial infections between those receiving IV iron (28.4 %) or placebo (22.9 %; $p = 0.44$). These data were confirmed by a recent meta-analysis of anemic ICU patients, which found that iron administration did not reduce the transfusion requirements but also did not alter the frequency of infection [279].

However, there is strong evidence from clinical studies that IO is an important risk factor for specific infections and outcome in immune-compromised patients as discussed above for MDS patients. As another example, bone marrow iron stores have been found to be positively associated with the risk of infection with invasive molds, such as *Aspergillus*, in patients after allogenic SCT [280]. Molds are highly iron-dependent fungi and their pathogenicity is linked to the expression of iron acquisition systems and a sufficient availability of the metal [155, 156]. In addition, iron compromises antifungal immune responses, which may be in part traced back to the effects of the metal on pro-inflammatory and IFN-γ driven effector pathways and T cell differentiation [130, 281, 282]. A similar observation was made in patients with HIV infection in the pre-HAART (highly active antiretroviral therapy) era where increased bone marrow iron concentrations were associated with a significantly shortened survival [283]. Of note, increased Ft levels or elevated hepcidin concentrations have been associated with an increased risk of infection in transplanted patients, however, both parameters are not only affected by iron levels but also by inflammation, and thus the cause effect relationship of iron loading needs to be confirmed by prospective studies using additional biomarkers of body iron homeostasis [284–286]. Accordingly, in allogenic SCT patients increased levels of NTBI were a good predictor of blood stream infection [287] which is in agreement with data indicating that enhanced liver iron stores as determined by magnetic resonance imaging are associated with increased non-relapse mortality in patients after allogenic bone marrow transplantation [288, 289]. All these data indicate that in patients with an impaired immune control of infection the excess of iron results in increased infection-related morbidity and mortality.

4. CONCLUDING REMARKS AND SUGGESTIONS FOR FUTURE RESEARCH

The continuous access to iron and the maintenance of iron homeostasis are central processes in host-pathogen interaction, significantly impacting the outcome of infections. During evolution, host processes have emerged with the aim to reduce the access of iron for invading pathogens. This includes diversion of iron traffic during infection as well as genetic mutations of iron-binding proteins which make the metal less accessible to microbes, a process for which the term 'nutritional immunity' has been coined [290–292]. The close linkage between iron homeostasis and immune function further points to the importance of iron in infectious diseases susceptibility and outcome [247]. On the other side of the coin, pathogenic microbes have evolved strategies to acquire iron in tissues, in the circulation or within cells by producing or releasing iron-binding compounds such as siderophores or by pirating the metal from host resources such as Tf. In line with that, we have witnessed the cloning and functional characterization of numerous proteins that orchestrate iron metabolism either in the host or the microbe, which determine the balance of host-pathogen interaction.

We are still missing though, a comprehensive understanding of the fine-tuning of iron metabolism in the microenvironment of an infectious focus in which different cell types interact to recognize, contain, and kill the pathogen, to limit tissue damage and to restore organ microanatomy and function. Also, evidence is accumulating that host, pathogens, and commensal microbes form a triangle around iron at its center. Similarly, in scenarios of co-infection or super-infection with more than one agent, iron can affect the proliferation of and the immunity against either of the microbes. Sophisticated technology such as single-cell analysis, unbiased omics approaches including metagenomics and modern biostatistics is available and awaits being employed to answer the questions that have arisen in these fields.

Population-based studies may allow us to identify subjects who will benefit the most from IS and those who are at risk for adverse side effects such as exacerbation of a latent or chronic infection. Again, iron and immune indices measured in peripheral blood may enable a predictive assessment of the benefit-to-risk ratio before any intervention is started, in line with the principals of individualized medicine.

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ABBREVIATIONS

Bmp	bone morphogenetic protein
CD	cluster of differentiation

C/EBP	CCAAT enhancer-binding protein
CI	confidence interval
CKD	chronic kidney disease
CRP	C-reactive protein
DcytB	duodenal cytochrome B
DFO	deferoxamine
Dmt1	divalent metal transporter-1 AKA Slc11a2
Epo	erythropoietin
Erfe	erythroferrone
Fpn1	ferroportin-1 AKA Slc40a1
Ft	ferritin
Fth	ferritin heavy chain
Ftl	ferritin light chain
Hb	hemoglobin
Hfe	hemochromatosis-associated gene/protein
HIF-1α	hypoxia-inducible factor-1α
HIV	human immunodeficiency virus
Hjv	hemojuvelin
Hmox	heme oxygenase
Hpx	hemopexin
ICU	intensive care unit
ID	iron deficiency
IFN-γ	interferon-γ
IL	interleukin
IO	iron overload
IRE	iron-responsive element
IRP	iron-regulatory protein
IS	iron supplementatiom
IV	intravenous
JAK	Janus kinase
Lcn2	lipocalin-2 AKA 24p3 AKA Ngal
LIP	labile iron pool
LPS	lipopolysaccharide
MDS	myelodysplastic syndrome
MHC	major histocompatibility complex
MPS	mononuclear phagocyte system
mRNA	messenger ribonucleic acid
NADPH	nicotinamide dinucleotide phosphate, reduced
NF-IL6	nuclear factor interleukin-6 AKA C/EBP-β
NF-κB	nuclear factor-κB
Ngal	neutrophil gelatinase-associated lipocalin
NO	nitric oxide
Nos2	nitric oxide synthase-2 AKA inducible Nos
Nox2	NADPH oxidase-1 AKA phagocyte oxidase
Nrf2	nuclear factor erythroid 2-related factor-2
NTBI	non-transferrin bound iron

OR	odds ratio
PAMP	pathogen-associated molecular pattern
PCBP2	poly(rC)-binding protein-2
PDGF	platelet-derived growth factor
PRR	pattern recognition receptor
RBC	red blood cell
RCT	randomized controlled trial
ROS	reactive oxygen species
SCT	stem cell transplantation
SEC	sinusoidal endothelial cell
SR	scavenger receptor
STAT	signal transducer and activator of transcription
sTfr	soluble transferrin receptor
TBI	transferrin-bound iron
Tf	transferrin
Tfr1	transferrin receptor-1 AKA CD71
T_H	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
UTR	untranslated region

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6

Iron Oxide Nanoparticle Formulations for Supplementation

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Abstract: Intravenous (IV) iron is widely used to provide supplementation when oral iron is ineffective or not tolerated. All commercially available intravenous iron formulations are comprised of iron oxyhydroxide cores coated with carbohydrates of varying structure and branch characteristics. The diameter of the iron-carbohydrate complexes ranges from 5–100 nm and meets criteria for nanoparticles. Clinical use of IV iron formulations entered clinical practice beginning of the late 1950s, which preceded the nanomedicine exploration frontier. Thus, these agents were approved without full exploration of labile iron release profiles or comprehensive biodistribution studies. The hypothesis for the pathogenesis of acute oxidative stress induced by intravenous iron formulations is the release of iron from the iron-carbohydrate structure, resulting in transient concentrations of labile plasma iron and induction of the Fenton chemistry and the Haber-Weiss reaction promoting formation of highly reactive free radicals such as the hydroxyl radical. Among available IV iron formulations, products with smaller carbohydrate shells are more labile and more likely to release labile iron directly into the plasma (i.e., before metabolism by the reticuloendothelial system). The proposed biologic targets of labile-iron-induced oxidative stress include nearly all systemic cellular components including endothelial cells, myocardium, liver as well as low density lipoprotein and other plasma proteins. Most studies have relied on plasma pharmacokinetic analyses that require many model assumptions to estimate contribution of the iron-carbohydrate complex to elevations in serum iron indices and hemoglobin. Additionally, the commercially available formulations have not been well studied with regard to optimal dosing regimens, long-term safety and comparative efficacy. The IV iron formulations fall into a class defined by the Food and Drug Administration as "Complex Drugs" and thus present considerable challenges for bioequivalence evaluation.

Keywords: intravenous · iron · nanoparticle · oxidative stress · regulatory science

1. INTRODUCTION

Intravenous (IV) iron formulations provide a clinical treatment option for patients when iron supplementation is required but oral administration is not suitable due to intolerance or lack of efficacy. The commercially available IV iron products are considered first generation nanoparticle formulations synthesized by co-precipitation of iron oxide with carbohydrates of varying molecular weight and branch chain characteristics [1]. Because many of these formulations were approved 30–40 years ago, before contemporary nanomedicine science was well-defined, this class of complex drugs was not required to undergo extensive biodistribution and *in vivo* characterization consistent with the current standard for nanomedicine checkpoints [2].

IV iron use is increasing worldwide, in a broad spectrum of disease states including heart failure, inflammatory bowel disease, cancer, post-bariatric surgery and post-partum hemorrhage. However, the largest population receiving chronic IV iron supplementation are chronic kidney disease (CKD) patients. More aggressive IV iron use in the CKD population has been driven by several trials demonstrating adverse safety signals with the other pharmacotherapeutic

mainstay to treat anemia; erythropoiesis stimulating agents (ESAs). Increased risk of stroke, cardiovascular death and a trend toward higher risk of solid organ cancers were observed in these trials, prompting a "black box" label change by the Food and Drug Administration (FDA) in 2010 [3–6]. The now ubiquitous administration of large cumulative doses of IV iron has also driven health care policy, in the US, specifically. In 2011, the Centers for Medicare and Medicaid Services instituted a partially capitated payment system (also known as "the bundle") for dialysis services, which included both ESAs and IV iron, which were previously separately billable [7].

The recombinant human ESA therapies are clearly far more expensive than IV iron products, which accelerated a national trend to use larger cumulative doses of IV iron. Doses of ESA began to decline and IV iron doses began to rise several months in advance of bundle implementation [8]. The Dialysis Outcomes Practice Patterns study showed mean ferritin concentrations (which represents a marker of stored iron) increased from 640 to 826 ng/mL pre-bundle to post bundle (January 2012) and remained stable through December 2013 [8]. The percentage of patients with ferritins > 1200 ng/mL increased from 8.6 % to 18 % of patients [8]. On average, dialysis patients receive 3.6 grams of elemental iron annually [9]. However, blood loss associated with contemporary dialysis procedures, routine phlebotomy, and gastrointestinal losses have been estimated to be only 1.3 grams annually [10]. Thus, most dialysis patients are presumed to be in a positive iron balance [10]. Given the complex and variable pharmacokinetics and biodistribution of these agents, it is necessary to invest in further studies of these agents that are administered in a widespread and chronic manner in the CKD population.

2. STRUCTURE AND BIODISTRIBUTION OF INTRAVENOUS IRON FORMULATIONS

2.1. Evolution of Intravenous Iron Formulations

Early IV iron compounds were formulated as inorganic iron oxyhydroxide complexes. With little relative protection of the inorganic ferric iron, these formulations were highly toxic and were associated with high incidence of severe hypotension [1]. In currently available nanoparticle formulations the iron oxyhydroxide core is surrounded by carbohydrate shells of different sizes and polysaccharide branch characteristics (Table 1) [2, 11].

The first formulation to be approved by the Food and Drug Administration (FDA) in the United States was iron dextran (InFed®) in 1974 [12]. Iron dextran has a large molecular weight with dextran moieties varying in size. While the iron dextran formulation allowed successful administration of large total dose infusions of iron (e.g., 2.5 grams elemental Fe), this formulation has caused anaphylactic-type reactions. This necessitated a boxed warning labeling change by the FDA, which required a test dose (25 mg) before administration of the remainder of the IV doses. A higher molecular weight formulation, Imferon®, was

Table 1. Comparison of physicochemical characteristics and pharmacokinetics of reference listed drug intravenous iron formulations [1, 11, 15, 16].

PROPERTIES	Feraheme®	Injectafer®	InFed®	Venofer®	Ferrlecit®
Molecular weight (Da)	731,000	150,000	410,000	252,000	200,000
Carbohydrate shell	Polyglucose sorbitol carboxymethylether	Carboxymaltose	Dextran polysaccharide	Sucrose	Gluconate, loosely associated sucrose
Median shell/ particle diameter (nm)	26.3	23.1	12.2	8.3	8.6
Relative labile Fe release	+	+	++	+++	+++
Relative stability of elemental Fe within the carbohydrate shell	High	High	High	Medium	Low
Relative osmolalities	Isotonic	Isotonic	Isotonic	Hypertonic	Hypertonic
Administration (intravenous push) rates	30 mg/sec	Bolus push	50 mg (1 mL)/ min	~20 mg/min	12.5 mg/min
Half-life (hrs)	~15	7–12	5–20	6	~1

associated with higher rates of anaphylactic-type reactions and was voluntarily discontinued by the manufacturer. This prompted interest in IV iron formulations with less immunogenicity. New drug applications were subsequently filed for two newer, smaller molecular weight formulations (sodium ferric gluconate complex and iron sucrose) and approved by the FDA in 1999 and 2000, respectively [12]. The carbohydrate coating of the iron oxyhydroxide core is very weakly bound for these products compared to the previous dextran formulations [13]. These formulations were shown to be less likely to induce anaphylactic-type reactions, even when administered to patients with previous iron dextran reactions [14]. However, due to the loosely bound carbohydrate protecting the iron core, a larger proportion of labile iron is released directly into plasma after injection limiting the maximal single doses to < 200 mg [13, 15].

The increasing use of iron across a wide variety of disease states treated in the outpatient clinic setting has renewed interest in larger molecular weight formulations that can be administered with higher single doses. Ferumoxytol (Feraheme®) is a superparamagnetic iron oxide nanoparticle that was originally approved for magnetic resonance imaging [16]. The manufacturers of ferumoxytol subsequently filed a new drug application as an IV iron supplement and was approved by the FDA in 2009 to be dosed as 510 mg elemental iron pushed IV over 17 seconds [12]. Despite being marketed as a "non-dextran" formulation, numerous post-marketing reports of anaphylactic-type reactions were filed with the FDA [17]. This prompted the FDA to require a boxed warning label change in 2015 that mandated that a description of the risks of hypersensitivity/anaphylaxis be added, that ferumoxytol only be administered as an IV infusion over a minimum period of 15 minutes and patients be monitored for 30 minutes post-infusion. These new labeling changes limited the utility of ferumoxytol in the ambulatory outpatient setting.

More recently, ferric carboxymaltose (Injectafer®) underwent North American registry trials and was approved by the FDA in 2013 [12]. Ferric carboxymaltose can be administered to patients weighing more than 50 kg as 1,500 mg elemental iron (750 mg doses separated by 7 days). Iron maltoside (Monofer®) is a formulation only available in Europe that is structurally described as non-branched, non-anaphylactic carbohydrate-coated iron oxide nanoparticle, distinct from dextran coatings [18].

2.2. Physicochemical Characteristics

The size of all commercially available IV iron-carbohydrate complexes ranges from 5 to 100 nm, and thus meets the defined parameters for nanoparticle formulations [2]. The large-scale production of iron-carbohydrate complex formulations is highly sensitive to pH, temperature, and other conditions in the manufacturing process. This presents significant challenges to reproducible manufacturing, characterization, and synthesis of generic formulations.

Iron oxide nanoparticles with magnetic particle cores are well-established magnetic resonance imaging agents and have been used safely; however, different

carbohydrate shell structures determine the relative uptake by endothelial and lymphatic cells as well as by the reticuloendothelial system (RES) [2]. The clinical use of iron-carbohydrate nanoparticle formulations has not been well studied with regard to immediate labile iron appearance and potential long-term toxicities. Because commercially available IV iron formulations meet the criteria for nanoparticles, their pharmacodynamic profile with regard to direct cell uptake and subsequent physiological effects needs to be better characterized [19].

2.2.1. Particle Size

Nanoparticle size and uniformity dictate biodistribution and cellular uptake [2]. Gel permeation chromatography (GPC) and dynamic light scattering (DLS) are two approaches to determining the IV iron nanoparticle size. Commercially available IV iron formulations have nanoparticle diameters ranging from 5–40 nm by GPC [11]. Most of the formulations have homogeneous particle diameter distributions, except ferumoxytol and ferric carboxymaltose, which show additional smaller and larger diameter peaks [11]. DLS measures the hydrodynamic diameter of the carbohydrate shell coating the iron core and therefore reports larger diameters than those techniques, which evaluate the core size (transmission electron microscopy and X-ray diffraction). Jahn et al. showed the range of nanoparticle sizes were similar for iron sucrose/sodium ferric gluconate (median 8 nm), low-molecular-weight iron dextran/iron maltoside (median 9–12 nm), and ferumoxytol/ferric carboxymaltose (median 23 nm) [11]. Polydispersity indices ranged from 0.07 to 0.244 suggesting monodispersity; however, it should be acknowledged that DLS results can be driven by larger particle sizes.

The FDA recently evaluated the reference listed drug (RLD) Ferrlecit® and the generic formulation of Ferrlecit (sodium ferric gluconate complex, Watson Pharma, Inc). Results varied slightly (10.5–11.3 nm) with diluent (deionized water, 10 mM NaCl, saline) and varied more with Z-average, intensity-averaged, and volume-weighted reported diameters (8–14 nm) [20]. Recently, DiFrancesco and Borchard have suggested that size distribution by number is most relevant in characterization of iron sucrose formulations by DLS and that a certain percentage of the carbohydrate might be recognized as a contaminant by the instrument, leading to a misrepresentation of the iron sucrose particle size when using Z-average and the polydispersity index [21].

2.2.2. Size and Structure of Core

Transmission electron microscopy (TEM) imaging of IV iron formulations has shown that most of the formulations do not contain spherical nanoparticles, with the exception of iron maltoside [11]. Additionally, most formulations tend to aggregate; this is especially pronounced with ferric carboxymaltose [11]. Median core sizes ranged from 4.1 to 6.2 nm and increased in accordance with reported molecular weight (Table 1), suggesting some measurement errors may occur due

to the cores being surrounded by a less dense matrix which may be attributed to the carbohydrate fraction.

When TEM images were compared between Ferrlecit® and the sodium ferric gluconate complex, the average core size was similar (2.0 ± 1.4 nm). However, evaluation of the distribution of core sizes measured showed that Ferrlecit® core sizes were less normally distributed [20]. Analysis of core size by X-ray diffraction showed core sizes between 3.3–6.4 nm for the small and larger molecular weight IV iron formulations studied [11]. Among the products studied, all showed broad regions of high intensities at similar angle values with the exception of ferumoxytol where the diffractogram showed interference with crystallized mannitol in the formulation [11].

2.2.3. Iron Content

Measurement of total iron content in the iron nanoparticle formulation can be determined by various techniques including inductively coupled plasma mass spectrometry (ICP-MS). All IV iron formulations are labeled as ferric iron. Valence state can be determined by Mössbauer spectroscopy; however, the clinical relevance of slight differences in the ratio of Fe^{2+} to Fe^{3+} is unknown with regard to either efficacy or safety [13]. At the nanoparticle level, the size of the iron nanoparticle has important implications for the core surface area available for the dissociation and release of the reduced ferrous iron from the ferric oxyhydroxide cores [1].

2.2.4. Labile Iron Release

All IV iron products have the potential to have direct release of labile (nontransferrin bound) iron into plasma, a physicochemical characteristic that appears to be directly related to molecular weight [1, 22–24]. Labile iron, an important regulator of cell signaling, induces the Fenton-Haber-Weiss reaction where ferric iron is reduced to ferrous iron in the presence of hydrogen peroxide, yielding a hydroxide radical ($OH^{\cdot}$) [10]. Because of potential toxicity, labile or nontransferrin bound iron (NTBI) is tightly regulated and has several important physiologic roles in the body including its involvement in mitochondrial electron transport, cytochrome P450 activity, and in the transfer of oxygen by heme [10]. The efficacy of these carbohydrate molecules coating the iron nanoparticle's core in shielding iron and preventing iron-based free radical and cytotoxic reactions is variable and dependent on the size of the carbohydrate shell (e.g., molecular weight of the iron-carbohydrate complex) [1]. Short-term studies indicate all available formulations are capable of directly releasing unbound iron into the circulation from the iron-carbohydrate complex.

However, there is a clear relationship of stability of the complex to appearance of labile iron with the likely rank order based on available data: sodium ferric gluconate > iron sucrose > low molecular weight iron dextran > ferumoxytol = ferric carboxymaltose = iron maltoside 1000 [11, 15, 23, 25, 26]. Determining

labile iron release from IV iron formulations *in vitro* is challenging due to the destabilization of these formulations in diluents. A systematic evaluation of chelatable and redox iron assay methods to measure the amount of labile iron released from IV iron formulations was performed in biorelevant matrices *in vitro* [27]. The majority of published labile iron assays evaluated were not suitable for use *in vitro* due to the potential for interference by the IV iron products. However, an optimized HPLC-based method performed well for use *in vitro* for labile iron detection in a biorelevant matrix (rat serum) [27].

2.3. Pharmacokinetics

Pharmacokinetic analysis of IV iron-carbohydrate complexes is limited by difficulties in distinguishing IV iron formulations from endogenous iron. Using ^{59}Fe to radiolabel the formulations is one approach that can yield data on how much iron is incorporated into the red blood cells, which is the desired target cell for biodistribution to treat anemia [28]. Despite the red blood cell being the desired pharmacodynamic target, the carbohydrate shell clearly also determines the relative uptake by endothelial and lymphatic cells as well as the RES [2]. The carbohydrate moiety size drives clearance and resultant plasma residence times with higher doses, especially with larger molecular weight formulations exhibiting capacity-limited or zero-order metabolism [22, 28]. Doses above the RES capacity for uptake will remain circulating until the concentration falls below the capacity limit at which point the pharmacokinetics become linear (first-order) or concentration-independent.

For ferumoxytol administered as two 510 mg intravenous doses 24 hours apart to healthy subjects, the metabolism does not appear to become linear until approximately 96 hours after the first dose [22]. Henderson and Hillman showed that radiolabeled iron dextran exhibited capacity-limited metabolism at 500 mg whereas the 250 mg dose appeared to have a linear pharmacokinetic profile [28]. Ultimately, the complexity of IV iron-carbohydrate complex nanoparticle formulations has important implications with regard to both efficacy and safety. These agents have not been well studied with regard to comparative biodistributions, metabolic fates, and potential extracellular and intracellular toxicity profiles, and further evaluation of these agents is urgently needed. Current regulatory guidance has limited requirements for physicochemical characterization and pharmacokinetics of these agents, especially for abbreviated new drug applications (ANDA) for generic formulations, which necessitates independent clinical and translational studies to elucidate comparative product characteristics.

3. THERAPEUTIC EFFICACY IN THE DIALYSIS POPULATION

The introduction of ESAs and IV iron were important additions to the anemia treatment options as the requirement for frequent blood transfusion in the pre-

ESA era was associated with side effects and poor outcomes including iron overload, infections (especially hepatitis C), pre-transplant allo-sensitization, and the inability to safely transfuse highly immunized patients. However, whether IV iron treatment results in improved health outcomes is largely unknown. Randomized controlled clinical trials have not sufficiently evaluated definitive, patient-centered outcomes such as mortality, hospitalization or quality of life. In contrast, surrogate endpoints such as hemoglobin and impact on ESA dose have been fairly well-studied.

3.1. Intravenous *versus* Oral Application

There is a significantly large body of literature that supports the efficacy of IV iron in dialysis patients. The first published randomized clinical trial (RCT) comparing IV and oral iron in hemodialysis patients found a 46 % ESA dose reduction with IV iron therapy [29]. Subsequent studies have been very consistent in finding that regular (often weekly) doses of IV iron reduce ESA, although there is less literature with other dialysis modalities such as peritoneal dialysis. The dose of iron to administer, what dosing regimen is optimal and targets for clinical iron status tests, remain largely unanswered questions despite several decades of widespread use of these medications.

3.2. Loading *versus* Maintenance Dosing Approaches

One important trial was the Dialysis Patients' Response to IV Iron with Elevated Ferritin (DRIVE) Study [30]. This study pushed conventional upper limits of ferritin by testing the efficacy of IV iron when administered to individuals with serum ferritin > 500 ng/mL (normally 12–300 ng/mL) and transferrin saturation (TSAT) ≤ 25 % (normal 20–25 %), and allowed enrollment of individuals with ferritin between 800–1200 ng/mL – a level typically used to exclude enrollment in previous IV iron trials [31, 32]. Randomization to IV iron resulted in an increase in hemoglobin compared to placebo. A follow-up observational study of DRIVE participants demonstrated IV iron led to a significant reduction in ESA dose requirements and the cost of anemia management [33, 34]. The study extended previous knowledge by suggesting that IV iron treatment improves erythropoietic response to ESA therapy even with fairly high baseline ferritin, but the small number of participants (n = 134) and short duration (6 weeks) limits the power to make definitive safety conclusions. Additionally, results may be biased by the fact that all doses of ESA were increased before IV iron was administered, therefore "priming" the IV iron group for a larger increase in hemoglobin.

It is clear that raising ferritin and TSAT with IV iron reduces ESA doses, lowers costs, and it is possible that increasing hemoglobin levels with more IV iron rather than with more ESAs improves outcomes. However, this has not been tested in clinical trials, and improving hemoglobin levels by using aggressive doses of IV iron may not actually be beneficial. For example, increasing hemo-

globin levels did not improve clinical outcomes in ESA treatment studies (except in the treatment of severe anemia). In contrast, secondary analyses of trial data suggest that higher ESA doses are associated with adverse cardiovascular outcomes, suggesting through association that using IV iron to reduce ESA doses could have important cardiovascular benefits [35].

In a non-dialysis heart failure population, the Ferinject® assessment in patients with iron deficiency and chronic heart failure study randomized 459 patients with congestive heart failure to treatment with IV ferric carboxymaltose or placebo [36]. After 24 weeks, the IV iron group experienced improvements in measures of functional status and quality of life. There were no significant differences in mortality, but there was a trend towards fewer heart failure hospitalizations (P = 0.08). Findings were similar in a recent study evaluating ferric carboxymaltose in patients with chronic heart failure and iron deficiency. The study randomized 304 heart failure patients to ferric carboxymaltose or placebo and demonstrated significant reductions in heart failure hospitalization (hazard ratio (HR) 0.39, 95 % confidence interval (CI) of 0.19–0.82, P = 0.009) [37]. Extrapolation of these findings to hemodialysis patients is difficult, but it does support the potential for important clinical benefits from IV iron in the hemodialysis population; however, as noted previously, the long-term effects of such dosing strategies are unknown. IV iron induced ESA dose reduction and hemoglobin increase may improve health outcomes, but the putative benefits remain speculative and untested in RCTs. Until the effect of IV iron in hemodialysis on mortality, cardiovascular events, hospitalizations, quality of life or other outcomes is better understood, whether IV iron treatment is actually beneficial will remain unknown.

A study currently being conducted in the United Kingdom (PIVOTAL) is powered for a primary endpoint of time to all-cause death or a composite of non-fatal cardiovascular events and may prove helpful in this regard. PIVOTAL, which will recruit 2080 patients from more than 50 sites, will compare the effect of a proactive high-dose (loading) with a reactive low-dose (maintenance) regimen of iron sucrose in ESA-treated hemodialysis patients with ferritin < 400 µg/L and TSAT < 30 % [38]. With a primary endpoint of time to death or a composite of non-fatal cardiovascular events and secondary endpoints which include infection and infectious hospitalizations, it is expected that results will provide crucial insights into the true clinical benefits of IV iron in the setting of hemodialysis.

3.3. Comparative Efficacy

There are very few studies directly comparing dosing and efficacy of the commercially available IV iron compounds in a prospective controlled trial. Fundamentally, this has been due to a limited number of formulations on the market and lack on competitive initiative. However, with recent IV iron formulation approvals and a piqued interest in both efficient dosing and safety, more studies have been initiated in this area. A randomized study comparing ferumoxytol with its originally approved dosing recommendations (510 mg $\times$ 2 doses over 17 seconds) *versus* iron sucrose (10 $\times$ 100 mg doses at hemodialysis or 5 $\times$

200 mg for non-dialysis patients) was conducted in 162 patients with varying stages of kidney disease (43 % on dialysis) [39]. Patients received a single dosing course of ferumoxytol or iron sucrose and were followed for 5 weeks. Changes in hemoglobin were similar among both treatments; however, adverse reactions were more commonly reported in the iron sucrose-treated patients compared to those who received ferumoxytol (65 % *versus* 48 %, respectively). A randomized trial comparing the safety and efficacy in patients with iron deficiency of any etiology treated with ferumoxytol (n = 997) or ferric carboxymaltose (n = 1000) was conducted and patients were followed for 5 weeks [40]. Total doses administered were 1.02 grams and 1.5 grams, respectively. Ferumoxytol was determined to be non-inferior to ferric carboxymaltose with regard to hypersensitivity reactions (< 1 % reported for both formulations) and hemoglobin change (mean increase 1.4 g/dL and 1.6 g/dL, respectively). An ongoing study is the first to examine comparative efficacy of ferumoxytol and iron sucrose during long-term treatment. This randomized open label study will assess adverse effects and hemoglobin changes over the 11 months observation period. Two substudies will evaluate biomarkers of oxidative stress and magnetic resonance imaging to determine tissue deposition [41].

4. ADVERSE SAFETY SIGNALS

4.1. *In Vitro* Safety Signals

The hypothesis for the pathogenesis of acute oxidative stress induced by IV iron formulations is the release of labile iron from the iron-carbohydrate structure resulting in transient concentrations of labile plasma iron and induction of the Fenton chemistry and the Haber-Weiss reaction promoting formation of highly reactive free radicals such as the hydroxyl radical [42]. Labile plasma iron represents the oxidative reactive fraction of NTBI, i.e., iron that is not tightly bound to transferrin. Among available reference listed drug IV iron formulations, products with smaller carbohydrate shells are more labile and more likely to release labile iron directly into the plasma (i.e., before metabolism by RES) (Table 1) [15, 23].

The proposed biologic targets of labile iron induced oxidative stress include nearly all systemic cellular components including endothelial cells, myocardium, and liver as well as low density lipoprotein and other plasma proteins. Because of the extremely short half-lives of free radicals and the rapidity of the ensuing oxidative stress reactions produced by labile iron appearance, *in vivo* evaluation of this toxicity profile can only reasonably be accomplished by using biomarkers as surrogates.

Recently, a systematic review of widely used biomarkers to assess oxidative stress in chronic kidney disease was conducted. The authors applied scores for commonly used biomarkers for relationships to other biomarkers and clinical indicators, reliability and characterization in the CKD literature [43]. Many of the identified “robust” biomarkers have been evaluated in the context of potential IV iron toxicity in CKD (malondialdehyde, protein carbonyl and F2-isopro-

stane), however, it should be noted that none of the identified biomarkers have specificity for iron-induced oxidative stress [44, 45]. An additional concern regarding appearance of labile plasma iron is the potential for easily accessible iron to impair innate immunity and augment bacterial growth, increasing the risk of infection [46]. The transient release of labile iron directly into plasma (i.e., before metabolism by RES) is more likely with smaller carbohydrate shells and results in transient concentrations of labile plasma iron and formation of highly reactive free radicals such as the hydroxyl radical that uniquely limits the maximum dose that can be administered with each formulation [15, 23, 42]. Not surprisingly, IV iron formulations have been shown to induce oxidative stress, inflammation, and cellular toxicity, pro-oxidant cell signaling, tissue inflammation, cellular iron deposition, and cytotoxicity in cell culture models, animal models, and acutely in human subjects [23, 47, 48–50], with more labile compounds inducing more toxicity than those with larger carbohydrate shells [45, 46] Differential toxicity profiles have been observed among the available IV iron products *in vitro*, with more labile compounds inducing more toxicity than compounds with larger carbohydrate shells that exhibit better stability [46, 47]. IV iron has also been associated with immune dysfunction, augmentation of bacterial growth, and increased Gram-positive bacteria growth *in vitro* [51, 52]. Taken collectively, these studies underscore the need for comprehensive clinical and translational investigations to evaluate the impact of differences in formulation and whether repeated induction of oxidative stress from IV iron has long-term sequelae.

4.2. *In Vivo* (Animal) Safety Signals

In animal models, similar observations have been reported with administration of IV iron compounds inducing labile iron appearance, pro-oxidant cell signaling, tissue inflammation, cellular iron deposition, and cytotoxicity [43, 48, 53]. In similar rat models, increased tissue oxidative stress has been observed with several iron sucrose similar (i.e., generic) products compared to the branded product [48]. A caveat to interpretation of these *in vivo* animal model data is the wide variation in doses administered in the experiments (1.4 mg/kg to 500 mg/kg). [48, 53]. While the dose in the rat should be higher, based on allometric scaling, the optimal dose to model human IV iron toxicity has not been determined.

4.3. Safety Signals in the Dialysis Population

4.3.1. Translational Studies

Very few translational studies have been conducted in the dialysis population, however, the majority have aimed to evaluate changes in biomarkers of oxidative stress and inflammation. A study compared oxidative stress and inflammation response in hemodialysis patients receiving single 100 mg elemental Fe doses of

iron sucrose and iron dextran in a cross-over design [54]. Labile iron concentrations measured over 6 hours were 60 % higher after iron sucrose administration compared to iron dextran. Serum IL-6 increased more after iron sucrose treatment and both treatments induced intracellular reactive oxygen species and loss of mitochondrial membrane potential. In another study iron sucrose was administered alone and with 300 mg of ascorbic acid in a crossover design. Co-administration with this reducing agent, intended to improve efficacy of IV iron, increased cytokine activation measured by serum interleukins (IL) IL-1, IL-6, IL-10, and tumor necrosis factor α [55]. An elegantly conducted study by Kuo et al. evaluated iron sucrose effects on atherogenesis across cell culture animal and human experiments [50]. The leukocyte-endothelium interaction was evaluated in a human aortic endothelial cell/macrophage co-culture model. An *in vitro* mononuclear-endothelial cell adhesion assay showed that iron sucrose-treated mononuclear cells showed more adhesion to endothelial cells as well as increasing reactive oxygen species and cellular adhesion molecules. In mice, iron sucrose treatment increased adhesion molecule expression. This effect was even greater in mice that had surgical nephrectomies. An *ex vivo* assay also showed increased leukocyte adhesion to endothelium after iron sucrose treatment which was further increased in kidney failure mice. The investigators also administered iron sucrose to 20 healthy controls and 20 patients receiving hemodialysis. The hemodialysis patients had significantly increased intracellular reactive oxygen species production and plasma soluble adhesion molecule concentrations.

4.3.2. Clinical Trials

Although well-powered studies of sufficient duration are not available, examination of previously reported studies provides at least a topical understanding for whether any safety signal is present with IV iron treatment. The easiest adverse effect of IV iron to assess is anaphylaxis, because of its immediate presentation and severity. In a study of 2,534 hemodialysis patients who were directly observed after double blind exposure to IV sodium ferric gluconate (SFGC) or placebo [32], one patient in each of the SFGC and placebo group experienced anaphylactoid reactions. Additional cases with characteristics possibly consistent with anaphylaxis occurred in 0.4 % of IV SFGC patients and 0.1 % of placebo-treated patients. The results suggest that there is a relatively low rate of anaphylaxis with non-dextran iron formulations and that the reactions are generally easily managed. Relatively large, but non-randomized, clinical trials are consistent with a similar safety profile for iron sucrose therapy, although this and other studies have been less helpful for assessing anaphylaxis due to the lack of placebo controls, direct observation, under-reporting or use of inappropriate data sources to assess endpoints [31].

Assessing long-term safety is more challenging, although some information on end-organ effects, cardiovascular events and infection risk can be evaluated from available clinical trials, which were underpowered and/or had insufficient duration of follow up for reliable conclusions. For example, in the recently reported

Ferinject® assessment in patients with iron deficiency anemia and non-dialysis-dependent chronic kidney disease study, 626 patients with pre-dialysis CKD were treated with IV ferric carboxymaltose (with a high and low ferritin target) or oral iron for 52 weeks. The percentage of deaths, myocardial infarction, and infections was not significantly different between oral and IV iron-treated patients. However, the study was not powered for safety [39]. Similarly, a 2008 meta-analysis comparing IV iron to oral iron by Rozen-Zvi et al. included 7 dialysis studies, but only one had >100 patients and none had treatment periods >6 months [56]. The authors noted after analysis that data related to safety (mortality rate) was limited. Finally, the 6 weeks DRIVE trial and its 6 weeks follow-up DRIVE II randomized only 57 subjects with ferritin above 800 ng/mL [30, 33]. Taken collectively, the clinical trial literature on IV iron in hemodialysis has an insufficient number of patients followed for an insufficient length of time to fully assess the long-term safety of IV iron treatment, particularly when used with individuals with serum ferritin >800 ng/mL.

Observational studies provide an important alternative to randomized clinical trials for assessing the safety of IV iron, despite a greater susceptibility to confounding, bias and inability to conclude causality. A 2002 study by Feldman et al. utilized the United States Renal Disease System (USRDS) Dialysis Morbidity and Mortality Studies waves 1, 3, and 4 to analyze the safety of IV iron. Among 10,169 patients, those with bills submitted for >10 vials of iron dextran over a 6 months period were found to have increased risks of death (adjusted relative risk (RR) 1.11, 95 % CI: 1.00–1.24) and hospitalization (adjusted RR 1.12, 95 % CI: 1.01–1.25) than those without any submitted bills [57]. A subsequent analysis of nearly 33,000 Fresenius Inc. hemodialysis patients by the same authors did not confirm an association between IV iron dose and risk of death, after adjusting for time-varying measures of iron treatment and fixed and time-varying measures of morbidity. In contrast, a study by Feldman et al. of nearly 60,000 prevalent hemodialysis patients found that use of IV iron was associated with a 22 % reduction in mortality, while Kaysen et al. found that iron administration was associated with a lower risk of death, independent of hematocrit [58, 59].

More recently, Kalantar-Zadeh et al. studied 58,058 DaVita Inc. dialysis patients. For patients who received less than 400 mg of IV iron per month, the risk for death was lower compared to patients with no IV iron administered [60]. In contrast, doses >400 mg/month were associated with an increased risk of death [60]. Kshirsagar et al. studied 117,050 hemodialysis patients and found no association between dose of IV iron and short-term risk of myocardial infarction, stroke or death [61]. In contrast, a much smaller observational study found dose-related increases in risks of cardiovascular events and death with IV ferric chloride hexahydrate – a product that is not available in the US [62].

The relationship between IV iron and infection is of concern. Previously published studies have had equivocal results. In a small retrospective study, 132 dialysis patients receiving their first course of IV iron were followed for one year after therapy initiation for time to first bacteremia episode [63]. Patients with transferrin saturation values ≥20 % and ferritin ≥100 were defined as iron-

replete; this group had a 2.5-fold higher risk of bacteremia compared to patients with functional iron deficiency and those who were iron-deficient. These data may infer that iron availability is increased when additional iron is administered to these already iron-replete patients, promoting bacterial growth and subsequent bloodstream infections.

More recently, a large epidemiologic study examined the risk of infection-related hospitalization with bolus *versus* maintenance or high *versus* low IV iron-dosing patterns [64]. Bolus dosing of IV iron was associated with a higher risk of infection-related hospitalization (25 additional events per 1000 patient-years) and increased risk of mortality. Differences in infection rates between iron formulations are difficult to evaluate. In two studies evaluating United States Renal Data System data, the short-term infection risk in hemodialysis patients with sodium ferric gluconate was marginally lower than with iron sucrose [65]. In contrast, a study by the same group showed that the longer-term infection risk was modestly lower in iron sucrose treated hemodialysis patients [66]. A prospective observational study by Hoen et al. [67] followed 988 hemodialysis patients from 19 French centers for 6 months. There were 51 episodes of bacteremia, but no association was found with either IV iron dose or serum ferritin concentration [67]. Recently, Brookhart et al. used a large dialysis consortium's data to study 117,050 hemodialysis patients [64]. Patients with higher compared to lower doses had a slightly but statistically significantly greater risk of infection-related hospitalization or death (HR [7], 1.05, 95 %: 1.02–1.08), while individuals with the combination of high serum ferritin and high TSAT at baseline had the highest risk of infection-related hospitalizations [64]. They also found that compared to maintenance therapy, loading dose treatment was associated with a greater risk of infection (risk difference, 25 additional events/1000 patient-years; 95 % CI: 16–33) [64]. More prospective studies are needed to elucidate whether risk and predictors of infection differ among formulations.

These studies have conflicting signals and cannot establish a clear relationship between IV iron use and infection and mortality. Better studies of the associations of outcomes, particularly infection, with dose and pattern of administration are needed. Dose-related safety information is particularly necessary given that the epidemiologic studies analyzed data that predate contemporary increases in IV iron utilization and serum ferritin. The long-term impact of IV iron administration to patients with elevated baseline serum ferritin levels on surrogate markers of atherosclerosis, immune function, inflammation, and vascular reactivity also remain unstudied. However, a study found 19/21 (90.5 %) long-term dialysis patients with serum ferritin >1000 ng/mL had evidence of increased oxidative stress as well as splenic and hepatic tissue iron overload which suggests caution is warranted [68].

Thus, although the biological plausibility and available *in vitro* and animal model data are generally compelling, controversy remains regarding whether iron-induced oxidative stress manifests long-term toxicity such as cardiovascular disease and infection in CKD patients. The complex biochemical milieu in CKD coupled with multiple inciting factors for oxidative stress and inflammation com-

plicates translational investigation of potential IV iron safety concerns. Given that the impact of iron-induced oxidative stress on cardiovascular disease likely takes extended periods of time, immediate correlation of iron dose to cardiovascular events is not likely possible. Evaluating the relationship between iron and infection risk from an epidemiologic perspective could be more easily evaluable given that the risk of infection would be in close proximity to the dose administered when labile iron is released from the formulation.

4.4. Challenges with Generic Formulations

There is a plethora of generic iron sucrose products (iron sucrose similar, (ISS)) on the global market. When compared to the RLD Venofer®, several ISS formulations have been shown in translational models to have significantly more tissue iron deposition, induce greater tissue cytokine expression and cause endothelial dysfunction [48–50]. Ultimately, the complexity of IV iron-carbohydrate complex nanoparticle formulations has important implications with regard to both efficacy and safety in chronic kidney disease. These agents have not been well studied with regard to comparative biodistribution, metabolic fate, and potential extracellular and intracellular toxicity profiles and further evaluation of these agents is urgently needed as long-term clinical use is widespread.

Current regulatory guidance provides some recommendations for physicochemical characterization and pharmacokinetics of these agents. This is especially relevant for abbreviated new drug applications for generic formulations, which necessitates independent clinical and translational studies to elucidate comparative product characteristics [69]. Even slight changes (temperature, pH, polymer content) in the co-precipitation reaction to synthesize iron-carbohydrate nanoparticles can alter the properties of the final product, presenting challenges to reproducible manufacturing of IV iron formulations being considered for generic approval [70]. These formulations have been referred to as “similars” as exact copies cannot be formulated [48, 69]. Although, it has been shown that if the iron complex is thermodynamically stable, complexes of similar molecular weight can be synthesized using multiple different manufacturing procedures, this may or may not translate to similar disposition *in vivo* [71].

Simple fold dilutions in polymer content during iron oxide-dextran co-precipitation have yielded particles with similar hydrodynamic diameters determined by dynamic light scattering; however, the cellular iron uptake and cell viability are markedly different among the particles [11]. Several ISSs available outside the United States have been shown to not meet United States Pharmacopeia (USP) Reference Standards [48, 49]. Differences in molecular weight, titratable alkalinity, kinetics of degradation have also been shown between lots of the same generic formulation [72]. Toblli et al. characterized the physicochemical characteristics of the RLD Venofer® and compared these to several of the compounds available and in clinical use in Europe and Asia [48]. Notably, only one generic product in these comparative analyses complied with USP criteria. Four of the seven products (57 %) evaluated had markedly higher molecular weights

measured by gel permeation chromatography. In animal studies using 40 mg/kg single IV doses, generic iron products have been shown to be associated with higher tissue concentrations of pro-inflammatory cytokines, higher intracellular antioxidant enzyme activity, adverse effects on the basic metabolic profiles (elevated liver function tests), and kidney dysfunction (elevated serum creatinine and proteinuria) [48, 73].

It has been hypothesized that labile iron is principally involved in these observed deleterious effects by generating reactive oxygen species via the Fenton-Haber-Weiss reaction. In a systematic series of experiments, *in vitro* labile iron release profiles were evaluated for six IV iron formulations [27]. The formulations studied included the only approved generic IV iron in the United States (sodium ferric gluconate complex) and the RLD Ferrlecit®. Labile iron release in both saline and rat serum matrices was higher for the RLD *versus* the generic SFGC, indicative of some formulation variability. To date no published studies comparing RLDs with generic IV iron formulations have evaluated labile iron release profiles in human subjects. Because generic iron-carbohydrate complex formulations may differ with regard to molecular weight, carbohydrate shell chemistry, shell and particle diameter, and osmolality, these agents require additional considerations for bioequivalence testing [1, 11].

As discussed previously, biologic plausibility of labile iron being a fundamental cause of adverse drug events (excluding immunogenetic reactions) related to IV iron formulations is strong and supported by translational research evaluating several of the RLD products [71, 74]. The higher incidence of hypotension reported with some generic formulations, including different lots of the same formulation, is likely attributable to formulation-based free iron release [75, 76]. Thus, labile iron measurement is both a relevant and practical candidate to further evaluate bioequivalence (BE) of generic IV iron formulations [27, 77]. Assessment of labile iron release profiles extends data provided by physicochemical characterization (PCC) to better understand how the disposition of generic formulation compares to the RLD. As mentioned previously, despite evidence of similar PCC, these complex formulations may behave differently in *in vivo* systems [78, 79], underscoring the need for a multi-pronged approach in evaluation of BE among complex drug formulations.

Animal studies evaluating generic iron sucrose formulations have evaluated serum iron concentrations and TSAT and found values to be higher in animals receiving the generic formulations *versus* the RLD [48, 49, 73]. However, TSAT is not a direct measurement of the reactive labile iron species and does not adequately represent the potential for deleterious redox reactions. Although TSAT values greater than 100 % do strongly infer the presence of labile iron, we (and others) have shown that labile iron is present at TSAT values less than 100 % limiting the utility of this parameter [15, 42]. An optimal approach for BE for generic IV iron formulations would be the development of an *in vitro* to *in vivo* correlation (IVIVC) model. A validated IVIVC model would allow *in vitro* labile iron release kinetics under physiologic relevant conditions to support BE evaluation in addition to rigorous PCC with standards [77, 80]. Several assays have been developed and validated to measure labile plasma iron, mainly em-

ploying redox active or chelatable methodologies, however many of these assays are not viable for *in vitro* determination of labile iron release from the formulation [27, 42]. DiFrancesco et al. utilized the Ferrozine® assay to determine iron release among six available IV iron formulations *in vitro* incubated in human serum [81]. Smaller molecular weight formulations and higher concentrations representing clinically relevant doses were associated with higher concentrations of iron release. However, it should be noted that this assay measures NTBI (labile reactive iron plus iron weakly bound to other plasma proteins) and may overestimate formulation-based labile iron release.

In interventional clinical trials and observational reports, when compared to the RLD, different formulations and lots of ISS have been associated with intracellular reactive oxygen species generation, increases in biomarkers of endothelial dysfunction, and adverse drug events including hypotension and phlebitis [50, 75, 76, 82, 83]. Labile iron release in the immediate post administration period (directly from the formulation) from RLD iron-carbohydrate complexes has been shown to induce oxidative stress, cytokine activation and endothelial dysfunction [15, 54]. Therefore, the biologic plausibility strongly implies that differences in labile iron release is fundamentally responsible for the higher rates of adverse drug events reported with generic iron sucrose formulations.

The difficulties in evaluating non-biologic complex drugs such as IV iron formulations are appreciated by scientists and regulatory agencies [69, 77, 84, 85]. However, most clinicians who utilize these formulations across a wide spectrum of acute and chronic disease states are not aware of their complicated pharmacokinetic and pharmacodynamics profiles nor do they appreciate the challenges in BE evaluation of generic formulations. A survey administered to 140 pharmacists in France and Spain was designed to provide insight into the current decision-making process by pharmacists regarding IV iron products in the hospital [86]. Substitution of RLD iron sucrose for an ISS ranged from 38–47 %. However, only 19 % and 7 % of pharmacists in France and Spain, respectively, thought there were relevant differences between RLD and ISS formulations.

Taken collectively, there is a need for comprehensive clinical and translation investigations of IV iron formulations to mechanistically evaluate and understand the biodistribution, safety, and toxicity profiles of these agents. Such studies would be useful in moving the needle forward on BE evaluation to ensure safe and effective generic IV iron products.

5. CONCLUDING REMARKS: GAPS IN REGULATORY SCIENCE FOR INTRAVENOUS IRON FORMULATIONS

IV iron nanoparticle formulations fall into a class the FDA defines as “complex drugs” while the European Medicine Agency refers to this class as “non-biologic complex drugs” [69, 77]. The emergence of generic IV iron formulations has revealed significant challenges and gaps in evaluation of these products. Because of the complexity of the formulation and stability issues, physicochemical characterization, including advanced techniques such as DLS, cannot be used alone

to provide sufficient assurance of similarity between the RLD and the generic formulation. Thus, a combination of non-clinical and clinical studies need to be conducted to assess similarity of these complex drugs. The optimal combination of these studies has not been defined by regulatory agencies. The collection of bioequivalence studies may differ between the various IV iron formulations because size and surface properties vary widely and are the key determinant of iron release.

The FDA's current draft bioequivalence guidance remains vague which has been frustrating for pharmaceutical companies seeking to file abbreviated new drug applications for IV iron formulations. Despite Venofer® being the most widely used in the United States, the FDA has not approved a generic iron sucrose formulation.

The FDA reported the most common deficiencies among the eleven abbreviated new drug applications submitted that were ultimately denied approval. Among other manufacturing reporting deficiencies, the FDA cited inadequate bioanalytical methodology for the suggested *in vivo* pharmacokinetic study, using a crossover designed study instead of the parallel design suggested without justification and missing formulation and comparative physicochemical characterization properties [87]. Yet, the FDA does not provide detailed guidance for the physicochemical characterization or justifies the advantage of a parallel design for the *in vivo* pharmacokinetic study.

In January of 2018, the US Government Accountability Office (GAO), released the results of the study in response to congressional requesters asserting that the FDA has not been transparent, specific, and directive in their bioequivalence guidance and that this is delaying or preventing entry of generics on the market [88]. The report details several issues relevant to IV iron formulations, including modification of guidance post ANDA submission and holding the company submitting accountable for a change that occurred after the submission. Ultimately, the GAO concluded that "FDA should announce their plans to issue and revise product-specific guidance for drugs that are non-biological and complex". Adding more physicochemical characterization and clinical study requirements to guidance for generic formulations brings up a vexing controversy for RLDs because these approved formulations did not undergo the advanced testing being suggested for generics.

In summary, IV iron formulations are complex and have potential toxicities that are highly biologically plausible. These widely used agents need to be more carefully studied in the context of the widespread use and contemporary dosing approach, which is typically off-label. The newer advanced physicochemical characterization techniques need to be applied to the approved RLDs as well as to future generic formulations. These findings need to be considered in the context of more comprehensive pharmacokinetic and biodistribution studies coupled with biomarkers that can provide more clinical information about toxicity from oxidative stress. This will require significant investment from scientists, funding agencies and pharmaceutical industries to ensure the use of these formulations in widely varied populations has an acceptable balance of safety and efficacy.

ABBREVIATIONS

BE	bioequivalence
CKD	chronic kidney disease
CI	confidence interval
DL	dynamic light scattering
DRIVE	Dialysis Patients' Response to IV Iron with Elevated Ferritin
ESAs	erythropoiesis stimulating agents
FDA	Food and Drug Administration
GAO	Government Accountability Office
GPC	gel permeation chromatography
HAECs	human aortic endothelial cells
HPLC	high performance liquid chromatography
HR	hazard ratio
ICAM-1	intracellular adhesion molecule
ICP-MS	inductively coupled plasma mass spectrometry
IL	interleukins
IP	intraperitoneal
ISS	iron sucrose similar
IV	intravenous
IVIVC	*in vitro* to *in vivo* correlation
NTBI	non-transferrin bound iron
PCC	physicochemical characterization
RCTs	randomized clinical trials
RES	reticuloendothelial system
RLD	reference listed drug
ROS	reactive oxygen species
RR	relative risk
SFGC	sodium ferric gluconate
TEM	transmission electron microscopy
TSAT	transferrin saturation
USP	United States Pharmacopeia

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7

Building a Trojan Horse: Siderophore-Drug Conjugates for the Treatment of Infectious Diseases

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Abstract: Antimicrobial resistance is a major global health problem, and novel approaches to solving this crisis are urgently required. The 'Trojan Horse' approach to solving this problem capitalizes on the innate need for iron by pathogens. Siderophores are low-molecular-weight iron chelators secreted extracellularly by pathogens to scavenge iron. Once bound to iron, the iron-siderophore complex returns to the pathogen to deliver its iron treasure. "Smuggling" antimicrobials into the pathogen is accomplished by linking them to siderophores for transport. While simple in concept, it has taken many decades of work to accomplish the difficult hurdle of transporting antimicrobials across the cell membranes of pathogens. This review discusses information learned about siderophore structure, production, and transport, and lessons learned from the successes and failures of siderophore-conjugate drugs evaluated during the development of novel agents using the 'Trojan horse' approach.

Keywords: antimicrobial · iron · siderophore · siderophore-conjugate · Trojan horse

1. INTRODUCTION

Worldwide, infections caused by multidrug-resistant (MDR) strains of bacteria are increasingly common. Given their remarkably diverse mechanisms of resistance, they present chemical and biological challenges to the treatment of infections. Unfortunately, though urgently needed, few pharmaceutical companies remain invested in pursuing the development of novel antibacterial agents to treat these highly resistant pathogens [1].

During infection, the increased need for iron required for growth results in a dramatic increase in the production of siderophores by pathogens. However, the vertebrate host tightly controls the serum concentration of free iron to $\sim 10^{-24}$ M. There have been numerous attempts to exploit this increased need for iron by 'hijacking' the iron uptake machinery of bacteria by harnessing siderophores to various antimicrobials. Utilizing the siderophore uptake systems allows antimicrobial drugs, in a 'Trojan Horse' approach, to circumvent the barrier of Gram-negative outer membranes, as importation of the siderophore-antimicrobial com-

plex increases during infection, when upregulation of the iron acquisition machinery, including siderophore production, is highest [1].

Three main approaches are currently being pursued in the development of novel antimicrobial therapies that exploit siderophore iron uptake systems: (1) iron starvation via competitive chelation; (2) small-molecule inhibition of siderophore biosynthesis; and (3) siderophore-mediated 'Trojan Horse' drug delivery of antibiotics [2–4]. This review will focus on the 'Trojan horse' approach to solving the problem of antimicrobial drug resistance and the lack of novel agents to address this crisis.

The 'Trojan horse' strategy has been under investigation for several decades, and a vast number of such compounds has been designed, and tested *in vitro* [1]. However, to date, none are licensed and available for human use, though several are currently undergoing late stage clinical trials [1, 5]. In this review, we provide an overview of siderophore chemistry, focusing on its application to the development of 'Trojan horse' antimicrobials, and a brief overview of selected siderophore-antimicrobial conjugates that have been developed, highlighting concepts learned during the study of these potentially useful additions to the rapidly dwindling armamentarium of antimicrobials targeting multidrug resistant human pathogens.

2. OVERVIEW OF SIDEROPHORES

Iron acquisition and regulation is a key step for promoting successful microbial growth and survival. Deficiency can compromise vital cellular processes such as respiration and proliferation, while an excess may exert toxic effects via induction of oxidative damage by reactive oxygen species produced in the Fenton reaction [6]. Although iron is abundant in the Earth's crust (as the second most abundant metal), in the aerobic environment it exists as insoluble ferric hydroxide, largely inaccessible to microorganisms. The free Fe(III) concentration in solution at neutral pH (10^{-18} M), is far too low for what is required by microorganisms for optimal growth (10^{-6} to 10^{-3} M) [7]. The natural Fe(III) abundance is further limited by the mammalian proteins, e.g., transferrin, which keep free iron in human serum at the level of 10^{-24} M [8].

In order to acquire and regulate iron, and overcome host nutritional immunity, microorganisms have developed intricate iron acquisition and trafficking systems. Although many of these mechanisms share functional similarities, it is clear that far more is known about bacterial systems [8]. Both pathogenic bacteria and fungi have developed reductive iron assimilation (RIA) for the uptake of ferrous iron, direct Fe(II) uptake, as well as a piracy from iron-loaded hosts' storage/transport proteins such as heme and heme-containing proteins, ferritin, ferroportin, lactoferrin or transferrin [8, 9].

Under iron depleted conditions, Gram-negative and Gram-positive bacteria, and some fungi, produce and excrete hydrosoluble low-molecular-weight chelators (500–1500 daltons) termed siderophores [5]. Siderophores possess a high binding affinity for ferric iron and are capable of solubilizing the ferric hydroxide

and sequestering iron from a host's iron storage, transport, and metabolic proteins. The biosynthesis of siderophores is regulated by intracellular iron concentrations. Low iron levels trigger a "signal" to induce gene expression and start biosynthesis of the appropriate siderophores and the proteins involved in the siderophore uptake machinery. Once sufficient iron is transported and accumulated inside the cell, the iron acquisition system is turned off [10].

2.1. Naturally Occurring Siderophores

Since the early 1950s, when the first three siderophores were isolated and identified as growth factors of bacteria, 270 different siderophores have been structurally characterized (from over 500 identified) [5]. Numerous reviews have appeared in the literature describing the types of siderophores produced by microbes, their properties, functions, as well as their acquisition machineries; for a comprehensive discussion, the readers are referred to recent reviews and the references therein [2, 10–13]. In this chapter, we will focus on selected principles illustrated by most prominent examples for bacterial and fungal pathogens, especially the ones relevant to the subject of the Trojan horse strategy.

Natural siderophores can be classified according to their producing organisms (bacterial, fungal or plant), or the chemical properties of functional groups that chelate ferric iron, and their structural arrangement (molecular architecture). The most common siderophore-chelating units are catecholate, hydroxamate, and α-hydroxycarboxylate. Other binding units comprise phenol, carboxylate, hydroxyphenyloxazoline, α-aminocarboxylate, or α-hydroxyimidazole, among others. Apart from single-chelate unit-type siderophores, a number of 'mixed-type' iron carriers are known, with elements of two or more siderophore families. The majority of siderophores are hexadentate chelators, and their ferric complexes are among the most stable coordination compounds of Fe(III) known in nature. Siderophore-chelating units may be arranged in diverse topologies: linear, cyclic or a template pendant with chelating "arms" [14].

2.2. Environmental Aspects of Siderophore Choice

Catecholate siderophores are predominantly produced by Gram-negative bacteria (primarily the Enterobacteriaceae and the genus *Vibrio*), while hydroxamate based siderophores are produced mostly by fungi (the Ascomycetes and Basidiomycetes) and some Gram-positive bacteria (e.g., Streptomycetes). Carboxylate siderophores are produced by few bacteria (e.g., Rhizobium) and fungi (Mucorales, belonging to the Zygomycota) [14]. Complex stability, high environmental pH, lipophilicity, and weak nitrogen metabolism favor catechol production, while acid-stability and versatile nitrogen metabolism favor hydroxamate production. Organic acid secretion by fungi is probably the reason why most fungal siderophores are hydroxamates (e.g., the fusarinines and ferrichromes), which are sta-

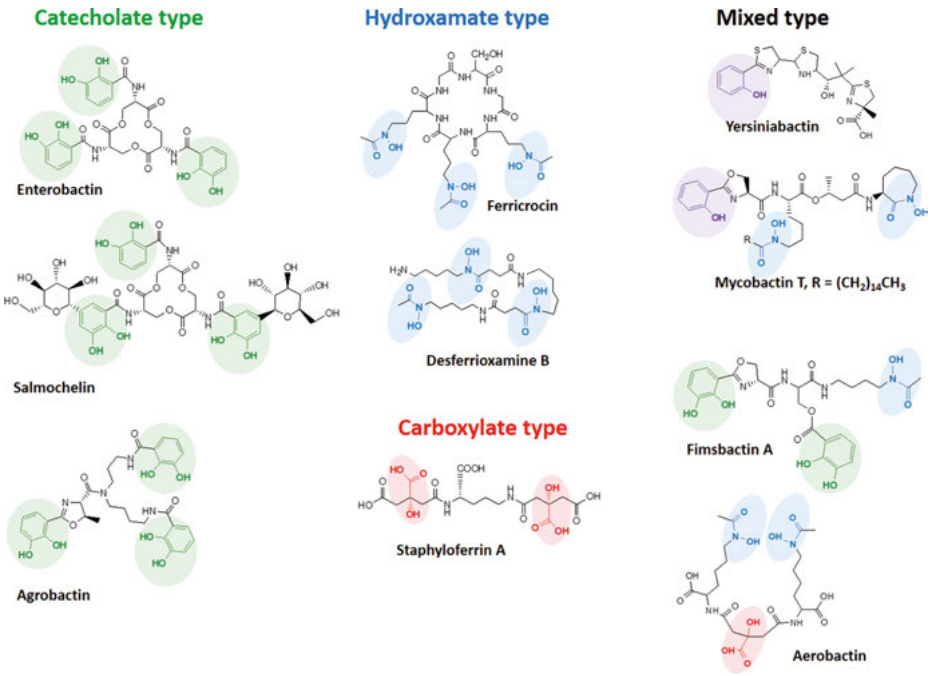

Figure 1. Selected siderophore structures.

ble down to pH 2 [4, 14]. Carboxylate siderophores such as staphyloferrin A (Figure 1) or rhizoferrin also exhibit a superior affinity for ferric iron at acidic pH and hence, likely contribute to enhanced fitness of microbes in more acidic milieus. In contrast, in physiologic environments, catecholate siderophores such as enterobactin and salmochelin (Figure 1) reveal higher affinity for ferric iron, relative to carboxylate or hydroxamate siderophores.

The ability to utilize a number of siderophore systems operating at different pH and iron conditions enhances efficacy of iron uptake by an organism, facilitating its adaptation to various environments. Indeed, most pathogens are able to produce several structurally different siderophores. For example, uropathogenic *E. coli* (UPEC) recovered from patients with urinary tract infections exhibit enhanced survival in urine, where pH varies from 4.6–8, via synthesis of 3 different siderophore types: enterobactin, aerobactin, and yersiniabactin (Figure 1) with a range of pH optima for iron chelation [15]. Aerobactin is maximally produced at more acidic pH than are yersiniabactin or salmochelin (pH 5.6 *versus* pH 7.0 or 7.6, respectively), and thus, demonstrates better iron scavenging ability in low pH environments. Enterobactin is less well suited as a siderophore for *E. coli* to enhance its virulence in bloodstream infections as it binds to serum albumin, and (with high affinity) to the host immune response-derived protein siderocalin, which acts as a growth inhibitor of *E. coli* and other pathogens. Whether siderocalin will be able to recognize enterobactin- or other catechol-based siderophore conjugates in the extracellular milieu, to prevent its

uptake by the pathogen, is unknown [16]. This may be circumvented by the development of conjugate drugs based on 'stealth siderophores' which can evade siderocalins, such as those constructed by Chairatana et al. [17], who prepared glucocylated enterobactin-β-lactam conjugates, which were successful in increasing activity against UPEC by 1000-fold.

In order to gain a competitive growth advantage, some microorganisms have developed receptors able to recognize and transport heterologous siderophore–iron complexes (xenosiderophores), produced by other bacterial and fungal species. For example, *P. aeruginosa* produces two major endogenous siderophores, pyoverdine and pyochelin, but it is capable of using at least five heterologous siderophores: cepabactin, ferrichrome, enterobactin, ferrioxamine, and citrate, and its genome encodes for >25 siderophore receptors [18]. Many of these non-native siderophore receptors are highly conserved with the primary siderophores of clinically relevant Gram-negative pathogens such as *E. coli* and *Klebsiella pneumoniae*.

Selected representatives of siderophore structural families, relevant to the topic of this chapter, are depicted in Figure 1, and are briefly described below.

2.3. Fungal Siderophores

Fungal hydroxamate siderophores are most widely studied on *Aspergillus fumigatus*, a ubiquitous saprophytic fungus that causes invasive pulmonary aspergillosis [19]. The elementary structural unit in fungal hydroxamate siderophores is N^5-acyl-N^5-hydroxy-L-ornithine, which, either through cyclization or substitution by different acyl residues (acetyl, anhydromevalonyl, or methylglutaconyl), leads to elucidation of four structural classes: rhodotorulic acid, fusarinines, coprogens, and ferrichromes [20]. *A. fumigatus* produces four siderophores: two extracellular fusarinine-type siderophores, fusarinine C, and its N-acetylated derivative, triacetylfusarinine C, secreted to mobilize extracellular iron, as well as two intracellular ferrichrome-type siderophores, ferricrocin, and hydroxyferricrocin, used for iron distribution, storage, and oxidative stress resistance [19]. Nevertheless, *A. fumigatus* is able to take up ferrichrome or bacterial ferrioxamine E siderophores [19].

Fusarinine C consists of three N^5-anhydromevalonyl-N^5-hydroxy-L-ornithine residues cyclically linked by ester bonds. Triacetylfusarinine C is the N^2-acetylated fusarinine C. This siderophore is able to remove iron from its complexes with transferrin, which makes it an important virulence factor [2]. Ferricrocin (Figure 1) is a cyclic hexapeptide with the structure Gly-Ser-Gly-(N^5-acetyl-N^5-hydroxy-L-ornithine)$_3$ and hydroxyferricrocin is the hydroxylated ferricrocin with a single unidentified hydroxylation site [19].

The polycarboxylate-type siderophore rhizoferrin, produced by Mucorales (in particular, *Rhizopus*, *Mucor*, and *Lichtheimia*), [21] and the catecholate pistillarin [22] are the only two fully characterized non-hydroxamate siderophores produced by fungi.

2.4. Bacterial Siderophores

Bacteria produce solely extracellular siderophores. The most widely studied bacterial species for siderophore metabolism is *E. coli*, which predominantly produces enterobactin (Figure 1), a quintessential representative of catecholate siderophores, with the highest affinity towards Fe(III) of any siderophore (log β of 10^{49}), enabling bacteria to thrive on iron obtained from transferrin [7, 11]. Enterobactin is a triscatechol derivative of cyclic triserine lactone, for which the clearest picture of siderophore-mediated iron transport has been obtained [7]. However, in a host-invading pathogen battle for iron, both apo and ferric bound forms of enterobactin are sequestered by the host-derived defense protein siderocalin [23]. To overcome this host defense strategy, bacteria produce salmochelins (Figure 1), which are C-glucosylated enterobactin analogues also known as "stealth" siderophores, whose structural modifications result in steric hindrance that precludes recognition and binding by siderocalin [24].

Bacterial hydroxamate siderophores are made up of acetylated and hydroxylated alkylamines (N^6-acyl-N^6-hydroxylysine) [14]. For example, the spore-producing, Gram-positive member of the family Streptomycetaceae *Streptomyces* produces desferrioxamine siderophores, such as desferrioxamine B (Figure 1) and E, the trihydroxamate siderophores with linear and cyclic structures, respectively. Ferrioxamine B is one of the most striking siderophores, as it is produced by bacteria, but consumed by fungi. Fungal pathogens, including *S. cerevisiae*, *C. neoformans*, *Geotrichum candidum*, *C. albicans*, and *R. oryzae*, are capable of utilizing ferrioxamine B [25]. Ferrioxamine B is used clinically for iron chelation therapy [26].

In response to iron starvation, two α-hydroxycarboxylate-type siderophores may be produced by Gram-positive staphylococci: staphyloferrin A (with a D-ornithine backbone, Figure 1) and/or staphyloferrin B [9]. Both staphyloferrin A and staphyloferrin B are able to acquire iron from host transferrin and/or lactoferrin [9].

Many bacteria produce extracellular "mixed-type" siderophores. The tuberculosis-causing mycobacteria (*M. tuberculosis*) secretes carboxymycobactins, which, in addition to hydroxamate moieties, possess a hydroxyphenyloxazoline ring [27]. *Yersinia pestis*, the causative agent of deadly plague, produces yersiniabactin (Figure 1), a siderophore whose phenolate, thiazole, oxazoline, and carboxylate groups participate in ferric iron coordination and removal from transferrin and lactoferrin [28]. *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen responsible for nosocomial infections, produces two principal siderophores: pyoverdine and pyochelin [2, 29]. Pyoverdine is a complex chromopeptide, based on a dihydroquinoline-type chromophore bound to an oligopeptide possessing 4–14 amino acids produced by all pseudomonads [30]. It has three bidentate chelating sites: a catechol and two hydroxamates form 6-coordinated octahedral Fe(III) complexes [31]. More than 100 pyoverdins have been identified from different strains and species of *Pseudomonas*, representing a very significant group among all siderophores characterized thus far [30]. Aerobactin (Figure 1), a "mixed-type" citrate-hydroxamate siderophore [32] produced by many patho-

genic strains of *E. coli*, is an important virulence determinant. Fimsbactin A (Figure 1) is a bis-catechol, mono-hydroxamate siderophore produced by aggressively pathogenic Gram-negative *Acinetobacter baumannii*, causing problematic and antibiotic-resistant infections [33].

2.5. Siderophore Uptake Systems

2.5.1. Gram-Negative Bacteria

Gram-negative bacteria, with two membranes and a peptidoglycan cell wall, require a multistep process for iron-loaded siderophore uptake (Figure 2). First, ferric-siderophores are recognized by specific outer membrane, ligand-gated, energy- and TonB-dependent receptors, called TonB-dependent transporters (TBDT) [6, 18, 34]. Each TBDT recognizes and transports a specific ferric siderophore, or in some cases, a few structurally related siderophores, but never siderophores with different chemical structures. The binding site is always located in the lumen on the extracellular side of the transporter; its specificity results from the nature of the amino acids involved in the binding site [18].

Some TBDTs are also able to bind and transport ligands other than siderophores. Hydroxamate Fhu transporters were shown as mediators of entry of natural ferrichrome- and ferrioxamine-antibiotic conjugates, albomycin and salmycin, respectively, into several different Gram-negative and Gram-positive bacterial pathogens [9, 35, 36]. Resistance to sideromycins typically occurs by mutation of the siderophore transporter or the intracellular enzyme required to cleave (and thus activate), the antibiotic from the conjugate [9].

To accomplish translocation of the ferric-siderophore complex through the lumen, TBDTs interact with the inner membrane protein TonB. After transport across the outer membrane, the ferric-siderophore is trafficked to the inner membrane by a periplasmic binding protein, and then imported into the cytoplasm via either ABC transporters or proton-motive force-dependent permeases [18]. Bacterial ABC transporters are generally organized into five structural domains: a periplasmic binding protein, two transmembrane polypeptides forming a channel for the ferric-siderophore complex passage, and two nucleotide binding subunits that hydrolyze ATP. These permeases and ATPases are usually assembled from four separate polypeptides [34]. Permeases work as single-subunit transporters [34]. Ferric-siderophore transport across the cytoplasmic membrane reveals less specificity than that across the outer membrane, and periplasmic binding protein-dependent ABC transporters may have some flexibility in their ligand specificities. The ferric hydroxamate uptake system of *E. coli* facilitates the transport of various hydroxamate siderophores, including ferrichrome, coprogen, ferrioxamine B, and aerobactin, each of which requires its cognate TBDT at the outer membrane [37].

Once in the cytoplasm, the release of iron requires degradation of the molecule, proton-assisted dissociation of the complex, or reduction of the metal center (siderophores have a lower affinity for Fe(II) than for Fe(III)), with differences

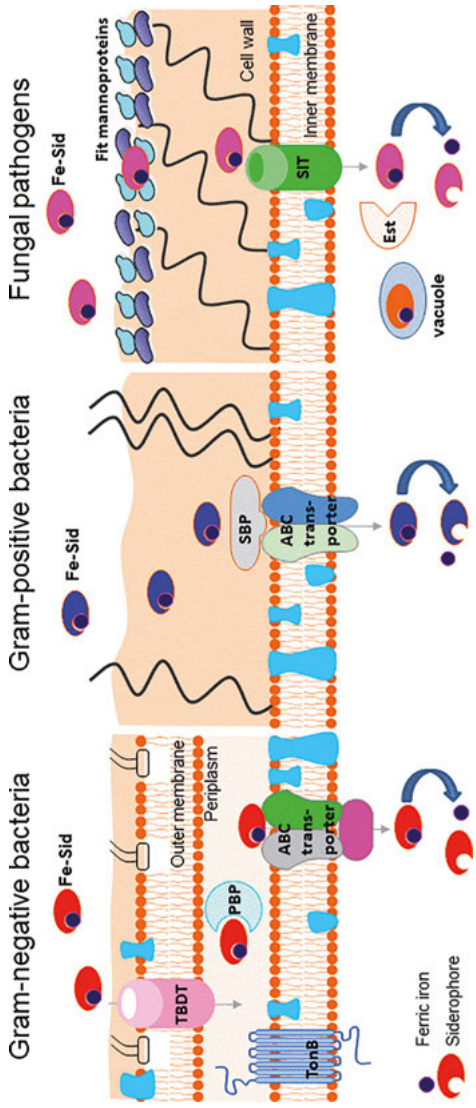

Figure 2. Schemes for ferric iron uptake via siderophores in Gram-negative and Gram-positive bacteria, and fungi. **Gram-negatives** (e.g., *E. coli*), with two membranes and a peptidoglycan cell wall, require a multistep process for Fe(III)-loaded siderophore (Fe-Sid) uptake. First, ferric-siderophores are recognized by specific outer membrane, ligand-gated, energy- and TonB-dependent receptors, called TonB-dependent transporters (TBDT). Next, the ferric-siderophore is trafficked to the inner membrane by a periplasmic binding protein (PBP), and then imported into the cytoplasm via either ABC transporters or proton-motive force-dependent permeases. Once in the cytoplasm, release of iron requires degradation of the molecule, proton-assisted dissociation of the complex, or reduction of the metal center, with differences depending on the nature of the siderophore and the bacterial species. Subsequently, the iron-free siderophore can be re-secreted outside bacteria, via specific efflux pumps, and reutilized. In **Gram-positives** (e.g., *Staphylococcus aureus)* the relatively simpler uptake mechanism involves ABC transporters that consist of three components, a lipoprotein siderophore-binding protein (SBP) found on the external surface of the membrane, one or two transmembrane permease proteins and an ATPase that provides the energy for the transport process. In **filamentous fungi** and **yeast**, siderophore uptake occurs via siderophore iron transporters (SITs), located in the cell membrane, which belong to a subfamily of the major facilitator protein superfamily unique to fungi, but which are absent in prokaryotes and other eukaryotes. Facilitator of iron transport (Fit) mannoproteins appear to trap siderophore-bound iron in the cell wall, and facilitate its uptake via a siderophore-transporter present in the plasma membrane. Following the uptake, Fe-siderophore complexes are hydrolyzed by esterases (Ests), the free siderophores are excreted and iron is delivered to metabolic pathways or stored in a siderophore-bound form in vacuoles.

depending on the nature of the siderophore and the bacterial species. Subsequently, the iron-free siderophore can be re-secreted outside of the bacteria, via specific efflux pumps, and re-utilized [34]. From selected siderophores, such as pyoverdine in *P. aeruginosa*, iron can be released in the periplasmic space with subsequent translocation of siderophore-free iron into the cytoplasm and recycling of the empty siderophore to the extracellular medium [34].

2.5.2. Gram-Positive Bacteria

As Gram-positive pathogens only have a single cell membrane and lack a periplasmic space, the mechanism by which iron, or iron-containing siderophores, are internalized must be different compared to that of Gram-negatives. The relatively simpler uptake mechanism involves ABC transporters that consist of three components, a lipoprotein siderophore-binding protein (SBPs) found on the external surface of the membrane, one or two transmembrane permease proteins and an ATPase that provides the energy for the transport process [8, 9].

Of the major Gram-positive pathogens, iron uptake has been most intensively investigated in *Staphylococcus aureus*. Similar to *P. aeruginosa*, *S. aureus* can obtain iron via a direct heme uptake system, or by the siderophore system. Uptake of ferric staphyloferrin A or staphyloferrin B siderophores in *S. aureus* occurs by way of the dedicated ABC-type transporters HtsABC and SirABC, respectively [38]. Interestingly, *S. aureus* can also utilize a variety of heterologous siderophores, including the catecholate enterobactin, catecholamine hormones such as epinephrine, norepinephrine, and dopamine as 'pseudosiderophores', and hydroxamate siderophores [9, 39]. Several SBPs bind not only Fe-siderophores but also apo-siderophores [40]. The binding affinity of *Bacillus cereus* YxeB for desferriferrioxamine and desferriferrichrome is similar to the affinity for the Fe-siderophore [40]. It was shown that transport may proceed through two mechanisms: (i) a shuttle mechanism, where a metal exchange between ferric-siderophore and apo-siderophore is facilitated by the receptor and increases the uptake rate of iron, and (ii) a displacement mechanism, where a ferric-siderophore can be transported without a metal exchange step [23, 40].

2.5.3. Fungi

In filamentous fungi and yeast, siderophore uptake occurs via siderophore iron transporters, (SITs), which belong to a subfamily of the major facilitator protein superfamily unique to fungi, absent in prokaryotes and other eukaryotes [12]. SITs act as proton symporters, i.e., the energy for active transport is obtained from the electrochemical potential gradient obtained by pumping ions across the membrane rather than from ATP [12]. The SIT system is common and generally well conserved in the fungal kingdom, both in siderophore producers (e.g., *Aspergillus* spp.) and non-producers (e.g., *S. cerevisiae*, *Candida* spp., and *C. neoformans*) [12, 41–43].

Most fungal species produce multiple SITs located in the cell membrane. *S. cerevisiae* possesses four types, each of which is characterized by different ligand specificity [19]. Of importance, in *S. cerevisiae* the cell wall seems to play an additional role in iron-siderophore uptake; significant quantities of siderophores are found in the cell wall and periplasm [44]. Moreover, the cell wall of *S. cerevisiae* contains the facilitator of iron transport (Fit) mannoproteins. While the exact mechanisms of Fits in enhancing iron uptake remains unknown, they appear to trap siderophore-bound iron in the cell wall, and facilitate its uptake via a siderophore-transporter present in the plasma membrane [45]. *A. fumigatus* encodes seven SITs to acquire iron, and *A. nidulans* encodes ten SITs, including MirB and MirA, which are highly specific for triacetylfusarinine C and enterobactin, respectively [12, 19, 46, 47]. Following uptake, the complexes are hydrolyzed by esterases [48]. The free siderophores are excreted and iron delivered to metabolic pathways, or stored in a siderophore-bound form [12, 48]. In *A. fumigatus,* active siderophore-mediated iron transport is crucial for delivering iron into the cell, as the fungus does not possess a heme-utilization system for iron acquisition, and RIA is inefficient during host infection [8, 19]. Recognition of siderophores by SITs is stereospecific and binding to the transporter is likely dependent on specific binding sites, not on the size or hydrophobicity of the complex [4, 12].

Apart from the siderophore shuttle mechanism employing SITs, fungi utilize another mechanism to exploit siderophores for iron acquisition. In the 'taxicab' mechanism, iron is transported through the membrane from extracellular scavenging siderophores to intracellular siderophores [49], which is an unusual phenomenon, since in other species intracellular siderophores are mostly involved in accumulation of iron supplies, not in iron assimilation *per se* [50].

3. SIDEROPHORE ANTIMICROBIAL CONJUGATES

3.1 Construction of Siderophore Drug Conjugates

As discussed previously, the earliest known siderophore antimicrobials include the natural product sideromycins (originally termed sideramines or siderochromes), which include albomycins, salmycins, and microcins, which are produced by bacteria. These agents demonstrated potent, broad-spectrum activity against a number of Gram-negative and Gram-positive bacteria [5]. However, while these agents have provided a basis for understanding the structural and chemical properties needed for construction of 'Trojan horse' antimicrobials, their *in vivo* activity proved limited, due to extracellular hydrolysis of the labile ester linkage of albomycins, and by exclusion of salmycins resulting from specificity of the outer membrane transporters [4]. Since then, assembly of siderophore-drug complexes has been primarily directed at the use of siderophores created semisynthetically (as derivatives of natural siderophores) or by totally synthetic methods. The semisynthetic approach has the advantage of being able to induce large scale synthesis and excretion of siderophores by microbes grown

under iron-poor conditions. However, many of these siderophores lack functional sites for conjugation with antimicrobials. Total synthesis of siderophores, while more complex to accomplish, offers the advantage of a wider range of structures to which drugs can be linked, and have provided investigators with valuable tools for the study of ferric iron recognition and transport into microbes [5, 13].

Siderophore-antibiotic conjugates typically contain three components: (i) an iron-chelating moiety, (ii) a linker, and (iii) a small molecule with antibacterial activity. The use of siderophore-antimicrobial conjugates offers several advantages over use of the antibiotic alone: (1) it allows antimicrobials to bypass membrane-associated resistance mechanisms such as decreased permeability barriers and antimicrobial efflux pumps, thus increasing their potency dramatically relative to passive diffusion of drug; (ii) it can limit the selection of resistance by excluding pathogens deficient in a specific siderophore receptor, and increasing the ability to attack multiple drug targets [3]. The majority of antibacterial siderophore conjugates have utilized agents whose target sites of action are located in the pathogen's periplasm, and have targeted primarily Gram-negative bacteria. For example, successful drug conjugates have been developed with β-lactams, monobactams, and monocarbams, which affect bacterial penicillin-binding proteins (PBPs). Interestingly, catechol-coupled spiramycin conjugates had no better *in vitro* activity than the parent drug. However, drug conjugates have also been developed with activity against Gram-positive bacteria, fungi (*Candida* and *Cryptococcus neoformans*), but not, as yet, against *Aspergillus* species, the Mucorales, mycobacteria, or plasmodia [51].

More recently, the marked increase in MDR Gram-negative pathogens, combined with a lack of novel agents in the pipeline with which to combat them, has led to renewed interest in their development. Siderophore conjugates have proved effective in circumventing common antibiotic resistance mechanisms. Rather than providing a comprehensive listing of all siderophore-antimicrobial conjugates thus far developed, the reader is referred to several excellent reviews [2, 5, 10, 52]. Herein, we will focus on the important findings gleaned from research in the use of these agents in the *in vitro* and *in vivo* setting.

3.2. β-Lactams

3.2.1. Overview of β-Lactam Conjugates

While the earliest rationally designed siderophore-antimicrobial drug conjugates were prepared with sulfonamides linked directly to ferricrocin or ferrioxamine B, by far, the largest group of siderophore-drug conjugates have been developed with members of the β-lactam class of antibiotics: penicillins, cephalosporins, monobactams, and carbapenems [10, 13].

There are several advantages of exploring β-lactams as conjugate drugs (a) since PBPs are located in the periplasm, compounds need to cross only the bacterial outer membrane to access the drug target; (b) there is no requirement for drug release from the siderophore moiety in order to have antimicrobial activity,

since attachment of the β-lactam to PBPs is at a site different from that of the linker to the siderophore [10]. While a number of these early β-lactam conjugates demonstrated excellent *in vitro* activity against *P. aeruginosa*, none progressed beyond early *in vitro* studies, likely due, in part, to their higher cost and lack of Gram-positive activity. More recently, it was demonstrated that conjugates of the catecholate siderophore enterobactin with stable polyethylene linkers to ampicillin or amoxicillin required the presence of FepA (the outer membrane ferric enterobactin transporter) for the enhanced antibacterial activity observed.

Several valuable lessons were learned during early development of hydroxamate and catecholate siderophore drug conjugates. First, it was shown that drug conjugates were successful in being able to cross bacterial membranes, and to attack desired microbial targets. Secondly, while conjugates often initially inhibited growth of microbes, mutants quickly developed that lacked TonB and outer membrane receptor proteins to the siderophore type (hydroxamate or catecholate) utilized in the drug conjugate. This disappointing finding led to the development of rationally designed, 'mixed' siderophore system conjugates, in which the siderophore contained both a catechol and a hydroxamate. Investigators postulated that the development of mutations and hence, resistance to a mixed system would be more difficult, as it would require mutation of two different iron acquisition mechanisms. While resistant mutants lacking receptors to both catecholate and hydroxamate siderophores still occurred, their development was slower, and they were unable to grow under the iron-poor conditions used to simulate human serum [13].

3.2.2. Monocarbams/Monobactams

Several siderophore-conjugated monobactams (BAL30072, MB-1, MC-1) and a monocarbam (SMC-3176)) (Figure 3) have demonstrated potent antibacterial activities against MDR Gram-negatives, including *P. aeruginosa,* when standard *in vitro* minimum inhibitory concentration (MIC) and resistance frequency methodologies are utilized. However, resistance to BAL30072 was quickly observed, due to the increased expression of drug efflux pumps. Initially, MB-1 appeared resistant to standard resistance mechanisms (e.g., porin changes, destruction by β-lactamase). However, further development of MB-1 and SMC-3176 proved problematic due to a lack of correlation between *in vitro* activity and *in vivo* efficacy and the development of adaptive type resistance (the observation of regrowth of the organism despite drug exposure far in excess of the minimum inhibitory concentrations of the test isolates) [53].

MC-1 proved to be very active against MDR pathogens, even those strains with resistance due to porin mutations, upregulation of bacterial membrane efflux pumps, or to destruction by almost any of the multiple classes of β-lactamases. Similar to the monobactam aztreonam, MC-1 displays a high affinity to PBP 3, giving it potent activity against *P. aeruginosa.* MC-1's excellent stability to β-lactamases was postulated to be due to steric hindrance caused by the positioning of the hydroxypyridone siderophore moiety and by the triazolone-derived

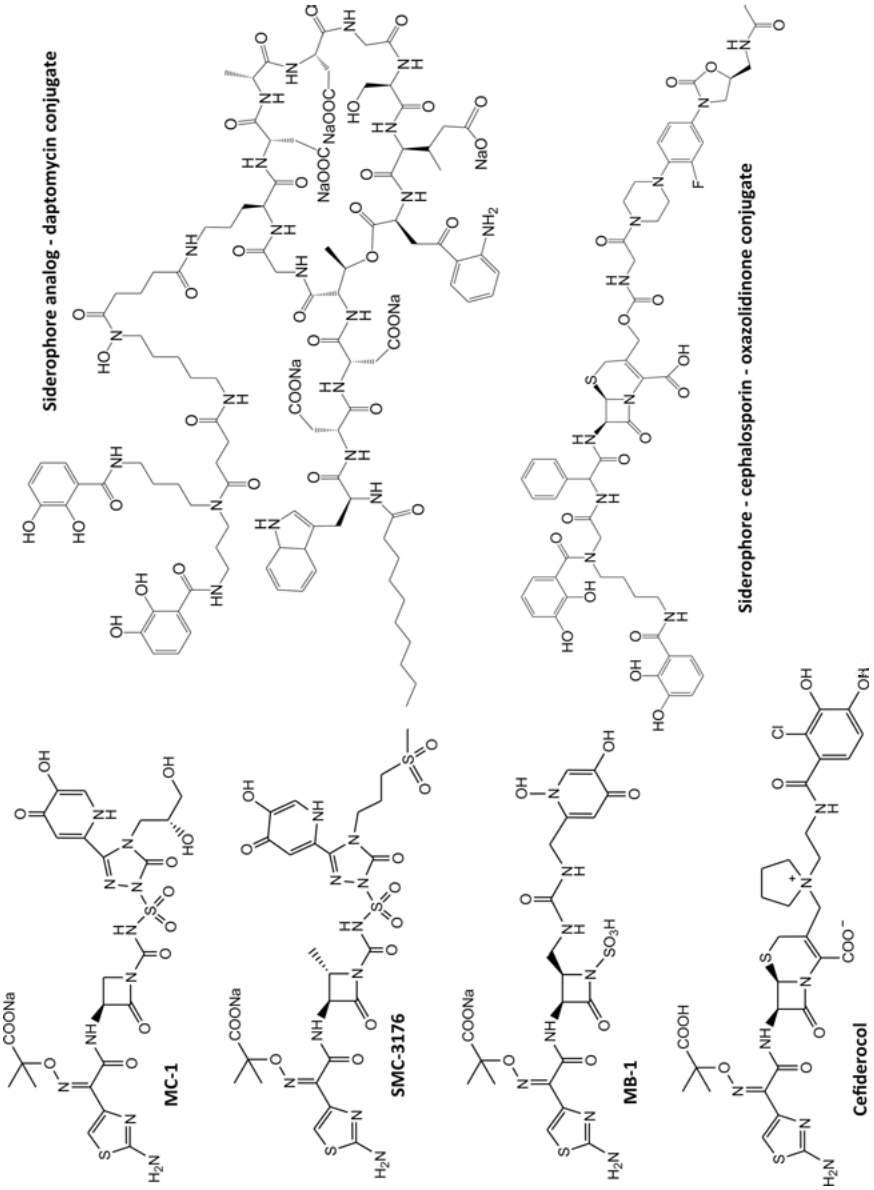

Figure 3. Structures of selected siderophore-conjugate antimicrobials.

linker joining the siderophore to the monobactam, which prevented docking by β-lactamases. Initial studies suggested that uptake of both MC-1 and BAL30072 into *P. aeruginosa* appears to be via the PiuA and PirA TonB-dependent outer membrane siderophore receptors [53]. Not surprisingly, given the dependence of siderophore receptor-mediated uptake of this compound for activity, the primary mechanism of resistance to MC-1 was due to decreased transport of the siderophore-drug conjugate across the bacterial outer membrane. During early *in vitro* work with MB-1, it was discovered that native siderophore production led to modified expression and utilization of siderophores and siderophore receptors [53]. In animal models, native siderophores competed with the drug conjugate MB-1. Under iron-poor conditions, *P. aeruginosa* reduced the use of the outer membrane receptors responsible for the uptake and transport of MB-1, while upregulating production of pyoverdine [54]. Subsequent *in vitro* studies were able to demonstrate restoration of antimicrobial activity with the use of reserpine, a siderophore efflux pump inhibitor. Although reserpine is an unacceptable agent for clinical use in this setting, due to safety and tolerability issues, this proof-of-concept suggests that the development of other efflux pump inhibitors may prove beneficial [53].

3.2.3. Cephalosporins

In the early 1990s, catechol derivatives of ureido-penicillins, cephalosporins, and cephamycins demonstrated significantly enhanced activity against *P. aeruginosa*, which was speculated to result from their ability to bind iron and be transported into cells [26]. Cefidericol (S-649266; Figure 3) is a recently developed 'siderophore cephalosporin' (rather than a siderophore conjugate), whose catechol side chain functions as a siderophore [55]. Cefidericol is a potent ferric ion chelator, which under iron-depleted conditions appears to undergo active transport into *P. aeruginosa* via iron transporters [55]. It displays potent *in vivo* and *in vitro* activity against many MDR Gram-negatives and in *in vivo* studies it does not develop the adaptive resistance observed with other siderophore-conjugate drugs. Clinical trials with this siderophore antibiotic are in progress [56].

3.3. Fluoroquinolones

In most early studies of siderophore-fluoroquinolone conjugates, the activity of conjugates was no greater than that of the parent antimicrobial, and in some cases, activity was completely lost. Loss of activity was hypothesized to be due to an inability to cross the cytoplasmic membrane, lack of target recognition by the pathogen for the siderophore-modified fluoroquinolone, premature release (prior to reaching the cytoplasm), or lack of a release mechanism for the antimicrobial agent upon achieving the target site in the pathogen [5]. Recently, Ji and Miller [57] incorporated 'trimethyl lock' linkers designed to selectively release ciprofloxacin from siderophore conjugates within the bacterial cytoplasm, by

harnessing the reductive pathway of iron metabolism. Antimicrobial activity was increased, suggesting that drug release occurred within the bacterial cells; however, conjugates were still less active than the parent drug. Similarly, the stabilities of several enterobactin-based conjugates of ciprofloxacin with hydrolyzable ester linkages varied >370-fold, depending upon the substituents close to the ester linkages [58].

3.4. Antifungal Agents

The increasing resistance of *Candida* species, and more recently, of *Aspergillus* species, to currently available antifungal agents, coupled with a lack of novel antifungals, has sparked interest in the 'repurposing' of currently available agents in the form of siderophore-antifungal conjugate drugs. These agents have also been proposed as imaging agents to improve detection of these difficult to diagnose pathogens, and demonstrate significant potential as a means of pathogen-selective detection in biological samples [4].

As noted previously, the majority of successful siderophore-drug conjugates have β-lactams, whose target sites of action are the PBPs, located in the periplasm. Antifungal agents utilized in the treatment of human fungal infections primarily target either ergosterol, found in the fungal cell membrane (azoles, polyenes, and allylamines) or the formation of β-glucan cross-linking of the fungal cell wall (echinocandins) [4]. Thus far, development of siderophore-antifungal drug conjugates has been limited to the synthesis of ferrichrome (hydroxamate)-based agents with 5-fluorouridine as neoenactin and several of its congeners. While none of these agents are currently used clinically as antifungal agents, the investigators were able to demonstrate 'proof of principle' successfully, increasing the activity of the native drugs against *C. albicans* and *C. neoformans* but not, as yet, against *Aspergillus* species, or the Mucorales. Of note, however, is that some conjugates displayed iron transport-dependent species selectivity [13].

Szebesczyk et al. [4] recently reviewed the potential for development of siderophore-antifungal drug conjugates. They noted that while the need for development of these drugs is great, there are obstacles to overcome in accomplishing their development and it is important to keep in mind key differences in bacteria *versus* fungi. First, unlike bacteria, fungi are metabolically similar to human cells; thus, pathogen-specific targets are extremely limited. Fortunately, since fungi (like bacteria) use siderophores as a key means of iron acquisition, while humans do not, the use of siderophore-antifungal conjugate drugs offers a plausible means of accomplishing drug delivery. While most fungi synthesize siderophores (mainly hydroxamate-type) which are excreted into the environment, and bind ferric ions with high affinity and selectivity, notable exceptions to siderophore synthesis include *C. albicans, C. glabrata,* and *Cryptococcus neoformans.*

Fortunately, virtually all fungi appear to accept a wide variety of xenosiderophores. In fungi, as in bacteria, different siderophores have different modes of release and accumulation. For example, in *S. cerevisiae*, ferrichromes accumulate in the cytoplasm while ferrioxamine B appears to accumulate in the vacuole. Thus, drug targets (and thus, delivery) must be tailored to match those of siderophores.

Finally, in addition to iron uptake via siderophores, during iron-poor settings such as during infection, fungi possess several other mechanisms by which they can acquire iron, including assimilation from ferritin, transferrin, or from heme.

4. USE OF SIDEROPHORE-DRUG CONJUGATES TO ALTER THE SPECTRUM OF ACTIVITY OF AN ANTIMICROBIAL

Siderophore conjugates can be designed to limit the spectrum of activity of the parent drug, allowing the conversion of an antimicrobial with a broad spectrum of activity into one with a narrow spectrum. This can prove beneficial in settings where the specific pathogen has been identified, and the clinician wants to target only that pathogen, without disruption to other local flora (potentially of the same species, but with less virulence). For example, investigators have targeted UPEC strains of *E. coli*, which possess an iroA gene cluster that codes for the salmochelin receptor IroN. Conjugation of the stealth siderophore salmochelin to ampicillin or amoxicillin increased the parent β-lactam activity against UPEC (but not of non-pathogenic *E. coli*) by up to 1000-fold; similarly, siderophore conjugates of enterobactin and ciprofloxacin are capable of selective targeting of UPEC strains [17, 59].

Transport of ferric siderophore-conjugate complexes can provide increased penetration of drugs previously unable to attain cytosolic targets and have enabled drugs to gain activity against pathogens to which they did not previously demonstrate antimicrobial activity. Proof of concept was demonstrated for artemisinin, an antimalarial and anti-schistosomal agent (also discussed in Chapter 2) which lacks activity against *M. tuberculosis.* Conjugation of artemisinin to mycobactin, a mycobacterial-specific siderophore, enables the drug to gain activity against *M. tuberculosis* (even MDR strains), while retaining its antimalarial activity. Killing of malaria parasites by artemisinin is thought to be mediated by oxy radicals, formed by reductive cleavage of the peroxide bond in artemisinin in the presence of ferrous iron. The conjugate drug is hypothesized to allow delivery of the peroxide drug to the pathogen, with subsequent reduction of the bound ferric iron, and the generation of free radicals via Fenton-like chemistry [60].

Similarly, daptomycin, which has activity against Gram-positive but not Gram-negative pathogens due to its inability to penetrate Gram-negative bacterial outer membranes, was combined with a specifically recognized siderophore analog to allow its active transport into *A. baumannii* [61]. In another recent example, Liu et al. [62] created a ‘dual drug sideromycin’ (a siderophore–cephalosporin−oxazolidinone conjugate) with a releasable linker between the two antimicrobial agents. Generally, oxazolidinones lack activity against Gram-negatives, either due to an inability to permeate the outer cell membrane, or due to rapid efflux upon entry. As with other ‘Trojan horse’ siderophore drug conjugates, this design allows delivery of both a cephalosporin and an oxazolidinone (eperezolid) to the pathogen.

However, once inside the bacteria, destruction of the cephalosporin by periplasmic bacterial β-lactamase results in intracellular release of the oxazolidinone [62].

5. LESSONS LEARNED FROM THE DEVELOPMENT OF ANTIBACTERIAL SIDEROPHORE DRUG CONJUGATES

5.1. The Choice of Siderophore Must Match the Target Pathogen

As noted above, different pathogens synthesize different siderophores; some do not synthesize any. Similarly, the ability to accept xenosiderophores differs among pathogens, as does their ability to acquire iron by other sources (e.g., from ferritin or transferrin, or by heme assimilation). In constructing drug conjugates, the choice of siderophore 'carrier' must match its ability to deliver the antimicrobial agent to its intended target pathogen(s) [3].

5.2. The Role of Drug Release in Siderophore-Mediated Drug Delivery

Whether drug release in the cell is required is not fully understood [3]. In some instances, the drug conjugate is active, while in other instances, only the antimicrobial itself is the active component; this likely depends on the structure-activity relationships of the drug conjugate, and the drug target. Release of iron (and drugs) differs between pathogens and siderophores; in some pathogens, iron is released in the cytoplasm (e.g., for *E. coli* with ferrichrome and ferri-enterobactin) while in others, release is in the periplasm (e.g., for *P. aeruginosa* via the ferric-pyoverdine system).

Drug release from its siderophore 'Trojan Horse' is not necessary for the β-lactam or daptomycin antimicrobial conjugates (*vide infra*, Section 3.2.1). Most siderophore-β-lactam conjugates target bacterial PBPs, located in the periplasm [3]. Investigators have hypothesized that antimicrobials whose targets are located in the cytosol may require release from their siderophore carriers in order to achieve antimicrobial activity [52]. The choice of 'linker' between the siderophore and the antimicrobial depends both on whether drug release is important (or not) and whether release is possible in cells [3]. For example, since binding of β-lactams to target PBPs in the periplasm is maintained in the face of significant side chain modifications, the entire β-lactam conjugate can bind to PBPs, and drug release is not necessary. However, for most antimicrobials whose target site is in the cytoplasm, drug release may be required. Recent dual drug combinations have expanded the ability of conjugates to access drug targets located in the cytoplasm; drug release was required for the 'dual drug' siderophores and the 'trimethyl lock' fluoroquinolone, as discussed in Sections 3.3 and 4.

5.3. The Siderophore-Conjugate Must Deliver the Antimicrobial to the Drug Target Site

The main goal of siderophores is to deliver iron to the bacteria; in doing so, they must get iron across the cell wall and plasma cell membrane of bacteria, as discussed in Section 5.2. In delivering a siderophore-drug conjugate, the antimicrobial agent must be delivered to the appropriate target site in the pathogen.

The cell wall of Gram-positive bacteria, which is thicker than those of Gram-negatives, consists of a thick outer layer of peptidoglycan and teichoic acid and an inner plasma membrane, which sandwich the periplasmic space. Gram-negatives have an additional outer lipid membrane with a second periplasmic space sandwiched between it and the peptidoglycan layer. While the structure of fungi is similar to that of Gram-positives, with an outer cell wall sandwiching the periplasm between it and the inner plasma membrane, the cell walls of fungi consist of chitin, glucans, and a layer of mannoproteins, rather than peptidoglycan. Some clinically important pathogenic bacteria (including *Hemophilus influenza*, and some species of *Bordetella*, *Yersinia*, and *Neisseria*), do not produce siderophores, and obtain their iron from other sources, including hemin, transferrin, or lactoferrin. In these species, the use of siderophore-conjugate antimicrobials is likely to be less effective.

5.4. Practical Measurement of Siderophore-Conjugate Antimicrobial Activity in the Clinical Laboratory

During early work with β-lactam conjugates, it was discovered that the standard media used to determine *in vitro* activity of traditional (non-conjugate) antimicrobial agents could not be employed for conjugate drugs. In standard media, MICs tend to be 4–32 times higher than those observed during testing under iron-depleted conditions, because iron concentrations are not representative of physiological concentrations of ferrous or ferric ions [1]. Results in animal models differed significantly, due to differences in iron availability *in vivo*. Current guidelines from the Clinical and Laboratory Standards Institute recommend that *in vitro* testing of bacteria to siderophore antibiotics and siderophore-conjugate antimicrobials be conducted in iron-depleted (with 2,2′-dipyridyl or Chelex), cation-adjusted Mueller Hinton broth [17, 53] so that conditions mimic those in an infected host, in which siderophore production is upregulated.

6. CONCLUDING REMARKS

Despite many failed attempts over several decades, to design and build Trojan horse drug delivery, the future appears promising that the remaining obstacles will be overcome. Failures have generally been found to result from (1) use of siderophores with relative low affinity for Fe(III), or that cannot effectively com-

pete with native siderophores; (2) selection of siderophores with poor recognition by outer membrane siderophore receptors; (3) loss of antibacterial activity due to altered target affinity in the presence of the bulky siderophore; (4) use of an inappropriate linker between the siderophore and the drug, resulting in either premature drug release, or a lack of release at the necessary site of action; (5) development of adaptive resistance, and (6) assays that fail to correlate *in vitro* and *in vivo* activity of the conjugates [57]. Many of these obstacles can (and have been) overcome by the use of thoughtful design and evaluation of siderophore-drug conjugates.

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ABBREVIATIONS

Fit	facilitator of iron transport
MIC	minimum inhibitory concentration
MDR	multidrug-resistant
PBP	penicillin-binding proteins
RIA	reductive iron assimilation
SITs	siderophore iron transporters
TBDT	TonB-dependent transporters
TonB	protein complex: cytoplasmic transmembrane complex
UPEC	uropathogenic *Escherichia coli*

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8

Developing Vanadium as an Antidiabetic or Anticancer Drug: A Clinical and Historical Perspective

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Abstract: Vanadium has been known for centuries to have beneficial effects on health and has the potential to be used as an alternative to other diabetic and anticancer medicines. The beneficial effects of vanadium salts or organic compounds have been explored *in vitro*, *ex vivo*, and *in vivo* in animal and human studies. A consensus among researchers is that increased bioavailability of these compounds could markedly increase the efficacy of this class of compounds. In addition, because many commercially available vanadium derivatives are being used by body builders to enhance performance, more understanding of their mode of action is desirable. Future studies of various vanadium compounds need to evaluate their biodistribution, biotransformation, and the effects of food and formulation on the bioavailability of the compounds. To date, most studies in humans have employed vanadium salts, mainly vanadyl sulfate, and dose-limiting side effects were reported at therapeutic doses. One organic vanadium compound, bis(ethylmaltolato)oxovanadium(IV), had improved efficacy compared to the vanadyl sulfate and was selected for Phase 1 and 2 clinical trials.

Future studies should be conducted as randomized, placebo controlled trials lasting several months, with monitoring of both fasting blood glucose and hemoglobin A1c. Now, the most promising potential uses of vanadium compounds are as nutritional supplements to control glucose levels and perhaps, as an anticancer agent potentiated by immunotherapy.

Keywords: anticancer agent · clinical studies · *Diabetes mellitus* · formulation · insulin-enhancing agent · nutritional supplement · vanadium · vanadyl sulfate

1. INTRODUCTION: ANTIDIABETIC VANADIUM-CONTAINING COMPOUNDS

For centuries, vanadium has been known to improve the quality of life of diseased humans and animals [1, 2]. Historically, the salts were originally explored early for a range of different biological properties [1, 3, 4]. Later, the organometallic compounds were investigated for their anticancer activities [5–7], and finally, in the 1980s the insulin-like effects of salts [3, 4, 8], peroxovanadium(V) [3, 4, 8] and coordination compounds were discovered [9–14]. In addition to these systems, selected additional vanadium compounds have been reported of relevance to this review and will be briefly described.

Current interest in vanadium results from its multiple biological effects and specifically, its ability to lower elevated blood glucose and lipid levels in diabetic animal models and in humans [8–13, 15–39]. The effects are reported on animal or human subjects with either insulin-dependent (IDDM, Type 1 or DMI) or non-insulin dependent diabetes (NIDDM, Type 2). Some of the animal models

have phenotypic symptoms of both IDDM and NIDDM, so studies are not always clearly associated with either type. Early studies with simple vanadium (V) salts describe symptomatic improvement in patients and animals suffering from diabetes, cancer, and cardiovascular disease. In this review, we describe some of the promising effects of different forms of vanadium and provide some history on the development of vanadium as a potential drug as well as information on its biological properties. Due to the focus in this chapter on clinical issues, the emphasis will be on human studies as well as relevant information for appreciation of the application of vanadium compounds as nutritional additives.

Vanadium is a trace element in the biosphere but is the 21st most abundant element in the earth's surface [40]. It is a known component in many minerals both as a simple salt and as a polyoxovanadate [41, 42]. As a trace element vanadium is a component of the human diet, in addition, vanadium is often present with iron and thus is present as a trace metal contaminant in iron supplements. Vanadium is commonly taken up by crops grown in vanadium-rich soils, so it is found in a number of grains, as well as in peas, corn, tomatoes, radishes, carrots, pears, dill seed, and black pepper [40, 43]. It is also found in meat and fish, with particularly high concentrations in the liver [43, 44]. Some brands of "vitamins" contain vanadium, and nutritional additives with different forms of vanadium are available to athletes and the general population (see below Section 4) [45, 46].

Vanadium is a first-row transition metal with element number 23. Beneficial effects of vanadium are mainly observed when the vanadium is in high oxidation states such as $+4$ or $+5$. Thus, vanadium is present in the form of oxovanadate salts (vanadate or vanadyl ions) and/or metal chelates under physiological conditions [47–54]. The versatility of vanadium is related to its large number of oxidation states; however, even when vanadium exists in oxidation states $+4$ or $+5$ (as found under physiological conditions), it can still act as a metal cation or as an anion. When vanadium acts as an anion, it is in the form of a phosphate analog [47, 51, 55]. The vanadate-phosphate analog (VO_4^{3-}, HVO_4^{2-}, $H_2VO_4^-$) is particularly important because of its interactions with proteins such as phosphatases and phosphorylases [8, 55–58]. The many known forms of vanadium that exist under physiological conditions have been shown to readily interconvert through redox and hydrolytic reactions and it is therefore difficult to determine which are the active species [49]. The Crans group demonstrated that enzymes could recognize each vanadium(V) species, suggesting that different forms would exert correspondingly different responses on cells [59, 60]. In yeast, the different cellular responses to vanadium oxometalates [59, 60] were determined to be related to a membrane transport protein [61, 62]. Since then, studies have been designed to distinguish effects of different forms of vanadium even if the compounds are rapidly interconverting [59, 60]. Under physiological conditions and low concentrations, the species likely to be found *in vivo* are the monomeric forms of oxovanadium derivatives, i.e., the vanadyl cation for V(IV) and the vanadate anion for V(V) (see below) [47–53] or as protein-bound species [48, 63, 64].

Interest in vanadium increased significantly in the mid-1980s and early 1990s, as is evident when plotting of the number of publications per year concerning vanadium, vanadate or vanadyl sulfate. The number of publications increased

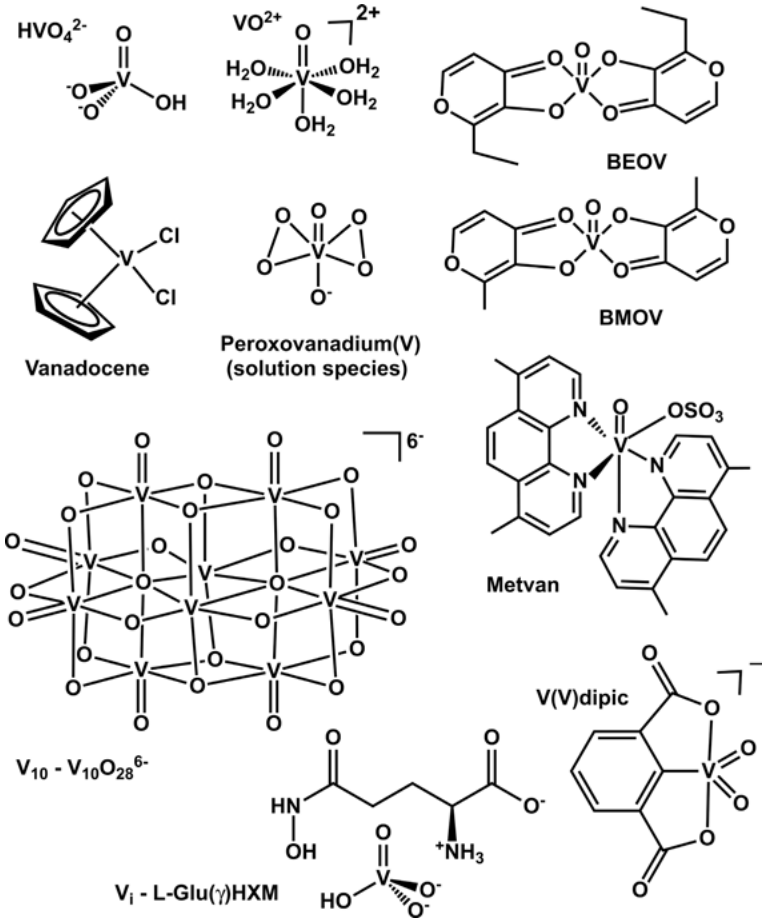

Figure 1. Structures of the vanadium compounds used in human clinical trials as well as a few vanadium compounds for which a significant amount of animal and/or *in vitro* data exist.

because of major discoveries in vanadium science, several of which were directly related to the biological effects of vanadium compounds in cell culture, animal models, and human beings during this period. The series of discoveries began with the initial report that vanadate is a potent inhibitor of the Na^+,K^+-ATPases [65–67], and ribonucleases [68]. These results led several groups to begin exploring vanadium salts and related compounds, leading to reports of "insulin mimetic" vanadium salts [3, 8, 69, 70] or to the "insulin-like" properties of these salts [13, 71], which described the observation of effects mimicking some of the functions of insulin. More recently, the term "insulin-like" has been used for simple salts and chelated vanadium compounds [13, 71] because it was discovered that the affect of vanadium compounds required some insulin to be present in a patient to achieve the synergetic and enhancing effects of vanadium.

Peroxovanadium compounds are a distinct class of vanadium(V) compounds in which, following the addition of H_2O_2 to a vanadate solution, at least one peroxo group binds to V(V) (see Figure 1), and this changes the reactivity completely [51, 70, 72, 73]. Great excitement was generated upon the finding that peroxovanadium complexes significantly (at least 10-fold) enhance the effects of simple vanadate salts; these complexes dramatically increase protein tyrosine phosphorylation both *in vitro* and *in vivo,* as observed with insulin treatment (see Figure 2) [3, 8, 69, 70, 72, 74–77]. The enhanced phosphorylation of the insulin receptor kinase (IRK) by peroxovanadium(V) compounds in the presence and absence of insulin is visualized by ^{125}I-insulin binding as a function of time in panel B in Figure 2. The representative peroxovanadium compound used in this case was bpV(phen) (bisperoxo(1,10-phenanthroline)oxovanadate(V)). In addition to the simplest form of the peroxovanadium(V) salt that forms in aqueous solution, the Posner-Shaver team tested several chemically synthesized peroxovanadium(V) complexes to optimize the potency of these compounds as well as to explore their mode of action [72–78].

During this same period, the anticancer properties of two very different classes of vanadium compounds were explored. One line of investigation involved the organometallic compounds, the vanadocenes [5, 6, 78–83]. These compounds contain two cyclopentadienyl groups that are known to stabilize the vanadium oxidation state. Studies with the simplest parent complex dichlorodicyclopentadienylvanadium(IV), also referred to as "vanadocene", showed promise as antitumor agent [5, 6, 79–81]. At about the same time, other studies reported the anticancer effects of peroxovanadium(V) compounds [83–86]. These compounds were of great interest to both chemists and biologists alike because of their unusual coordination geometries and chemical properties as well as their potent biological effects. Mechanistic studies with both simple and complex peroxovanadium(V) species identified the inhibition of protein tyrosine phosphatases (PTPases) as one of the mechanisms of action of peroxovanadium(V) compounds [8, 55, 87]. Despite the initial excitement over the observed insulin-like effects, the inhibition of PTPase was non-specific, indicating a potential for augmenting growth factor signaling and hence, augmenting oncogenesis. Further compound design is needed to enhance specificity while reducing potential toxic side effects.

During *in vivo* model system studies, vanadium salts were investigated in cell culture, animal models [8–13, 15, 17, 19–36] and human beings [88–91]. Initial studies with vanadium salts demonstrated promise and additional clinical trials were conducted with a chelated organic compound run by Kinetek, a start-up company, in collaboration with the McNeill/Orvig team [12, 14, 17]. Indeed, patents were obtained for the broad class of organic vanadium compounds (coordination complexes), and the lead compound, bis(ethylmaltolato)oxovanadium(IV) (BEOV), was investigated in Phase 1 and 2 clinical trials [12, 14]. Patents have a limited 20-year lifetime, and because the "BEOV" patent ended Sept 30, 2011, there is no potential for generating revenue on these materials, limiting future development of these compounds as therapeutic agents for diabetes [12, 14, 27, 92]. Despite this, BEOV exists as a nutritional additive and may have potential as a weight loss agent, although the mechanism for this effect is un-

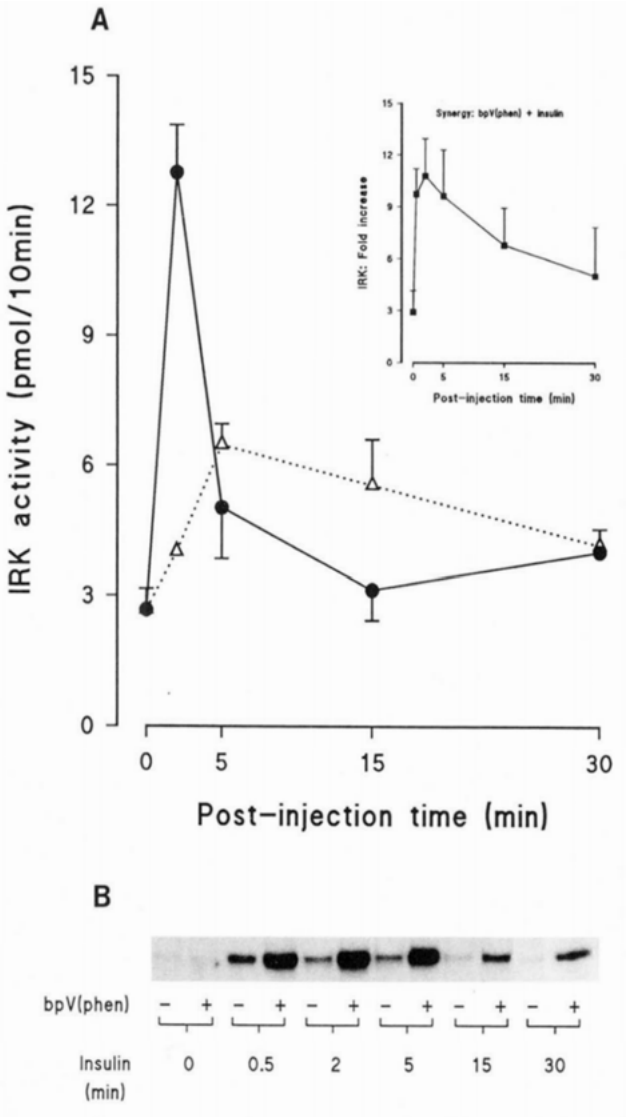

Figure 2. Panel **A**: Time course of rat liver microsomal insulin receptor kinase (IRK) activation after the *in vivo* administration of insulin (●), a peroxovanadium compound (△, bpV(phen), potassium bisperoxo(1,10-phenanthroline)oxovanadate(V)), and the combination (in the inset). Fasted rats received an intrajugular injection of 0.6 μmol/100 g body weight of bpV(phen) (△) or 1.5 g/100 μg body weight insulin (●), and were killed at the indicated times thereafter. Animals treated with a combination of insulin and the peroxovanadium compound are shown in the inset (solid squares). Panel **B**: Tyrosine phosphorylation of IRK. Equal amounts of receptor binding (assessed by ^{125}I-insulin binding) were loaded for SDS-PAGE and immunoblotting with antibodies against phosphotyrosine on the insulin receptor as described previously [78]. Adapted with permission from [72]; copyright 1994 American Society for Biochemistry and Molecular Biology.

known [12, 14]. In this review, we provide a brief account of the more clinical and economical aspects involved with investigating antidiabetic vanadium compounds, in addition to topics generally highlighted in most reviews, focusing on the chemistry, mechanistic, and medicinal aspects of the compounds [10–14, 24, 28, 31, 32, 92–94].

2. BIOLOGICAL ACTIVITIES OF VANADIUM COMPOUNDS

The biological activities of vanadium salts and many vanadium compounds have been investigated and reviewed extensively with regard to their characterization and biological effects in cell culture as well as in *ex vivo* and *in vivo* studies [8–13, 15, 17, 19–32, 35, 36, 88, 91, 95–97]; *in vivo* studies were carried out using well known and documented protocols. The results of *in vivo* and human studies with vanadium compounds are summarized in Section 2.2, while Section 2.1 details aspects of the development of vanadium compounds as a potential treatment of diabetes as well as describing the studies with different classes of vanadium compounds.

Prior to studies in animal models, there are generally numerous studies done in cell culture because such experiments are readily performed and relatively inexpensive in a comparative sense. It is beyond the scope of this review to describe this area in detail, but in general, the effects of vanadium compounds have been found to be dependent on the biological system being used. Because the *in vivo* biological effects observed for vanadium compounds may not be repeated in *in vitro* systems, several research groups work directly in animals and regardless of the insulin-like effects of vanadium compounds studies derived from one model system should be interpreted cautiously and not be extended to other animal systems [8–13, 15, 17, 19–32, 35, 36, 39, 88, 91, 95–97]. In the following, we will briefly summarize the animal models used and the classes of vanadium compounds investigated, and in the process, highlight some of these results and observations in each system.

2.1. *In vivo* Model Systems for Studies of the Antidiabetic Effects of Vanadium

The selection of an animal model is critical for the success of an *in vivo* study and there are three fundamentally different ways to obtain animals with IDDM and NIDDM for laboratory studies. The mechanism for induction of diabetes in the animals can be (1) to use an animal breed that is genetically predisposed to become diabetic, (2) to induce diabetes with drugs (such as streptozotocin, STZ), or (3) to induce diabetes with diet. Although studies have been done using all three approaches and will be briefly described in the following, we will focus mainly on the use of STZ to induce diabetes, the reproducibility of *diabetes mellitus* development and treatment using the STZ model, and other pros and cons of using this model in evaluating V-based antidiabetic agents.

Most animal studies with vanadium derivatives were carried out with the STZ-induced Wistar rat model, but some studies were also reported with the alloxan-induced Wistar rat model, or selected genetically modified inbred models [4, 12, 14, 17, 19, 98–101]. Regardless of the model system, induction of diabetes results in changes in glucose and lipid metabolism, with the latter showing 10 to 20-fold level changes. Subjects are experiencing elevations in clinical (excessive thirst and urination) and biochemical parameters (fasting blood glucose (FBG), hemoglobin A1c (HbA1c) and lipids) as well as changes in transcription of cholesterol and fatty acid biosynthesis and uptake [102]. STZ is a glucosamine-nitrosourea molecule derived from *Streptomyces achromogenes* which is clinically used to destroy the pancreatic β cells in the animal models. Alloxan, a derivative of pyrimidine, induces diabetes in rat models by acting as a glucose analog to selectively destroy pancreatic β cells [103, 104], resulting in hypoinsulinemia and hyperglycemia. Since the clinical state of IDDM involves a lack of insulin production and the onset of DM is triggered when the cells can no longer make insulin, the STZ model can be considered a model for IDDM. Depending on the dose of STZ, the phenotype may mimic NIDDM. Thus, STZ-induced diabetes is not an ideal model system for either IDDM or NIDDM [105].

In the STZ-induced diabetic rat model system, healthy animals treated with STZ become diabetic two weeks after treatment. HbA1c is a reliable target with a normal range of 4–5.6 %; an individual whose HbA1c level is between 5.7 % and 6.4 % has a higher chance of becoming diabetic. HbA1c levels of greater than 6.5 % reflect long-term poor glycemic control; individuals with this level are diagnosed as having diabetes. As an outbred animal model system, Wistar rats are genetically homogeneous [98], but the animals can differ, depending upon the particular supplier and even from the same supplier at different times. Perhaps not surprisingly, responders and non-responders to vanadium treatment have been reported in Wistar rats [13, 52, 98, 106]. Specifically, the different responses have been demonstrated genetically with microarray studies, documenting the heterogeneous population in these rats and the complications of data interpretation with this non-homogeneous model system [52, 106].

Animal models other than the outbred Wistar rat system have been evaluated to explore whether less genetically heterogeneous animals might provide more consistent responsivity. Specifically, the Willsky lab studied STZ-induced diabetes in several inbred rat models, including the Wistar Kyoto and Wistar Furth strains [13, 98, 106]. Although several additional inbred rat strains such as Dahl, ACI, PVG, Buffalo, Lewis, Brown Norway, and F344 were investigated, and despite observing differences in STZ-induced diabetic rats, no significant differences in blood glucose levels were demonstrated between control and vanadyl sulfate-treated diabetic animals for all these inbred rat strains using similar doses of treatment [13, 107].

Some model systems have demonstrated, that it is possible to develop diabetes using diet in animal systems such as cats [10], and Wistar rats [14, 103, 108]. Although there were variations in the physical parameters monitored in each study, some similarities exist [103, 108]. In these studies, some animals were subjected to a high-caloric diet for several months. This group developed metabolic disorders of carbohydrates and lipids, and a significant weight increase [103, 108].

Other genetically well studied hyperglycemic, hyperinsulinemic rodent models for NIDDM (i.e., with a reduced response to exogenously administered insulin) include the ob/ob and db/db mice, BB rats, and fa/fa rats [11]. However, the ob/ob and db/db mice model systems display improved insulin responsivity to oral vanadate treatment, indicating that these are suitable models for further study. Specifically, studies in ob/ob mice showed that administering sodium-orthovanadate (Na_3VO_4) improved oral glucose tolerance tests [109]. When tested intravenously, the mice demonstrated an increase in insulin-induced reductions in blood glucose and normalization of malate dehydrogenase and glucose-6-phosphate dehydrogenase [110].

2.2. Types of Vanadium Compounds Used in *in vitro*, *in vivo*, and in Clinical Studies

Vanadium is a versatile element that can exist in many oxidation states; however, oxidation states 4 and 5 are most common in antidiabetic applications. As displayed in Figure 1, a wide range of vanadium oxidation states and salts, organometallic and peroxovanadium(V) compounds, and coordination complexes have been evaluated in cell culture, animal models, and human studies [111].

Several vanadium salts have been studied, including sodium orthovanadate (Na_3VO_4) and ammonium or sodium metavanadate (NH_4VO_3 and $NaVO_3$). $NaVO_3$ was the vanadium(V) compound chosen for clinical trials [4, 7, 96, 97, 112, 113]. Of vanadium(IV) compounds, vanadyl sulfate ($VOSO_4$) was selected for use in clinical studies [22, 88, 90, 113–115]. Twenty years ago, the counter ions to vanadate were limited to ammonium and sodium salts and vanadyl sulfate was the most readily available vanadium(IV) compound; it is therefore possible that these compounds were chosen for the early work because of availability rather than consideration of the optimal form [46]. Before the many different vanadium sources were available, researchers used V_2O_5 and, when a specific base was added, one could form a desired salt. This would, however, generate the vanadium in oxidation state V unless a redox reagent such as ascorbate was added. We will describe the clinical studies that were done using sodium as a counter ion [11, 22, 31, 88, 90, 97, 112–116]. Of more complex vanadium(V) compounds, two decavanadates including the alkylamine and the sodium compounds [31, 32] have also been tested in animal studies. Additionally, vanadyl sulfate, combined with a complexing amino acid [11], was used in animal studies, and was for some time championed by a start-up company (Figure 1, V_i-L-Glu(γ)HXM) [11]. In addition, chelated vanadium [91] was employed in clinical trials [91]. Figure 3 displays the significant glucose-lowering effects observed following administration of bis(maltolato)oxovanadium(IV) (BMOV) to diabetic and non-diabetic rats [17] which further demonstrates that administration of BMOV to healthy, non-diabetic rats does not lead to hypoglycemia [17].

In the early 1980s, organometallic metallocenes were evaluated as anticancer agents [5–7]. These included cellular studies and subsequent compound development. Titanocene [83, 117] was chosen for evaluation in human clinical trials

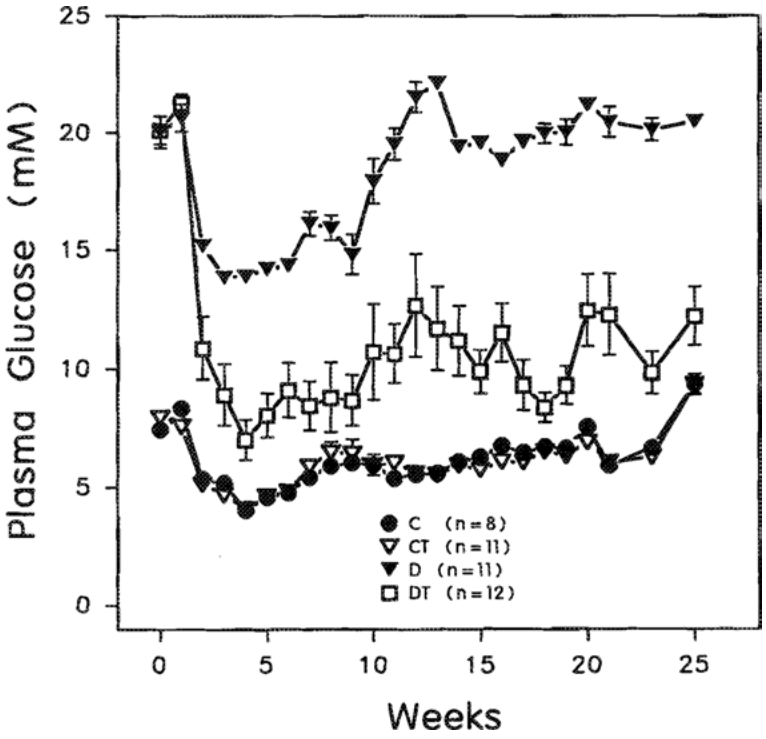

Figure 3. Plasma glucose concentrations are shown as a function of time of streptozotonin (STZ)-induced diabetic and normal Wistar rats with chronic administered BMOV in the drinking water. Four groups are treated and the key is control (C), control treated (CT), diabetic (D) and diabetic treated (DT). Time 0 is the day of STZ-injection and treatment with 0.5 mg/mL for 3 weeks was started 7 days after STZ-injection. The dose was increased to 0.75 mg/mL after 4 weeks and for the rest of the treatment period. Adapted with permission from [17]; copyright 1993 NRC Research Press.

for breast and renal cancer. Unfortunately, this compound did not demonstrate sufficient efficacy to warrant continuation of clinical trials. Since the vanadocene compounds were reported to have promise [83, 118, 119], but titanocene was selected for human trials [83, 117], there are no reports with vanadocene. Reinvestigation of vanadocene would presumably require some improvements in compound design or delivery to enhance its efficacy.

The peroxovanadium complexes are compounds in which at least one peroxo group is coordinated to vanadium(V) (Figure 1), which alters the reactivity of the metal ion [51, 70, 72, 73]. These complexes can facilitate protein phosphorylation and dramatically increase the autophosphorylation of the insulin receptor tyrosine kinase observed during treatment with insulin (see Figure 2) [69, 72, 74, 75]. In contrast to simple vanadium salts and chelated vanadium, peroxovanadium compounds do not require insulin for their insulin-like action. Posner and colleagues first described a key role for PTPases in regulating receptor tyrosine

kinases. The peroxovanadium complexes possess a high PTPase-inhibiting affinity that greatly exceeds that observed for vanadate or H_2O_2, respectively [70]. This finding is important because it establishes that the high activity demonstrated by this class of compounds originates from the intact complex, and not from the sum of the components [70, 72]. The use of these compounds in *in vitro* and *ex vivo* work was continued with great success [72], but when the compounds were tested in animal studies *in vivo*, they were found to be non-specific in their inhibition of PTPs, and hence, promoted the phosphorylation of growth factor tyrosine kinases along with a potential to promote oncogenesis.

Several crucial early discoveries increased interest in vanadium, including the finding that vanadate is a potent inhibitor against Na^+,K^+-ATPases [65–67], ribonucleases [68], and (in the form of peroxovanadium) can oxidize the cysteine group in protein phosphatases [55, 87]. These results led several groups to explore the vanadium salts and other compounds in various types of studies leading to reports of their "insulin-mimetic" [69, 70] or "insulin-like" properties. The former term was used initially to describe the effects observed that mimicked some of the functions of insulin, whereas more recently the term "insulin-like" is being used [13, 71], because it was discovered that the effect of simple vanadium salts and chelated vanadium compounds required a basal level of insulin for its effects to be observed. The reports on peroxovanadium(V) in *in vitro* systems and follow up studies created excitement for the therapeutic efficacy of vanadium because this species significantly enhanced the effect of vanadium. It was the first compound for which this dramatic enhancement was observed [70, 76, 77].

Finally, the largest group of vanadium compounds for which the biological effects have been explored are coordination complexes [11–14, 19, 27, 28, 33, 39, 52, 70, 71, 92, 94, 111, 120]. Coordination complexes are diverse and contain vanadium in different oxidation states, as well as a wide range of ligands and two, three, four or five different coordinating groups. This class of compounds is particularly promising, because a member can be effective depending on structure-activity relationships [12–14, 39, 120]. Coordination complexes involve both ligands and metal ions, and the combination (that is, formation of a coordination complex) can modify the efficacy of the vanadium complex [8–15, 17, 19–33, 35, 36, 111]. Importantly, these coordination complexes are reported to be more efficacious than the simple salts in cell culture and animal studies and these effects reduce the dose needed, thus, an observed lower toxicity results [8–15, 17, 19–33, 35, 36, 111]. Several groups were reporting different derivatives. For example, the Sakurai group, one of the first groups initially reporting utilizing vanadyl sulfate and picolinatooxovanadadium(IV) more recently presented a much more potent natural product-derived alloxan derivative [15, 18, 21, 24].

Several of the compounds shown in Figure 1 deserve brief mentioning at this point. One of these classes of compounds is the dipicolinato oxovanadium(V) series [13, 71]. These materials, explored extensively by the Crans/Willsky team, demonstrated for the first time that the antidiabetic properties of the vanadium(V) coordination chemistry compounds can be improved over the vanadium(IV) compounds [13], contrary to findings with the BMOV series of compounds [71]. Importantly, redox cycling does take place in all these systems, and

the reactive oxygen species are likely to play a role in how vanadium compounds are metabolized [13, 71, 121–123]. In traditional drug development studies, many complexes must be investigated to identify one complex suitable for human studies. At this time, one class of coordination complexes has advanced beyond animal studies based on BEOV [12, 14].

Studies and commercialization of a very different class of vanadium compounds was also explored V_i-L-Glu(γ)HXM (see Figure 1) but less information is available on these studies, because this work was done mainly in a private startup company [11, 124, 125]. A vanadium-peptide coordination complex was developed around the concept of facilitating entry of the vanadium compound into the cell presumably allowing for lower dose in *in vivo* studies [11]. This concept was reported in several papers with very convincing *ex vivo* and *in vivo* animal data demonstrating that vanadium(V) improved glucose uptake and glucose metabolism by 4–5 fold and 5–7 fold, respectively [124, 125]. However, the financial demands for funding clinical trials are continuously increasing and generally little or no information is available on the impact of formulation on the biological activity. To date no human studies have been reported with these compounds. Only BEOV underwent studies in humans that have been reported in the literature [91], although most of the literature studies describe studies of the close counterpart BMOV [17]. This will be summarized further below in this review.

3. HUMAN STUDIES WITH VANADIUM COMPOUNDS

3.1. Description of the Three Phases of Clinical Trials

Human studies (referred to as clinical trials) are generally grouped as three types: In Phase 1 clinical trials, healthy humans are administered a new drug, in order to evaluate potential toxicities. When no or limited toxicity is observed, the compound advances to Phase 2 trials, in order to define the effective dosage to be used for the final clinical trial study. In Phase 3 trials, patients receive therapy with the drug for a specific clinical indication (e.g., diabetes). Upon completion of all the studies, the data are presented to the Food and Drug Administration (FDA) for approval for marketing in humans. Often, a successful compound may still need to satisfy additional minor safety concerns, and thus, further studies (referred to by some as "Phase 4 trials" [126]) may need to be completed prior to marketing but after the approval of the drug.

3.2. Vanadium(IV) and Vanadium(V) Salts

In the early 1990s, an NIH RFA (request for funding from National Institutes of Health) was announced calling for clinical studies with vanadium salts, and resulting in several NIH-funded human studies [11, 31, 46, 88, 90, 97, 112–116]. Although typically, such clinical studies are co-funded by industry, in this unusual

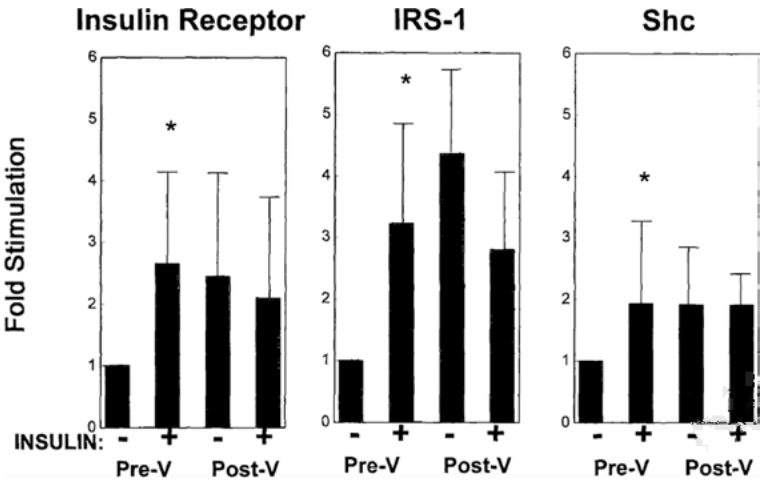

Figure 4. Protein phosphorylation in human muscle before and after treatment with 150 mg vanadyl sulfate administration. Insulin stimulates tyrosine phosphorylation of the insulin receptor, IRS-1, and the Shc proteins (2.7-, 3.2-, and 1.9-fold) in the basal state. Adapted from [113]; copyright 2000 Elsevier.

case funding resulted from the negotiations of a congressman to obtain 1 million dollars allocated specifically to explore new and promising treatments for diabetes. In the early human studies with Type 2 diabetic patients and healthy humans, oral tablets of a placebo or sodium metavanadate at three dosage levels: 25, 50, and 100 mg were administered [14, 97]. Unfortunately, the lowest doses did not have sufficient efficacy and the highest dose caused side effects such as diarrhea. The Goldfine/Kahn team modified their study design, and in their subsequent study the results with vanadyl sulfate showed improved glucose uptake and metabolism (Figure 4) [113]. Results equivalent to Phase 1 and 2 clinical trials with vanadyl sulfate were therefore achieved by several groups [88, 90, 97, 113–115]. The studies with vanadyl sulfate (summarized in Table 1), while showing improved tolerance as compared to previous studies with sodium metavanadate [14, 97], were carried out following the FDA guidelines at the time of the studies but with fewer subjects than required currently. Requirements have increased in the past 20 years and a double-blind study with more subjects is now needed to substantiate the demonstration of safety and efficacy [127].

Recommendations and support for vanadium treatments found in the literature are therefore based on older studies with outdated study designs. Specifically, current FDA guidelines require a randomized, placebo-controlled trial with treatment of oral vanadium compounds, of at least a 2 months duration, with a minimum of 10 adults with diabetes per study group [127]. While such studies are not available for vanadium, those that come closest to satisfy the current guidelines are summarized in Table 1; most were too short and while changes in FBG and HbA1c were observed, the desired magnitude of changes was not achieved [88, 90, 97, 114, 115, 127].

Table 1. Glycemic outcomes before and after vanadium treatment.[a]

Glycemic Outcome Measure	Dose administered	Before (mean, SD)	After (mean, SD)	Reported P-value	Ref.
HbA1c (%)		8.1 (0.4)	7.6 (0.4)	< 0.01	[115]
		9.4 (0.5)	8.8 (0.5)	< 0.01	[114]
	75 mg	Not stated	Not stated	Not stated	[97]
	150 mg	7.8 (1.7)	6.8 (1.1)	< 0.07	[97]
	300 mg	7.1 (2.3)	6.8 (2.1)	= 0.05	[97]
		9.6 (0.6)	8.8 (0.6)	< 0.002	[88]
Fasting Blood Glucose (mmol/L)		10.8 (0.9)	8.6 (0.9)	< 0.01	[115]
		12.3 (1.3)	10.6 (0.9)	< 0.01	[114]
	75 mg	Not stated	Not stated	Not stated	[97]
	150 mg	Not stated	Not stated	'Not significant'	[97]
	300 mg	9.3 (4.0)	8.0 (3.7)	< 0.02	[97]
		9.3 (1.8)	7.4 (1.4)	< 0.05	[90]
		11.7 (1.1)	10.0 (0.8)	< 0.05	[88]

a Adapted with modification from Ref. [127].

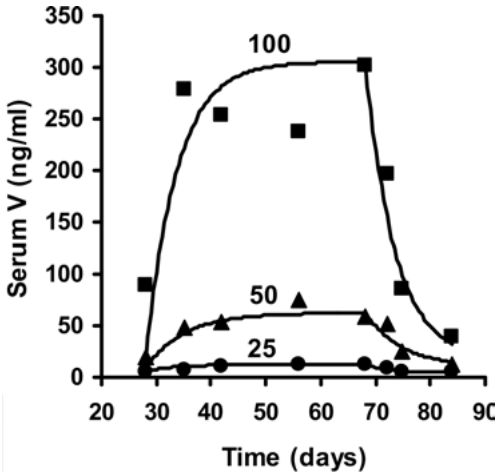

Figure 5. Composite serum vanadium accumulation and decline curves describing the pharmacokinetic data for patients dosed 25, 50, and 100 mg V administered in the form of vanadyl sulfate. The mean concentrations of for each time point of the decline phase were used to generate the first order elimination constant for a one-compartment open model using the equation C_t = baseline concentration + C_o e^{-kt}. The mean serum V levels are indicated by (●) for the 25 mg V dose, (▲) for the 50 mg V dose, and (■) for the 100 mg V dose. These data show non-linear association of effects with total vanadium concentration. Adapted with permission from [112]; copyright 2013 Royal Society of Chemistry.

The vanadium(IV) salt, vanadyl sulfate, has been used most frequently not only in the studies listed in Table 1 but also in subsequent mechanistic studies. Recent reports documented that the activity of this compound does not correlate with total serum vanadium levels [112]. The work by Willsky et al. continued for a decade, exploring not only the efficacy of vanadium, but the nature of the active species in human subjects. They assessed its pharmacokinetics examining the vanadyl sulfate administered to NIDDM and IDDM patients (Figure 5) showing that the pharmacokinetics could be explained by a one-compartment model [112]. Because the efficacy of the vanadyl sulfate does not relate to the total vanadium concentration in the serum or the blood, the active pool of compound has not yet been identified, supporting the notion that the bioprocessing and coordination chemistry takes place once the material is administered.

3.3. Chelated Vanadium: Bis(ethylmaltolato)oxovanadium(IV)

Animal model data suggest that glucose uptake and metabolism are facilitated by administration of vanadium [127, 128] and the effect is greater and with less side effects when using chelated vanadium instead of the salts [13, 96]. Phase 1 and 2 clinical trials were carried out with the chelated vanadium compound, bis(ethylmaltolato)oxovanadium(IV) (BEOV) [9, 12, 14] (Figure 3) which was

developed as a collaboration between McNeill and Orvig at the University of British Columbia and the start-up company Kinetek. They explored a coordination complex formed between vanadium and a bidentate ligand, maltol, which is a naturally occurring flavor enhancer. The complex between vanadium and maltol had been reported earlier by Stewart and Porte [9, 129]. Maltol is a ligand on the FDA's list of permitted substances and much work has been reported about the complex formed between maltol and vanadyl sulfate, bis(maltolato)oxovanadium(IV). BMOV has been described in the literature as the gold standard reference compound. However, the compound selected for clinical trials was based on a related ligand, the ethyl-substituted version of maltol, eatol. Eatol combined with vanadyl sulfate forms BEOV. The Orvig/McNeill team documented that this compound had potential by (1) demonstrating that V was taken up from the BMOV treatment and (2) that plasma glucose normalized upon treatment with vanadium (Figure 3) [17, 130]. This team also patented the application of these compounds for diabetes [9, 131] and for a decade, this discovery was important to the development of vanadium [91, 132–135].

The team continued studies characterizing the chemistry [132] as well as the details of how vanadium interacts in the biological system. For example, studies were done examining the interaction and transport by blood proteins (transferrin and human serum albumin) [133]. The team also demonstrated that uptake of V from BMOV was more than 3-fold that found for $VOSO_4$ and that V accumulated in the liver, kidney, and bone after administration to animals [134, 135]. As explored by ID and 2D electron spin echo envelope modulation (ESEEM) spectroscopy, V concentrations were highest in bone. Specifically, the 2D hyperfine sub-level correlation (HYSCORE) spectrum of diabetic V-treated rat bone shows a spectrum consistent with binding of the vanadium to triphosphate in bone [136], confirming biological studies reporting accumulation of vanadium in the bone. After the treatment commenced, vanadium continued leaching out of bone resulting in continued antidiabetic activity. Further, they documented that the ligand could dramatically improve V uptake.

Structure-activity relationship studies were carried out with a range of other complexes using ligands related to maltol, systematically replacing parts of the ligand in BMOV [91]; since, as BEOV was found to be the best compound identified, it was selected to be used in the clinical trials. However, as pointed out above, while the randomized controlled trials carried out with vanadium compounds have been promising, these studies did not utilize an optimally rigorous design satisfying FDA regulations in 2018 [127, 128].

3.4. Treatment Parameters Involving Oral Administration of Vanadium Compounds

Diabetes is a manageable disease when control of blood-glucose levels is established [137, 138]. To effectively achieve glucose control in Type I diabetics, the most common treatment is to administer subcutaneous administration of insulin

to achieve blood-glucose homeostasis. However, most subjects would prefer to consume a tablet or to utilize other slowly delivered means of insulin administration, such as the newly developed insulin patches rather than administering insulin injections [139]. Many factors are considered when electing alternative drug use particularly those that require different routes of administration.

A standard management approach is to use blood-glucose monitors and either oral hypoglycemic agents or injections of insulin with the aim of avoiding hyperglycemia, while ensuring that treatment does not lead to hypoglycemic episodes between meals or overnight [140, 141]. Since insulin treatment is costly and eventually loses efficacy, and injections are uncomfortable, it is usually desirable to initiate management of Type 2 DM with oral agents (tablets or capsules which might eventually include a vanadium compound). But, it must be recognized that for all oral agents the pharmacokinetic profile is very different from administered insulin.

Many orally consumed medications are sensitive to the effects of food on drug dissolution and absorption. Ideally, oral formulations of vanadium should be developed, preferably as forms that can be taken with food, in order to minimize side effects such as gastrointestinal discomfort [115, 142]. In several studies, vanadium administration was tolerated better with food and adverse gastrointestinal effects generally disappeared within one week [90, 142].

4. COMMERCIALIZATION OF VANADIUM AS ANTIDIABETIC AGENT

4.1. Formulation and Commercialization of Vanadium Compounds

In our modern and technological world driven by free enterprise, the protection of a drug by patents is required for development of novel drugs [12, 24, 54, 92, 143]. The topic is further complicated by worldwide differences in regulations. Although most countries have some type of regulation, it is simpler to obtain an international patent than a patent to distribute a drug in the USA; thus, potential uses are often championed by a powerful interest group. For example, since athletes may view vanadium-containing supplements as advantageous for optimal performance, commercial enterprise is likely to continue their development as a nutritional supplement [40, 127, 144]. Since there are large differences in having a compound approved as a drug satisfying the regulations and clinical trial studies *versus* being licensed as a nutritional additive, the compounds are sold as nutritional additives, where the requirements are much less strict. Unfortunately, available information on the formulation is very limited, and researchers must examine the publications reporting human and animal studies as well as infer from the many commercial forms of the compound most commonly used as a nutritional additive, as is the case for vanadyl sulfate [45, 46].

Clinical trials are multibillion dollar endeavors, and because clinical trials need investor backing they are dependent on the state of the global economy. It is a rare event for a drug to be developed sufficiently at exactly the same time that funds for clinical trials are available. The problem in timing may be part of the reason why the success rates of compounds that enter clinical trials are so low. Both a favorable global economy and availability of a sufficiently advanced drug (with respect to studies defining the efficacy, toxicity, bioavailability, and formulation) are required in order for a drug to emerge as a successful candidate at the end of a clinical trial. For V, formulation of the compounds as drugs was not optimized at the time most of the human studies commenced [46]. This is a problem because it is well recognized that most of the vanadium administered in animal studies have a very low bioavailability (< 1 %). However, a number of approaches to improve bioavailability have been employed including using the polymer carboxymethyl cellulose to improve uptake [12, 14, 46, 71, 145, 146]. The bioavailability and efficacy of the vanadium compounds could have been improved if the formulation had been optimized prior to administration in the human studies [89, 97, 113]. To illustrate the magnitude of this issue, vanadyl sulfate is sold as a nutritional additive, and there are a range of different formulations with vanadium levels per pill ranging from 5 mg to 100 mg [45, 46]. All the pills contain vanadyl sulfate, but both the amounts and the accompanying material to make up the tablets/capsules, which can be proteins or nutrients, are different between the tablets [45]. If the human studies were to be carried out with the current level of knowledge, it is likely that different forms of vanadium and formulations would have been chosen for the studies probing the use of vanadium as a drug in humans.

Based on the number of commercially available forms of vanadyl sulfate as a nutritional additive, there are clearly many options to what is the most efficacious vanadium compound for treatment. It is widely accepted that a high dose is needed because bioavailability is so low, but if a lower dose of a more bioavailable compound could be taken on a full stomach, the discomfort from taking vanadium supplements or drugs could be significantly decreased. Unfortunately, although studies may have been done with some of these nutritional compounds shown in Figure 1, the results have not been reported in the peer reviewed literature, and clinicians and consumers are left to try for themselves what is the best formulation by trial and error. Although this may seem random, perhaps considering that human wellbeing is individualized, and the concept of personalized medicine, the "try it and see" approach, may be more acceptable today than it was even 10 years ago particularly for a material that enhances athletic performance. Although the need for data to justify human treatments cannot be ignored, the fact remains that access to the compound will continue to allow self-administration of these materials.

The initial studies provided documentation early on describing the beneficial effects in this review, but the specific methods and materials used have less to do with what would be best and how such studies should be done presently. It is important to recognize that a very different set of requirements exist for a nutritional agent or drug today [127, 144] than 50 or even 10 years ago, and that

the practice and protocols needed for bringing a compound to the clinic for public use as a therapeutic agent have dramatically changed [11, 91, 147, 148]. It is not only the marketing and financial aspects of the use that have changed, but the type of scientific documentation that a compound is safe and efficacious has changed. Thus, what may have been valid 30 years ago may no longer hold due to increasing rigor required in human studies [127, 144]. In summary, early studies in humans and animals document the long history of therapeutic application of vanadium and its importance [28], and explain the fact that "vanadium" remains on the list of potential treatments that are described as promising even when applying the rigor currently required for clinical applications [28, 91, 127]. Based on current knowledge identification of the optimum vanadium-containing material for treatments, should such studies be done today, would require a more detailed analysis of formulation than has currently been reported.

5. WHAT IS NEXT?

5.1. Vanadium as a Weight Loss Agent

A major symptom of diabetes is the loss of control of glucose metabolism which often involves control of weight. Weight gain is one of the physical parameters investigated in studies administering vanadium compounds to animals or humans [12, 16, 24, 115, 144, 149]. Administration of vanadium compounds to diabetic animals will often result in weight loss, and it has been suggested that the vanadium compounds be considered for use for weight loss [16, 24]. Indeed, BEOV is still under consideration for such purposes and for some of the vanadyl sulfate nutritional additives on the market, weight loss is the advertised goal for its application [45, 46].

5.2 Human Trials Exploring a Protective Role of Vanadium for Development of Diabetes

In addition to the human studies described above, one recent study investigated whether vanadium might protect humans against the development of diabetes [150]. This study, carried out in China, explored if blood levels of vanadium correlated with the pathogenesis and complications of newly diagnosed NIDDM. Plasma vanadium concentrations were significantly lower in subjects with newly diagnosed NIDDM than in control subjects ($p = 0.001$). Mean plasma vanadium concentration levels in subjects with and without diabetes were 1.0 μg/L and 1.2 μg/L, respectively. The subjects in the highest quartile in a plot of vanadium plasma concentration had a significantly lower risk of newly diagnosed NIDDM (odds ratio = 0.26, 95 % confidence interval: 0.19, 0.35; $p < 0.001$), compared with subjects in the lowest quartile. Known risk factors were considered and the trend remained significant after adjustment in the statistical

analysis. The conclusions show that plasma vanadium concentrations were inversely associated with newly diagnosed NIDDM in the Chinese Han ethnicity population investigated in the study. These findings are important since for the first time data from a large clinical study documented the potential importance of vanadium in the serum [150].

5.3. Vanadium as an Oncovirogenic Agent

Oncolytic viruses (OVs) are an emerging class of anticancer bio-therapeutics that induce antitumor immunity through selective replication in tumor cells (Figure 6). The efficacy of oncolytic viruses as single agents remains limited.

In collaboration with the Diallo group we have recently reported an approach that boosts the therapeutic efficacy of oncolytic viruses by combining their activity with an immuno-modulating, small molecule PTPase inhibitor [35, 36, 151]. The enhancement of oncolytic virus infection by vanadium-based phosphatase inhibitors *in vitro* and *ex vivo* was investigated and the effect was particularly useful in resistant tumor cell lines (Figure 7). Thus, vanadium compounds were found to increase antitumor efficacy in combination with oncolytic viruses in several tumor models, leading to systemic and durable responses, even in models otherwise refractory to oncolytic viruses and drug alone.

Mechanistically, studies demonstrated that this effect involves the antiviral type I interferon response towards a death-inducing and proinflammatory type II interferon response. Such a response leads to improved proliferation of oncolytic virus thus increasing bystander killing of cancer cells, and enhancing antitumor immune stimulation [36]. Overall, a new application in medicine, using vanadium compounds to maximize viral oncolytic and systemic anticancer immunity,

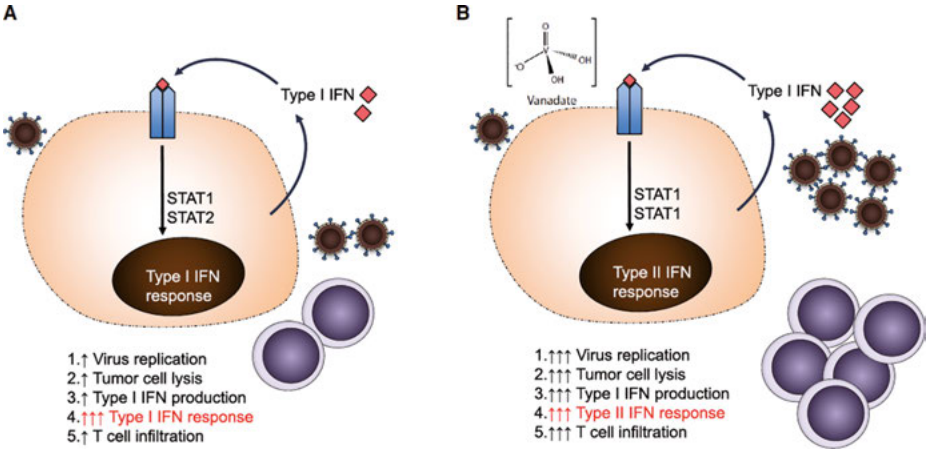

Figure 6. Oncolytic viruses: (**A**) a new biological tool to combat cancer; (**B**) vanadate, an effective stimulator of the oncolytic RNA-based viruses. Type 1 interferon is abbreviated IFN. Adapted with permission from [151]; copyright 2018 Cell Press.

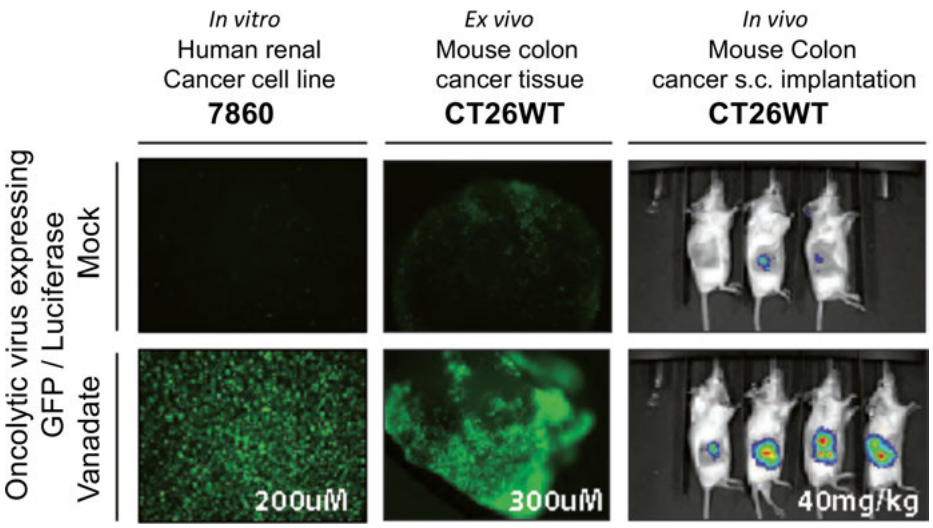

Figure 7. The effect of oncolytic virus (labeled as Mock virus-treated control) and oncolytic virus in the presence of vanadate *in vitro*, *ex vivo*, and *in vivo*. The *in vitro* effects are demonstrated on the human renal cancer cell line 7860. The *ex vivo* effects are demonstrated on the mouse colon cancer tissue CT26WT and the *in vivo* oncolytic virus expression is demonstrated in live mice. In all cases the expression is visualized using the green fluorescent protein luciferase. See [36] for details. Adapted with permission from [36]; copyright 2018 Cell Press.

offers new avenues for the development of improved immunotherapy strategies. This is particularly of interest considering that the mechanisms and the work carried out with vanadium as an antidiabetic agent remain timely and relevant to the novel mode of action recently discovered.

5.4. Vanadium in the Biosphere and as a Nutritional Supplement

Although vanadium is often described as a potentially essential nutrient, this statement is highly controversial [43, 147]. If essential, V would likely be required in only minute quantities in human and mammals. Organisms that require vanadium do exist; for example: sea squirts, also known as tunicates, have been reported to contain vanadium-binding proteins. These organisms are known to accumulate vanadium from seawater despite living in an environment with a very low vanadium concentration (only 10^{-9} to 10^{-10} M of vanadium) [46, 93]. Such accumulation against a gradient is very rare and has generated interest in these organisms. Importantly, this process involves reduction of the high oxidation state vanadium(V) that is being accumulated. Seaweed and algae contain a class of vanadium-containing proteins known as vanadium haloperoxidases. Vanadium chloro and bromoperoxidases are enzymes that utilize vanadium as a

cofactor enabling the oxidation of resistant substrates [50, 93]. Although it can be speculated that perhaps humans have such an enzyme in the thyroid, no such report has appeared to date.

Despite the fact that no randomized clinical trials supporting its use have been reported, vanadium (mainly in the form of vanadyl sulfate) is a popular nutritional additive which is available for purchase by the general public in supermarkets in the US and online [45]. Therefore, regardless of the lack of studies adhering to current legal licensing as a drug, the vanadium-containing nutritional additives are used by athletes and recommended by some physicians. The ability to sell a "supplement" on the market, remains a loophole in legislation that many wish could be closed. However, now distribution of vanadium as a nutritional additive is the only avenue for these compounds until more studies have been done [127, 128].

6. CONCLUSIONS AND OUTLOOK

Vanadium has been known for centuries to have beneficial effects in humans, including normalizing elevated glucose levels in diabetic subjects. The beneficial effects of vanadium have been explored in animals and humans. Most human studies have utilized vanadium salts, sodium metavanadate or vanadyl sulfate and in each trial, antidiabetic effects were observed. However, side effects were also observed at the doses needed for beneficial efficacy which approached toxic concentration. Recent human studies demonstrated that in a large (1500 subject) clinical study in newly diagnosed Type 2 patients, blood levels of vanadium were lower in diabetic patients than in healthy subjects, raising the possibility that vanadium compounds have some protective effects, and providing fuel to the concept that vanadium compounds could ultimately led to a successful drug for treatment of diabetes.

Other vanadium derivatives were investigated, and these investigations were important for the development of vanadium compounds as potential drugs. The peroxovanadium(V) compounds were discovered and showed superior enhancement in protein phosphorylation. These studies led to the discovery that the vanadium salts inhibit protein phosphatases.

Other compounds that were investigated as potential antidiabetic drugs include the vanadium(V) complex with monohydroxamate of l-glutamic acid, and decavanadate. A few other compounds have been investigated for treatment against cancer, including dipicolinatodioxovanadium(V) complexes, dichlorovanadocene, and Metvan (Figure 1). These compounds have not been submitted for human studies yet. Since ligand coordination to the vanadium increases the efficacy, it seems reasonable to expect that organic vanadium compounds would be superior to the salts and one organic vanadium compound, bis(ethylmaltolato)oxovanadium(IV), that has undergone Phase I and II clinical trials. This material showed very favorable effects, but the combination of economic factors and the patent expiration made it difficult to fund Phase III clinical trials.

Since BEOV has gone off patent, there have been few mechanistic studies, aimed at understanding the vanadium-facilitated inhibition of additional promising compounds. Now, the most promising uses of vanadium compounds may remain as a nutritional additive or a weight control agent designed to control glucose levels. In addition, new application of vanadium as a stimulant for oncolytic virus treatment has been reported to combat cancer. The latter preclinical effect has just been published at the beginning of 2018. Since the combination treatment showed potent effects against a series of resistant human and murine cancer cell lines with no effect on healthy tissue, this approach is particularly promising.

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ABBREVIATIONS

2D	electron spin echo envelope modulation
2D HYSCORE	hyperfine sub-level correlation
BEOV	bis(ethylmaltolato)oxovandium(IV)
BMI	body mass index
BMOV	bis(maltolato)oxovanadium(IV)
bpV(phen)	potassium bisperoxo(1,10-phenanthroline)oxovanadate(V)
DMI	insulin-dependent *Diabetes mellitus*
FBG	fasting glucose level
FDA	United States Food & Drug Administration
HbA1c	glycated hemoglobin
IDDM	insulin-dependent diabetes
IRK	insulin receptor kinase
Maltol	3-hydroxy-2-methyl-4H-pyran-4-one
NIDDM	non-insulin dependent diabetes
NIH	National Institute of Health
NMR	nuclear magnetic resonance
OV	oncolytic viruses
PTPase	protein tyrosine phosphatase
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
STZ	steptozotocin

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Chromium Supplementation in Human Health, Metabolic Syndrome, and Diabetes

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Abstract: After 40 years of significant work, it was generally accepted that chromium in its trivalent valence state, Cr(III), is an essential micronutrient for humans. This view began to be challenged around the turn of the millennium. Some investigators argue that its effects on glucose and lipid metabolism reflect a pharmacological rather than a nutritional mode of action while yet others express concern about the toxicity and safety of supplemental chromium. Understanding the conjectures requires a reflection on the different definitions of "essential" and a perspective on the development of the field, which in itself is a remarkable snippet of science history and education. At the center of the discussion is our failure to have established a molecular structure and a specific site of action of a biological chromium complex. Instead, many different types of Cr(III) complexes, in particular chromium picolinate, but also those with nicotinate, propionate, histidinate, chloride, and other ligands, all with different chemical properties and biological activities, are being used in laboratory investigations and supplementation. Without knowledge of the metabolic transformations and the specific chemical properties that biological ligands impart on chromium, many of these investigations, in particular those *ex vivo*, have limited value for understanding chromium's biological function. Whether a chromium deficiency exists in humans and who is affected is poorly defined. There is evidence for the efficacy of chromium supplements in improving conditions in metabolic syndrome and in some diabetes Type 2 patients, but there are no effects on body composition in healthy individuals. Chromium is present in human tissues and in our food and Cr(III) compounds are given in (total) parenteral nutrition, taken as a supplement by athletes and bodybuilders, are ingredients of vitamin pills consumed by the general population, and are employed in animal nutrition. Another contentious issue is whether Cr(III) complexes are safe, as chromium in its hexavalent state, Cr(VI) (chromate), is genotoxic and a group I carcinogen for humans with sufficient evidence for inhalation and lung cancer. For the benefit of human health, there is a continuing need for a balanced view and informed and robust experiments to determine the specific biological molecules that are involved in the metabolism of Cr(III), the activity of biological Cr(III) complexes at specific sites of action, and the amount of supplemental Cr(III) that potentially causes long-term toxicity.

Keywords: chromium · Cr(III) · diabetes · glucose tolerance · metabolic syndrome

1. THE NATURE OF ESSENTIAL CHEMICAL ELEMENTS

A discussion of chromium in human health and disease serves as a prime example of the subject matter addressed in this book, as the status of chromium as an essential metal for humans has been questioned recently. Some general remarks about the history of the field are necessary to understand the basis of the present controversy, in particular what defines an essential metal ion and that essential metal ions cause toxicity at higher concentrations in the diet or when other factors compromise their homeostatic control and they accumulate in the body. Protagonists from traditionally separated disciplines, nutrition, pharmacology, toxicology, chemistry, and others inform the discussion with opposing viewpoints that engender continuing controversy.

The biological sciences around the middle of the last century witnessed major efforts to intensify investigations that began in the 19th century, namely to define which chemical elements are essential for life. This search may not have been completed, though, not even for humans. It was realized that the mere presence of an element in tissues is a poor indicator of its importance. For example, the presence of cadmium, a congener of zinc in the same group of the periodic

system of the elements (PSE), in horse kidneys led to the discovery of the metalloprotein metallothionein [1]. The quest to find an essential function of cadmium in mammals turned out to be a red herring. Cadmium is an element with considerable toxicity for humans and accumulates with age in metallothionein, which is involved in zinc and copper metabolism [2, 3]. Conversely, zinc was found in human tissues and it was speculated that it is not a contaminant [4]. It took several decades to show its essentiality for humans [5], and its real significance as a major element in cellular regulation is emerging only now with recognition of its functions as a signalling ion. With finding functions for many chemical elements, the biological PSE expanded considerably so that we now count at least 20 of them as essential for humans [6]. However, there is a difference between the elements that are essential for humans and the ones that are essential for other living organisms. For instance, nickel and vanadium seem to be essential for only some species. Many additional chemical elements are present in human tissues, some even at higher concentrations than the essential ones [7]. Their presence has functional, either beneficial or adverse, consequences. Non-essential does not mean non-functional. A characteristic feature of many essential elements is nutritional deficiency, extant or induced by experimentation. Some chemical elements have pharmacological responses above their physiological concentrations and most express toxicity at higher concentrations.

Another important point to consider for the following discussion is what "essential" means for a chemical element. There is considerable confusion about such a seemingly simple concept and the semantics involved. "An element is essential when a deficient intake consistently results in an impairment of function from optimal to suboptimal and when supplementation with physiological levels of this element, but not others, prevents or cures this impairment." [8]. This definition includes improvement of all functions that support health. Some elements are perhaps only beneficial under certain conditions as they have functions other than supporting our immediate survival, growth or reproduction, e.g., fluoride for tooth health or boron, silicon, nickel, and vanadium [9]. Another definition states: "An element is generally considered essential if it has a defined biochemical function or its lack results in death or failure to reproduce and addition to the diet can prevent these effects." [10]. This definition is narrower and differs from the criteria originally employed to define "essentiality". It links function to more specific outcomes and stipulates that a biochemical function must be known. Clearly, some elements were proven to be essential long before their molecular functions were elucidated, or vice versa, some elements, e.g., zinc, were found as cofactors of enzymes before their essentiality for humans was proven. A more comprehensive definition is given in the classic textbook "Trace Elements in Human and Animal Nutrition" by Underwood [11]: "A trace element can be considered essential if it meets the following criteria: (i) it is present in all healthy tissue of all living things; (ii) its concentration from one animal to the next is fairly constant; (iii) its withdrawal from the body induces reproducibly the same physiological and structural abnormalities regardless of the species studied; (iv) its addition either reverses or prevents these abnormalities; (v) the abnormalities induced by deficiency are always accompanied by

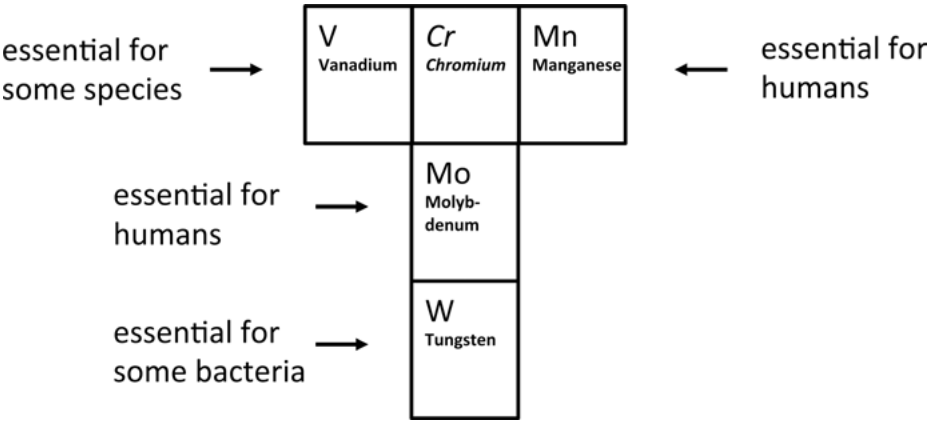

Figure 1. The chemical elements next to chromium in the periodic system of the elements. All chemical elements around chromium are essential, though vanadium has been shown to be essential for only some species.

pertinent, specific biochemical changes; and (vi) these biochemical changes can be prevented or cured when the deficiency is prevented or cured." In this definition, the requirement for presence in all living things is no longer valid. Thus, scientists do not seem to agree on the definition of "essential." We will resume this discussion later in the context of the criteria employed by the European Food Safety Authority (EFSA) in evaluating the essentiality of chromium. In their glossary, EFSA defines an essential nutrient as "any substance which a living organism must consume from the diet to support normal health, development and growth."

Similar issues in fulfilling all the above criteria for essentiality existed or still exist for the elements surrounding chromium in the PSE: vanadium, manganese, and molybdenum (Figure 1).

Chromium is present at a total amount of 1.8 mg in a 70 kg human, similar to Mn (12 mg) and Mo (9.3 mg), both of which are essential and were found to be so only rather recently [7]. Also, the blood serum concentrations of the three elements are rather similar (in μg/L): Mn (0.5–1), Mo (0.1–0.5), Cr (0.1–0.2) [12]. However, the liver concentrations are in the order Mn > Mo > Cr, demonstrating the much lower tissue concentrations of Cr. The total amount and liver and blood concentrations of Cr are similar to those of cobalt. Cobalt is an essential metal as part of the vitamin B_{12} cofactor, which is required in only two enzymatic reactions in humans. The total amount of vanadium present in the human body is only in the microgram range. It is essential for some organisms but apparently not for humans, though it has beneficial effects [9]. The concentrations of all of these elements are very low and it is difficult to establish deficiencies and their consequences in humans. They all have toxic effects at higher concentrations, but there is a much wider range of concentrations for Cr and Mo than for Mn before signs of toxicity appear. Even as recently as 1996, questions lingered as to whether or not manganese is nutritionally essential [12]. An article in a series

called “nutrition information” concludes that “Manganese deficiency is exceptionally rare and has not been reported in the literature under nonexperimental settings” and “toxicity from dietary exposure has not been reported”, perhaps with one exception [13]. However, manganese deficiency has been associated with numerous diseases and poor health outcomes [14]. Manganese is used in several enzymatic reactions, albeit the total number of proteins that employ it for function is unknown. Molybdenum in the same group of the PSE as chromium is essential. Only four human enzymes use it when bound to a pterin cofactor. Nutritional deficiency of molybdenum has never been observed in humans, again perhaps with one exception [15]. The work on chromium’s functions in biology should be seen in this context, namely that both essential and non-essential elements are present in human tissues and have different functions at different concentrations and in different chemical forms. Did biology then use chromium for a critical biological function?

2. CHROMIUM IN MAMMALIAN BIOLOGY

Continuing the historic perspective is necessary in order to understand the present state of the field of chromium biology and how the biological function of chromium was discovered through seminal observations by the founders of the field. The requirement for specific dietary “factors” was addressed by feeding rats diets that induce deficiencies, and then identifying diets that rescue the phenotypes, i.e., cure the impairments of function. In this way, many vitamins were discovered, e.g., vitamin E (originally called factor X), which plays a role in preventing liver necrosis [16]. With relevance to the discovery of chromium, a semi-purified Torula yeast-based diet impaired intravenous glucose tolerance in rats before they developed necrotic liver degeneration [17]. The diets resulting in liver necrosis led to the identification of factor 3, which turned out to be the essential element selenium [18]. But there was another factor, which affected the glucose tolerance, called a glucose tolerance factor (GTF), which differed from factor 3 [19]. In an attempt to identify this factor, it was noted that this soluble, low-molecular-weight substance was present in brewer’s yeast and pork kidney powder as diets based on such feeds cured the impairment [20]. The active preparation in this curative assay contained chromium, which in its trivalent form (but not in its hexavalent form) was active [21]. This finding led to a significant number of biochemical investigations on the role of chromium in carbohydrate and lipid metabolism, which were summarized ten years later in a major review [22]. The article concludes that

(i) chromium is detected in biological material and present in its trivalent state,

(ii) “low-chromium states are associated with impaired glucose tolerance in rats and monkeys” and severe deficiencies have additional adverse health effects,

(iii) a "diabetes-like syndrome has been observed in chromium-deficient rats raised in a controlled environment",

(iv) chromium increases the survival and growth rates of rodents and prolongs survival of old rats,

(v) a diet deficient in chromium leads to opacity and neovascularization of the cornea in 10–15 % of the cases investigated,

(vi) chromium stimulates the insulin response in a narrow range suggesting an interaction with the hormone and/or its receptor,

(vii) "physiological amounts of the element are transported to the tissues bound to siderophilin" (another name for transferrin),

(viii) radiochromium is excreted primarily via urine but may not represent the nutritionally important form of chromium,

(ix) the organic chromium complex from yeast, i.e., GTF, has quantitatively a much higher effect on glucose metabolism than other Cr(III) compounds and is transported through the rat placenta,

(x) regarding humans, low-chromium states exist and "chromium supplementation has been shown to improve or normalize the impaired glucose tolerance of some diabetics, old people, and malnourished children, but not of others",

(xi) "low-chromium states … do not constitute the sole cause for impairment of glucose tolerance."

The article ends with criteria that would need to be fulfilled if one were to consider chromium to be an essential element. The identification of chromium-deficient populations and the nature of the biologically active chromium complex were outlined as the most pressing research needs. They remain so today.

The next decade (1970–1980) saw major advances in analytical instrumentation with atomic absorption spectrophotometry becoming commercially available. It lowered the detection limit considerably and demonstrated that the chromium concentrations are lower than previously measured with less sensitive methods. For example, in the case of urine, the chromium concentration was found to be in the parts per trillion (ppt) range (ng/L). At the end of the decade, the accumulated knowledge required books to summarize the field [23–25]. A 1993 review [26] discussed the efficacy of chromium in some humans with impaired glucose tolerance and identified three challenges: identification of GTF, determining chromium status, and definition of the mode of action. In another summative perspective 30 years after the initial review [27], among the major challenges emphasized was the need to develop methods for determining chromium deficiency. It also was concluded that (i) earlier absolute measurements were too high but the conclusions drawn from relative comparisons in the earlier literature hold, (ii) that the difficulties associated with eliciting deficiency states in experimental animals may well be related to interaction with other dietary factors, similar to selenium where immediate effects occur only when vitamin E and sulfur amino acids are limiting, and (iii) at present the only way of looking at status is retrospective, i.e., curative

effects when chromium is given as a supplement. As with other elements, chromium in the circulation is not in equilibrium with tissue concentrations and the lack of robust biomarkers to determine status continues to be a perennial problem in trace element research, not only for chromium.

The view of nutritionists to consider chromium to be an essential nutrient was summarized again in a major review in 1999. The author concludes that chromium supplements have health benefits for some people with glucose intolerance but no benefit for the healthy in terms of body composition or body mass [28]. It is important to keep in mind that the inability to characterize the biologically active chromium compound and the finding of nicotinic acid in preparations of GTF led to the wide use of another compound with a similar ligand, namely picolinic acid, chromium picolinate [tris(picolinate)chromium(III)], in supplementation. A statement by the US Food and Drug Administration (FDA) followed concerning health claims of widely available chromium picolinate supplements [29]. It concluded "the relationship between chromium picolinate intake and insulin resistance is highly uncertain". A Cochrane review in 2013 concluded that there is no evidence that chromium picolinate affects body composition [30]. The beneficial effects of chromium supplements in some patients with some form of insulin resistance, however, continued to be observed until today.

The tone changed at the turn of the millennium with claims that (i) chromium(III) should not be considered to be essential, (ii) its biological activities may be a pharmacological rather than a nutritional effect, (iii) the potential for toxicity exists in chromium supplementation. Coincidentally, it was at this time that Walter Mertz, a pioneer in trace element research and in establishing the physiological roles of chromium, passed away [31]. Thus, at the same time when comprehensive reviews discussed chromium as an essential element, other reviews in the literature of the chemical and toxicological sciences claimed that chromium is not essential [32]. The last 20 years then became dominated by discussions of definitions and the relative significance of functions in different regions of the action spectrum of chromium. It did not solve the major issues of chromium metabolism or action and distracted from further exploring its beneficial biological effects.

3. REDOX AND COORDINATION CHEMISTRY OF CHROMIUM RELEVANT TO BIOLOGY

Most methods of metal analysis determine the total amount of a metal. In the case of chromium biology, speciation analysis to distinguish the valence states is critically important because chromium(III) is considered to be essential whereas chromium(VI) exposure clearly engenders toxicity (Figure 2).

Equally important is an analysis of the coordination environment as the type of ligands influence the biological chemistry. Chemical species are usually presented in Pourbaix diagrams as a function of both pH and redox potential. In the absence of ligands, at physiological pH and depending on the redox environment (from most reducing to most oxidizing), elemental chromium, Cr(0), chro-

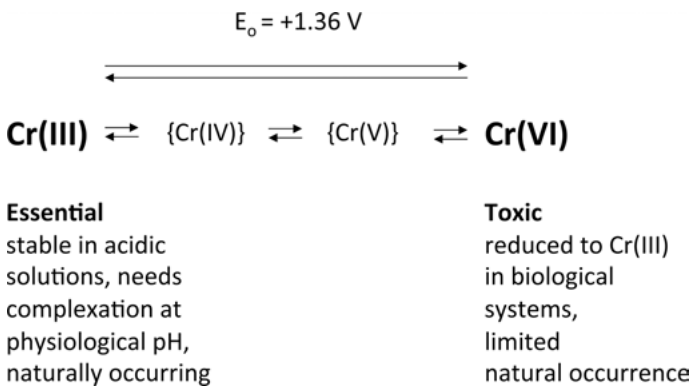

Figure 2. Trivalent chromium, Cr(III), is the major oxidation state of chromium in biology. There is discussion whether the generation of reactive species from Cr(IV) and Cr(V) intermediates during oxidoreduction contributes to the biological actions of chromium and the toxicity of hexavalent chromium, Cr(VI).

mium hydroxide, Cr(III), and dichromate ($Cr_2O_7^{2-}$), Cr(VI), are the three valence states of chromium [33]. Chromium hydroxide with the overall formula $Cr(OH)_3$ has a polymeric structure (pK_L $[Cr(OH)_3]_8$ = 30.2) and is rather insoluble. Cr(III) is the most stable species and the most important redox state in the reducing environment of the cell. The Cr^{3+} aqua complex $[Cr(H_2O)_6]^{3+}$ is stable only in acidic solutions and undergoes olation if the pH increases, forming $[Cr(OH)(H_2O)_5]^{2+}$, $[Cr(OH)_2(H_2O)_4]^{+}$, and polymeric species [34]. Thus, similar to Fe(III), Cr(III) is soluble only in a complexed form in the physiological pH range. Characteristic for the coordination chemistry of Cr(III) are the remarkably slow ligand exchange reactions compared to other transition metal ions, with the exception of Co(III). For instance, the water exchange rate of $[Cr(H_2O)_6]^{3+}$ is $k_1 = 5 \times 10^{-7}$ sec^{-1} [35], giving a half-life of 16 days for the exchange. On a purely speculative note, one could envisage that biology availed itself to this remarkable kinetic inertness of chromium(III) complexes for some function. Chromate (Cr(VI)) is sparingly available from natural sources; MnO_2 is the only oxidant thought to produce it in water [33]. Our exposure to chromate is therefore limited to primarily man-made sources of chromate, e.g., paints, wood preservatives (chromated arsenicals such as chromated copper arsenite), leather tanning products, or mordants in wool and other textile dying. Chromate is reduced to Cr(III) in the stomach and in tissues. Furthermore, many bacteria have chromate reductases, NAD(P)H-dependent flavoenzymes, that form Cr(III) from chromate, apparently without the reactive Cr(IV) and Cr(V) intermediates [36]. Therefore, the intestinal microbiome in different species needs to be considered in the metabolism and possible toxicity of chromate.

The chemical biology of chromium depends on how chromium is complexed [37]. Chromium complexes with different ligands have different biological responses and perhaps even target different biomolecules [38]. Without knowledge which specific properties biological ligands impart on chromium, discussions about its redox chemistry in tissues and cells remain rather hypothetical.

4. CHROMIUM METABOLISM

Only about 0.2 % of Cr(III) is absorbed in the intestine, i.e., > 99 % of supplemental chromium is excreted in feces. Intestinal uptake is by non-saturable passive transport [39]. In the blood, Cr(III) is bound to transferrin. Cells were thought to take up Cr(III) by transferrin receptor-mediated endocytosis. However, recent observations that Cr(III) bound to transferrin is not internalized via the transferrin receptor suggest a role of transferrin in keeping chromium out of the cell [40]. In contrast, owing to the similarities with anions such as sulfate, cells take up chromate much more readily through anion transporters. However, because of the reduction of Cr(VI) to Cr(III) already in the stomach, it is unknown how much chromate is available to cells for uptake. In the cell, chromium(III) binds to high and low molecular weight ligands. The latter will be discussed in the next section.

There is evidence for some type of regulation that could indicate homeostatic control of chromium: chromium levels decline with age, a surplus is excreted in urine, chromium is excreted under conditions of stress and increased sugar intake, and the absorption efficiency increases with decreasing intakes. Key steps in metabolism are summarized in Figure 3. One is prompted to conclude that

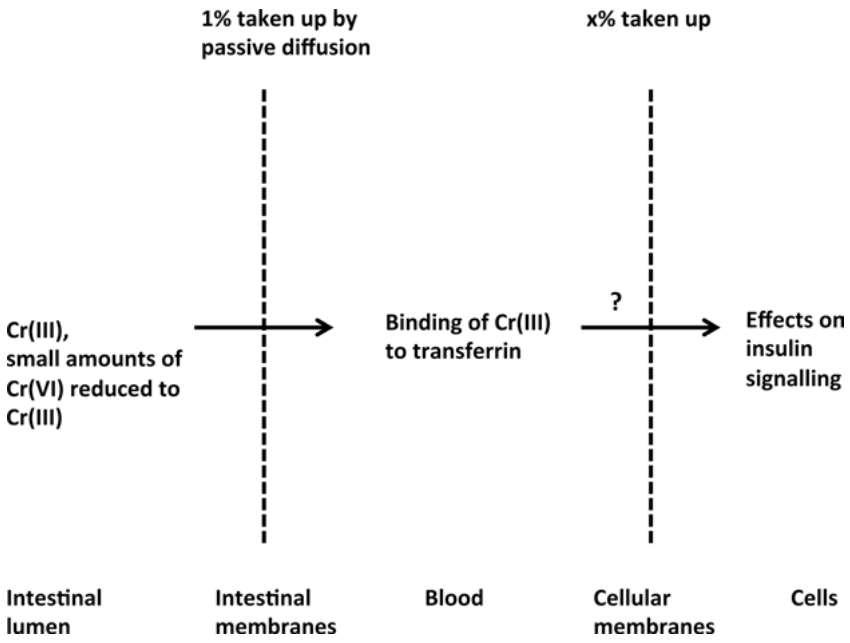

Figure 3. Chromium metabolism. The majority of texts focuses on Cr(III) because of the capacity for non-enzymatic and enzymatic reduction of Cr(VI), which is a toxicant predominantly introduced by human activities. As discussed in the main text here, it is doubtful whether cells take up transferrin-bound Cr(III) [40]. Some investigators maintain that there is no cellular uptake of Cr(III) [41], which would suggest that chromate is the source of any cellular Cr(III). How chromium is exported from cells is unknown.

despite 60 years of research our knowledge about chromium metabolism is rather rudimentary.

5. LOW-MOLECULAR-WEIGHT BIOLOGICAL CHROMIUM COMPLEXES

Active preparations containing Cr(III) were isolated from several sources, but complete structures of the respective chromium complexes are not available. Knowing the biological ligands of chromium is crucial. As with molybdenum, cobalt or iron biological control may be provided by a chelating ligand.

5.1. Glucose Tolerance Factor from Brewer's Yeast

Isolation of the chromium compound with biological activity from Brewer's yeast resulted in a preparation that contained glutamate (Glu), glycine (Gly), and cysteine (Cys). Based on very limited spectroscopic evidence, it was thought that it contains nicotinic acid, too. A synthetic chromium-nicotinic acid complex showed very similar chemical and biological properties [42]. The putative identification of nicotinic acid critically influenced the ensuing investigations as it led to the use of chromium(III) picolinate instead. Conditions that hydrolyze peptide bonds were applied in the isolation of GTF, thus the structure of a possible peptide could not be determined though it was noted that the amino acids found are the ones of the tripeptide glutathione [42]. GTF activity is not restricted to yeast, though, as pig kidney powder was also curative in the original assay.

5.2. Chromodulin: A Low-Molecular-Weight Chromium-Binding Substance from Liver

A low-molecular-weight chromium-binding substance (LMWCr) was found in mouse liver and biological fluids such as human urine and bovine colostrum and it was shown that chromium is necessary for its biological activity [43, 44]. Fractionation of the material from livers of chromate-injected mice or rabbits resolved two fractions with molecular masses of 1500 and 2300 Da. The former has a composition similar to the one reported for GTF with a substance absorbing at 260 nm. Several attempts were made to characterize this material further. Another characterization demonstrated that the complex contains the amino acids Glu, Gly, Asp, and Cys as major constituents, i.e., with the exception of Asp, the ones found originally in GTF [45]. The material isolated from bovine liver was compared with a reaction mixture of chromate, glutathione, aspartate, and glutamate and found to be a mixture of Cr(IV) or Cr(V) species, including a dimeric Cr(V) glutathione complex [46]. Based on a comparison with the protein calmodulin, which regulates proteins in a Ca^{2+}-dependent way, the oligopeptide was called chromodulin [47]. It binds four chromium ions with an overall K of 10^{21} M^{-4}. A

mechanism was proposed, in which insulin leads to chromium uptake into cells, chromium binds to apochromodulin, and holochromodulin binds to the insulin receptor with an affinity of 250 pM to activate it. The structure remains unknown. A heptapeptide with the composition EEEEGDD (but given as EEEEDGG in the abstract) from bovine liver was then further characterized [48]. Neither the exact structure nor the origin of the peptide is known. If indeed a peptide with no spectroscopic signature of the ligand is needed to form a cofactor for the biological activities of Cr(III), additional work will be challenging experimentally.

Despite similarities in composition, GTF is a yeast factor and chromodulin is a factor in other tissues or body fluids. The yeast factor has not been further characterized and it remains unknown whether it is the same complex if conditions are used without hydrolyzing peptide bonds. The problem with not knowing whether the heptapeptide is critically involved in chromium's function is that all the experiments are performed with non-biological chromium complexes with different properties. Both chromodulin and GTF contain biological ligands. Both have been dubbed artifacts, first chromodulin and then GTF owing to the methods of isolation [10, 49].

6. CHROMIUM TOXICITY

The toxicity of chromium in the hexavalent state adds another level of complexity and uncertainty to the discussion.

The carcinogenicity of Cr(VI) for the lung in occupationally exposed workers has been known for over 80 years [50]. Chromates are group I carcinogens for humans with sufficient evidence for lung cancer [51]. The toxicity of chromate received widespread attention with the year 2000 film 'Erin Brockovich', which is based on groundwater contamination on a large scale through chromate-containing discharges in Hinkley, CA, where chromate was used as a rust suppressor in pipelines operated by the Pacific Gas and Electric Company. The presence of chromate in drinking water and in food, largely from anthropogenic sources, raises issues about its genotoxicity. A 2-year long exposure of rats and mice under the National Toxicology Program showed that oral exposure to chromate is carcinogenic while that of chromium(III) picolinate is not [52, 53]. Because of the toxicity of chromate, the World Health Organization (WHO) suggests a maximum allowable concentration in drinking water of 50 μg/L [54]. The US Environmental Protection Agency sets a value of 100 μg/L for total chromium [55]. In contrast, Cr(III) is considered much less toxic – by about a factor of 1000. The EFSA CONTAM (contaminants in the food chain) panel "derived a Tolerable Daily Intake of 300 μg Cr(III)/kg body weight per day" [56], which amounts to 21 mg for a 70 kg adult.

Chromate is unreactive with DNA and needs reductive activation with either ascorbate or thiol-based reductants to form binary complexes of Cr(III) with DNA or ternary complexes with DNA and glutathione/cysteine [34, 57]. The prevailing opinion is that chromium is present in its trivalent state in tissues and any small amounts of chromate in the diet will already be reduced to Cr(III) in

the stomach. It has been suggested, however, that some chromate escapes reduction and may be responsible for carcinogenesis in the digestive tract and for epigenetic changes [41, 58]. Recent work has refuted the view that ingested chromium causes gastrointestinal tumors [59]. We are exposed to some chromate in our diet. For instance, bread toasting results in some oxidation of Cr(III) to Cr(VI) [60]. Also, chromium leached from stainless steel cookware becomes part of our food [61]. Whether any chromate reaches and enters cells depends on food composition, in particular the amount of reducing capacity in relation to the total amount of chromate in our diet. Conditions of oxidative stress may be conducive to forming some chromate.

As the genotoxic species have been identified as Cr(III) adducts of DNA [34], the issue of the potential carcinogenicity of Cr(III) supplements lingers. In 1995, work from the laboratory of the late Karen E. Wetterhahn, who worked on the molecular mechanisms of Cr(VI) toxicity, showed that chromium picolinate is genotoxic for cultured Chinese hamster ovary (CHO) cells while chromium nicotinate and chloride do not share this property [62]. The finding emphasizes the role of the picolinate ligand. In 2013, it was summarized that chromium picolinate at oral doses considered safe is not mutagenic for humans or carcinogenic for rodents over a period of two years (see above), and that conclusions from work with cells are not translatable to the situation in the whole organism [39]. After all, toxicity is a concept developed to examine an effect on an entire organism. Investigations of cellular toxicity contribute to elucidating mechanisms how toxicity may be expressed.

The paradox that Cr(VI) is much more toxic than Cr(III) and yet Cr(III) adducts of DNA are thought to be the genotoxic species may be resolved by considering the following. The transport of chromate, if available, into cells is much higher than that of Cr(III) and the cellular handling of chromate in a reductive pathway may bring it in a chemical state that is different from that of endogenous biologically active Cr(III). Some go as far as stating that there is no cellular uptake of Cr(III) [58]. Whether or not chromium enters cells as Cr(III) and whether it is protected in the cell by a specific ligand chemistry that avoids possible genotoxicity appears to be a crucial research question to be answered.

In addition to DNA adduct formation, the genotoxicity of chromium has been linked to its cellular redox chemistry, namely the reactive Cr(IV) and Cr(V) species transiently formed from reduction of Cr(VI) and/or from spurious oxidation of Cr(III) through some redox signalling. In isolated adipocytes, Cr(VI) can be formed from the same Cr(III) compounds used as dietary supplements [63]. This observation led to the suggestion that the reactive species formed are responsible for the oxidation of the thiol of the catalytic cysteine of protein tyrosine phosphatases and consequently, that the antidiabetogenic effects of chromium are expressed indirectly through the role of the reactive species generated. Likewise one can argue that the effects are due to chromate releasing zinc from metallothionein [64] and the released zinc ions inhibiting protein tyrosine phosphatases [65]. Whether any of this chemistry indeed occurs in living organisms will depend on whether or not cells are ever exposed to sufficiently high chromium concentrations, a specific ligand exists to handle cellular Cr(III) and protects the cell from the toxic effects of possible redox cycling or DNA/adduct formation, and how

Cr(III) species reach the nucleus to become mutagenic or clastogenic. Such investigations will need exquisite methodology to distinguish Cr(VI) and Cr(III) species in living tissue and addressing the specific solution chemistry of Cr(III) with formation of polymeric species and kinetically rather inert complexes.

Very little is known about mechanisms of toxicity other than direct genotoxicity, in particular the interaction of chromium with specific cellular proteins. Chromate inhibits thiol-dependent redox enzymes such as thioredoxin reductase, thioredoxin, and peroxiredoxin [66].

The viewpoint that chromium is basically a non-essential element that happens to have some medicinal effect but otherwise causes toxicity similar to the biological inorganic chemistry of arsenic is at the other extreme of interpretations concerning the role of chromium in biology [32].

7. CHROMIUM IN DIABETES AND METABOLIC SYNDROME

The basis for discovering a role of chromium in animals was its effects on glucose tolerance and the metabolic consequences of compromised insulin function. In 1977, it was noted that infusing a human patient on TPN (total parenteral nutrition) with TPN solutions containing 250 μg chromium(III) chloride for two weeks abolished the requirement for insulin and lowered blood glucose and free fatty acid levels [67]. The patient was followed for 18 months and remained healthy when maintained on 20 μg/d chromium(III). The efficacy of chromium in diabetic patients, in the elderly and in malnourished children was summarized in 1993 and the relationship between chromium and syndrome X, now generally called metabolic syndrome and associated with cardiovascular disease and diabetes type 2, was pointed out [26]. Five years later, another major review summarized that out of 23 supplementation studies of individuals with impaired glucose tolerance, all but 5 demonstrated improved glucose tolerance and positive effects on lipids (cholesterol and triglycerides) when 200 μg chromium(III) per day were given. The treatments increased insulin efficacy or improved the blood lipid profile [68]. Diabetic patients needed at least 400 μg per day, however. They lose chromium in urine but compensate for the loss by taking up more from dietary sources. The beneficial response seemed to depend on the degree of glucose intolerance. The response times varied from 10 days to 3 months. It is not clear whether any of the patients treated were chromium-deficient as estimated daily intakes are 25 μg for females and 33 μg for males and are usually met by chromium in the diet [69]. The amounts given to diabetic patients are above the ESADDI (estimated safe and adequate daily dietary intake), which is given for children as >7 and for adults as 50–200 μg per day [70]. Reports on chromium supplementation of diabetic patients with positive outcomes continued to appear, albeit some non-responders were usually observed. A series of investigations culminated into the "most comprehensive, double-blinded, placebo controlled chromium supplementation study" [71]. Improvement of insulin sensitivity was noted when 1 mg chromium picolinate per day was given to diabetic patients for 24 weeks. Some of them did not respond.

A systematic review and meta-analysis reported positive effects of chromium supplementation on glycemic control in patients with diabetes [72]. An analysis of 20 randomized controlled trials noted the limited efficacy of chromium supplements for glycemic control in type 2 diabetes patients [73]. Thus, to fully exploit the potential of chromium supplementation for treatment in the clinics, it is necessary to address the phenotype of non-responders. One approach suggested is to examine how chromium's glucoregulatory effects are associated with neurobiological behavior, i.e., to consider the roles of the neurotransmitters dopamine in binge eating and serotonin in depression [74].

With regard to the effects of chromium on pre-diabetic states and insulin-sparing, cholesterol- and triglyceride-lowering effects, a recent prospective study showed that higher chromium levels in young urban US American adults were associated with a lower incidence of metabolic syndrome later in life [75]. Another potentially significant observation is that heart rate was significantly reduced upon supplementation with chromium-enriched yeast in a prospective, randomized, double-blind and placebo controlled study of patients with metabolic syndrome and impaired glucose tolerance [76].

The development of severe symptoms of diabetes in patients on TPN and the resolution of these symptoms without further need for insulin when chromium was supplemented are cited as a key reason for considering chromium to be an essential nutrient [77]. For short treatment (1–3 months) 10–20 μg per day were required; for longer treatment, the variable, contaminating chromium present in the TPN solutions sufficed. It was suggested that chromium concentrations in the fluids should be monitored as variable degrees of ambient (contaminating) chromium is present. Concerns were expressed that the amount of chromium in TPN fluids causes kidney damage when it was noted that the glomerular filtration rate decreases with the increasing serum (up to 40-fold higher) and tissue (10 to 100-fold higher) chromium concentrations during TPN [78]. In a study of adult patients on parenteral nutrition at home, it was noted that the serum chromium concentrations were up to 23-fold increased and that there is considerable variation in chromium content of the solutions depending on manufacturer and lot number [79]. Accordingly, it was suggested that serum chromium should be monitored and that chromium (and manganese) in the solutions needs to be restricted to avoid accumulation. The American Society of Parenteral and Enteral Nutrition reviewed the multi-trace element products available in the clinics in 2009 and concluded that the amount of Mn and Cr are considered too high in neonatal and pediatric care and need to be lowered [80]. Pre-existing kidney conditions should be evaluated before any chromium supplementation is considered. Moreover, lead and cadmium exposure should be considered as they have been identified as cumulative risk factors for the nephrotoxicity of chromium [81].

7.1. Mechanism of Action

The original investigations indicated an interaction between chromium and insulin/the insulin receptor. Later, molecular and cellular investigations focused on an intracellular action, notwithstanding the largely unresolved issue of how

Cr(III) enters cells. Chromium enhances the insulin receptor tyrosine kinase activity with an estimated binding constant of 250 pM for LMWCr [82]. However, several Cr(III) compounds do not directly activate recombinant insulin receptor kinase or inhibit protein tyrosine phosphatase, yet enhance the tyrosine phosphorylation of the insulin receptor in cells [83]. With regard to chromium's action on insulin receptor activity/phosphorylation, it is worthwhile considering that many other essential and non-essential elements modulate insulin signalling, notably vanadium compounds that have significant insulinomimetic properties as inhibitors of protein tyrosine phosphatases. Many metal ions in their cationic form and as oxoanions inhibit protein tyrosine phosphatase 1B, which controls the insulin and leptin receptor [84]. A most significant activator of insulin signalling is the essential element zinc. Zinc ions inhibit protein tyrosine phosphatases at picomolar to nanomolar concentrations [65, 85]. Therefore, any investigations of the effect of chromium complexes on phosphatases should determine the concentrations of zinc and consider the possible effects of intracellularly released zinc ions [86]. Stringent control of possible contamination by other metals is mandatory. For example, vanadate has been identified in preparations of ATP owing to its inhibition of (Na,K)-ATPase with an IC_{50} value of 40 nM [87].

Additional cellular investigations with Cr(III) compounds showed an effect on cellular cholesterol and glucose transporter GLUT-4 translocation [88]. More recently, investigators focused on the effect of chromium on the activity of protein kinase B (AKT) and AMP-activated protein kinase (AMPK) [38, 89]. Many of these downstream effects, of course, could be due to binding of chromium to a single site upstream in the pathway. In conclusion, the specific site(s) of chromium's action remain(s) elusive. Further progress will depend on identifying the cellular chromium complex involved in the interactions and determining whether such a complex is an essential cofactor mediating chromium's effect.

A well balanced diet is rich in substances that have beneficial effects and have been referred to as nutraceuticals. One could ask: If chromium complexes are metallodrugs, why isn't more attention given to this subject matter for the purpose of treating metabolic disease and diabetes? It seems a pressing issue with regard to the dire need for additional therapeutic approaches for treating the epidemic of diabetes worldwide. Chromium(III) compounds have a significant advantage over vanadium compounds, which have been used as insulinomimetics in clinical trials, because the former have a much larger therapeutic index. Given chromium's effectiveness in some conditions of insulin resistance and metabolic syndrome, it is remarkable that neither pharmaceutical companies nor diabetes organizations have paid more attention.

7.2. Other Clinical Observations

The discussion provides the background to address other issues in medicine and in the clinics, namely whether chromium, in addition to the cobalt released from MoM (metal-on-metal) hip replacements (arthroplasties) and other surgical or-

thopedic steel implants, and fixed orthodontic appliances, contributes to the metallosis associated with medical procedures.

Next to nickel and cobalt, chromium is the third major skin irritant. 1 to 3 % of adults in Europe are allergic to chromium present in a large number of items and may develop contact dermatitis [90, 91].

8. POSSIBLE RESOLUTION OF CONTROVERSIES IN CHROMIUM BIOLOGY

A rather substantial literature during the last two decades attempts to debunk the notion that chromium is an essential element [10, 32, 92, 93]. A key document that is cited is a scientific opinion from the expert panel NDA (nutrition, novel foods and food allergens) of EFSA, which concluded in 2014 [94]: "Evaluating the possibility of chromium(III) as an essential element for humans, the evidence from reported improvements associated with chromium supplementation in patients on total parenteral nutrition was considered to be the most convincing, but overall the data do not provide sufficient information on the reversibility of the possible deficiencies and the nature of any dose-response curve in order to identify a dietary requirement for humans." The panel considered the following criteria for "essentiality": (1) absence from the diet causes reproducible and consistent functional and structural abnormalities; (2) reintroduction or addition to intake reverses or prevents these abnormalities; (3) the abnormalities associated with deficiencies are accompanied by specific biochemical physiological changes; (4) these biochemical and physiological changes are prevented or reversed by preventing or curing the deficiency. Implicit in these criteria is the need for organisms to have systems to ensure acquisition, systemic regulation and utilization of the trace element, as well as a means to prevent its excessive acquisition [95]. The panel concluded that there is not enough evidence for considering chromium to be an essential element for humans − notwithstanding their definitions of "essential." Another EFSA panel FEEDAP (additives and products and substances in animal feed) concluded in 2009 [96] "that there is no evidence for essentiality of Cr(III) as a trace element in animal nutrition." In contrast, the dietary supplement fact sheet "chromium" of the Office of Dietary Supplements (National Institutes of Health), last updated in March 2018, states "Chromium is a mineral that humans require in trace amounts, …" [97]. The scientific basis for establishing the essentiality of trace elements was discussed in detail with specific reference to chromium when an expert consultation of WHO/FAO (Food and Agricultural Organization)/IAEA (International Atomic Energy Agency) formulated yet another definition in 1996 [12]: "An element is considered essential to an organism when a reduction of its exposure below a certain limit results consistently in a reduction in a physiologically important function, or when the element is an integral part of an organic structure performing a vital function in the organism." The discussion of this definition is particularly instructive for the discussion in this article in that it draws attention to the total dose-response of trace elements and the conclusion

that the old classification in either one category, toxic or essential, has impeded progress [98, 99].

The present situation is that beneficial effects of Cr(III) are established, but some believe that they reflect pharmacological rather than nutritional responses while yet others suggest that they are side effects of a toxic metal. The rather unbalanced discussion is unproductive as it hinders progress, is marred by elements of negativity and occasionally becomes acrimonious, makes extrapolations from isolated systems to whole organisms, and contains uncertainties of definitions that distract from the most important issue, namely to define under which conditions the biological activity of Cr(III) can be employed to improve human health. It is inappropriate to criticize early research in the field of chromium biology on the basis of what present methodology can or could achieve. Many recent experiments are inconclusive because the major goals of describing a chromium-deficient state in humans, characterizing the cellular active chromium species, and obtaining consistent dose-dependent responses with chromium supplementation in patients with metabolic disease have not been reached. Without understanding important aspects of chromium metabolism, and in lieu of knowing the cellular chromium complex, experiments are being performed with compounds that are active at different concentrations or have a different spectrum of actions, e.g., chromium(III) picolinate.

It is unfortunate that the discussion is waged with using terms that are not sufficiently clear to scientific communities and the public, and with one major flaw best described by an adage that underlies discovery research in general: *Absence of evidence is not evidence of absence.* The postulate that specific chemical structures and modes of action must be known before an element classifies as essential is a laudable aim but not a *conditio sine qua non*. The issue of essentiality is linked to inducing a deficiency of chromium or defining populations that are chromium-deficient, which, as discussed, has been difficult or impossible to demonstrate for humans, not only for chromium but also for other trace elements. Are the beneficial effects of chromium then nutritional or pharmacological? A *nutrient* is a substance that allows an organism to survive, grow, and reproduce. The doses used for chromium may appear pharmacological but this in part is a matter of the low bioavailability of chromium. Broadly defined, a *drug* is a man-made, natural, or endogenous substance. In order to classify chromium as a drug, one would need to know the function of chromium already present in our body and whether or not it is a function that differs from that of any added chromium.

The biological activity of essential compounds is often not optimal. Thus, augmenting the normal physiological function by supplementation would be a nutritional and not a pharmacological effect. One should bear in mind that a typical action spectrum of an essential substance is described by three phases: nutritional, pharmacologic, and toxic. Hence, the actions of chromium are likely "all of the above", and not "either/or" as presently suggested by some scientists. Already Paracelsus noted 500 years ago that the difference between a compound being a drug or a toxin is a matter of dose. The literature is replete with reviews of past or shifted paradigms, opinions of experts, recommendations reached by

committee consensus, and meta-analyses of scientific reports. What we need are solid new data from experiments based on established facts. With chemical elements, we are in a peculiar and paradoxical situation: We have the incredible capacity to sequence the entire human genome but we do not even know with certainty how many chemical elements are required for optimal health.

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ABBREVIATIONS

AMP	adenosine 5′-monophosphate
ATP	adenosine 5′-triphosphate
EFSA	European Food Safety Authority
FDA	US Food and Drug Administration
GTF	glucose tolerance factor
LMWCr	low-molecular weight chromium-binding substance
NAD(P)H	nicotinamide adenine dinucleotide (phosphate), reduced
PSE	periodic system of the elements
TPN	total parenteral nutrition
WHO	World Health Organization

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Manganese: Its Role in Disease and Health

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Abstract: Manganese is an essential dietary element that functions primarily as a coenzyme in several biological processes. These processes include, but are not limited to, macronutrient metabolism, bone formation, free radical defense systems, and in the brain, ammonia clearance and neurotransmitter synthesis. It is a critical component in dozens of proteins and enzymes, and is found in all tissues. Concentrated levels of Mn are found in tissues rich in mitochondria and melanin, with both, liver, and pancreas having the highest concentrations under normal conditions. However, overexposure to environmental Mn via industrial occupation or contaminated drinking water can lead to toxic brain Mn accumulation that has been associated with neurological impairment. The objective of this chapter is to address the biological importance of Mn (essentiality), routes of exposure, factors dictating Mn status, a brief discussion of Mn neurotoxicity, and proposed methods for neurotoxicity remediation.

Keywords: essentiality · manganese · neurotoxicity · sources · therapy

1. INTRODUCTION

Manganese is the twelfth most abundant element on the planet, comprising a significant amount of the earth's crust [1], with soil concentrations ranging from 40 to 900 mg/kg [2]. Necessary for a variety of industries, Mn is present in numerous products, including steel, batteries, animal feed, pigments, and ceramics [1]. It is also a trace element essential for the normal biology of humans and mammals [3–6]. As a constituent of phosphoenolpyruvate carboxykinase, Mn aids in the conversion of oxaloacetate to phosphoenolpyruvate during gluconeogenesis. Mn superoxide dismutase is a valuable enzyme in combating reactive oxygen species (ROS) in the mitochondria. Manganese is key in maintaining ammonia levels in the body via the activity of arginase (reviewed in [3]). Glutamine synthetase, a Mn-containing enzyme responsible for the conversion of glutamate to glutamine, is thought to account for 80 % of Mn in the brain and is critical for handling ammonia in the brain.

Although Mn has a diverse role in the human body, dietary requirements for this micronutrient have been modestly established at 1.8 and 2.3 mg per day for healthy adult women and men, respectively [7]. This recommendation accounts for relatively low absorption rates of Mn (≤ 5 %), but still fulfills the biological requirements for this trace metal ion. Because Mn is a ubiquitous metal found in a variety of foods (fruit, nuts, legumes, whole grains, and brewed tea) dietary Mn deficiency is not of public concern; however, because it is abundant in the earth's crust and is widely used for industrial purposes, excessive exposure to Mn does happen and can lead to dire neurological consequences (reviewed in [3]). Overexposure to Mn most commonly happens via inhalation to excessive amounts of airborne Mn particulate, but has also been reported to occur after consumption of Mn-polluted drinking water [8]. Symptoms from these exposure routes manifest in a slightly different manner, *vide infra*, but the end result is Mn accumulation in the brain (most notably the basal ganglia) interfering with neurotransmitter systems that result in cognitive and movement disorders similar to Parkinson's disease. Due to the occurrence and clinical relevance of Mn neurotoxicity, exposure thresholds have been es-

tablished by the Environmental Protection Agency (EPA) at 0.05 mg/L in drinking water and 0.2 mg/m^3 for chronic air exposure [8]. Even with these guidelines in place, Mn neurotoxicity still occurs predominantly in factory or mining industries where Mn is procured for welding, smelting, or battery manufacturing. Workplace guidelines for airborne Mn limits have been established for these settings; however, chronic exposure may still result in toxic accumulation. Similarly, populations that rely on well water from ground water sources with propensity for Mn contamination have reported learning impairment in children consuming unfiltered well water [9].

2. MANGANESE ESSENTIALITY

Manganese is an essential element that is required for many biological processes including bone health, macronutrient metabolism, and defense against ROS (reviewed in [3]). The beneficial effects of Mn are due to the incorporation of the metal into metalloproteins. A few well known Mn metalloproteins are arginase (rate-limiting enzyme in urea synthesis), acetyl-CoA carboxylase (critical catalyst for endogenous fatty acid synthesis), phosphoenolpyruvate decarboxylase, and pyruvate carboxylase (gluconeogenesis), Mn superoxide dismutase (mitochondrial antioxidant), glutamine synthetase (critical for brain ammonia metabolism), and glycosyltransferases (bone health). Tissue contents in mammals are in the range of 0.3–2.9 μg Mn/g wet tissue weight [10], making Mn one of the most common metals in tissues.

2.1. Requirements

The Institute of Medicine's Dietary Reference Intakes (DRI) for Mn cites approximately 2 mg/day as adequate intake for adults, and 1.2–1.5 mg/day for children [7]. The adequate intakes were established based on bioavailability and typical dietary sources for each particular age-range and gender. In general, females will absorb more dietary Mn than males due to iron status differences, specifically, iron and Mn are inversely related due to a shared transporter in the gut, so if iron status is low, Mn absorption is greater (see Section 2.2). Therefore, females have lower adequate intakes of 1.8 mg/day (female 19 years or older) *versus* 2.3 mg/day (male 19 years or older) than males; infants will range from 0.003 mg/day (0–6 months) to 0.6 mg/day (6–12 months) and 1.2 mg/day to 1.6 mg/day for boys and girls ages 1–8 years old based on typical food sources (the very low adequate intake for infants 0–6 months is based on human milk having trace amounts of Mn). The DRI has also established Tolerable Upper Limits for Mn as 2 mg/day for infants; 6 mg/day for older children, and 9–11 mg/day for adults (females and males, respectively). Absorption of Mn is tightly regulated in the gut and therefore toxicity from dietary exposure has not been reported.

2.2. Dietary Sources

Manganese is commonly found in the food supply but is particularly abundant in whole grains (oat bran and wheat), fruits (pineapple), vegetables (spinach), and red meats. Plant sources have much higher Mn concentrations than animal sources. For a thorough list of food sources and their Mn concentrations, see the review by Freeland-Graves et al. [11]. The daily average intake of Mn has been estimated at 2–6 mg for adults with vegetarians consuming slightly greater amounts, estimated around 11 mg/day [11]. These quantities are adequate for ideal health and fall slightly above the National Academy of Sciences recommended intakes. Fortunately, because absorptive mechanisms for Mn in the gut are tightly regulated, excess dietary Mn rarely results in toxicity. Dietary supplements and vitamins are another source of Mn, some of which contain up to 20 mg of the element. Manganese is taken as a supplement for a variety of conditions, including osteoarthritis and osteoporosis [10].

Manganese does not undergo metabolism in the body, being both absorbed and excreted in the same form. On average, only about 1 to 5 % of dietary Mn is absorbed in the gut [12]. This is in contrast to the absorption of calcium, which ranges from about 25 to 35 % of dietary intake [13, 14], illustrating that Mn is indeed a trace metal. Absorption of dietary Mn is influenced by several different factors including intestinal pH, the presence of the divalent metal transporter (DMT1), other divalent metals competing for absorption (e.g., iron, copper, zinc, or calcium), and chelating agents such as phytic acid [15, 16]. DMT1 is the primary metal transporter (iron and Mn) in the intestinal tract [17], but it is also a key transporter allowing Mn to cross the blood-brain barrier (BBB) [18]. In the gut, DMT1 expression is influenced primarily by systemic iron status [19] with duodenal biopsy data showing increased protein levels of DMT1 correlating with decreased serum ferritin [20]. In studies evaluating Mn concentrations in males and females following ingestion of a test dose of ^{54}Mn, serum ferritin levels were associated with prolonged Mn retention in males, but females with lower ferritin levels had increased Mn absorption [21]. There is an inverse relationship between iron and Mn with regard to absorption and tissue distribution where, generally, iron deficiency (ID) increases Mn absorption [20], and in cases of Mn overexposure, ID accelerates its tissue accumulation [22, 23].

Recently, the zinc transporters ZIP-8 and ZIP-14 (SLC30A10), the cation transporting ATPase ATP13A2, and the calcium ATPases SPCA1 and SPCA2 have been shown to play important roles for Mn transport into and within the brain. Defects in SLC30A10 have been linked to Parkinson's disease and are likely involved in familial manganism [24].

2.3. Environmental Sources

Occupational exposure to Mn is the most common cause of Mn neurotoxicity. Several cases of Mn neurotoxicity have been linked to its exposure within mining, manufacturing, and welding industries [25, 26]. Inhalation of dense Mn par-

ticulate, up to 100-fold higher than established safe limits, has been reported for workers who display neurological symptoms [26]. While acute exposure manifests neurotoxic symptoms fairly quickly (within months of exposure), chronic exposure to low dose airborne Mn in the form of methylcylopentadienyl Mn tricarbonyl or Mn-contaminated drinking water may present future health concerns. This is particularly true in vulnerable infants and iron-deficient populations where chronic low-grade exposure to Mn has been associated with cognitive impairments [9, 27]. In Bangladesh, children drinking from wells with a high Mn content (793 μg/L, compared to the EPA established safe limit of 50 μg/L) had significantly reduced verbal scores and overall intellectual performance compared to children consuming water Mn concentrations under the EPA's established safe limit [9]. The concentration of Mn in drinking water varies by location, ranging between 1 and 100 μg/L (but can exceed 200 μg/L in well water). A recent study found that more than 1 million people who rely on well water living in parts of Virginia, North Carolina, South Carolina, and Georgia reside in an area where soil Mn concentrations are exceptionally high and most of the wells tested have Mn levels that are considered unhealthy [28].

3. FACTORS INVOLVED IN MANGANESE STATUS

3.1. Manganese Deficiency

Because of its widespread presence in human diets, frank Mn deficiency is generally not clinically recognized in humans. Manganese deficiency has been observed in laboratory animals and has been associated with impaired growth, skeletal defects, reduced reproductive function, birth defects, and abnormal glucose tolerance, as well as altered lipid and carbohydrate metabolism [5, 29]. Reduced Mn status may also be observed in individuals with osteoporosis and epilepsy [3, 10]. People with exocrine pancreatic insufficiency, chronic hemodialysis, Perthes' disease, and phenylketonuria may also possess inadequate Mn levels [3]. The pathology of these diseases may be impairing normal Mn metabolism leading to inadequate levels, but since these are isolated cases and not all people suffering from these diseases have inadequate Mn levels, Mn deficiency is not considered a health concern.

3.2. Liver Disease

Individuals at risk for Mn toxicity include those suffering from liver disease [30–32], which may affect the normal process of Mn excretion via the biliary system [12, 33]. In healthy individuals the liver is able to accumulate higher levels of Mn due to biliary excretion. Individuals suffering from cholestatic liver disease and cirrhosis may display hypermanganesemia and T1-weighted MRI hyperintensity in the globus pallidus, hallmarks of Mn toxicity [34–36]. During chronic

Mn exposure, liver failure (in particular in the presence of hyperammonemia) can affect the substrate supply for the synthesis of monoamine neurotransmitters, such as dopamine and norepinephrine [37].

3.3. Milk and Infant Formulas

Human milk is generally low in Mn (1.8–27.5 μg/L); however, concentrations in infant formulas can vary dramatically (33–300 μg/L) [38–41]. In human (and animal) milk, Mn concentrations vary with the stage of lactation [3, 39, 41–43]. For example, Stastny and coworkers reported that mean ($\pm$ SD) human milk Mn concentrations in the fourth week of lactation were 6.6 $\pm$ 4.7 μg/L and these levels were higher than those collected during the 12th week of lactation (3.5 $\pm$ 1.4 μg/L) [39]. Notably, in human milk, Mn is in the trivalent oxidation state where it is bound to lactoferrin, the major iron-binding protein in milk. Receptors for this protein are abundant in the brush border membranes of epithelial cells throughout the length of the small intestine, thus allowing for regulation of the uptake of Mn across the gastrointestinal tract. Since in formula, Mn is in the divalent oxidation state, absorption through the gastrointestinal (GI) tract cannot be regulated by lactoferrin receptors. Accordingly, transport of infant formula-derived Mn is likely governed by mechanisms different from those from Mn in human breast milk. It should be noted that while most infant formulas do not contain lactoferrin, in the past few years, formula companies have produced some formulas that add bioactive components including lactoferrin based on emerging research [44]. Thus, in these new, lactoferrin-containing formulas, Mn delivery may be more similar to human milk.

In general, infant formulas contain much higher Mn concentrations compared to those observed in human milk [3]. Given the risk posed by high Mn concentrations in infant formulas [45] their composition has significantly changed over the last 20 years. For example, Enfamil™ (Mead Johnson and Company) contained 1289 μg Mn/L in 1983; levels dropped to 105 μg/L shortly thereafter [39]. It has been suggested that consumption of soy-based infant formulas is a potential area of concern for human infants. For comparison of Mn concentrations in different infant formulas see [3].

3.4. Parenteral Exposure

Risk for Mn neurotoxicity is associated with total parenteral nutrition (TPN), as these nutritional solutions are commonly formulated to include Mn along with other essential trace metals and they can contain Mn as a contaminant [46, 47]. A report by Wilson et al. found that Mn concentrations in TPN solutions ranged from 5.6 to 8.9 μg/L, in the absence of any supplementation [48].

Few studies have addressed Mn kinetics upon TPN administration [49], but most note that body levels of Mn increase significantly due to parenteral nutrition and high levels may persist after ceasing Mn supplementation from TPN

[50, 51]. Furthermore, no clear standard has been recommended for the daily dose of parenteral Mn, with the published literature indicating a broad, 200-fold range in the recommended daily Mn dose for adults on TPN ranging from a low dose of 0.18–0.91 μmol (0.01–0.05 mg) to a high dose of 40 μmol (2.2 mg). In 2012, the American Society of Parenteral and Enteral Nutrition lowered their recommended parenteral Mn concentration from 0.06–0.1 mg/day to 0.055 mg/day [52] to account for the risk of Mn neurotoxicity. It should be acknowledged that infants, especially premature infants, receiving TPN, may not have intact or mature homeostatic control of the metal. In addition to bypassing the homeostatic barrier of the GI tract where Mn absorption is normally tightly regulated [53], many infants and children on TPN solutions suffer from hepatic dysfunction and cholestasis, compromising their biliary excretion of Mn. Finally, further risk of Mn-induced toxicity is associated with the overall neurophysiological immaturity of the developing brain, perhaps allowing entrance for more Mn than would occur in the adult brain.

Manganese intoxication associated with TPN solutions providing ≥ 0.1 mg Mn/day is well established [54–56]. These patients developed elevated serum Mn levels [48], and they exhibit symmetrical high intensity MRI lesions in the globus pallidus consistent with the preferential accumulation of Mn at this site, in association with characteristic psychiatric symptoms and clinical signs of Mn-induced parkinsonism-like syndrome. Discontinuation of TPN therapy significantly decreases Mn levels in both the blood and central nervous system (CNS) [54]. In children chronically receiving TPN, high Mn blood levels and abnormal neurological signs have been reported, along with MRI findings indicative of Mn deposition in the brain [57], specifically the globus pallidus [58]. Once removed from Mn-supplemented TPN, brain levels of Mn tend to decline over time, blood Mn levels normalize and some case reports suggest a good prognosis without long-lasting neurodevelopmental sequelae [57].

3.5. Manganese Toxicity

While Mn deficiency is rare, Mn toxicity resulting from environmental exposure has been documented since the early 19th century, when a small group of workers grinding Mn oxide developed an unsteady gait and muscular weakness [59]. At present, Mn toxicity is most often associated with occupational exposure of welders, miners, and steel workers to chronic high levels of airborne particulate Mn [60, 61]. When inhaled, the Mn can lead to inflammation in the lungs and respiratory symptoms, including cough, bronchitis, pneumonitis, and impaired pulmonary function [62]. Manganese toxicity eventually manifests as a neurological disorder. Cases of impaired cognitive function in individuals drinking Mn-contaminated well water have also been reported in several countries [27, 63, 64]. Another potential environmental source for Mn exposure of current interest is the recently approved gasoline additive methylcyclopentadienyl Mn tricarbonyl, which releases Mn compounds, in the form of phosphates, sulfates, and oxides,

into the atmosphere upon combustion [65–67], with more than 90 % of these particles being of a respirable size [68].

4. MANGANESE NEUROTOXICITY

4.1. Mechanisms

Many of the transport proteins involved in brain Mn transport are also involved in dietary absorption (discussed above in Section 2.2). Briefly, transferrin-transferrin receptor and DMT-1 mediated transport are the primary mechanisms involved in the transport of Mn across the BBB. Recently, it has been revealed that the zinc transporters ZIP-8 and ZIP-14 (SLC30A10), the cation transporting ATPase ATP13A2, and the calcium ATPases SPCA1 and SPCA2 have been shown to play important roles for Mn transport into and within the brain too. Details of the pharmacokinetics of Mn transport into and within the brain can be found in [3, 10, 24].

Proposed mechanisms of Mn neurotoxicity range from functional changes in neurotransmission, to cellular organelle damage, and oxidative stress caused by Mn accumulation. The effect of Mn on the brain is also influenced by route of exposure and magnitude of accumulation. Inhalation of Mn is generally associated with oxidative stress and increased neuronal apoptosis [69, 70], whereas ingestion of Mn has more of a subtle effect, altering neurochemistry and cognition [71–73]. These differences are likely due to inhaled Mn having a more direct and concentrated delivery to the brain [3, 4, 6, 70] *versus* ingested Mn passes through the gut and much of this Mn is stored in the liver [22, 23, 73] and the delivery to the brain is much less than inhaled Mn [70, 73].

Manganese is known to localize in the mitochondria of cells where its cytotoxic properties have been linked to inhibition of complex I and II of the electron transport chain [74], increased production of ROS [75], disruption of mitochondrial membrane potential [76], and caspase-3 activation leading to apoptosis [69]. Other than energy production, one of the key physiologic functions of the mitochondria is to sequester cellular calcium [77], a function shared with the endoplasmic reticulum (ER) [78]. Similarly, Mn has been shown to induce ER stress [79] which is associated with the release of calcium into the cytosol [80, 81]. Altered cytosolic calcium may in turn trigger caspase-3 mediated apoptosis [82]. Additionally, increased ROS produced by Mn has also been associated with lipid peroxidation [83]. These data display the breadth of damage instilled by Mn on several cellular compartments. Cells (astrocytes or neurons) damaged by Mn have an undoubtedly hampered ability to respond to the brain's dynamically changing environment. Linking mechanistic changes (due to Mn) to functional outcomes of toxicity is the next step in understanding the progression of Mn neurotoxicity. For more detailed discussion of Mn neurotoxicity, please see the reviews by Horning et al. and Peres et al. [10, 24].

4.2. Proposed Therapies for Manganese Neurotoxicity

4.2.1. Chelation Therapy

Few treatment options have been proposed for Mn neurotoxicity. Removal from the Mn toxic environment is the first course of action, but only two clinical treatments have been tested; calcium disodium EDTA ($CaNa_2EDTA$) and para-aminosalicylic acid (PAS), each yielding success in small sample cohorts. $CaNa_2EDTA$ is a synthetic compound used in detergents and food preservatives that is known to bind divalent and trivalent metal ions. A study by Hernandez et al. used $CaNa_2EDTA$ to treat seven welder/foundry workers presenting with Mn-induced Parkinson's symptoms [84]. Five of the seven workers showed improvement in muscle rigidity and postural tremor. The use of PAS as treatment for Mn intoxication was investigated in a case study of a 50 year old woman who had been exposed to airborne Mn for 21 years. All Mn-induced symptoms were significantly alleviated upon receiving PAS therapy, and the patient presented close to normal clinical, neurologic, MRI, and handwriting scores in a follow up examination 17 years post treatment [85].

Though $CaNa_2EDTA$ and PAS treatments have shown positive results, the sample sizes in these studies are small, and to date, no progress has evolved from these putative therapies. Additionally, these treatments are intended to relieve or improve symptoms secondary to Mn toxicity when neuronal damage may have already occurred. It is imperative that treatment strategies shift to prevent the onset of Mn neurotoxicity rather than the treatment of its symptoms.

4.2.2. Bioactive Food Components as Therapy

Recently, the use of bioactive food components in cancer and cardiovascular research fields has gained notoriety as a preventative treatment [86]. Bioactive food components are non-nutritive compounds found in foods that have immunoprotective properties within the plants themselves. These compounds are frequently pigments of plants and provide protection from free radical damage and other environmental insults. Emerging evidence suggests that a specific subclass of these bioactive components, the polyphenolic compounds, may have additional neuroprotective properties (for a more detailed review see [87]).

Quercetin is the most abundant polyphenolic compound in the American diet, and is particularly abundant in onions and blueberries [88]. With its ability to cross the BBB, quercetin has emerged as a promising neuroprotective agent. Rats ingesting quercetin (in the form of blueberry extract) had decreased hippocampal neuron loss in a model of excitotoxic neurodegeneration [89]. Similar decreases in hippocampal neuron loss and improvement in learning and memory were observed in an Alzheimer's disease rodent model administered quercetin in an inhaled liposome [90]. Quercetin is also known to inhibit protein kinase C (PKC) activation, and to decrease inositol-3-phosphate activity [91, 92]. These properties of quercetin making it a candidate for neuroprotection due to the role

of PKC activation in γ-amino butyric acid (GABA) transporter and dopamine transporter internalization.

Once ingested, quercetin is metabolized by the liver and other tissues to form several bioactive variants. Isorhamnetin (ISO) is a methylated quercetin metabolite that is capable of crossing the BBB [93]. In a study where rats and pigs were administered oral quercetin and examined for quercetin metabolite content in plasma and various tissues, ISO was the predominant metabolite in the brain with concentrations reported at 200 nM [89]. Plasma ISO levels were reported around 15 μM [93]. While ISO is a slightly less potent PKC inhibitor than quercetin [91], ISO has superior bioavailability and tissue distribution [94]. Recently, ISO has been shown to improve GABA transporter functioning in astrocytes impaired by Mn exposure [95].

Identifying a specific dietary food component of protective value against neurodegeneration would change the approach of therapeutic interventions. Until now treatments for Mn toxicity and neurodegenerative diseases have focused on treatments after the onset of symptoms. A dietary treatment utilizing a relatively ubiquitous bioactive food component could be a proactive approach to mitigate disease prevention. Alternatively, quercetin may also provide an answer for therapeutic intervention alternative to pharmaceutical administration in progressing neurodegenerative disease.

5. CONCLUSIONS

Although it is an essential element and its dietary deficiency is uncommon, Mn overexposure is a known neurotoxicant that causes neurodegeneration and is of concern for vulnerable populations (iron-deficient individuals, people dependent upon well water, ferroalloy workers). Sources of Mn exposure include the diet, drinking water, airborne exposures (automobile exhaust, welding fumes, smelting) and parenteral feeding and each of these, except diet, are directly linked to overexposure. In the last two decades, the neurotoxicity associated with Mn overexposure has been well characterized; however, the treatment of the neurodegeneration that ensues is still being developed.

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ABBREVIATIONS

BBB	blood brain barrier
DMT1	divalent metal transporter 1

DRI	Dietary Reference Intake
EDTA	ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
GABA	γ-amino butyric acid
GI	gastrointestinal
ISO	isorhamnetin
PAS	para-aminosalicylic acid
PKC	protein kinase C
ROS	reactive oxygen species
TPN	total parenteral nutrition

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11

Cobalt-Schiff Base Complexes: Preclinical Research and Potential Therapeutic Uses

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Abstract: The use of metals in medicine has grown impressively in recent years as a result of greatly advanced understanding of biologically active metal complexes and metal-containing proteins. One landmark in this area was the introduction of cisplatin and related derivatives as anticancer drugs. As the body of literature continues to expand, it is necessary to inspect sub-classes of this group with more acute detail. This chapter will review preclinical research of cobalt complexes coordinated by Schiff base ligands. Cobalt-Schiff base complexes have a wide variety of potential therapeutic functions, including as antimicrobials, anticancer agents, and inhibitors of protein aggregation. While providing a broad introduction to this class of agents, this chapter will pay particular attention to agents for which mechanisms of actions have been studied. Appropriate methods to assess activity of these complexes will be reviewed, and promising preclinical complexes in each of the following therapeutic areas will be highlighted: antimicrobial, antiviral, cancer therapy, and Alzheimer's disease.

Keywords: Alzheimer's disease · antimicrobial · cancer · cobalt complex · coordination complex · Schiff base

1. INTRODUCTION

Transition metal complexes offer a diverse array of geometries and oxidation states, making this class an excellent platform for new and innovative therapeutics [1, 2]. Since the discovery of cisplatin as a potent but toxic anticancer drug [3], the field has focused on developing less toxic and increasingly effective transition metal complex therapeutics. One option for mitigating toxicity of transition metal complexes is to choose trace essential metals that humans are known to tolerate well [4]. Cobalt is one example, and despite its pharmaceutical promise it remains relatively ignored by pharmaceutical chemistry. Several excellent reviews of cobalt-based therapeutic research exist [5–8], but the biological properties of cobalt complexes vary widely depending upon the chelation strategy. As a result, the scope of this review is narrowed to cobalt complexes of Schiff bases ligands. However, it is notable that non-Schiff base cobalt complexes are having success as redox-activated prodrugs and drug delivery vehicles [9, 10].

Schiff bases are a synthetically flexible class of imines typically formed by condensation of a primary amine with an aldehyde or ketone (see Figure 1a below). First described by Hugo Schiff in 1864 [11], Schiff bases have since been studied as antifungals, antibacterials, antimalarials, antiinflammatories, antivirals, and antitumor agents [12–14]. In addition, Schiff bases are selective metal chelators whose biological properties are often enhanced upon complexation with a transition metal [15]. This review will highlight preclinical applications of cobalt-Schiff base complexes as potent antimicrobials, effective antivirals, specific and non-specific anticancer agents, and as inhibitors of amyloid-β.

2. ANTIMICROBIAL ACTIVITY

Metal complexes of Schiff bases have long been of interest as potential antimicrobial agents, including as both antifungals and antibacterials. In 1952, the first biological investigation with cobalt complexes demonstrated bacteriostatic and bacteriocidal activity in the μM range, while also exhibiting low systemic toxicity in mice [16]. Since then, low-cost methods for testing antifungal and antibacterial activity have become commonplace, and concern over antibiotic-resistant bacteria has increased drastically [17]. This combination of low cost and rising need has yielded a prolific field of research into transition metal-Schiff base complexes as antimicrobial agents [15, 18]. The closely related hydrazone family of ligands (Figure 1b) demonstrates similar electron donating properties as Schiff bases, and hydrazone complexes are mentioned where noteworthy.

Although Schiff base complexes are well studied as antimicrobial agents, their antimicrobial mechanism is not fully understood. It has been noted that three normal cellular processes are disrupted: (1) enzymatic metal binding site activity, (2) cellular respiration, and (3) protein production [19–21]. Uncomplexed Schiff bases can affect these processes, but complexation usually enhances their overall cytotoxic effect. This is attributed to Tweedy's chelation theory which states that chelation allows for electron delocalization and charge sharing between the metal center and its donor ligands [22], and increases the overall lipophilic character of the complex, favoring cell membrane permeability. However, it is important

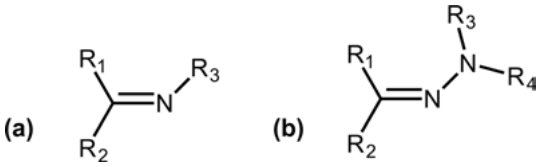


Figure 1. (**a**) General chemical structure of a Schiff base, where R_1 and R_2 can be alkyl groups, aryl groups, or hydrogens, and R_3 is an alkyl or aryl group. (**b**) General chemical structure of the related hydrazone family of ligands, where R_1, R_2, R_3, and R_4 can be alkyl groups, aryl groups, or hydrogens. Sometimes considered a sub-category of Schiff bases, hydrazone ligands demonstrate the same electron donating properties as Schiff bases via the lone pair residing in nitrogen's sp^2-hybridized orbital.

to note that the measured lipophilicity does not always correlate with increased antimicrobial potency, suggesting more complex mechanisms are indicated [23].

The successful evaluation of cobalt-Schiff base complexes as antimicrobials requires understanding the strengths *and* weaknesses of common assay methods. In much of the literature, minimum inhibitory concentrations (MICs) are calculated incorrectly, or are not compared against known positive controls, making it difficult to draw reliable conclusions. To obtain trustworthy and repeatable results, synthetic chemists should work more closely with microbiologists and follow established performance standards in biological assays [24]. Section 2.1 will introduce these concepts, discussing the methods that are most commonly applied and best practices for assessing antimicrobial activity. Section 2.2 will highlight antimicrobial cobalt-Schiff base complexes with demonstrated antimicrobial activity, herein defined as a MIC <1 mg/mL or favorable comparison with a known positive control. It is important to note that clinical antimicrobial potency cannot be assessed from MIC values independently. Clinical potency depends upon blood concentration levels of the microbe in question. Thus, a clinically potent MIC value varies from species to species.

2.1. Common Methods for Measuring Antimicrobial Activity

Methods of measuring antimicrobial activity can be broadly grouped into two categories: diffusion and dilution. While diffusion assays are typically simpler to perform, the results are qualitative and vary widely with materials used. Dilution methods require more product, but quantify minimum inhibitory concentrations and enable comparisons across studies. However, neither diffusion *nor* dilution techniques are sufficient to clinically distinguish between bacteriostatic and bactericidal mechanisms. To gain more detailed mechanistic insights, time-kill tests and fluorescent flow cytometry are recommended. Time-kill tests provide information on time- *versus* concentration-dependence, and fluorescent flow cytometry assesses the extent of cell damage [25].

Diffusion assays are overwhelmingly utilized in the inorganic chemistry laboratory due to their low cost and ease of use [25]. In these protocols, agar media is inoculated with bacteria and treated with antimicrobial agent in a localized well or on a paper disk. As antimicrobial agent diffuses out of the treatment site and into the agar, a circular zone of no bacterial growth will result. The radius of the zone of inhibition is measured after 16–24 hours (depending on the microbial species being tested [24]) and directly correlates with antimicrobial activity [25]. Diffusion methods are appropriate for qualitative screening, but should not be used to calculate MIC, as small variations in materials and protocols used yield large differences in radii of zones of inhibition [26].

For laboratories without an automated setup, dilution assays are more labor-intensive and require more of the antimicrobial agent. However, they are more accurate for quantitation MIC and are commonly used in clinical settings. In broth dilution protocols, serial dilutions of antimicrobial agent are treated with a fixed number of bacterial cells. Turbidity (indicative of cell growth) is measured

after 16–24 hours, and MIC is defined as the lowest concentration of antimicrobial agent that visually inhibits growth [25]. Further subculture of non-turbid samples can determine whether small amounts of live bacteria are still present. For both diffusion and dilution assays, careful attention and adherence to standard protocols is required for reproducibility and accuracy [24]. Failure to follow standard protocols results in unreliable or unrepeatable results.

In the laboratory, diffusion and dilution methods are generally used to quantify growth inhibition rather than cell death, and do not provide mechanistic insights. To ascertain microbicidal activity, a time-kill curve experiment is recommended, where live bacterial suspensions are treated with antimicrobial agent and assayed for viability at intervals over 24 hours. If a series of concentrations are tested, dilution methods can be used to determine whether the agent acts via a time- or concentration-dependent mechanism. This provides dynamic information about the interaction of microbe with agent over time, and can be used to gage *in vivo* dosing [25].

For further mechanistic insight, flow cytometry with appropriate fluorescent dyes is used. Propidium iodide (PI) is an intercalating dye used to determine whether an antimicrobial agent disrupts the bacterial cell membrane. In bacteria with an intact cell membrane, PI is membrane-impermeable and will not be found within cells. However, if an antimicrobial agent compromises cell membrane integrity, PI can permeate and is therefore found intracellularly [27]. A complementary technique uses carboxyfluorescein diacetate, a membrane-permeable dye that is only activated within viable cells with esterase function [28]. The vast majority of literature citing cobalt-Schiff bases as antimicrobials does not include mechanistic investigations, likely due to lack of cross-talk between the fields of inorganic chemistry and microbiology. This represents a gap in the understanding of fundamental mechanisms and must be addressed by future work.

2.2. Preclinical Complexes with Demonstrated Potency

While cobalt-Schiff bases are routinely investigated as antimicrobials, relatively few studies determine MICs and/or compare against known antimicrobial standards. The cobalt complexes mentioned here have potent MICs (defined as < 1 mg/mL [29]) or were shown to be more effective than an antibacterial or antifungal control. Given that MICs may vary widely depending on the method used, comparison against an appropriate positive control should be considered the most broadly reliable indicator of potency. While the complexes cited here have not been mechanistically studied, they exploit a variety of ligand types to achieve antimicrobial activity. These types include azido ligands, large lipophilic ligands, modifications of clinically approved agents, and halogen-substituted ligands.

2.2.1. Cobalt-Schiff Bases Incorporating Azido Ligands

Two studies have successfully utilized cobalt-Schiff bases with azido ligands as antimicrobials [30, 31]. First, a series of three pyrrole-based ligands (Figure 2a)

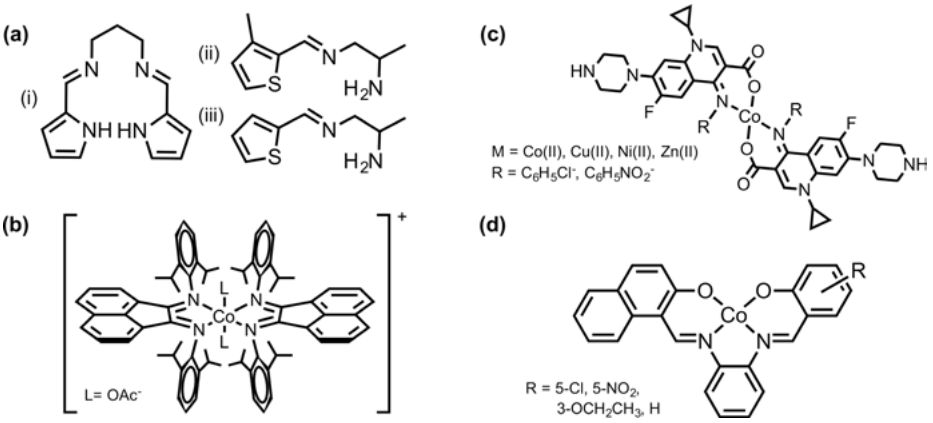


Figure 2. Schiff base ligands and complexes reported to have promising antimicrobial activity. (**a**) This series of pyrrole-derived ligands [30] was complexed to Co(III) and utilized azido ligands to bind remaining coordination sites, which was hypothesized to increase antimicrobial activity. Co(III) has six coordination sites total, and complexes with (i) followed the form $[CoLN_3]_2$, while complexes with (ii) and (iii) followed the form $[CoL_2(N_3)_2]$. (**b**) The proposed structure of a cobalt complex with large lipophilic ("bulky") ligands, where "bulkiness" was correlated with antimicrobial activity [23]. (**c**) A rationally designed series of complexes [32] modified ciprofloxacin to chelate transition metals, and the complexes were more potent antibacterial agents than ciprofloxacin alone, likely due to increased cell permeability upon complexation. (**d**) A series of complexes in which overall antimicrobial activity was correlated with having a halogen or other electrophilic substitution on the ligand [35]. This trend held true in a number of studies [35–38], and warrants further attention as a mechanism of antimicrobial potency.

were prepared and complexed with Co(III); azido ligands were used to chelate the remaining open coordination sites [30]. Co(III) has six available coordination sites, most commonly forming octahedral complexes. Thus, ligand (i) formed a dinuclear $[CoLN_3]_2$ complex with each azido group coordinating both Co(III) centers, while ligands (ii) and (iii) formed mononuclear $[CoL_2(N_3)_2]$ complexes. Complexes with ligands (ii) and (iii) had a higher azido:cobalt ratio, and these proved more potent than the complex with ligand (i). Complexes with (ii) and (iii) had MIC values of < 0.25 mg/mL (where potency is typically defined as MIC < 1 mg/mL) for three or more of the bacterial species *S. aureus* and *B. subtilis* (Gram-positive), and *P. aeruginosa* and *E. coli* (Gram-negative). However, the prepared complexes were not more potent than ciprofloxacin. These results suggest that increasing the number of azido ligands per complex increases antimicrobial activity, though variations in complex stability and kinetic lability were not investigated.

In the second study, two bidentate phenol-based ligands were complexed with Co(III) and end-on azido ligands coordinated the axial positions for a composition of $[CoL_2(N_3)_2]$ [31]. These two complexes demonstrated excellent antibacterial ability, most potently against *B. subtilis* with MIC values of 0.004 and 0.009 mg/mL. These MIC values were higher than that of penicillin (0.002 mg/mL), but were lower than sodium azide alone (0.125 mg/mL). Although the experimen-

tal conditions and controls used in this study varied from those in the first, the results still suggest that cobalt-Schiff base complexes with two azido ligands are potent antimicrobials [31].

2.2.2. Cobalt-Schiff Bases with Large Lipophilic Ligands

Ligands that are large and sterically hindered are considered "bulky," and this property can be exploited to increase antimicrobial activity. Ligands that are both bulky and lipophilic increase the hydrophobicity of the overall complex, thereby enhancing bacterial cell penetration. One example from the literature that directly correlates the "bulkiness" of Schiff base ligands with antimicrobial activity utilized bulky N-bisimine derivatives (Figure 2b) [23]. The orthodisubstituents of these derivates were found to contribute to antibacterial function, with the most hydrophobic substituent (2,6-diisopropylphenyl) leading to the highest biological activity. The MIC values for Co(III) complexes did not meet the 1 mg/mL limit of potency at 1.9–2.6 mg/mL, depending on the species being tested. However, antibiotic controls had a similar potency range at 1.3–2.5 mg/mL under the conditions used. In fact, the Co(III) complexes outperformed nystatin, ampicillin, and streptomycin in bacterial species, and were only bested by clotrimazole in fungal species [23]. These results suggest that increasing complex lipophilicity through the use of large, sterically hindered ligands is a promising route for developing cobalt-Schiff base complexes as antimicrobials.

2.2.3. Cobalt-Schiff Bases Containing Modifications of Clinically Approved Agents

A rational approach to antimicrobial complex design is to chemically modify a clinically approved agent so that it becomes suitable to coordinate a metal. For example, Schiff bases derived from ciprofloxacin have been complexed with a series of transition metals, including Cu(II), Co(II), Ni(II), and Zn(II) (Figure 2c) [32]. Remarkably, the transition metal complexes (including cobalt) outperformed ciprofloxacin for all antimicrobial species tested [32]. This is likely because chelation allows for delocalization of π electrons over the whole chelate ring, increasing the lipophilicity of metal-ciprofloxacin complexes *versus* ciprofloxacin alone. Increased lipophilicity yields better membrane permeability and better antimicrobial activity.

Similarly, antipyrine-derived Schiff bases have been prepared in two studies [33, 34]. Antipyrine is clinically approved as a nonsteroidal antiinflammatory, analgesic, and antipyretic agent. Condensing antipyrine with aldehydes or ketones to form Schiff bases has been shown to yield products with antibacterial properties [33]. In the first study, an antipyrine derivative was condensed with benzil, and the resulting Schiff base was complexed with transition metals [33]. The transition metal complexes were found to have more antimicrobial activity than free ligand, but less than uncomplexed metal salts alone. This was an unexpected result, but it is likely that the metal salts are better able to inhibit cell

respiration and enzymatic processes. In the second study, an antipyrine derivative was condensed with 2-aminophenol and 2-aminothiophenol [34]. The resulting Schiff bases and all metal complexes (including cobalt) were more potent than ampicillin and amphotericin in many bacterial species. The antibacterial potency is attributed to lipophilicity of the complexes and hydrogen bonding of the azomethine group with enzymatic centers of activity. Unfortunately, MIC values were not reported in either study, making direct comparisons difficult.

2.2.4. The Effect of Halogen Substitution on Cobalt-Schiff Base Antimicrobial Activity

Many studies have found halogen substitution on Schiff base complexes to increase antimicrobial activity. It is postulated that metal complexes with electron-withdrawing groups such as halogens have a higher binding affinity for intracellular oxygen and increased ability to disrupt cellular respiration [35]. In a series of asymmetrical Co(II) complexes (Figure 2d), all four were found to have significant antimicrobial activity. However, the presence of a halogen or other electrophilic group increased potency significantly, fitting the hypothesized mechanism [35]. Using the qualitative disk diffusion assay, cobalt-Schiff bases worked comparably well to chloramphenicol. However, quantitative tests revealed that chloramphenicol had significantly lower MICs in eight of ten bacterial strains studied, while the cobalt-Schiff bases performed better against *E. cloacae* and *B. subtilis* [35]. As these are Gram-negative and Gram-positive strains, respectively, the mechanism is unclear, but is not related to cell membrane structure.

In a similar study, a series of five Co(II) complexes were prepared and tested for antifungal activity against three species (*A. alternata*, *F. oxysporum*, and *M. roridum*) [36]. The closely related series displayed a surprisingly wide range of activity (MIC = 0.017 mg/mL to >1 mg/mL), demonstrating the *substantial biological effect of ligand character*. The cobalt-Schiff base series was not more effective than the indofil M-45 standard against two of three species, but the halogen-substituted complex was among the best performing [36]. Although the authors did not postulate a mechanism, it is possible that the halogen-substituted complex is once again better able to inhibit cellular respiration.

In line with this trend, a cobalt complex with two halogen substitutions demonstrated antibiotic and antifungal activity at 0.5 mg/mL [37]. However, the MIC was estimated based on a diffusion experiment, casting uncertainty on the reported values. Similarly, a cobalt complex with one halogen substitution was more effective than amikacin and ketoconazole in seven of eight bacterial strains tested in a diffusion experiment [38]. Halogen substitution was not the primary focus of these studies, and was therefore not mentioned by the authors as being mechanistically important. However, a summary of the literature shows a broader trend: metal complexes of Schiff bases with halogen substitution show rich potential as an antimicrobial class. Future mechanistic investigations of this class are warranted, and can enable rational design of antimicrobials going forward.

3. ANTIVIRAL ACTIVITY

Despite the extensive body of research employing cobalt-Schiff base complexes as antimicrobial agents, this class of complexes has made significant clinical progress as an antiviral [39–41]. CTC-96, a $[Co(acacen)(L_2)]^+$ complex (Figure 3) where axial ligand L = 2-methylimidazole, is the only cobalt-Schiff base to have entered clinical trials. Its synthesis was first described in 1997 [39], and it was found to inhibit replication of herpes simplex virus type 1 (HSV-1) in a rabbit eye model at concentrations 1000-fold lower than the clinically approved standard [40]. Just as significantly, a targeted version of this complex demonstrated selectivity toward Sp1 zinc finger transcription factors (ZFTF) for potential implication in treating human immunodeficiency virus (HIV) [42]. Selective targeting against ZFTF proteins drastically increases the potential for pharmaceutical applications of this class of complexes, and this method was later exploited to target pathways implicated in cancer (for mechanism and use in anticancer studies, see Section 4.2).

The pharmaceutical properties of $[Co(acacen)(L_2)]^+$ complexes have prompted many mechanistic investigations [43–47]. While the antiviral mechanism of CTC-96 is still not fully elucidated, it is known to coordinate histidine (His) residues through dissociative exchange of its labile 2-methylimidazole axial ligands (Figure 3) [48]. Biologically, it is known to inhibit the membrane fusion events that allow for viral penetration of HSV-1 [41]. Thus, the most likely antiviral mechanism is via direct targeting of a histidine-containing herpes virus serine protease. For further mechanistic details on $[Co(acacen)(L_2)]^+$ complexes, see Section 4.2.

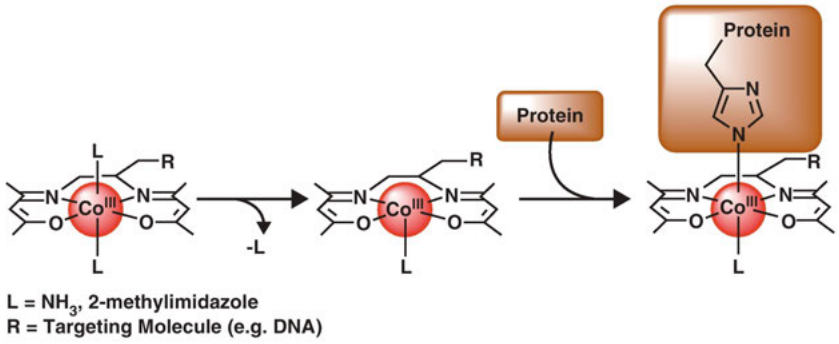


Figure 3. $[Co(acacen)L_2]^+$ complexes where axial ligand L = 2-methylimidazole or NH_3 have displayed extraordinary antiviral activity *in vivo*. The complex undergoes dissociative ligand exchange of its labile axial ligands to irreversibly bind His residues in biological settings. This mechanism can be exploited for therapeutic use where His residues are structurally or catalytically necessary for protein function. Reprinted by permission from [7]; copyright 2012 Elsevier Ltd.

4. ANTICANCER ACTIVITY

Schiff base complexes with anticancer activity can be grouped broadly into two categories: (i) those exhibiting non-specific cytotoxic activity, and (ii) those that selectively target a cancer-associated protein or pathway. The majority of published literature belongs to the former category, with therapeutic success largely relying upon making fortuitous discoveries as with cisplatin [3]. While targeted cobalt-Schiff base complexes comprise a much smaller body of literature, they can be rationally designed for greater likelihood of biological success. Section 4.1 will discuss non-specific cytotoxic cobalt-Schiff bases that are studied for their interaction with DNA, while Section 4.2 will discuss targeted complexes that rely on protein recognition for biological specificity.

4.1. Non-specific Cytotoxic Cobalt-Schiff Bases

Metallodrugs have been found to accumulate in cancer cells, a property which contributes to their success as cytotoxic agents [8]. Accumulation is attributed to cancer cells' requirement for increased concentrations of vitamin B12, which functions as a metallodrug carrier ligand [49]. Given that preferential accumulation of a drug in cancer cells *versus* normal cells is crucial for therapeutic success, cytotoxicity should always be tested in cancerous and non-cancerous cell lines. Metallodrugs display a wide variety of cytotoxic mechanisms [50], but the most commonly studied involve interaction with DNA. In Section 4.1.1, the most commonly used methods for assessing DNA interaction will be discussed. In Section 4.1.2, several promising examples of cobalt-Schiff base complexes as cytotoxic or DNA-binding agents will be described.

Although beyond the scope of this review, excellent work is being done with non-Schiff base cobalt complexes as anticancer prodrugs [51–53]. The reduction potential of Co(III) complexes can be tuned for selective release in hypoxic environments, such as the tumor microenvironment, allowing for *activation* of a Co(II) agent, or release of an active ligand into cancerous cells. These agents have been further modified to incorporate fluorophores, allowing visualization of hypoxia selectivity in spheroid cell culture tumor models [54].

4.1.1. Methods for Assessing DNA Interaction Mechanism

Metal complexes can interact with DNA by means of intercalation (between the base pairs), major or minor groove binding (between turns of the double helix), or surface stacking via electrostatic interactions. The least ambiguous way to distinguish between intercalative and non-intercalative interactions is to perform viscosity measurements [55]. Intercalating agents increase the axial length of DNA as they separate its base pairs. This makes the structure of DNA more rigid, yielding a concomitant increase in viscosity. Partial intercalators, on the other hand, may bend the DNA structure causing shortening and a reduction in

viscosity, while groove-binding causes little to no change in viscosity [55]. Because changes in viscosity result from *physical* changes in the structure of DNA, they provide the most direct information about intercalative *versus* non-intercalative binding modes.

Electronic absorption titration can be used to investigate the binding mode when the ligands of interest contain aromaticity. Intercalation allows for strong π-π stacking when aromatic ligands interact with the base pairs of DNA. This causes a hypochromic shift in DNA's absorbance spectrum, and the magnitude of shift is correlated with binding strength [56]. The binding constant (K_b) can be determined using Equation (1):

$$[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/K_b(\varepsilon_b - \varepsilon_f) \quad (1)$$

where ε_a is the apparent absorption coefficient of the complex, ε_f is the extinction coefficient of the free complex, and ε_b is extinction coefficient of the metal complex fully bound to DNA [57]. Because electron absorption studies allow for quantitation of K_b, they are considered a gold standard when aromatic ligands are involved.

In an analogous method to electronic absorption titration, fluorescence titration can be used when the metal complex exhibits autofluorescence [57–59]. The emission intensity of an intercalating agent increases when titrated with DNA, while emission intensity of a surface stacking agent is quenched. Upon intercalating, a suitable metal complex penetrates into hydrophobic regions of DNA, yielding less quenching from surrounding water and a concomitant increase in emission intensity. Conversely, the fluorescence of a stacking agent is quenched due to electron transfer from DNA to the excited MLCT state of the complex. Depending on the mechanism, equations are available to determine K_b from these experiments [55, 60].

An alternative fluorescence method exploits the strongly intercalating fluorophore ethidium bromide (EtBr). When pre-incubated with DNA, EtBr can be off-competed by an intercalating complex but not a groove binding or surface stacking complex. Since the emission intensity of EtBr (rather than the complex) is being monitored, this experiment provides indirect evidence of binding mode, and is therefore useful as a secondary confirmation of prior results [57].

Circular dichroism (CD) uses circularly polarized light to investigate DNA secondary structure, specifically interrogating changes in base pair stacking interactions and helicity. Free DNA exhibits a positive band near 275 nm and a negative band near 245 nm due to base pair stacking and right-handed helicity, respectively. Intercalators show strong base pair stacking and stabilize the right-handed B conformation of DNA. This causes an increase in intensity at both 275 and 245 nm. Groove binders and surface stackers cause little to no change in CD spectra [55, 61].

Upon interacting with DNA, some metal complexes will cause DNA cleavage. To assay for cleavage, gel electrophoresis is performed on a pre-incubated solution of DNA and agent. After staining to visualize, multiple bands of DNA indicate that cleavage has occurred. As DNA cleavage is often mediated by

oxidation, oxidants and reductants can be added to the reaction to further examine their roles [62].

These techniques are routinely used to investigate metal complex-DNA interactions. Viscosity measurements and electronic or fluorescence absorption studies are considered the gold standards, while EtBr competition and CD are often used as secondary techniques to confirm prior results. It is always necessary to employ multiple methods, as this grants greater certainty to the proposed mechanism.

4.1.2. Preclinical Complexes with Demonstrated Cytotoxicity or DNA Interaction

A group of hydrazone complexes has been investigated for cytotoxicity in five cell lines: HL-60, Caov-3, HeLa, MCF-7, and MDA-MB-231 [63]. Cytotoxicity (IC_{50}) is calculated as 50 % of the dose required for cell death. A low IC_{50} is obviously desirable, but because nonspecific toxicity is cisplatin's major shortcoming, a lower IC_{50} than that of cisplatin is considered a disadvantage. Of the studied complexes, a Co(II) complex showed cytotoxic activity against MCF-7 cells (IC_{50} = 1.8 μg/mL) that approached that of the anticancer drug tamoxifen (IC_{50} = 1.5 μg/mL). Metal chelation was found to slightly inhibit cytotoxic activity in this series, with the free ligand demonstrating a lower IC_{50} than any of its complexes or tamoxifen. Despite demonstrating such toxicity towards MCF-7 cells, little to no activity was observed in other cell lines [63]. These results demonstrate the importance of using *multiple* cell lines for preliminary cytotoxicity studies, although this is frequently overlooked in reports of novel complexes.

For most metal complex-DNA interactions it is difficult to predict specific binding mode based on structure alone. Intercalators are nearly ubiquitously square planar or octahedral complexes with aromatic ligand systems [64]. Planarity is necessary to meet the steric requirements of intercalation, while aromaticity supplies favorable π-π interactions with base pairs [64]. One such complex is an octahedral Co(II) coordination polymer (Figure 4a), where each planar aromatic ligand coordinates to the Co(II) center in the next subunit [57]. Despite the unique polymeric nature of the complex, an intercalative binding mode was confirmed with electronic absorption, fluorescence titration, and EtBr displacement. Moreover, the polymeric complex binds DNA more tightly than does free ligand ($K_b = 5.95 \times 10^5\ M^{-1}$ *versus* $2.59 \times 10^4\ M^{-1}$). The polymer also displayed toxicity against all four cancerous cell lines tested (HeLa, HEp-2, Hep G2, and A431), comparable to or exceeding the toxicity of cisplatin, while showing 100-fold lower toxicity against non-cancerous NIH/3T3 cells [57]. This study demonstrates that *polymeric* planar aromatic complexes can act as intercalators, and show preferential toxicity in cancerous *versus* non-cancerous cell lines.

A comparable dinuclear Co(II) complex is an intercalator that was confirmed by viscosity, electronic absorption, and fluorescence titration [65]. The study performed a head-to-head comparison of the dinuclear Co(II) complex *versus* a mononuclear Zn(II) complex with the same ligand. The Co(II) complex had a higher K_b than the zinc complex or the free ligand ($K_b = 8.02 \times 10^5\ M^{-1}$,

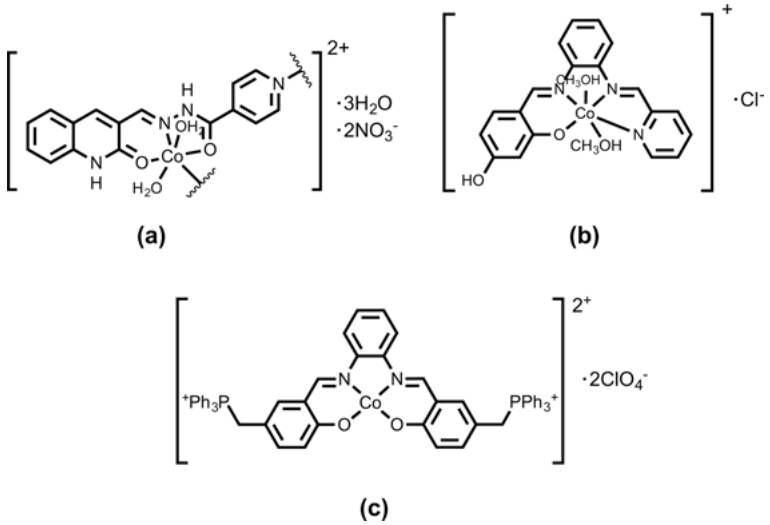


Figure 4. Schiff base ligands and complexes reported to have promising cytotoxic or DNA-binding activity. (**a**) A ligand-bridged, polymeric cobalt complex that is a strong intercalator [57]. Intercalators bind between nucleoside base pairs, and are usually square planar or octahedral complexes with aromatic ligands. (**b**) A cobalt complex that binds the minor groove of DNA, evidenced by both experimental and modeling data [61]. Groove binders may be sequence-specific, as different nucleoside combinations have different groove dimensions and properties. (**c**) A cobalt complex that surface stacks on DNA via electrostatic interactions, while the free ligand is an intercalator [55]. While electrostatic interactions are weaker than intercalation and groove binding interactions, surface stackers can still cause DNA cleavage and cytotoxicity.

$4.51 \times 10^5\ M^{-1}$, and $1.60 \times 10^5\ M^{-1}$, respectively). DNA cleavage was investigated using gel electrophoresis, and the Co(II) complex showed highest cleavage activity, likely due to higher Lewis acidity of the metal center [65]. Thus, Lewis acidity should be taken into consideration when designing metal complexes for interaction with DNA.

Unlike intercalators, major and minor groove binders of DNA are often sequence-dependent given that groove dimensions and presenting functional groups vary among the nucleoside base pairings [66]. For this reason, molecular docking simulations are often used as verification of groove binding. An octahedral Co(II) complex (Figure 4b) is proposed to strongly bind DNA in the minor groove (K_b = $3.29 \times 10^5\ mol^{-1}$ dm) based on electronic absorption, fluorescence titration, and CD [61]. Molecular docking experiments confirm minor groove binding with the double stranded DNA sequence CGCGAATTCGCG. The complex correspondingly exhibits significant *in vitro* cytotoxicity against three human cancer cell lines (IC_{50} = 7, 13, and 25 μg/mL against T cell leukemia, ovarian adenocarcinoma, and glioblastoma cells) and only moderate toxicity against non-cancerous cells (IC_{50} > 100 μg/mL) [61]. A Co(II) hydrazone complex and its Ni(II) analog were found to be groove binders, but major *versus* minor groove binding could not be determined [67]. Viscosity, electronic absorption, fluorescence titration, and CD all

confirmed a groove binding mechanism. The K_b values for the Co(II) and Ni(II) complex were 0.89×10^5 M^{-1} and 2.2×10^5 M^{-1} respectively, indicating that the Co(II) complex bound with lower affinity than the Ni(II) complex [67]. While the sequence specificity of groove binders limits their DNA strand interactions, this has not been shown to negatively impact their cytotoxicity.

Metal complexes that interact with DNA electrostatically via surface stacking are usually driven by *weak* π-π interactions. Based on structure alone, it is difficult to predict metal complexes that preferentially surface stack rather than intercalate as both contain aromaticity. One study found a Co(II)-Schiff base (Figure 4c) to interact with DNA electrostatically, while the same free ligand acted as an intercalator [55]. The binding modes of both the ligand and the complex were verified with viscosity, electronic absorption, fluorescence titration, and CD, and DNA cleavage was evaluated with gel electrophoresis. As expected, the intercalating free ligand had a higher DNA binding affinity ($K_b = 8.5 \times 10^5$ M^{-1}) than the complex ($K_b = 5 \times 10^4$ M^{-1}) [55]. Another study investigated a series of Co(II) and Co(III) hydrazone complexes for DNA binding and found all to interact via a surface mechanism, with a K_b range of 1.15 to 5.06×10^4 M^{-1} [62]. The mechanism was substantiated with electronic absorption, fluorescence titration, EtBr displacement, and CD. Notably, all complexes in the series efficiently cleaved DNA in the presence of hydrogen peroxide and the reducing agent 2-mercaptoethanol, demonstrating that a complex needs not have high binding affinity in order to cleave DNA [62]. Complexes that surface stack on DNA, therefore, have potential as cytotoxic anticancer agents.

4.2. Histidine-Targeted Cobalt-Schiff Bases

Inspired by the remarkable biological activity of CTC-96 (see Section 3), the Meade group has been investigating $[Co(acacen)L_2]^+$ complexes as targeted agents in cancer biology. In this context, further mechanistic investigations have enabled rational design of agents for targeting specific proteins and biological pathways. In particular, ZFTF proteins are a target of high interest for cancer research, and they can be specifically inhibited using $[Co(acacen)L_2]^+$ complexes. Section 4.2.1 will review what is known of the mechanism of inhibition, and Section 4.2.2 will discuss *in vitro* and *in vivo* biological studies that demonstrate specificity.

4.2.1. Investigating Mechanism of Action

$[Co(acacen)L_2]^+$ complexes selectively inhibit the activities of histidine-containing proteins through dissociative exchange of the labile axial ligands [45, 48]. Therefore, the kinetic and thermodynamic ligand exchange dynamics are important considerations in the rational design of metal-based therapeutics [45, 68]. There is a direct correlation between the observed axial ligand lability of the $[Co(acacen)L_2]^+$ derivatives and their ability to inhibit histidine-containing proteins [42, 44, 69–71]. Exploiting these findings, inhibitors of Cys_2His_2 ZFTFs

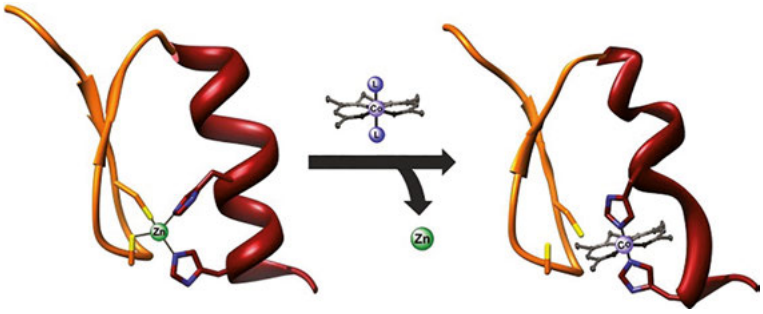


Figure 5. Proposed mechanism of inhibition of ZFTFs by Co(acacen). The complex coordinates to His residues in Cys_2His_2 zinc finger motifs through dissociative ligand exchange of axial ligands. Octahedral Co(acacen) displaces tetrahedrally coordinated Zn(II), disrupting protein structure and DNA binding function. Reprinted by permission from [46]; copyright 2013 Wiley-VCH, Weinheim.

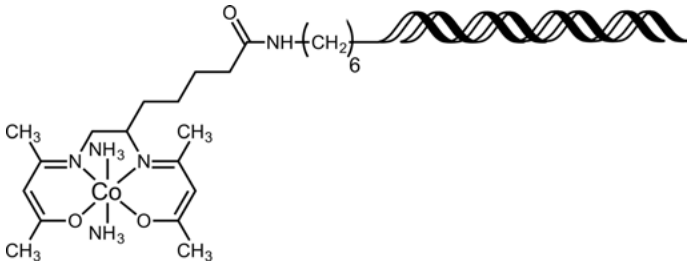


Figure 6. Co(acacen) conjugated to DNA, termed Co(III)-DNA. By conjugating the DNA consensus sequence of particular ZFTF, specific and potent inhibition can be achieved in cells and *in vivo*. The DNA serves as a reversible targeting moiety, while Co(acacen) provides irreversible protein inhibition.

have been developed using ammine (NH_3) axial ligands ($[Co(acacen)(NH_3)_2]^+$ hereafter termed "Co(acacen)").

The key roles that ZFTFs play in oncogenesis, tumor proliferation and growth, and metastasis make them highly desirable targets for therapeutic intervention [72]. A lack of hydrophobic binding pockets makes these proteins difficult to target with traditional organic molecules, but their coordination chemistry can be exploited for potential therapeutic effect [73]. A large class of ZFTFs tetrahedrally coordinate Zn(II) ions through a Cys_2His_2 structural motif. Moreover, Zn(II) coordination is required for sequence-specific DNA recognition and gene regulatory function [74]. Octahedral Co(acacen) complexes are able to displace Zn(II) and bind to the Cys_2His_2 domain, thereby disrupting protein structure and impairing DNA recognition and transcriptional activity (Figure 5). Since ZFTFs bind their consensus DNA with sequence-specificity, selective targeting can be achieved by conjugating Co(acacen) to oligonucleotides with high affinity for the protein of interest (Figure 6) [69–71].

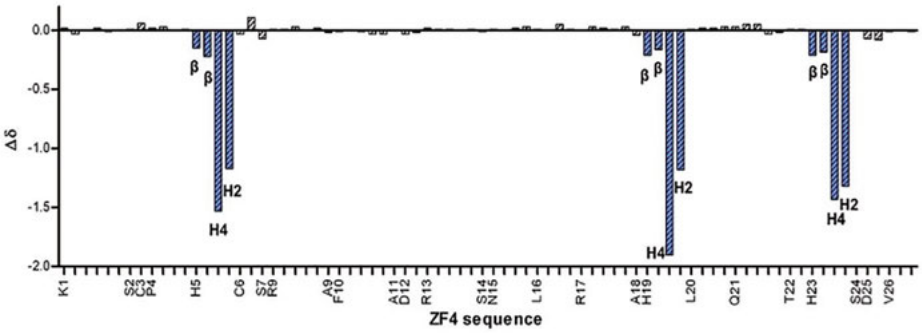


Figure 7. NMR data showing the specific binding of Co(acacen) to His residues in ZFTF model peptides. Upon treating a ZFTF model peptide (KSCPH CSRAF ADRSN LRAHL QTHSD V) with Co(acacen), the change in resonances of protons in the amino acid side chain were recorded. The Δδ Chemical Shift profile demonstrates Co(acacen)s selectivity for His residues, as significant effects were only observed on the His imidazole ^{1}H. (Reprinted by permission from [46]; copyright 2013 Wiley-VCH, Weinheim.

To further investigate the mechanism of ZFTF inhibition, model peptides of the zinc finger motif were treated with Co(acacen) and monitored by ^{1}H NMR and 2D NMR spectroscopy [46]. Upon treatment with the complex, protons of His residues *but no other residues in the peptides* underwent significant changes in ^{1}H resonances (>1 ppm) (Figure 7). CD and electronic absorption studies provided confirmation of structural perturbations of the zinc finger motif, supporting the hypothesis that the octahedral Co(III) complex distorts the tetrahedral Zn(II) binding pocket and therefore the local secondary structure. Taken together, these data reveal Co(acacen) complexes inhibit the activity of ZFTFs by coordinating His residues in the zinc finger domain via dissociative ligand exchange, thereby disrupting the structure required for gene regulation [46].

4.2.2. Investigating in vitro and in vivo Biological Activity

Specific inhibition of transcription factors has been achieved by employing a targeting method (Figure 6) where the conjugated oligonucleotide mimics the native binding partner of the protein (targeted complexes termed "Co(III)-DNA") [69]. The remarkable effectiveness of these agents has been demonstrated *in vivo* with inhibition of the Snail and Ci transcription factors in *Xenopus* and *Drosophila* embryonic models, respectively [70, 71, 75]. Inhibiting Snail transcription factors may have a direct impact on epithelial-to-mesenchymal transition (EMT), thought to be a key factor in driving cancer metastasis. Ci transcription factor is the *Drosophila* analog of human Gli proteins, which are known oncogenes for a variety of cancers.

Co(III)-DNA containing the Ebox DNA sequence (Co(III)-Ebox) binds selectively and irreversibly to Snail ZFTFs [69]. Specificity of binding was investigated using electrophoretic mobility shift assays (EMSA) in *X. laevis* embryo lysates.

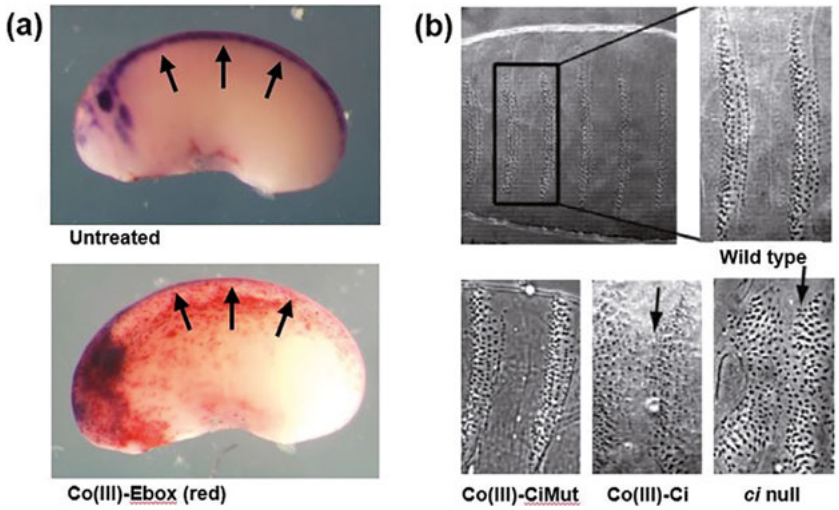


Figure 8. (**a**) *X. laevis* embryos treated with Co(III)-Ebox show dramatically impaired neural crest formation and migration. Embryos were injected with Co(III)-Ebox prior to neural crest migration. At stage 25 of embryo development, untreated (top) *versus* treated (bottom) embryos show that Co(III)-Ebox results in failure of the neural crest cells to migrate. *In situ* hybridization of Twist expression visualizes neural crest formation and cell migration with blue stain, while Co(III)-Ebox is stained red. Normal neural crest development is seen in untreated embryos, while little to no neural crest migration is observed in treated embryos, indicated with black arrows. For related work see [70]. (**b**) Co(III)-Ci inhibits Ci ZFTF function in *D. melanogaster* embryos. Embryos treated with Co(III)-Ci show impaired denticle belt formation relative to the untreated control and Co(III)-CiMut. Co(III)-Ci treatments mimics the phenotype of Ci null embryos, indicating that the conjugate disrupts the associated pathway through inhibiting the Ci ZFTF [71]. (b) Reproduced by permission from [71]; copyright 2012 American Chemical Society.

After overexpressing various control proteins in embryos, the resulting lysates were challenged with ^{32}P-labeled probes. Co(III)-Ebox bound to the Snail family proteins (Snail1, Snail2, and Sip1), but did not bind a *non-Snail* family ZFTF, *a non-ZFTF* that binds Ebox, or a *non-ZFTF/non-Ebox binding* protein. To investigate the irreversibility of inhibition, ^{32}P-labeled Co(III)-Ebox was incubated with lysates overexpressing Snail2 protein and challenged with an unlabeled control. Co(III)-Ebox remained bound to Snail2 even after being challenged with 100-fold unlabeled Ebox, while the same ^{32}P-labeled Ebox without Co(acacen) was displaced after being challenged by 33-fold unlabeled Ebox [69].

Co(III)-Ebox was subsequently tested *in vivo* with *X. laevis* embryos at varying stages of development to demonstrate its utility in live animals (Figure 8a) [70]. Co(III)-Ebox successfully inhibited known functions of Snail in the formation of neural crest cells and inhibited their migration. Specificity was demonstrated by a series of controls as well as retention of normal development of the central nervous system and mesoderm formation [70]. Snail ZFTFs have been linked to the formation of cancer stem cells and mediate EMT, making cancer cells more

invasive and migratory. The ability to inhibit Snail ZFTFs *in vivo* is a superb tool for better understanding Snail's roles in cancer, and targeted Co(acacen) complexes hold therapeutic promise as potential metastasis inhibitors.

Similarly, a Co(acacen) complex targeted to Ci (cubitus interruptus) ZFTF protein (complex termed Co(III)-Ci) was synthesized and tested *in vitro* and in a *Drosophila* embryo model [71]. The ability of Co(III)-Ci to inhibit Ci from binding its DNA target was evaluated *in vitro* with protein extracts and *D. melanogaster* cells (S2 cells). Consistent with the results of Co(III)-Ebox studies in *X. laevis*, Co(III)-Ci demonstrated potent and specific inhibition of its ZFTF target. Moreover, mutating the targeting sequence by one base pair or replacing it with a different sequence altogether precluded inhibition of DNA binding by Ci. Live cell studies demonstrated inhibition as well, wherein a luciferase reporter gene for Ci's target pathway was used. Co(III)-Ci was able to significantly decrease transcriptional activation by Ci [71]. This is a highly significant result demonstrating the utility of the Co(III)-DNA platform for broader applications. By simply changing the DNA sequence, the strategy can be used for a number of other cancer-associated ZFTF proteins.

Next, *in vivo* studies in developing *D. melanogaster* embryos were performed. In these embryos, Ci plays a major role in denticle belt formation, and genetically mutated ci^{94} null exhibits abnormal fusion of the segments. Treatment of embryos with Co(III)-Ci resulted in localized fusion where the agent was injected, mimicking the ci^{94} null phenotype (Figure 8b) [71]. In a complementary experiment, in which Ci is truncated into its repressor form but retains its ZF binding domain and sequence-specific DNA recognition, Co(III)-Ci could rescue denticle belt segmentation. This demonstrates that Co(III)-Ci can selectively inhibit Ci in both its activator or repressor form in an *in vivo* embryo model [71].

These biological investigations lay the groundwork for significant advances in the field of targeted cancer therapeutics. Both ZFTFs for which selective inhibition has been demonstrated (Snail family and Ci) are implicated in cancer-associated pathways. Overactivity of Snail proteins has been linked to EMT in cancer metastasis, while Ci regulates the hedgehog pathway associated with basal cell carcinoma and medulloblastoma. However, the modularity of the Co(III)-DNA platform allows for targeting of any ZFTF, granting the platform much broader applicability.

5. INHIBITING AGGREGATION OF AMYLOID-β

Alzheimer's disease (AD) is the most common form of age-related dementia and the 5th leading cause of death in the U.S.A. [76]. The etiology of AD is widely debated and likely multifactorial. As such, drug development has targeted a broad spectrum of biological pathways, but has yet to produce any disease-modifying therapeutics. Recently, a Co(III)-Schiff base complex has been utilized as an amyloid-β (Aβ) inhibitor [77]. This section will outline the relevant disease background (Section 5.1), detail the precedent of using metal coordination com-

plexes as Aβ inhibitors (Section 5.2), and describe the application of Co(III)-Schiff base to AD (Section 5.3).

5.1. Etiologic Factors in Alzheimer's Disease

5.1.1. Amyloid-β Aggregation

There is strong evidence linking disruption in the metabolism of Aβ to AD [78]. *In vivo*, Aβ is generated by sequential cleavage of the amyloid precursor protein by β-secretase and γ-secretase. The two primary isoforms of Aβ are $A\beta_{40}$ and $A\beta_{42}$. $A\beta_{40}$ is the most abundant, while $A\beta_{42}$ more readily aggregates and is therefore believed to be the more pathogenic isoform. Either through over-production or under-clearance, Aβ accumulates as amyloid plaques in the AD brain [79]. Pathologically, AD is characterized by both amyloid plaques composed of Aβ and intracellular aggregates composed of hyperphosphorylated microtubule-associated protein tau. Disruptions in tau processing are common to many neurodegenerative disorders, and in the case of AD, generally believed to be downstream of Aβ toxicity [80]. The precise mechanism by which these pathological changes induce synaptic dysfunction and neurodegeneration remains unknown.

Amyloid aggregation follows a sigmoidal growth curve and has been described as nucleation-dependent polymerization. An initial lag phase corresponds to nucleation, wherein monomers form oligomeric seeds for aggregation. This is followed by a rapid growth polymerization phase that eventually plateaus once equilibrium between Aβ monomers, oligomers, and fibrils has been reached [81]. A broad spectrum of oligomeric species has been identified both *in vivo* and *in vitro*. However, structural studies of Aβ oligomers are challenging due to their polymorphism and transient nature [82]. Although it remains unclear whether Aβ oligomers represent intermediates to fibril formation or distinct aggregation pathways, significant data has implicated oligomers as the toxic species of Aβ [80, 83–85]. While both monomers and fibrils are relatively inert toward neurons, oligomer toxicity has been demonstrated using both *in vitro* aggregation of synthetic Aβ and *ex vivo* isolation of soluble oligomers from AD brain [84]. Additionally, soluble Aβ oligomers better correlate to synaptic loss and markers of disease severity than insoluble plaques *in vivo*, making oligomeric species the most likely etiologic agent and a promising target for therapeutic strategies [86, 87].

Aggregation of Aβ is modulated by a variety of factors including metal binding, peptide concentration, buffer composition, temperature, agitation, molecular crowding, and pH [88, 89]. These factors affect generation of both oligomers and fibrils since both processes require the self-association of Aβ. In order to study the amyloid aggregation pathway, purified or synthetic Aβ can be aggregated *in vitro* [90]. *In vitro* aggregation kinetics have been shown to correlate to rates of disease progression in AD patients and animal models and represent a useful metric for testing Aβ-targeted therapeutics [91–93]. However, the dependence of aggregation assays on such a large number of variables and lack of standardi-

zation in protein preparation or assay setup has resulted in wide variability in published Aβ aggregation data. As such, it is generally accepted that multiple techniques should be employed for optimal characterization of Aβ aggregation [94, 95].

5.1.2. Metal Binding to the Amyloid-β Protein

Significant evidence implicates metal ions in the pathogenesis of AD [82, 96–99]. Early work identified drastic disruptions in metal homeostasis in AD patients and animal models [100]. For example, amyloid aggregates extracted from mouse models and human AD brain show high levels of Cu(II) and Zn(II) directly bound to Aβ [101, 102]. However, total brain levels of the metals remain unchanged, indicating significant miscompartmentalization [103]. Further, addition of chelators results in partial dissolution of the plaques, implicating metals in the aggregation process [104]. Experiments using animal models of AD also illustrate the importance of metal ions. Genetic manipulations perturbing zinc and copper metabolism alter Aβ metabolism in transgenic mice and chelators have shown cognitive benefits when used as therapeutics [105, 106]. Additionally, extensive *in vitro* work has demonstrated the ability of metal ions to alter aggregation and modulate toxicity of Aβ [107–109].

In order to better understand the role of metals in the AD brain, the specific binding interactions between Aβ and metal ions have been characterized by a variety of structural techniques *in vitro* [110]. Cu(II), Zn(II), and Fe(II) coordinate Aβ in a 1:1 stoichiometry at the same binding site [107]. Metal coordination is restricted to the N-terminal portion of the peptide, which is common to both $A\beta_{40}$ and $A\beta_{42}$ isoforms and remains outside of the β pleated core formed by residues 18–42 as Aβ aggregates. *In vitro* studies of metal ion binding to Aβ show that Cu(II) and Zn(II) bind to monomeric or fibrillar Aβ with identical affinity and coordination geometry, suggesting that metal binding is independent of the aggregation state of Aβ [111, 112].

As with studies of Aβ aggregation, reported metal ion binding affinities vary greatly due to variation in experimental conditions [113]. The binding affinity for Cu(II) ranges from attomolar to nanomolar, while the binding affinity for Zn(II) is in the low micromolar range [107, 112, 114–118]. These relatively low affinities make binding unlikely at the physiological Cu(II) and Zn(II) concentrations in cerebrospinal fluid (CSF) [119]. However, transient synaptic release of labile metal ions during neurotransmission can raise local concentrations significantly, allowing association with Aβ [120].

Metal ions have been shown to affect both the morphology of amyloid aggregates and the kinetics of aggregation. Possible mechanisms for metal-induced protein aggregation include amyloidogenic rearrangement of the peptide by metal coordination, intermolecular crosslinking between monomers, stabilization of species that facilitate aggregation, destabilization of non-pathogenic structures, and oxidative modifications of the peptide that increase aggregation due to metal-catalyzed redox reactions [82]. Initial studies showed that Zn(II) and Cu(II) markedly accelerate Aβ aggregation [107, 108]. Another study concluded

that Aβ aggregation *in vitro* does not occur if metal ions are rigorously excluded, further implicating the role of metal ions in Aβ aggregation [121]. However, more recent studies have reported diverse and contradictory results. Cu(II) has been shown to both accelerate fibrillation kinetics [122] *and* inhibit fibril formation by promoting amorphous aggregation [123–125], as well as stabilize oligomeric species [126, 127]. Similar contradictory results have been reported for Zn(II) [82, 115, 123, 128, 129]. A study demonstrating that the effect of metal binding depends on the stoichiometric ratio between Aβ and metal ions may partly explain the broad spectrum of reported results [122]. Additionally, variable experimental conditions and different assays for monitoring aggregation contribute to the poor agreement within the literature.

5.1.3. Metal-Mediated Oxidative Stress

Oxidative stress has been identified as a key component of AD pathology and a potential mechanism for Aβ-induced neurotoxicity. High oxygen consumption, relatively low antioxidant levels, and limited regenerative potential make the brain particularly susceptible to oxidative damage [97]. Oxidative stress in the brain is generated by the redox active metal ions Cu(II) and Fe(III) that activate molecular oxygen and generate reactive oxygen species (ROS) [96]. For this reason, these metals are rigorously regulated by numerous binding proteins [96].

In addition to altering aggregation, metal binding to the N-terminal His residues of Aβ has demonstrated importance in modulating its neurotoxicity [130, 131]. Metal-mediated production of H_2O_2 by Aβ has been observed for both Cu(II) and Fe(III) *in vitro*, but Fe(III) is not anticipated to play a significant role *in vivo* [132, 133]. Aβ-mediated reduction of Cu(II) to Cu(I) is thought to be the primary physiologic mechanism for Aβ generation of ROS *in vivo* through activation of molecular oxygen and formation of superoxide [134]. Superoxide is then rapidly converted to H_2O_2, which readily diffuses across membranes and can generate downstream highly reactive free radicals by Fenton and Haber-Weiss chemistry [97]. Aβ-mediated generation of ROS by reduction of Cu(II) induces oxidation of another species. The most likely candidate within the Aβ peptide is the sulfur of Met35. Studies isolating Aβ oxidized at Met35 and bound to copper support this redox scheme [135]. Alternatively, biological reducing agents such as dopamine, cholesterol, or ascorbate may be oxidized, allowing redox cycling of the metal ion without net oxidation of the Aβ peptide [133].

Unlike Cu(II) and Fe(III), Zn(II) is not redox-active and does not generate ROS. When Cu(II) and Zn(II) ions are co-incubated with Aβ peptides, redox inactive Zn(II) suppresses Cu(II)-mediated H_2O_2 production by competitively displacing Cu(II) ions from the metal binding site [136]. Concordantly, Cu(II) binding increases Aβ toxicity to cultured cells, while Zn(II) binding decreases toxicity in a concentration-dependent manner [97, 131]. Moreover, addition of metal chelators or the H_2O_2-degrading enzyme catalase decreases Aβ toxicity to cultured neurons, further supporting Cu(II)-generated H_2O_2 as a likely mediator for neuronal damage [133, 137].

5.1.4. Downstream Mechanisms of Amyloid-β Cellular Toxicity

The precise mechanism by which Aβ leads to cellular toxicity is widely debated and almost certainly multifactorial. There is evidence for involvement of many mechanisms including oxidative stress, disrupted biometal homeostasis, aberrant calcium signaling, impaired axonal transport, altered membrane integrity, mitochondrial dysfunction, and pathologic tau processing [138]. While Cu(II) binding is not thought to be exclusively responsible for Aβ cytotoxicity, it can be linked to many of the other pathologic mechanisms [122, 139]. Clearly a variety of both downstream and parallel processes contribute to the complex physiologic dysregulation leading to synaptic dysfunction and neuronal cell death in AD. While the precise mechanisms of neurotoxicity in AD are not elucidated, disruption of Cu(II) binding represents an ideal therapeutic target as ROS generation is an early pathologic event that is involved in other proposed mechanisms.

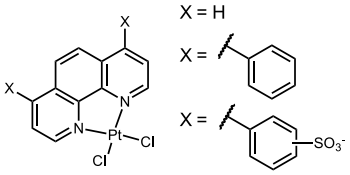
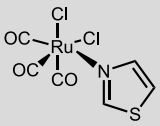
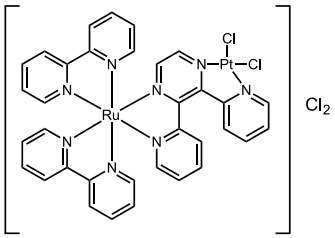
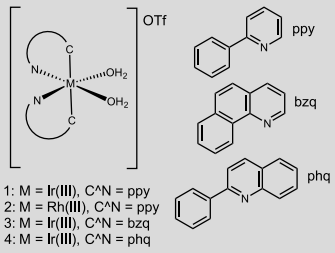
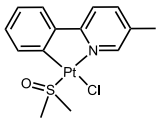
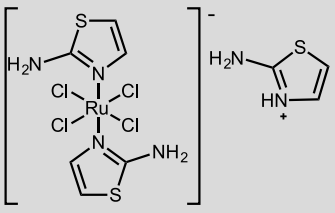
5.2. Metal Complexes as Amyloid Inhibitors

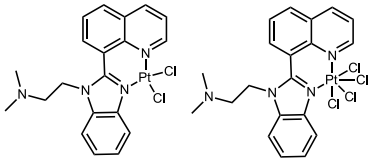
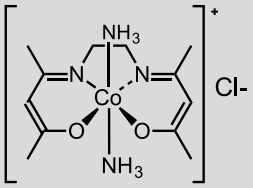
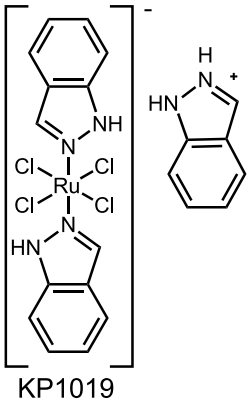
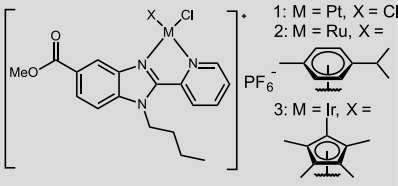
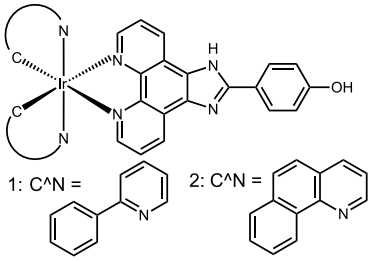
5.2.1. Chelation and Coordination: Strategies for Disrupting Metal Binding to Amyloid-β

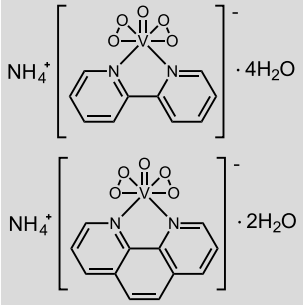
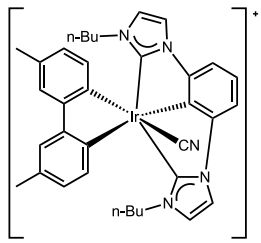
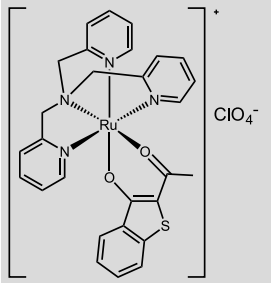
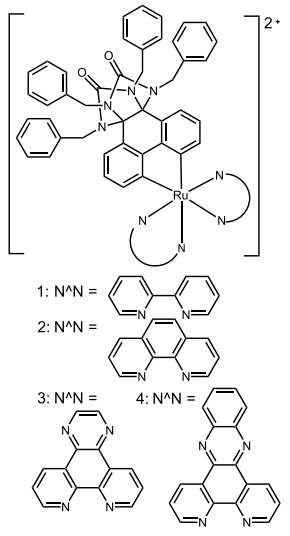
The current toolbox for studying and modulating Aβ-metal interactions is very limited. Early studies used metal chelation to experimentally control metal ion concentrations [121]. The use of organic chelators targeting Cu(II) and Zn(II) has allowed precise modulation of metal concentrations *in vitro* and facilitated much of the structural work on metal binding [104, 128]. Chelators have also been applied *in vivo* for mechanistic studies of metal binding [106]. Promising results of metal chelation in animal models led to the development of several chelating drugs, which unfortunately failed in clinical trials [140–143]. However, metal chelation both as a research tool and as a therapeutic approach has significant drawbacks including decreased bioavailability of necessary metal ions, poor metal selectivity, and inability to disrupt interactions between the metal binding site and unchelated metals [1, 144].

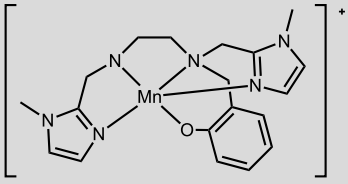
In 2008, Barnham et al. pioneered the first transition metal complex-based approach targeting the N-terminal His residues of Aβ. A class of $Pt(L)Cl_2$ complexes were used, where L represents bidentate phenanthroline ligands [145]. By coordinating to the His residues implicated in metal ion binding, these compounds effectively decreased Cu(II)-mediated aggregation and generation of ROS, rescued neuronal viability, and restored disruptions in long-term potentiation [145]. Since 2008, mechanistic work on $Pt(L)Cl_2$ complexes has revealed that the strong coordination of Pt(II) to Aβ is able to competitively displace Cu(II) and Zn(II) ions from Aβ and reverse metal-induced aggregation [146–148]. The efficacy of $Pt(L)Cl_2$ compounds stimulated the development and characterization of more cyclometallated Pt(II) compounds [148–150]. In addition, transition metal complexes have been expanded to include Ru(II), Ir(III), Rh(III), V(V), Mn(II), and Co(III) coordination complexes that have shown

Table 1. Coordination complexes for amyloid-β inhibition.

Structure	Effects	Ref.
	– Binds to $A\beta_{42}$ by SELDI-TOF MS and $A\beta_{40}$ by NMR – Alters $A\beta_{42}$ secondary structure by CD – Inhibits $A\beta_{42}$ aggregation by ThT fluorescence – Decreases $A\beta_{42}$: Cu(II) mediated H_2O_2 production – Increases cell viability following treatment with $A\beta_{42}$ by MTS assay – Rescues $A\beta_{42}$ inhibition of LTP in rodent hippocampal slice	[145]
	– Binds to $A\beta_{28}$ by NMR and ESI-MS – Alters $A\beta_{28}$ secondary structure by CD	[151]
	– Inhibits $A\beta_{42}$ aggregation by ThT fluorescence – Inhibits $A\beta_{42}$ formation of small oligomers by SDS-PAGE – Discovered distinct binding mode compared to Pt(II) complexes by SEC and MALDI-TOF MS	[152]
 1: M = Ir(III), C^N = ppy 2: M = Rh(III), C^N = ppy 3: M = Ir(III), C^N = bzq 4: M = Ir(III), C^N = phq	– Binds to $A\beta_{40}$ by ESI-TOF MS – Inhibits $A\beta_{40}$ aggregation by ThT fluorescence – Decreases fibril length and density by TEM – Ir(III) complexes show switch-on luminescence upon binding to $A\beta_{40}$	[153]
	– Binds to $A\beta_{28}$ by NMR and ESI-MS – Coordinates to $A\beta_{28}$: Cu(II) by EPR	[149]
 PMru20	– Decreases $A\beta_{42}$ toxicity in rodent primary cortical neurons by LDH assay – Binds to $A\beta_{42}$ by ESI-MS – Inhibits $A\beta_{42}$ aggregation by ThT fluorescence	[154]

Structure	Effects	Ref.
	– Inhibits $A\beta_{42}$ aggregation by ThT fluorescence – Decreases formation of $A\beta_{42}$ dimer by SELDI-TOF MS – Increases cell viability following treatment with $A\beta_{42}$ by MTS assay – Rescues $A\beta_{42}$ inhibition of LTP in rodent hippocampal slice – Reduces $A\beta_{42}$ levels and plaque number in APP/PS1 mouse model of AD	[150]
	– Binds to $A\beta_{16}$ by ESI-MS and NMR – Stabilizes formation of large $A\beta_{42}$ oligomers and reduces small oligomers by SDS-PAGE – Decreases binding of $A\beta_{42}$ oligomers to synapses in mouse primary neurons	[77]
 KP1019	– Inhibits $A\beta_{40}$ and $A\beta_{42}$ aggregation by ThT fluorescence – Coordinates to $A\beta_{28}$ by EPR – Stabilizes high MW oligomers by native gel electrophoresis and Western blotting – Rescues differentiated SH-SY5Y cells from $A\beta_{42}$-induced toxicity	[155]
	– Compounds 1–3 inhibit $A\beta_{42}$ aggregation by ThT fluorescence – Compound 3 rescues primary cortical neurons from $A\beta_{42}$-induced toxicity	[156]
	– Inhibits $A\beta_{40}$ aggregation by ThT fluorescence – Exhibits enhanced luminescence in the presence of $A\beta_{40}$ monomers or fibrils – Rescues SH-SY5Y cells and mouse primary cortical neurons from $A\beta_{40}$-induced toxicity	[157]

Structure	Effects	Ref.
	– Inhibits $A\beta_{42}$ aggregation by ThT fluorescence – Alters $A\beta_{42}$ secondary structure by CD – Alters $A\beta_{42}$ aggregate morphology by AFM – Reduces $A\beta_{42}$ particle size by DLS – Binds to $A\beta_{42}$ by NMR and ESI-MS – Rescues SH-SY5Y cells from $A\beta_{42}$-induced toxicity by MTT assay	[158]
	– Inhibits $A\beta_{40}$ aggregation by ThT fluorescence and TEM – Exhibits enhanced luminescence in the presence of $A\beta_{40}$ monomers or fibrils	[159]
	– Inhibits $A\beta_{40}$ aggregation by ThT fluorescence and TEM	[160]
	– Inhibits $A\beta_{40}$ aggregation by ThT fluorescence and TEM – Inhibits acetylcholinesterase activity	[161]

Structure	Effects	Ref.
	– Removes Cu(II) from Aβ via metal swapping – Decreases ROS production by ascorbate consumption assay	[163]

promise as anti-AD therapeutics *in vitro* [149–163]. A chronological summary of published literature in this field is provided in Table 1. Macromolecular compounds and nano-based strategies have been excluded for brevity. Metal-based imaging agents have been employed in the literature, but are beyond the scope of this review. The success of preliminary transition metal complexes validates the use of coordination to metal centers for amyloid inhibition and illustrates the necessity for further development (Table 1).

5.2.2. Cobalt-Schiff Base Complexes

In 2014, the Meade group introduced the first use of a Co(III)-Schiff base complex as an amyloid inhibitor (Figure 9) [77]. Mimicking the mechanism of the previously described platinum complex, it was designed to irreversibly bind His residues via axial ligand exchange, and therefore followed the form Co(acacen) previously used in antiviral and anticancer studies (see Figure 3). The complex was found to effectively coordinate the His residues of $A\beta_{16}$, the N-terminal fragment of Aβ often used in structural studies due to its solubility. Adducts between Co(acacen) and Aβ were observed by mass spectrometry, and ^{1}H NMR revealed the loss of free His protons (H6, H13, and H14) with increasing equivalents of Co(acacen) indicating His coordination (Figure 9a) [77].

In order to assess the effects of Co(acacen) on aggregation, Aβ oligomerization was measured using SDS-PAGE (Figure 9b). Gels were evaluated by silver stain and Western blot analysis using NU-2, an antibody specific for soluble Aβ oligomers. The data demonstrated that Co(acacen) binding to Aβ alters oligomerization by increasing the formation of large, SDS-stable (>30 kDa) oligomeric species and concomitantly decreasing the concentration of small oligomers in a concentration-dependent manner [77]. In order to determine what effect the Co(acacen)-mediated alterations in aggregation might have on cellular Aβ toxicity, mouse primary hippocampal neurons were treated with oligomers prepared with and without Co(acacen), and synaptic binding was assessed (Figure 9c). Treatment with 100 nM Co(acacen) decreased synaptic binding to neurons, illustrating the potential for Co(acacen) to inhibit Aβ synaptic toxicity [77].

Ongoing development of Co(acacen) as an amyloid inhibitor involves measuring its effects on aggregation with full kinetic assays targeting both fibril development with thioflavin T (ThT) fluorescence and CD spectroscopy, as well as small oligomeric distribution dynamics using fluorescence correlation spectroscopy.

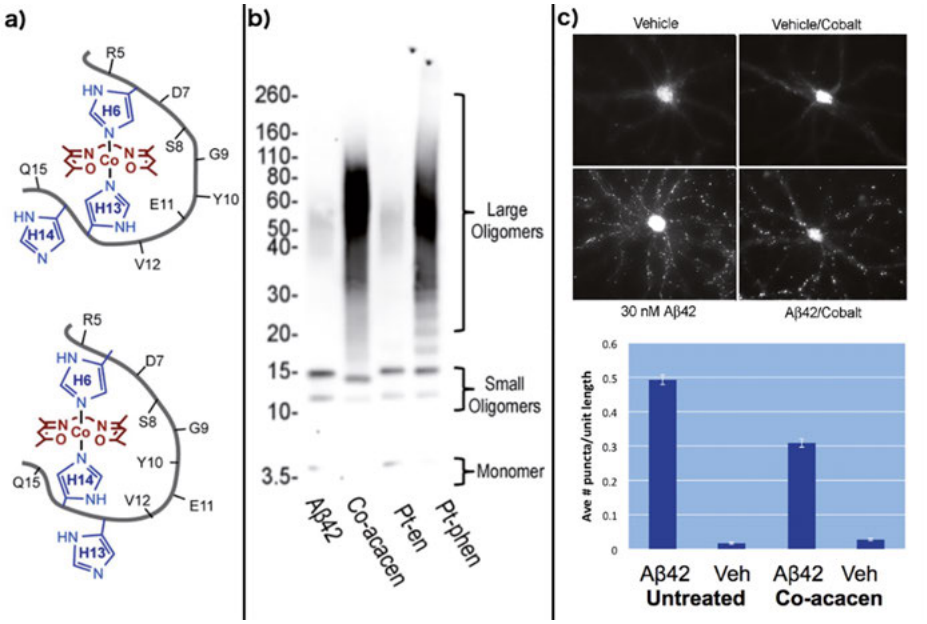


Figure 9. Co(acacen) coordinates to the N-terminal histidine residues of amyloid-β. (**a**) Bidentate binding to His 6 and His 13/14 was found to be the most energetically favorable binding conformation using DFT calculations. (**b**) Treatment of Aβ42 with Co(acacen) during aggregation results in the stabilization of large molecular weight oligomers (>30 kDa) by Western blot with the oligomer specific antibody NU-2. A decrease in small oligomers, which are regarded as the more toxic variant, is also observed upon treatment with Co(acacen). This stabilization of large molecular weight oligomers is also observed for the Pt(II)-phenanthroline complex developed by Barnham et al. indicating that the two coordination complexes are likely mechanistically similar [145]. (**c**) In addition to altering aggregate size, treatment with 100 nM Co(acacen) also decreases synaptic binding of Aβ oligomers in primary neuronal culture. Adapted and reproduced by permission from [77]; copyright 2014 Wiley-VCH, Weinheim.

Further biological characterization of the downstream effects of Co(acacen) on Aβ toxicity is underway in mouse primary hippocampal neuronal cultures. In addition, the modularity of the Co(acacen) platform is being utilized to attach an Aβ targeting moiety to the acacen backbone of Co(acacen), thus conferring specificity for future *in vivo* applications.

6. CONCLUSIONS

This chapter has attempted to review the wide variety of preclinical research employing cobalt-Schiff base complexes. While only one complex of this class has advanced to clinical trials thus far, this class of ligands and complexes boasts a *great deal of promise*. However, it is necessary to improve in several areas if

more clinical success is to be seen. In the field of cobalt-Schiff base complexes as antimicrobials, more mechanistic investigations of antimicrobial activity are required, including elucidation of structure-activity relationships. Concerning cancer research, promising cobalt-Schiff base complexes should begin to undergo *in vivo* testing, as even simple xenograft studies are largely absent from the literature. To successfully address AD, complexes must be targeted for specificity and made permeable to the blood-brain barrier. Addressing these unmet needs will meaningfully advance the use of cobalt-Schiff base complexes as potential therapeutic agents.

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ABBREVIATIONS AND DEFINITIONS

^{1}H NMR	proton nuclear magnetic resonance
$A\beta_{16}$	amyloid-beta 1–16
$A\beta_{28}$	amyloid-beta 1–28
$A\beta_{40}$	amyloid-beta 1–40
$A\beta_{42}$	amyloid-beta 1–42
acacen	acetylacetonato ethylenediamine
AD	Alzheimer's disease
AFM	atomic force microscopy
APP/PS1	amyloid precursor protein/presenilin 1
Aβ	amyloid-beta
bzq	benzoquinoline
CD	circular dichroism spectroscopy
Ci	Cubitus interruptus protein
Co(acacen)	$[Co(acacen)(NH_3)_2]^+$
CSF	cerebrospinal fluid
DFT	density functional theory
DLS	dynamic light scattering
EMSA	electrophoretic mobility shift assay
EMT	epithelial-to-mesenchymal transition
EPR	electron paramagnetic resonance
ESI-MS	electrospray ionization mass spectrometry
ESI-TOF	electrospray ionization time of flight

EtBr	ethidium bromide
H13	histidine 13 of Aβ
H14	histidine 14 of Aβ
H6	histidine 6 of Aβ
His	histidine
HIV	human immunodeficiency virus
HSV-1	herpes simplex virus type 1
IC_{50}	cytotoxicity, reported as 50 % of the dose required for cell death
K_b	binding constant of metal complex with DNA
L	ligand
LTP	long term potentiation
MALDI-TOF	matrix assisted laser desorption ionization time of flight
Met35	methionine 35 of Aβ
MIC	minimum inhibitory concentration
MLTC	metal-to-ligand charge transfer
MS	mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
NU-2	monoclonal antibody against Aβ oligomers
PAGE	polyacrylamide gel electrophoresis
phq	phenylquinoline
PI	propidium iodide
ppy	2-phenylpyridine
Pt-en	platinum(II)(ethylenediamine)Cl_2
Pt-phen	platinum(II)(1,10-phenanthroline)Cl_2
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SELDI-TOF	surface enhanced laser desorption ionization time of flight
TEM	transmission electron microscopy
ThT	thioflavin T
Veh	vehicle
ZFTF	zinc finger transcription factor

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12

Copper Depletion as a Therapeutic Strategy in Cancer

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Abstract: Copper is an essential trace element that plays a critical role in a variety of basic biological functions, and serves as a key component in a number of copper-dependent enzymes that regulate such processes as cell proliferation, angiogenesis, and motility. A growing body of preclinical work has demonstrated that copper is essential to metastatic cancer progression, and may have a role in tumor growth, epithelial-mesenchymal transition, and the formation of the tumor microenvironment and pre-metastatic niche. As a result, copper depletion has emerged as a novel therapeutic strategy in the treatment of metastatic cancer. We present a review of the physiologic role of copper with a discussion of relevant enzymes of the copper proteome in both normal tissue and in cancer. We conducted a comprehensive review of the available preclinical data of several copper chelation agents, including penicillamine, trientine, disulfiram, clioquinol, and tetrathiomolybdate (TM), across a variety of tumor types. We also present the existing early phase clinical trial data for the use of the copper chelator TM in the treatment of breast cancer and other malignancies.

Keywords: breast cancer · cancer · copper chelation · copper depletion · lysyl oxidase · tetrathiomolybdate

1. INTRODUCTION

Metals are endowed with unique characteristics that include redox activity, variable coordination modes, and reactivity towards organic substrates. Due to their reactivity, metals are tightly regulated under normal conditions and aberrant metal ion concentrations are associated with various pathological disorders, including cancer. For these reasons, coordination complexes, either as drugs or prodrugs, have been attractive targets for potential anticancer agents. Over the last fifty years, the development of anticancer drugs has moved away from conventional cytotoxic agents and towards the rational design of selective agents that act on specific cellular targets.

2. METALS

Metals, including heavy metals, constitute an important part of both normal biological processes and pathological states. Both essential and non-essential heavy metals, in either low or high concentrations outside of the physiological range, lead to disturbances in normal biological processes, and can induce cellular stress

which may cause disease progression. Trace elements are also known to affect the utilization and storage of other trace elements. For instance, copper-dependent ceruloplasmin (Cp) is involved in iron homeostasis, while zinc affects absorption of copper, and iron is known to be important for the absorption of zinc, cadmium, cobalt, and manganese [1]. Given their important roles in pathogenesis, manipulation of certain metals in the body has been used as a therapeutic strategy for the treatment of many diseases.

2.1. Copper Chemistry

Copper is a trace element, which plays various important roles in cell physiology, including enzyme catalysis, redox reactions, mitochondrial respiration, iron absorption, free radical scavenging, and elastin cross-linking [1]. Copper also contributes to many biological processes such as embryogenesis, growth, and metabolism [2]; it exists in two oxidation states, Cu(II) and Cu(I), within the body. These various states allow it to serve as a co-factor in redox reactions, where it acts as a catalyst for enzymes like cytochrome *c* oxidase (CCO), lysyl oxidase (LOX), and superoxide dismutase (SOD).

For healthy adults, the recommended daily dietary requirement of copper is 0.9 mg [3]. The primary sources include food and water. The total serum concentration of Cu in a healthy individual ranges from 63.7–140.12 μg/dL [4]. Once ingested, Cu is typically directed toward three distinct pathways [3]: (1) a *secretory pathway,* constituting the Cu chaperone ATOX1 that transports Cu to the Cu-dependent enzymes Cp and LOX, via the Cu-transporting P_{1B}-type ATPases ATP7A and ATP7B, (2) *free radical scavenging,* where Cu in cytoplasm is directed towards SOD via the Cu chaperone CCS, and (3) *mitochondria,* where cytoplasmic Cu is incorporated into cytochrome *c* oxidase with the help of COX17, SCOs, and COX11.

2.1.1. Homeostasis/Trafficking of Copper in the Body

As Cu exists in two oxidation states within biological systems, Cu(I) and Cu(II). it can act as a catalyst for a number of redox reactions involved in cellular, biochemical, and regulatory functions. Given its essential role in cellular physiology, it becomes important to study the absorption, distribution, and utilization of Cu in biological systems. The total Cu content of cells is divided into two groups (Fig. 1) [5, 6]: the easily available labile Cu pool (consisting of Cu chaperones that bind Cu with low affinity), and the immobilized Cu pool (consisting of metallothioneins that bind Cu with high affinity) [7–9].

Copper is almost entirely absorbed within the small intestine via a high-affinity Cu transporter, CTR1 (Cu transporter 1), a protein harboring three transmembrane domains and located at both plasma membranes and membranes of intracellular vesicles [10, 11]. CTR1 is highly specific for the reduced Cu(I) state, thereby making metalloreductases essential regulators of copper import into cells. These metalloreductases include Steap (six-transmembrane epithelial anti-

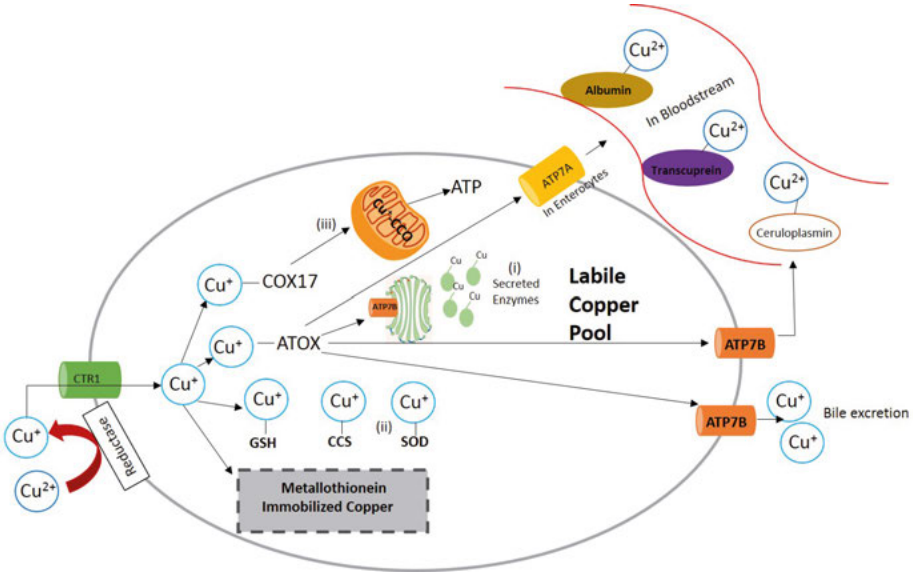

Figure 1. Copper Homeostasis. Copper enters cells via CTR1 in its reduced state. Within the cells it is bound to chaperones, and does not exist in its ionic forms. The labile pool of copper consists of Cu bound to proteins, which have lower binding affinity so that it can be transferred to other proteins for their metallation. The immobilized copper pool consists of copper bound to metallothionein, which has comparatively higher metal binding affinity, and this is important to avoid cellular toxicity due to Cu excess. The three important pathways requiring copper for their functionality are depicted (i) secretory pathway, (ii) free-radical scavenging, and (iii) mitochondria.

gen of prostate protein) proteins, located at both plasma and intracellular membranes [12] and dCytb (duodenal cytochrome b) which is expressed in the duodenum [13]. CTR1 allows both the import of copper into the cells and its transfer across intracellular vesicles for storage and release into the cytosol, where it is made available to cytosolic Cu chaperones.

CTR2 is a low affinity Cu transporter as compared to CTR1 [14, 15]. Once Cu is picked up by intestinal CTR1, it is transferred directly to the intracellular chaperone identified as antioxidant 1 Cu chaperone (ATOX1), which is encoded by the ATOX1 gene [16, 17]. Copper is then transported from the apical membrane to either intracellular vesicles (where CTR1 and/or CTR2 promote its uptake into the vesicles) or to the Cu-transporting P_{1B}-type ATPase (Cu-ATPase), ATP7A, located within the trans-Golgi network (TGN). Copper is then trafficked for ultimate export from the enterocyte to the blood [18, 19]. ATP7A transports Cu inside the TGN, where metallation of several secreted enzymes occurs. The majority of Cu homeostasis takes place in the liver. Dysfunction of ATP7A leads to reduced copper availability in tissues, which exhibits a lethal condition, Menkes disease [20]. Hepatocytes also contain another Cu-transporting P_{1B}-type ATPase, ATP7B, for exporting copper. Defects in ATP7B are associated with Wilson's disease, a disorder of excessive copper accumulation [21].

In the blood stream, copper is mainly transported via Cp, with contribution of other proteins including albumin. Cp is a ferroxidase enzyme, which is unstable in the absence of copper and thus is sometimes used for indirect measurement of Cu depletion in the body [22]. Cu in the cytoplasm of fibroblast-like mammalian cells may bind to the Cu chaperone for SOD, CCS. CCS serves an essential role in the free-radical scavenging pathway by the metallation and activation of Cu/Zn-superoxide dismutase (SOD1) [23]. For copper's role in mitochondria, the proteins important for the insertion of the metal to CCO include COX17, COX11, Sco1, and Sco2. Cu from COX17 is transferred to Sco1 in the intermembrane space of mitochondria, from where it gets loaded onto CCO [24].

Metallothioneins (MTs) are small, cysteine-rich proteins (6–7 kDa), capable of binding Cu(I) with high affinity in form of metal-thiolate clusters [25, 26]. There are two isoforms of MTs: MT-1/2 (expressed throughout the body) [27], and MT-3 (expressed primarily in brain) [28]. Due to their high Cu binding potential, they play an important role in Cu homeostasis by sequestering excessive Cu to avoid Cu toxicity. *In vivo* experiments have suggested that MT-1/2 knockout in Cu overload is lethal to animals [29].

2.1.2. Copper Proteome

The copper proteome has been found to comprise less than 1 % of the total proteome of an organism, in both eukaryotes and prokaryotes (Fig. 2) [30]. To avoid toxicity due to 'Cu overload' in the body, organisms have defined mechanisms to transfer Cu via chaperones, as Cu is not freely available in its ionic form in the body. In addition to Cu chaperones, there are Cu-dependent enzymes and other proteins in the body. Blockhuys et al. [31] analyzed the distribution of Cu-dependent proteins in humans. Apart from proteins involved in Cu homeostasis, Cu-dependent proteins are distributed across intracellular, transmembrane, and extracellular spaces. With the diverse Cu distribution, Cu-dependent proteins are involved in various cellular functions including proliferation, angiogenesis, and migration [32]. For instance, LOX is important for formation of the local metastatic microenvironment known as the metastatic niche [33], ATOX1 plays a role in inflammatory neovascularization [34], MEMO1 promotes metastasis [35], SPARC facilitates tumor cell invasion [36], and the mitogen-activated protein kinase kinase is involved in tumor growth [37].

3. COPPER HOMEOSTASIS IN PATHOPHYSIOLOGY

Due to its Fenton chemistry, copper is an ideal cofactor for various biological processes. Its redox states make it invaluable in the chemistry and formation of reactive oxygen species (ROS). It is necessary that copper is either protein-bound or well compartmentalized to avoid cellular toxicity. Derangements in copper utilization have been associated with multiple pathologic processes such as Wilson's and Menkes disease, Alzheimer's disease, diabetes, and polycystic ovary syndrome, among others [38–47].

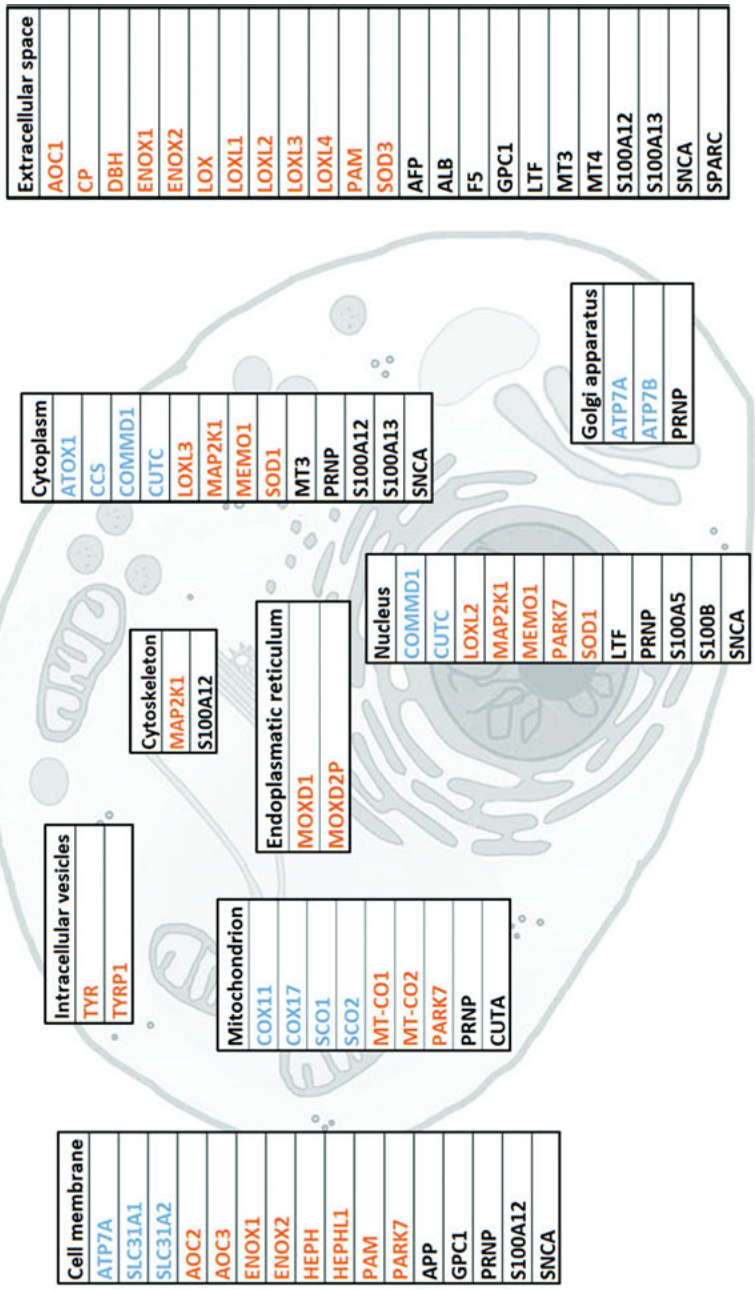

Figure 2. Copper Proteome. Copper, due to its Fenton Chemistry, serves as an important cofactor for numerous proteins and enzymes involved in both physiologic and pathologic processes. These proteins may be secreted, intracellular, or transmembranous. This figure demonstrates the distribution of these proteins, some of which are not only required by copper for its homeostasis, but also others that are copper-dependent for their functionality or expression. Reproduced by permission from [31]; copyright 2017 Royal Society of Chemistry.

4. COPPER IN CANCER

Cu is also known to play an integral role in the metastatic cascade within tumor cells and the tumor microenvironment [48], supporting the notion of Cu depletion as an attractive therapeutic strategy [49]. Cu regulates the energy balance of cells via oxidative phosphorylation within mitochondria [50], cell polarity, and the ability to transition between the epithelial-mesenchymal transition (EMT) and the mesenchymal to epithelial transition (MET) within the tumor [51], cell migration and invasion [52], and tumor growth through hypoxia-related genes including the hypoxia-inducible factor (HIF1α) [50, 53–55]. Cu is a critical component of various metalloenzymes including SOD1, vascular adhesion protein-1, matrix metalloproteinase (MMP-9) and LOX, which have been demonstrated to be integral to the metastatic process [49, 56–63]. Recently, Cu was shown to be necessary for BRAF signaling in the mitogen-activated protein kinase (MAPK) pathway [37]. Cu also plays an important role in angiogenesis, where it serves as a cofactor within pro-angiogenic molecules such as the basis fibroblast growth factor, the vascular endothelial growth factor (VEGF), and angiogenin [49, 56, 64–66]. While copper is important for normal immune function, Cu depletion has shown to cause conversion of tumor-associated macrophages (TAMs) from immunosuppressive to pro-immunogenic in mouse models by modifying the cytokine profile of TAMs and promoting an antitumor response of T cells [67]. Generally speaking, total Cu levels tend to be higher in the tumor tissue and serum of cancer patients when compared to controls. Cu levels are also measurably higher in tumors resistant to cisplatin/carboplatin and high Cu levels have been associated with inferior outcomes [2]. Given the important and diverse roles of copper and other metals in cancer biology, a new branch of cancer therapeutics has emerged that has focused on targeting these metals within the body and within cancer cells [68]

4.1. Breast Cancer

Higher copper levels are found in serum and tumor tissues of breast cancer patients as compared to healthy controls [69–72]. Several Cu-dependent proteins (termed the Cu proteome) are upregulated in breast cancer (approximately 26 % of the Cu proteome) including F5, ATP7B, SLC31A1, SCO2, hephaestin like 1, CUTA, ATOX1, COX17, tyrosinase-related protein 1, MT3, LOXL1–2, SPARC, and mono-oxygenase DBH like 1 [3, 31]. Cu-dependent LOX has been associated with bone metastasis in estrogen receptor (ER)-negative breast cancer patients [57]. LOX also promotes TWIST transcription, thus enhancing EMT [73], and stimulates cell adhesion and migration via FAK/Src signaling [74]. In addition, LOXL2 was found to be a prognostic marker for breast cancer [75]. It promotes EMT via its interaction with SNAIL and enhancing E-cadherin expression [76], and is known to interact with TIMP1 and MMP9 [75, 77]. LOXL2 is involved in various processes of tumor progression including cell invasion, tumor growth, and lung metastasis of basal-like breast carcinoma cells [51]. Cu-dependent MEMO1

forms the intersection between growth factor (heregulin and insulin growth factor (IGF)-1) and estrogen signaling pathway in breast cancer cells. It controls subcellular localization of estrogen receptor α (ERα), phosphorylation, and ErbB2/ER-, or IGFIR/ER-signaling, thereby activating the MAPK and PI3K signaling pathways to promote breast cancer cell migration and proliferation [78, 79]. MEMO1 also facilitates EMT, adhesion, and metastasis [35, 80, 81]. Cu-dependent SPARC occurs in basal-like breast tumors [82], and is associated with a more aggressive phenotype [83–85]. Cu chaperone ATOX1 is significantly upregulated in breast cancer compared to normal breast tissue [31]. ATOX1 is known to accumulate at lamellipodia borders of migrating breast cancer cells and thus enable breast cancer progression [86]. Copper is also known to induce estrogen-like responses including activation of ERα, transcription of ER-target genes, and cell proliferation in MCF-7 cells [87].

Given that an association between serum Cu levels and angiogenesis has been observed, metal chelation has been proposed as an antiangiogenic treatment in numerous types of tumors, including breast malignancy [88]. Disulfiram (DSF) is a pro-drug, which is converted to its metabolite DEDTC (N,N-diethyldithiocarbamate). DEDTC forms a metal complex (usually with Cu(II) or Zn(II)) that is responsible for the anticancer activity in various cancers including breast, prostate, glioblastoma, lung, melanoma, cervical, colorectal cancers (CRC), and leukemia [89].

4.2. Gastrointestinal Cancer

4.2.1. Colorectal Cancer

Several population-based studies have demonstrated an association of increased CRC incidence with elevated serum Cu levels compared to healthy controls [90–93]. LOX, a secreted Cu-dependent amine oxidase in CRC, synergizes with HIF-1 to promote tumor progression. LOX/HIF-1 leads to increased colonization of CRC cells to bone, and is associated with poor clinical outcome [94].

The influence of Cu and its transporters on chemosensitivity to 5-fluorouracil (5-FU)-based chemotherapy was explored with triple combination of 5-FU combined with metal chelators (Cu/Fe chelation: 1:1 mixture of the metal chelators di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone hydrochloride (DpC) and N,N,N′,N′-tetrakis-[2-pyridylmethyl]-ethylenediamine (TPEN)) and omega-3 fatty acid, docosahexaenoic acid), has been found to be effective in controlling disease progression of CRC and overcoming resistance of 5-FU via targeting the ubiquitin-proteasome system and antiapoptotic, myeloid cell leukemia (MCL)-1 [95]. TPEN was also found to exhibit selective cytotoxicity to the colon cancer cell line HCT116, both *in vitro* and *in vivo,* by forming a TPEN-Cu complex, which engages in redox cycling to generate hydroxyl radicals [96]. Disulfiram, a drug used for the treatment of alcohol dependence, chelates Cu (DS/Cu) and chemosensitizes gemcitabine-resistant cell lines by 12.2–1085-fold.

The DS/Cu complex also inhibits NFκB activity [97]. Tetrathiomolybdate is safe when added to the combination of irinotecan, 5-fluorouracil, and leucovorin used in the treatment of metastatic colorectal cancer patients [98].

4.2.2. Gastric/Esophageal Cancer

Serum Cu levels, Cp, ATP7A/7B, and the serum Cu:Zn ratio were found to be markers associated with outcome and resistance to therapy across a spectrum of gastrointestinal tumors including gastric and esophageal [99–102]. Expression of the Cu transporter, ATP7B, is frequently upregulated in gastric carcinoma and its expression may be utilized as a chemo-resistance marker against cisplatin in some patients with poorly differentiated/undifferentiated gastric carcinoma [101].

Lysyl oxidase, a hypoxia-responsive factor and a Cu-dependent enzyme, is a prognostic factor in gastric carcinoma. Recently, LOX was shown to induce EMT in two gastric cancer cell lines (OCUM-2MD3 and OCUM-12) under hypoxic conditions; and strong correlations between LOX expression and gastric cancer metastasis have been observed [103]. In another study analyzing immunohistochemical localization of Cu/Zn SOD, 34 of 70 cases demonstrated a positive correlation between the grade of Cu/Zn SOD immunoreactivity and the histological type of gastric cancer, with well-differentiated gastric cancer having more positive staining. It has also been observed that some types of gastric cancer may be resistant to Cu-dependent ROS [104]. Cellular prion protein (PrP(C)), a Cu-binding glycosyl-phosphatidylinositol, is overexpressed in gastric cancer and is found to confer multidrug resistance, to promote cancer metastasis and to inhibit apoptosis. Hypoxia induces PrP(C) expression in gastric cancer MKN28 cells, which is reduced by the MERK/ERK inhibitor PD98059, and PrP(C) knockdown led to hypoxia-induced drug sensitivity in these cells [102]. In a study analyzing the effects of DSF-Cu on esophageal squamous cell carcinoma cell lines, DSF-Cu caused significant cytotoxicity to cancer cells and its cytotoxic effect was enhanced by metformin, a molecule known to promote a reduced environment within cells. Copper was important for DSF activity, as the Cu chelator, bathocuproinedisulfonic acid, partially reversed the cytotoxic effects [105].

4.2.3. Pancreatic Cancer

In a study analyzing the pathogenesis of pancreatic cancer in relation to serum Cu levels in 100 cancer patients and 100 control subjects, high levels of serum Cu, defined as above a threshold of 1214.58 μg/L, were associated with an increased prevalence of pancreatic cancer [106]. Chronic exposure to elevated levels of Cu have been shown to increase cancer cell proliferation and pancreatic tumor growth *in vivo*. This process is impaired by systemic Cu chelation with TM, which leads to a reduction in Cu-dependent mitochondrial cytochrome *c* oxidase activity and reduced ATP levels [50].

4.3. Gynecologic Cancer

Higher Cu levels are found in the serum of ovarian and cervical cancer patients as compared to healthy controls [107–109]. CTR1, CTR2, ATP7A, and ATP7B are important mediators of cisplatin, carboplatin, and oxaliplatin intracellular uptake and efflux, and are clinically relevant as platinum-based chemotherapy is the first-line treatment for ovarian cancer, and other gynecological malignancies [110–116]. Numerous studies have identified the transport mechanisms by which platinum-containing drugs enter cancer cells, are distributed to various subcellular compartments, and are exported from cells via transporters [117]. A majority of the proteins involved in these processes are also involved in Cu homeostasis. CTR1 controls the tumor cell accumulation and cytotoxic effect of these compounds. There is a strong correlation between CTR1 expression and acquired cisplatin (cDDP) resistance among ovarian cancer cell lines, and genetic knockout of CTR1 renders cells resistant to cisplatin *in vivo*. CTR1 transcription is regulated by Cu via Sp1. Once expressed, it is post-translationally modified by the Cu proteosome. cDDP-resistance is reversible by Cu-lowering strategies in ovarian cancer. Cu chelation induces the expression of transcription factor Sp1, which upregulates the expression of Sp1 and CTR1 by binding to its promoter region. High CTR1 expression has been associated with favorable overall survival, progression-free survival, disease-free survival, and treatment response in cancer patients undergoing chemotherapy [116, 118]. It has also been shown that ovarian cancer patients with higher CTR1 expression in their tumors, had favorable outcomes after platinum drug treatment. Within cDDP-resistant cell lines and two ovarian cancer cell lines derived from patients who had failed in platinum-based chemotherapy, cDDP resistance was associated with reduced expression of CTR1. Resistant cells can be preferentially re-sensitized by Cu-lowering agents via upregulation of hCTR1 expression [119]. Deletion of CTR1 reduces the uptake of Cu, cisplatin, and carboplatin, and increases resistance to their cytotoxic effects by 2- to 3-fold. On the contrary, CTR2 knockdown increases cisplatin uptake in both CTR1(+/+) and CTR1(–/–) cells and proportionately enhances in ovarian cancer cell lines [120]. Claudin-3 (CLDN3) and claudin-4 (CLDN4) are major structural molecules forming tight junctions between epithelial cells with essential roles in mediating cDDP resistance in ovarian cancer cells. In ovarian carcinoma 2008 cells, knockdown of CLDN3 and CLDN4 (CLDN3KD and CLDN4KD, respectively) led to cDDP resistance both *in vitro* and *in vivo*. It also caused a reduction in endogenous mRNA levels of CTR1, baseline copper levels, Cu uptake, and Cu-dependent tyrosinase [121]. Another protein involved in Cu homeostasis and trafficking, COMMD1 (Cu metabolism MURR1 domain 1), plays an important role by sensitizing cancer cell lines to cisplatin [122].

4.4. Prostate Cancer

Studies have suggested that concentrations of copper are higher in prostate cancer samples compared to normal tissue in both xenograft mouse models and in humans [123–126]. Prostate cancer cells express high levels of CTR1 and other

chaperone molecules required to maintain intracellular Cu homeostasis. The expression levels of most of these proteins are increased further upon treatment of androgen receptor (AR) positive prostate cancer cells with androgens, thereby increasing CTR1-dependent uptake of Cu into cancer cells. A study utilizing TRAMP mice demonstrated that prostate cancer growth is dependent upon a functional supply of Cu from the liver [127], and that Cu is important for the development of prostate adenocarcinoma from prostatic intraepithelial neoplasia. Endogenous amyloid precursor protein is a Cu-binding protein upregulated in prostate adenocarcinoma and promotes cancer proliferation, thus serving as attractive therapeutic target [128].

4.5. Glioblastoma

Studies comparing serum Cu and Cp levels in patients with primary brain tumors found that the levels of both Cu and Cp were enhanced compared to healthy controls [129]. Furthermore, a study comparing autopsy specimens of intracerebral tumors from 29 patients observed that tumor tissues had higher levels of Cu (along with calcium, magnesium, and sulfur), as compared to normal tissues [130]. Studies of patients with glioblastoma multiforme (GBM) using laser ablation-inductively coupled plasma-mass spectrometry demonstrated that both copper and zinc are enhanced in a peritumoral zone [131, 132]. Metallothioneins were observed to be higher in patients with malignant neoplasms (glioblastoma multiforme) than benign neoplasms (astrocytoma) [129]. Sub-cytotoxic concentrations of Cu(II) sulfate have been shown to induce cellular senescence in GBM cells by downregulating the oncogene B lymphoma Mo-MLV insertion region 1 (Bmi-1) as well as causing reduced cell proliferation, cell enlargement, increased level of senescence-associated β-galactosidase activity, and overexpression of several senescence-associated genes, p16, p21, TGF-β1, insulin growth factor-binding protein 3, and apolipoprotein J [133].

4.6. Hematologic Malignancies

Serum Cu levels and Cp activity in patients with hematologic malignancies (including acute lymphoblastic leukemia (ALL), lymphoma, Hodgkin's disease) are significantly elevated as compared to healthy controls and are associated with poor prognosis in some studies [134–139]. A Cu chelator, ATN-224 (choline tetrathiomolybdate), induces cell death in DLBCL cells independent of Bcl-2, Bcl-xL, or MCL-1 status, and promoted mitochondrial dysfunction, release of apoptosis-inducing factor and induction of caspase-independent cell death [140]. In primary B-cell acute lymphoblastic leukemia patient samples, ATN-224 was shown to decrease cell viability. ATN-224 also caused downregulation of SOD1, cytochrome *c* oxidase activity, and mitochondrial membrane potential, leading to increased formation of intracellular oxidants and peroxynitrite-dependent cell death.

5. PRECLINICAL STUDIES

Numerous preclinical studies have investigated the effects of Cu depletion in a wide array of malignancies. Cu-depleting compounds such as penicillamine and trientine were tested in tumor models, and were shown to have antitumor effects via disruption of angiogenesis and impaired migration [141–143]. However, these agents demonstrated minimal clinical efficacy when tested in early phase clinical trials, and were largely abandoned as a cancer therapeutic strategy [144]. The subsequent development of the more potent and better tolerated anti-Cu agent TM renewed interest in Cu depletion as a therapeutic strategy and led to various areas of active research into its effects on cancer biology.

5.1. Penicillamine

Penicillamine (B-dimethylcysteine) is a Cu-chelating agent first isolated in 1953 and is a metabolic byproduct of the antibiotic penicillin. In addition to binding Cu, it has the ability to chelate several other divalent cations including zinc, nickel, and lead. Its chemical functions also include cleavage of unreduced double bonds in proteins and reactions with aldehyde groups on collagen chains formed by LOX. Its antineoplastic effects were first described in the literature in 1964 in experiments showing single-agent activity against sarcoma-180 tumor cells [145]. The initial studies of penicillamine in the treatment of breast cancer were published in 1981, when Okuyama and Mishina described dose-dependent killing of FM3A murine mammary adenocarcinoma cells after successful serum Cu depletion with penicillamine [146]. Early studies demonstrated that penicillamine has direct effects on p53 function and localization in MCF-7 models [147]. The observed cytotoxic effects of penicillamine appeared to be related in part to its regulation of intracellular nitric oxide levels and inhibition of thymidine incorporation [148, 149]. The formation of nitric oxide by penicillamine contributes to tumorigenesis via activation of the c-SRC kinase, leading to increased cell migration and tumor invasion [150]. *In vivo* studies demonstrated that intratumoral injection of D-penicillamine into mammary adenocarcinoma cells caused significant growth inhibition of tumors in murine models and increasing sensitivity to cyclophosphamide [151, 152]. Gupte and Mumpter and Leung et al. demonstrated that penicillamine increases the formation of intracellular ROS in the presence of elemental Cu, and this Cu-catalyzed oxidation is cytotoxic to MCF-7 breast cancer cells *in vitro* [153, 154]. This group later went on to develop a novel poly-L-glutamic acid conjugate of D-penicillamine with increased cellular permeability which demonstrated cytoxicity in the human breast cancer cell line MDA-MB-468 [155]. More recently, penicillamine has been shown to increase clonogenic cell death *in vitro* when combined with carboplatin or ionizing radiation, suggesting a possible future role as an adjuvant chemo- or radio-sensitizing agent [156].

Penicillamine was first described in Lewis lung cancer models in 1981, where experiments showed that treatment with penicillamine caused decreased tumor growth in C57/BL mice. However, its antineoplastic effects were attributed to

zinc chelation and the role of Cu depletion was not examined [157]. Later studies suggested that the nitrosylated variant of penicillamine, S-nitroso-N-acetyl-penicillamine (SNAP), demonstrated antiproliferative effects via induction of nitric oxide (NO)-mediated apoptosis and cell cycle deregulation [158, 159]. The antiproliferative effects of penicillamine were also demonstrated in mesothelioma tumor models, where Cu depletion led to decreased tumor growth, as well as decreased tumor vascularization, decreased intercellular adhesion molecule (ICAM)/CD54 expression, and increased CD4+ T-cell tumor infiltration [160]. Penicillamine was also shown to have primary protective effects against tobacco-induced carcinogenesis by reducing ROS formation and up-regulation of p53 in lung cancer cell lines [161]. More recently, a novel drug conjugate combining D-penicillamine with the chemotherapeutic idarubicin demonstrated increased cytotoxicity and decreased tumor growth *in vivo* in the NCI-H460 non small cell lung cancer xenograft tumor model [162].

Several studies have investigated the use of penicillamine in the treatment of primary brain tumors. Using a rat VX2 carcinoma brain tumor model, Brem et al. found that successful Cu depletion with penicillamine caused suppression of intracerebral tumor growth and impaired angiogenesis with decreased microvascular vessel formation [141]. Additional studies in a 9L gliosarcoma rat model demonstrated that hypocupric mice treated with penicillamine had impaired pseudopodia formation and decreased tumor invasion on histological examination [142]. Treatment with penicillamine also was associated with decreased vascular density as well as decreased tumor weight [163, 164]. Penicillamine also was able to induce cell adhesion and differentiation in human neuroblastoma cells via induction of NO [165]. Similarly, induction of NO by SNAP was shown to cause decreased cellular proliferation and increased sensitization to ionizing radiation in cultured glioma cells [166]. A recent randomized clinical trial found that treatment with penicillamine in combination with a Cu-restricted diet resulted in decreased serum VEGF in patients with recurrent glioblastoma multiforme undergoing stereotactic radiosurgery, suggesting a possible future role as an anti-angiogenic agent [167].

Penicillamine was demonstrated to have effects in gynecological malignancies. Szalay et al. demonstrated that treatment with penicillamine could inhibit collagenase activity in invasive cervical carcinoma [168]. Treatment with penicillamine also caused re-sensitization of oxaliplatin-resistant cervical cancer cells to platinum chemotherapy via downregulation of the Cu efflux transporter ATP7A in a p53-dependent manner [169]. This is important because p53 gene codes for a protein that is an essential part of the cell cycle, and acts as a tumor suppressor gene in multicellular organs. Similar effects were observed in ovarian cancer models, where treatment with SNAP increased p53 protein levels and induced apoptosis in both cisplatin-sensitive and cisplatin-resistant human ovarian cancer cells [154].

Early studies using a S-91 mouse melanoma model implicated D-penicillamine as having potential anti-neoplastic activity in the treatment of melanoma [170]. Chvapil and Dorr demonstrated that repeated injections of D-penicillamine caused regression of C57BI/J6 mouse melanoma tumors *in vivo*, and that intratumoral administration of the long variant benzyl-ester-D-penicillamine also

caused significant tumor growth inhibition [171]. Other studies using SNAP demonstrated it has dose-dependent cytotoxic effects in B16 melanoma cell lines *in vitro* [172, 173]. However, more recent data has demonstrated that treatment with SNAP causes upregulation of several matrix metalloproteinases (MMPs) in a NO-dependent manner, as well as activation of ERK/p38 [174]. Contrary to prior studies, these data suggest that this nitrosylated variant of penicillamine may promote disease progression of metastatic melanoma.

5.2. Trientine

Triethylene tetramine dihydrochloride (trientine) is a Cu-chelating agent initially developed in 1969, and is considered to have decreased Cu-chelating ability relative to D-penicillamine but with a more tolerable toxicity profile [175, 176]. It has a polyamine structure that allows for Cu chelation, and promotes Cu elimination via urinary excretion [177, 178]. Early studies showed that trientine had antineoplastic effects, as treatment with trientine led to significantly suppressed tumor development in a murine hepatocellular carcinoma (HCC models) [143, 179]. Yoshii et al. demonstrated that trientine caused suppression of tumor angiogenesis, with diminished CD31 expression and decreased endothelial cell proliferation within tumors [143]. Subsequent studies demonstrated that this antiangiogenic effect may be mediated via downregulation of interleukin-8 (IL-8), as treatment with trientine led to diminished IL-8 transcription and lower serum levels of IL-8 in an HUH-7 xenograft murine model [180]. Additional studies began to find antitumor effects of trientine in other tumor types. Hayashi et al. demonstrated that trientine caused delayed tumor growth and smaller tumor volumes in a murine xenograft fibrosarcoma model [181]. This effect appeared to be secondary to direct cytotoxic effects from trientine, leading to increased tumor cell apoptosis in a p38 MAPK-dependent manner [182].

Trientine also appears to have effects as a sensitizing agent to platinum chemotherapy. Experiments by Brown et al. demonstrated that incubation with trientine could resensitize platinum-resistant ovarian cancer cell lines to the cytotoxic effects of cisplatin [183]. These effects were mediated via inhibition of SOD1, a Cu-dependent metalloenzyme that reduces and inactivates ROS. Additionally, cellular uptake of cisplatin was increased in cisplatin-resistant ovarian cancer cell lines after treatment with trientine, suggesting possibly changes in uptake and efflux of platinum drugs [184].

Limited studies have explored the effects of trientine as a therapeutic agent in breast cancer. Lixia et al. demonstrated that trientine, specifically its variant triethylene tetramine, caused senescence of MCF-7 cells *in vitro* as well as dose-dependent cytotoxicity [185]. These effects were mediated via upregulation of the tumor suppressor genes p21 and p53, as well as senescence-associated β-galactosidase. Subsequent studies demonstrated that trientine also inhibits telomerase activity via decreased expression of human telomerase reverse transcriptase, and can act as a chemosensitizer to cyclophosphamide *in vitro* and *in vivo* [186]. More recently, studies have demonstrated that combining trientine with high dose ascorbic acid is capable of upregulating production of ROS in MCF-7

cells, in part via increased oxidation of ascorbic acid and downregulation of the RAS/ERK pathway [187].

5.3. Tetrathiomolybdate

Brewer and Merajver first described the anticancer effects of TM in breast cancer in the 1990s [188]. TM was shown to decrease angiogenesis and tumor growth in HER2-positive and inflammatory breast cancer via downregulation of pro-angiogenic growth factors and NFκB [189]. Subsequent studies demonstrated that TM could function as a chemosensitizer by increasing apoptosis of malignant cells when used in combination with cytotoxic chemotherapeutic agents like doxorubicin [190]. TM can also re-sensitize platinum-resistant breast cancer cell lines to platinum chemotherapy via down-regulation of the Cu-ATPase transporter ATP7A [191]. TM appears to have direct effects on the tumor microenvironment. In murine animal models, treatment with TM was able to prevent Her2/neu-induced mammary tumorigenesis via remodeling of the mammary gland and expansion of the mammary stem cell compartment [192].

TM also appears to have activity in a variety of other tumor types. Khan et al. explored use of TM in combination with cytotoxic radiotherapy in a Lewis lung high metastatic mouse model [193]. They demonstrated that TM could decrease tumor growth when administered prior to ionizing radiation therapy without significant toxicity and despite having no measurable effect of cell proliferation. The second generation compound ATN-224 was also tested in a non-small cell lung model, and was shown to induce cell death via H_2O_2-dependent activation of P38 MAPK and downregulation of the antiapoptotic gene Mcl1 [194]. These studies suggest the potential therapeutic use of TM as monotherapy or in combination with standard therapies in lung cancer.

Numerous studies suggest that TM may have therapeutic benefit in colorectal cancer. Cui et al. demonstrate that treatment with TM can potentiate the cytotoxic effects of oxaliplatin in human colorectal cancer cell lines via upregulation of the Cu uptake transporter 1 (hCTR1) [195]. TM also demonstrates cytotoxic effects as monotherapy in colorectal cancer. Sanjesh and McManus demonstrate that TM-mediated inhibition of SOD1 can cause preferential killing in selected BLM- and CHEK2-deficient CRC cells [196]. Similarly, RAD54B-deficient colorectal cells are selectively killed after treatment with TM, likely via inhibition of SOD1 and increased formation of ROS and DNA double strand breaks.

TM has also been studied for a number of genitourinary malignancies. Van Golen et al. demonstrated in a Dunning rat prostate cancer tumor model that pretreatment with TM prior to tumor implantation could achieve a statistically significant decrease in tumor weight as well as a non-statistically significant trend toward lower numbers of metastases [197]. TM was demonstrated to have chemosensitizing effects in ovarian cancer when used in combination with chemotherapeutic agents such as doxorubicin, mitomycin C, fenretinide, and 5-fluorouracil, with increased apoptosis via increased ROS generation and proapoptotic signaling [198]. TM also appears to impair angiogenesis in ovarian and

endometrial cancer by downregulating HIF-1α, as well as inhibition of the Cu-dependent mitochondrial complex IV and disruption of mitochondrial respiration [199]. In a cervical cancer model, Ishida et al. demonstrated that TM pretreatment caused increased cisplatin adduct levels and increased sensitivity to cisplatin [200].

Merajver and colleagues have conducted numerous preclinical studies exploring the effects of TM in head and neck cancer. Initial studies demonstrated that TM monotherapy could suppress growth and tumor vascularity of HNSCC in an orthotopic murine model [201, 202]. These effects appear to be related to TM's ability to downregulate various proinflammatory and proangiogenic cytokines in HNSCC, including interleukin 6 and basic fibroblast growth factor [203]. TM also appears to interfere with metastatic progression by impaired angiogenesis, decreased tumor cell motility, and invasiveness via inhibition of VEGF, LOX activity, FAK activation, and MMP2 [66, 204]. TM also demonstrated efficacy as a radiosensitizer in HNSCC, where the combination of TM with RT provided decreased tumor growth compared to either treatment alone [205]. Using a xenograft model of TNBC, Chan et al. demonstrated that although primary tumor growth was not affected by TM, lung metastases were markedly reduced, LOX in the pre-metastatic lungs abrogated, and collagen fiber length diminished [206].

Individual publications have investigated the effects of TM in several other malignancies, including mesothelioma, neuroblastoma, medulloblastoma, melanoma, and B cell acute lymphoblastic leukemia [160, 207–210]. Given the abundance of positive preclinical data, continued exploration of TM as a potential therapeutic agent should be pursued for malignancies with limited treatment options and overall poor prognosis.

5.4. Newer Copper Chelators

Disulfiram is a pro-drug, which is converted to its metabolite DEDTC (N,N-diethyldithiocarbamate). DEDTC forms a metal complex (usually with Cu(II) or Zn(II)) that is responsible for the anticancer activity in various cancers including breast, prostate, glioblastoma, lung, melanoma, cervical, CRC, and leukemia [89, 133, 211–214].

Clioquinol is a Cu-depleting agent, targets tumor proteasome in a Cu-dependent manner, to repress the growth of human prostate tumor C4–2B xenografts (by 66 %), and is also associated with *in vivo* proteasome inhibition, AR protein repression, angiogenesis suppression, and apoptosis induction [124].

6. CLINICAL TRIALS OF COPPER DEPLETION IN CANCER

To date, there have been seven phase II clinical trials that examined the effect of TM in the treatment of cancer of various different tumor types. Redman et al. studied TM in a cohort of 15 patients with advanced renal cell carcinoma [215]. Patients were treated with induction doses of TM 40 mg three times daily

with meals, 60 mg at bedtime, with all patients achieving successful Cu depletion as measured by serum Cp. TM was overall well tolerated, yet 11 of 15 patients required dose reductions for granulocytopenia. The clinical benefit was modest with a response rate of 31 % as defined by patients having stable disease. Using a similar dosing schedule of TM, Henry et al. evaluated the use of TM in 19 patients in hormone refractory metastatic prostate cancer [216]. Despite a high rate of successful Cu depletion (89 %), TM was ineffective, as 14 patients had progression of disease on TM therapy, and the study was ultimately discontinued due to lack of efficacy. A subsequent phase II study by Lin et al. trialed a second-generation version of TM, ATN-224 (choline tetrathiomolybdate), in 47 men with biochemically recurrent prostate adenocarcinoma [217]. Patients were randomized to receive either 30 mg or 300 mg of ATN-224 daily, and serum Cp levels were serially monitored. Patients on the high-dose arm had lower mean log Cp levels compared to the low-dose arm; however, no target level of Cp was pre-specified. Approximately 19 % of patients had severe grade 3–4 adverse reactions including neutropenia and leukopenia. Progression-free prostate-specific antigen (PSA) at 24 weeks was demonstrated in 59 % and 45 % of men in the low and high dose arms, respectively. Median PSA progression-free survival was 30 weeks (95 % CI 21–40) and 26 weeks (95 % CI 24–39) in the low- and high-dose arms, respectively, suggesting some potential clinical benefit for this compound in the treatment of prostate cancer.

TM was also investigated as adjuvant therapy in two unique trials. Schneider et al. enrolled 69 patients with locally advanced resectable esophageal cancer to receive adjuvant TM after completion of neoadjuvant chemoradiation and surgical resection [218]. Patients received TM 40 mg three times daily with meals and 60 mg before bed, and serum Cp levels were followed to target Cp level of 5–15 mg/dL. Unfortunately, results were again modest with a 3-year disease-free survival rate of 44 % and 3 year overall survival of 45 %, demonstrating only a slight improvement over historical controls. Grade 3/4 adverse effects were relatively infrequent (6 %). Pass et al. explored the use of TM as adjuvant monotherapy for patients with resectable mesothelioma [219]. This cohort of 30 patients received an induction dose of TM 180 mg daily in divided doses, and all patients successfully achieved the target Cu depletion. Interestingly, patients with stage I or II disease treated with TM demonstrated a time to progression of 20 months, which when compared to 10 months for historic stage I/II patients suggests a potential therapeutic benefit. No progression-free survival improvement was noted for patients with stage III disease. Dose reduction or holding of the dose was required in 54 % of patients, due to grade 3/4 adverse effects, of which granulocytopenia was most common (40 %).

Currently a single arm, Phase II trial led by Linda Vahdat has focused on the potential therapeutic use of TM in the treatment of breast cancer. The trial enrolled 75 patients with either stage III, stage IV NED, or stage II triple negative breast cancer (TNBC) to receive TM either in combination with standard hormonal therapy or as monotherapy [206, 220]. Prior to enrollment, all women had very high risk of relapse, as determined by the clinical stage and molecular subtype of cancer. Patients received an initial induction dose of TM 180 mg in 4 divided doses then transitioned to maintenance TM 100 mg in divided doses

once serum Cp levels were within the target range. At time of publication, 75 patients were enrolled, and 51 had completed at least 24 months of TM therapy. The cohort was comprised of 55 % of patients with stage III disease and 40 % stage IV NED, with a large subset of patients having TNBC (48 %). Treatment with TM was successful in reducing serum Cp levels, with 85 % of patients achieving at least one target range Cp within 8 weeks and 97 % within 16 weeks. Successful Cu depletion was significantly correlated with lower levels of circulating VEGFR2+ endothelial progenitor cells ($p = 0.002$). This study examined the effects of TM on various enzymatic mediators of the pre-metastatic niche microenvironment by measuring serum levels of the Cu-dependent enzyme lysyl oxidase 2 (LOXL-2) in this patient cohort. In preclinical models, LOXL2 was shown to promote invasive and metastatic phenotypes and to have significantly decreased activity after Cu depletion in mouse models. Consistent with this finding, circulating levels of LOXL2 were significantly lower in Cu-depleted patients at 12 months (55 % reduction, $p < 0.001$) and 24 months ($p < 0.001$) of TM treatment. TM was overall well tolerated in most patients, with only a 5.4 % incidence of grade 3/4 hematological toxicity, and no non-hematologic toxicity. Clinical outcomes were encouraging with an overall event free survival and overall survival of 72 % and 84 %, respectively, at a median follow-up time of 6.3 years. Of particular significance are the encouraging outcomes for patients with TNBC, with a 2-year event-free survival for stage II/III TNBC of 90 % and stage IV NED of 69 %. This data suggests improved clinical outcomes for TNBC patients treated with TM when compared to historical controls. Further randomized clinical trials are necessary to confirm this benefit.

7. CONCLUDING REMARKS AND FUTURE DIRECTIONS

Copper depletion, as a therapeutic strategy, has the ability to target multiple key processes important in the evolution of cancer and metastatic spread. Until now, despite strong pre-clinical data, the clinical data have not supported its development as a therapeutic strategy. We believe that this is because Cu depletion strategies were tested in the classic drug development paradigm, which we believe was not the optimal setting. We further believe that if Cu depletion is to be pursued as a therapeutic strategy, it appears to be most effective in reducing the risk of relapse in a high-risk population. The optimal chelator has yet to be determined. Repurposing drugs such as disulfiram and investigating new drugs like methanobactin are promising. Randomized phase 2 clinical trials with biomarker development are necessary to assess whether there is a role for Cu depletion as a therapeutic strategy in breast cancer and other malignancies.

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ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AR	androgen receptor
ATN-224	choline tetrathiomolybdate
ATOX1	antioxidant 1 copper chaperone
ATP	adenosine 5′-triphosphate
BMI	B lymphoma Mo-MLV insertion
BRAF	B-rapidly accelerating fibrosarcoma
CCO/COX	cytochrome *c* oxidase
CCS	copper chaperone for superoxide dismutase 1
cDDP	cisplatin
CI	confidence interval
CLDN	claudin
Cp	ceruloplasmin
CRC	colorectal cancer
CTR	copper transporter
DEDTC	N,N-diethyldithiocarbamate
DSF	disulfiram
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
5-FU	5-fluorouracil
GBM	glioblastoma multiforme
HIF	hypoxia-inducible factor
HNSCC	head and neck squamous cell carcinoma
ICAM	intercellular adhesion molecule
IGF	insulin growth factor
IL	interleukin
LOX	lysyl oxidase
MAPK	mitogen-activated protein kinase
MCL-1	myeloid cell leukemia
MEMO	mediator of cell motility
MET	mesenchymal to epithelial transition
MMP	matrix metalloproteinase
MT	metallothionein
NED	no evidence of disease
NO	nitric oxide
PrP(C)	cellular prion protein
PSA	prostate-specific antigen
ROS	reactive oxygen species
SCO	synthesis of cytochrome *c* oxidase
SNAP	S-nitroso-N-acetyl-penicillamine
SOD	superoxide dismutase
SPARC	secreted protein acidic and rich in cysteine

TAM	tumor-associated macrophages
TGN	trans-Golgi network
TIMP	tissue inhibitors of metalloproteinases
TM	tetrathiomolybdate
TNBC	triple-negative breast cancer
TPEN	N,N,N′,N′-tetrakis-[2-pyridylmethyl]-ethylenediamine
VAP	vascular adhesion protein
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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13

Metal Compounds in the Development of Antiparasitic Agents: Rational Design from Basic Chemistry to the Clinic

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Abstract: Metal compounds seem to be a promising approach in the search of new therapeutic solutions for neglected tropical diseases. In this chapter, efforts in the design of prospective metal-based drugs for the treatment of Chagas disease, human African trypanosomiasis, and leishmaniasis are discussed. Careful selection of the metal center (including organometallic cores) and the types and number of coordinated ligands is essential for controlling the reactivity of the complexes and hence, tuning their biological properties. In a target-based approach, some targets that have been validated for organic antiparasitic compounds are expected to remain targets for metal complexes of these compounds. In addition, specific targets for metal compounds, like parasitic enzymes or DNA, would also be included for these metal complexes leading to potential additive or even synergistic effects between organic ligand and metal ion. However, even though a good number of prospective antiparasitic metal-based drugs have been developed, further systematic efforts are needed for these metal compounds to accomplish the regulatory guidelines that let them reach the different stages of clinical trials.

Keywords: drug design · metal-based antiparasitic compounds · metallomics in parasites · neglected diseases · rational design strategies · trypanosomatid parasites · validation of parasite targets

1. INTRODUCTION

Tropical diseases strictly include those that occur solely, or principally, in the tropics. Nevertheless, the term is often used to refer to infectious diseases, most of them caused by parasites that prosper in hot and humid conditions, such as malaria, leishmaniasis, schistosomiasis, onchocerciasis, lymphatic filariasis, Chagas disease, African trypanosomiasis, and dengue [1]. Although they represent a tremendous burden to society, many of these infectious diseases have historically received little attention of the pharmaceutical industry due to little prospect of generating financial profit. They are considered neglected tropical diseases (NTDs) by the World Health Organization because of the restricted development of diagnostic methods, therapeutic treatments and/or control strategies. NTDs are a group of seventeen communicable and poverty-related diseases that affect about one billion people living in 149 countries. The worst affected populations are those living in poverty, without adequate sanitation and in close contact with infectious vectors, domestic animals and livestock [2].

This chapter focuses on general rational design strategies in the development of metal compounds as prospective agents against three parasitic illnesses that are major health concerns in the developing world: American trypanosomiasis, human African trypanosomiasis (HAT), and leishmaniasis. These NTDs were selected to be studied together because they are caused by genetically related parasites belonging to the trypanosomatid genus and kinetoplastida order [3].

The genoma of the three main trypanosomatid parasites responsible of these diseases show a high percentage of common proteins that could offer the opportunity to develop wide spectrum drugs that could affect more than one of these related parasites [4]. Having in mind that some of these diseases are simultaneously prevalent in the same regions of the world, like leishmaniasis and Chagas disease in South America or leishmaniasis and HAT in Africa, this kind of development would favor the availability of cheap drugs suitable for the treatment of more than one disease at the same time.

1.1. American Trypanosomiasis

American trypanosomiasis or Chagas disease is an ancient chronic illness caused by the flagellated protozoan parasite *Trypanosoma cruzi* (*T. cruzi*). It is transmitted to the mammalian host by blood-sucking triatomine bugs. The initial phase of the disease, the acute one, is often asymptomatic and remains undiagnosed leading to a prolonged chronic phase that can induce the development of cardiac and gastrointestinal syndromes. Chagas disease is endemic in rural regions of Latin America. There are around 8 million infected people, 10,000 annual deaths, and approximately 25 million people live in risk zones [5]. In Latin America and the Caribbean region, the burden of the disease is five times greater than that of malaria. Due to intense migration flow, in the last decades the number of cases in non-endemic regions, like United States, Australia, Europe, and Japan, has been increasing.

Non bug-associated transmission, i.e., transfusion of contaminated blood, organ transplants, and transmission from infected mothers to newborns, is the cause of the observed rise of infected patients in the mentioned countries. In

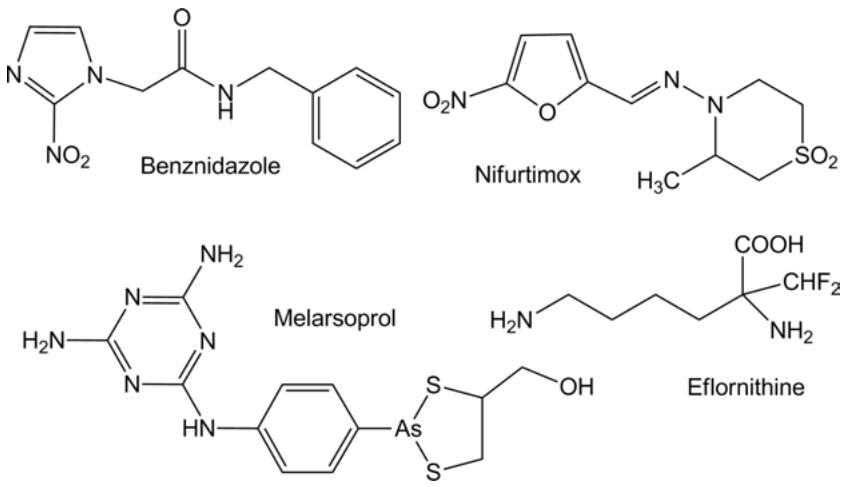

Figure 1. Current chemotherapeutic drugs for the treatment of American and African trypanosomiasis.

addition, this situation is favored by the lack of adequate controls together with a general lack of familiarity with the disease, in particular with the identification of its symptoms [6–9]. The life cycle of the parasite includes several stages of development in the insect vector and in the mammalian host. Some stages include replicative forms, i.e., epimastigotes within the midgut of the insect vector and intracellular amastigotes within the mammalian host. Others are non-replicative infective life forms, i.e., metacyclic trypomastigote in the feces of the insect vector and trypomastigotes in the bloodstream of the mammalian host [10]. Current chemotherapy is based on two drugs developed almost 50 years ago: Benznidazole and Nifurtimox (Figure 1). Both show high toxicity that leads to severe side effects together with variable results associated with different susceptibility and drug resistance of the different parasite strains [11].

1.2. Human African Trypanosomiasis

Human African trypanosomiasis or sleeping sickness is a parasitic disease caused by two strains of *Trypanosoma brucei* (*T. brucei*): *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. It is transmitted by the bite of an insect, commonly known as the tsetse fly, and it is one of the most neglected diseases. It is restricted to poor regions of Africa affecting mainly people living in rural areas. *T. brucei rhodesiense* causes the most virulent form of the disease and is usually fatal if left untreated [12]. In the first stage of the illness, the parasite is in the host's blood, but then the infective form crosses the blood-brain barrier causing weakness and finally death. Only very few active compounds are available for the treatment of this infection. Current therapies suffer from several drawbacks due to toxicity or emergence of resistant forms of the parasite. Some of the currently used drugs, Melarsoprol B and Eflornithine, are shown in Figure 1. The animal form of the disease (called Nagana) is also threatening as serious losses in domestic animals affect breeding within the rural areas [13–15].

1.3. Leishmaniases

Leishmaniases are a group of diseases caused by protozoan parasites from more than twenty *Leishmania* species. These parasites are transmitted to humans by the bite of an infected female phlebotomine sandfly insect vector. There are three main forms of the disease: cutaneous, visceral or kala-azar, and mucocutaneous. Cutaneous forms of the disease produce skin ulcers on parts of the body such as the face, arms, and legs. Mucocutaneous forms show lesions that can lead to partial or total destruction of the mucous membranes of the nose, mouth, and throat and surrounding tissues. Visceral leishmaniasis leads to fever, weight loss, swelling of the spleen and liver, and anemia and can lead to death. Leishmaniasis

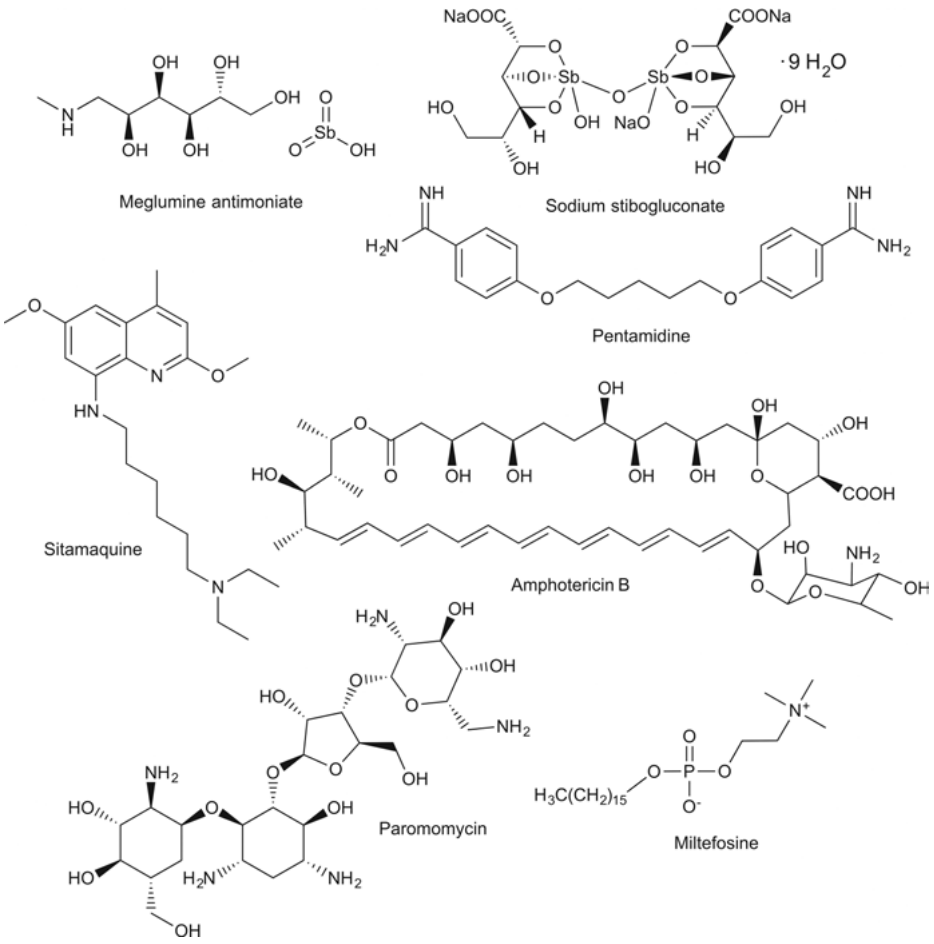

Figure 2. Current drugs for the treatment of leishmaniasis.

currently threatens 350 million people in 88 countries around the world being prevalent in four continents. Almost 90 % of mucocutaneous leishmaniasis cases occur in Bolivia, Brazil, and Peru. The majority of visceral and cutaneous leishmaniasis cases occur in regions of East Africa and South America. *Leishmania*–HIV co-infection intensifies the burden of visceral and cutaneous leishmaniasis by causing severe forms that are more difficult to manage. The spread of HIV infection brought the visceral form to new geographical areas. Indeed, co-infection has been reported in 34 countries in Africa, Asia, Europe, and South America. In Europe, intravenous drug users have been identified as the main population at risk. Currently used drugs are shown in Figure 2 [16, 17].

1.4. Metal Compounds as Antiparasitic Agents

The main common problems of current drugs for the treatment of diseases caused by trypanosomatid parasites are their severe side effects, the need for prolonged treatment regimes, and the emergence of resistance. New improved treatment options and new drugs for the chemotherapy of these three diseases are urgently needed [18].

In this sense, the development of metal-based drugs has shown to be a promising approach to find a pharmacological answer to parasitic diseases. Although many of the metal-based drugs currently on the market have been discovered by chance, attempts to get new prospective metallodrugs through a rational design approach are the current focus of medicinal inorganic chemists [19]. Most efforts have been made in the cancer therapy field while, in the case of antiparasitic metal-based drugs, this is an almost unexplored area that deserves intense investigation [20, 21].

In the search of new antiparasitic agents through a target-based approach, metal compounds seem to be particularly suitable as they have the potential of interacting with selected parasitic enzymes and biomolecules [18, 22]. On the other hand, metal complexes are ideal candidates for the design of a multi-target therapy using a single chemical entity that acts simultaneously on multiple targets through not only the metal but through different antiparasitic agents included in the molecule as ligands [23, 24]. In addition, it has been observed, for both organic and metal-based drugs, that a number of antitumor agents have shown antitrypanosomal effects and conversely, several antitrypanosomal compounds demonstrated activity against experimental tumors [25, 26]. This mutual action leads to think on the existence of common features and shared targets in tumoral cells and parasites. For example, the metabolism of the predominant bloodstream form of African trypanosomes is quite similar to those of some tumor cells. In particular, both undergo a high rate of aerobic glycolysis due to inefficient or non-functional mitochondrial systems [27]. These similarities support the potential of metal compounds, which have been widely studied in the cancer field, as antiparasitic drugs. As it will be stated below in this chapter, many of the strategies used for the rational design of prospective antiparasitic metallodrugs are based on the resemblances of tumor cells and parasite. In particular, most of the selected metal centers are related to those that have proved to be useful in cancer therapy, like platinum group metals (Pt, Pd, Ru, Rh, Os, Ir) while essential metal ions have been less explored.

In this frame, in the last twenty years, the identification of some prospective metal-based drugs against highly prevalent parasitic illnesses, such as Chagas disease, malaria, leishmaniasis, and amoebiasis has been accomplished [20, 21, 28–32].

This chapter will focused on all stages of the development of prospective antiparasitic metal-based agents. The basic chemical and biological bases of the rational design will be discussed as well as the identification and validation of the parasite target. The general features that could push a metal-based antiparasitic compound into the clinic will be also analyzed.

2. RATIONAL DESIGN OF PROSPECTIVE AGENTS

2.1. General Strategies

The rational design of prospective agents against the selected parasitic diseases has been mainly based on one of the following strategies:

(i) Coordination of an organic molecule bearing antiparasitic activity (an established drug or not) to different metal ions in order to improve its bioavailability, to achieve a metal-drug synergism through a dual or multiple mechanism of action and/or to circumvent resistance mechanisms. The metal complex could act by itself on different parasitic targets or the metal could act as a "chaperone" delivering the active organic compound [28, 33].

(ii) Developing metal complexes pointing to DNA as a potential parasite target. This strategy includes metal coordination of ligands with DNA intercalating capacity or the design of chemical structures that could allow the covalent interaction of the metal center with nucleobases or DNA phosphates. This approach was inspired, as previously stated, in that highly proliferative cells, tumors, and trypanosomatid parasites show many metabolic similarities [25].

(iii) Metal coordination of non-bioactive innocent ligands that could deliver metal ions and/or metal-containing fragments to parasite-specific enzymes in order to produce inhibition of the enzymatic activity. Metal ions can coordinate to active site residues blocking the interaction with the substrate or can cause conformational changes in the enzymatic structure by coordination to residues outside of the active site that lead to inactivation of the enzyme [29].

In order to accomplish the pharmacological objectives of the different metal complexes described above, a very strict control of all the variables that will affect their chemical behavior should be considered. The choice of the metal, its oxidation state, the types and number of coordinated ligands, and the coordination geometry are essential for controlling the reactivity of the complexes and tuning their properties. This selection should be done not only considering the biological aspects of the complexes' design but also the chemical ones. From a chemical point of view, not any organic molecule can be used as ligand for any metal ion. Both thermodynamic and kinetic stability of the prospective complexes should be considered as the compound will probably encounter biological ligands that can modify its composition through ligand exchange reactions. Even though some of these reactions could be beneficial for the action (as it is for the anticancer drug cisplatin) they should be known and controlled. The lability of metal–ligand bonds can be tuned by the choice of metal ion, its oxidation state, and the inclusion of auxiliary co-ligands [19, 34].

In addition to the variety and complexity that classical coordination complexes give to the design of prospective metal-based antiparasitic agents, more recently, organometallic compounds have been included in this field contributing with novel modes of action and high structural diversity [21].

In the following section we will describe, based on some representative examples, the basis of the rational design of prospective antiparasitic agents, in particular in those aspects related to the selection of the metal ions (or organometallic core), the ligands, co-ligands, and counterions.

2.2. Selection of the Ligand

The selection of the principal ligand in the rational design of a prospective metal-based antiparasitic agent will be mainly dependent on which of the strategies previously described is intended to be applied. On one hand, the coordination of ligands bearing antiparasitic activity could include those drugs that are being used to treat the corresponding illness or others (repositioning) or other compounds that have proved to present this activity *in vitro* and/or *in vivo*. On the other hand, when intending to target DNA, potential DNA intercalating ligands could be used. The role of ancillary ligands will also be discussed.

2.2.1. Antiparasitic Drugs

Benznidazole (N-benzyl-2-(2-nitro-1H-imidazol-1-yl)acetamide, Bz, Figure 1) is the most frequently prescribed drug for the treatment of Chagas disease. However, it exhibits undesirable side effects related to redox damage to the host's tissue and the appearance of resistance of some *T. cruzi* strains. Aiming to obtain a more active species that allows lowering the required dose and to circumvent resistance, two RuBz complexes were obtained. On one hand, in order to enhance water solubility, Bz was included as ligand in a ruthenium(II) tetraammine core [35]. The obtained complex $[Ru^{II}(Bz)(NH_3)_4SO_2](CF_3SO_3)_2$ (Figure 3a) was much more active *in vitro* against both epimastigote (IC_{50} = 127 μM) and trypomastigote (IC_{50} = 79 μM) forms of *T. cruzi* than the free Bz (IC_{50} > 1 mM for both forms). In addition, in an acute model of Chagas disease a very low dose (385 μmol/kg/day) of this complex was able to protect infected mice, eliminating the nests of amastigotes in myocardium tissue and skeletal muscles, without observed acute toxicity.

Based on a similar approach, $[Ru^{II}(NO_2)(bpy)_2(Bz)](PF_6)$ (bpy = bipyridine) was also obtained (Figure 3b) [36]. This complex was designed as a potential NO-donor intending to obtain a synergistic effect of two different antiparasitic actions in a single compound. NO is a major mediator produced by infected cardiac myocytes and macrophages in response to IFN-γ and TNF-α, with pronounced trypanocidal activity via an oxidative stress-dependent mechanism. The obtained complex resulted in being more effective than the same concentrations of Bz against both trypomastigote and the intracellular amastigote forms of the

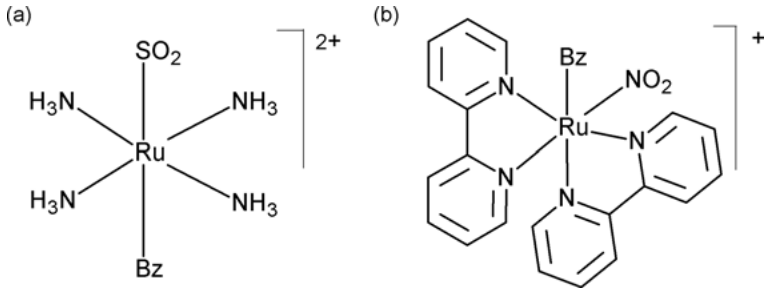

Figure 3. Ruthenium benznidazole complexes (**a**) $Ru^{II}(Bz)(NH_3)_4SO_2](CF_3SO_3)_2$ and (**b**) $[Ru^{II}(NO_2)(bpy)_2(Bz)](PF_6)$.

parasite, with no cytotoxic effect in mouse cells. *In vivo* treatment with the compound improved the survival of infected mice and significantly diminishing tissue inflammation and parasitemia.

2.2.2. Repositioning of Drugs

In order to face the high costs of developing new drugs, one of the approaches used in the search of new antiparasitic drugs is the repositioning of compounds that were previously developed for an alternative use. Because most repositioned drugs have already passed the early phases of development and clinical testing, they can potentially win approval in less than half the time and at one quarter of the cost. The repositioning or repurposing concept is not new for the treatment of neglected tropical diseases. Many drugs that are currently used to treat them were repositioned from other diseases like fungal, bacterial or helmintic infections as well as for cancer. For example, the antifungal amphotericin B and the anticancer agent miltefosine were both repurposed for the treatment of visceral leishmaniasis. Other compounds, like antifungal azoles (posaconazole and ravuconazole) have reached advanced clinical phases for the treatment of Chagas disease [18, 37, 38].

From the antiparasitic metal-based agents' point of view, some of these repurposed compounds have been used as ligands for the development of different metal complexes in order to improve their pharmacological properties related to their potential new use. Pioneering work by Sánchez-Delgado and Anzellotti [28] led to the development of metal complexes of the well-established antifungals clotrimazole (CTZ) and ketoconazole (KTZ) intended for antitrypanosome therapy. These drugs act on fungi by blocking the enzyme cytochrome P_{450} 14α-demethylase involved in the production of ergosterol that is also essential for the trypanosomatid parasites. In particular, $[Ru^{II}Cl_2(CTZ)_2]$ (Figure 4a) displayed *in vitro* an anti-*T. cruzi* activity a thousand times higher than free CTZ, with no toxicity to mammalian cells. Unfortunately, the low water solubility of the complex prevented its effects *in vivo*.

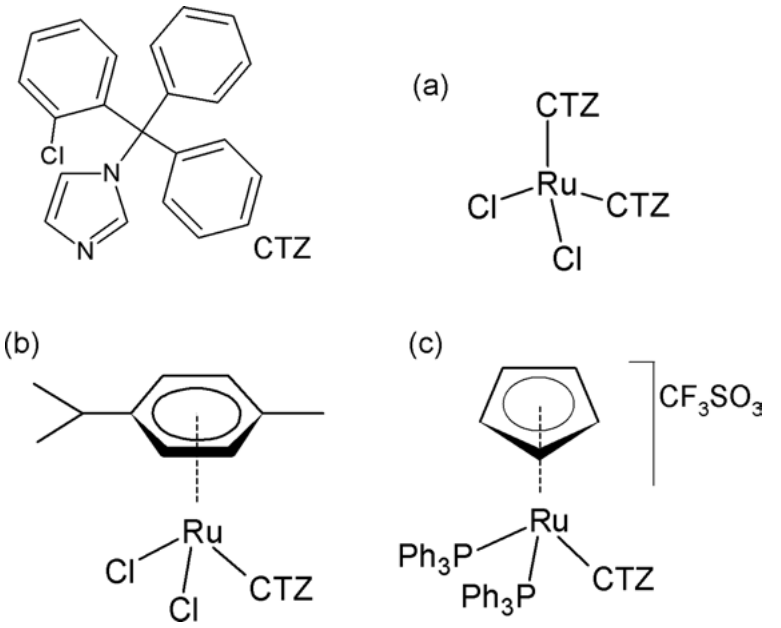

Figure 4. Ru-CTZ complexes: (**a**) $[Ru^{II}Cl_2(CTZ)_2]$, (**b**) $[Ru^{II}(\eta^6\text{-}p\text{-cymene})Cl_2(CTZ)]$, and (**c**) $[Ru^{II}Cp(PPh_3)_2(CTZ)](CF_3SO_3)$.

However, based on these promising results, Sánchez Delgado's group developed other Ru(II) and Ru(III)-CTZ coordination compounds as well as a series of organometallic $[Ru^{II}(\eta^6\text{-}p\text{-cymene})Cl_2(CTZ)]$ compounds [39]. In all those complexes, different ancillary ligands (dimethylsulfoxide, bipyridine, ethlylenediamine, acetylacetonate) were included in order to modulate the physicochemical properties of the complexes and in particular to improve their water solubility. The organometallic piano stool complex $Ru^{II}(\eta^6\text{-}p\text{-cymene})Cl_2(CTZ)$ (Figure 4b) was 110 and 58 times more active *in vitro* than free CTZ against *L. major* and *T. cruzi*, showing IC_{50} values in the submicromolar range and excellent selectivity indexes (SI) (greater than 500 and 75 for *L. major* and for *T. cruzi*, respectively. SI = IC_{50} mammalian cell model/IC_{50} parasite). *In vivo* studies showed that this compound significantly reduced the lesion size in mice exposed to *L. major* cutaneous infection. A mitochondria-dependent apoptotic-like death in the extracellular form of the parasite was observed [40]. Recently, a new Ru-CTZ organometallic compound $[Ru^{II}Cp(PPh_3)_2(CTZ)](CF_3SO_3)$ (Figure 4c) (*Cp* = cyclopentadienyl fragment) was described by our group. Results showed that complexation of the bioactive CTZ to the $\{RuCp(PPh_3)\}$ moiety also leads to a significant increase of the antiparasitic activity *in vitro* showing a 6-fold and 40-fold higher activity than free CTZ on *T. cruzi* and on the infective form of African trypanosomes, respectively [41].

Another example of this approach is the use of metal complexes of bisphosphonates. Nitrogen-containing bisphosphonates (NBP) are well-established drugs for

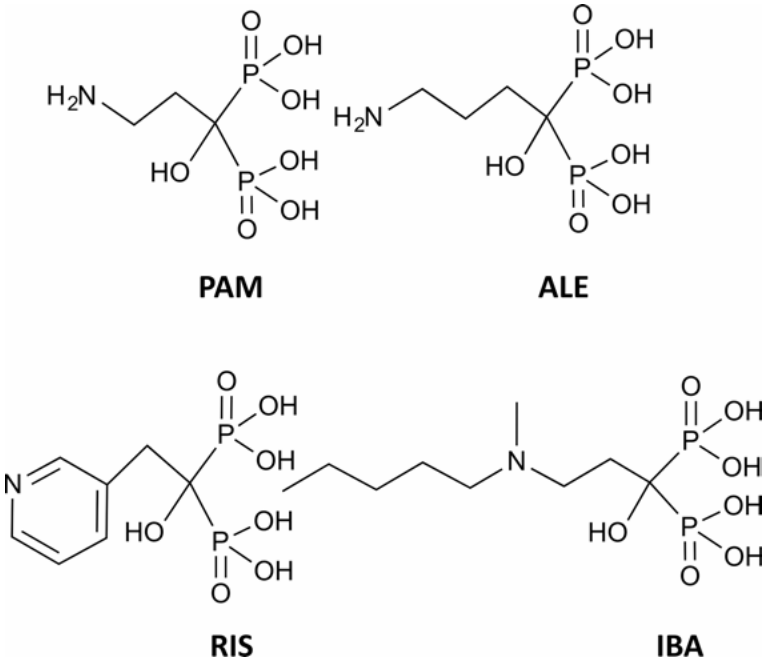

Figure 5. Some commercial bisphosphonates: risedronate (RIS), alendronate (ALE), pamidronato (PAM), and ibandronate (IBA).

the prevention and treatment of diseases associated with excessive bone resorption, including Paget's disease of bone, myeloma, bone metastases, and osteoporosis but they also showed activity against trypanosomatid parasites. The main mechanism of action of NBPs is the inhibition of the farnesyl diphosphate synthase (FPPS) enzyme of the osteoclastic cells. This enzyme is present in trypanosomatid parasites as well and, in addition, the accumulation of NBPs into parasitic specific organelles called acidocalcisomes would facilitate their action [42]. Our group developed first-row transition metal complexes with the NBPs risedronate (RIS), alendronate (ALE), pamidronato (PAM), and ibandronate (IBA) as ligands (Figure 5) [43–45]. Results showed that the coordination of these bisphosphonates to Cu(II), Co(II), Ni(II), and Mn(II) improved their antiproliferative effect against *T. cruzi* intracellular amastigotes exhibiting IC_{50} values in the low micromolar levels with no or little toxicity on mammalian *Vero* cells up to 100 μM.

2.2.3. Bioactive Ligands

In a similar approach to that presented in Sections 2.2.1 and 2.2.2, organic molecules that have shown antiparasitic activity could be selected as ligands for the development of metal-based prospective antiparasitic agents.

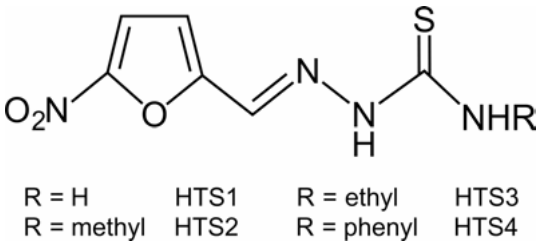

Figure 6. Bioactive 5-nitrofuryl-containing thiosemicarbazones.

Our group has extensively worked on the rational design of metal complexes with a series of 5-nitrofuryl-containing thiosemicarbazones (HTS) (Figure 6) as ligands. These compounds, containing the 5-nitrofurane pharmacophore, were obtained as nifurtimox (Figure 1) analogs and they showed very good activities *in vitro* against *T. cruzi*, but they were rather toxic against mammalian cells [46]. In order to improve the pharmacological properties of these compounds more than eighty classical and organometallic compounds have been obtained. These compounds have been designed in a rational manner in order to study the effect of performed modification on the antiparasitic activity and the mechanism of action of these compounds. Different metal centers (palladium, platinum, ruthenium, rhenium), different oxidation states (Ru(II) and Ru(III)) and the inclusion of different organometallic cores (Ru*p*-cym = Ru *p*-cymene, Ru*Cp* = Ru-cyclopentadienyl, $Re(CO)_3^+$) were assayed. In addition, different co-ligands (dimethylsulfoxide, triphenylphosphine, PTA = 1,3,5-triaza-7-phosphaadamantane, dppf = 1,1′-bis(diphenylphosphino)ferrocene) were included in the metal coordination sphere in order to modulate some of the physicochemical properties of the complexes like lipophilicity or water solubility [21, 47, 48].

Most obtained compounds were active *in vitro* against *T. cruzi* and in most cases an enhanced activity with respect to the free thiosemicarbazone ligand was observed because of complex formation. Among all the obtained compounds, those containing organometallic cores ($[Ru^{II}Cp(PPh_3)(TS)]$ and $[Ru_2^{II}(p\text{-cymene})_2(TS)_2]^{2+}$) showed the most promising results. These compounds were also tested as anti-*T. brucei* agents. In particular, $[Ru_2^{II}(p\text{-cymene})_2(TS4)_2]^{2+}$ (TS4 = monodeprotonated *N*-methyl derivative of 5-nitrofuryl-containing thiosemicarbazone) showed higher activity against trypomastigotes of *T. cruzi* than the reference drug nifurtimox and a very significant increase in growth inhibitory activity on *T. brucei brucei* with respect to the free HTS4 ligand ($IC_{50} > 100$ μM). Furthermore, selectivity was also evidenced for this compound, as it was about 50-fold more potent on this parasite than on the studied mammalian cell line [47, 48]. $[Ru^{II}Cp(PPh_3)(TS2)]$ (TS2 = *N*-methyl derivative of 5-nitrofuryl-containing thiosemicarbazone) showed very good activities against both parasites (IC_{50} *T. cruzi* = 0.41 μM; IC_{50} *T. brucei brucei* = 3.5 μM) as well as very good selectivity towards *T. cruzi* (SI > 49) and a good selectivity towards *T. brucei brucei* (SI > 6) [49].

On the other hand, all $[M(dppf)(TS)]^+$ (M = Pd^{II} or Pt^{II}) compounds were more active against both *T. cruzi* and *T. brucei* than the corresponding free

thiosemicarbazones (unpublished results). The magnitude of this increase appears to have resulted from the inclusion of the dppf co-ligand, as most complexes were more active than those previously reported [MCl_2(HTS)], [$M(TS)_2$] or [MCl(PTA)(TS)]. In addition, the presence of dppf also increased the selectivity of the compounds that were not toxic at concentrations up to 50 μM on EA.hy926 mammalian cells. It should be noted that a good correlation between antiparasitic activities against both *T. cruzi* and *T. brucei* was observed for these compounds. Based on these results, all of them are good candidates for *in vivo* studies.

2.2.4. DNA Intercalators

In order to include DNA as a potential target in the design of prospective antiparasitic metal-based compounds, ligands that can have the ability to intercalate DNA bases were selected. An example of this approach, includes various families of $V^{IV}O$-compounds with polypyridyl ligands (NN) (Figure 7) [32, 50].

Both homo- and heteroleptic families of compounds, [$V^{IV}O(SO_4)(H_2O)_2$(NN)] and [V^{IV}O(L-2H)(NN)], where L are tridentate salicylaldehyde semicarbazone derivatives, showed moderate to very good *in vitro* activities against *T. cruzi* epimastigotes. In both cases, the nature of the NN ligand was the main determinant for antitrypanosomal activity. Among the twentyfive heteroleptic compounds obtained, those including aminophen as intercalating ligand were the most active, showing IC_{50} values in the submicromolar range. It should be noted that these compounds were 9.6- to 18.5-fold more active than aminophen itself, so the effect of vanadium complexation is also relevant for the activity. For these compounds, a QSAR study was performed which confirmed the correlation between the nature of the NN ligand and the *in vitro* activity, and that lipophilicity of the compounds is the most significant parameter. On the other hand, DNA interaction of the complexes was assessed through different techniques: agarose gel electrophoresis, viscosity measurement of DNA solutions, and atomic force microscopy.

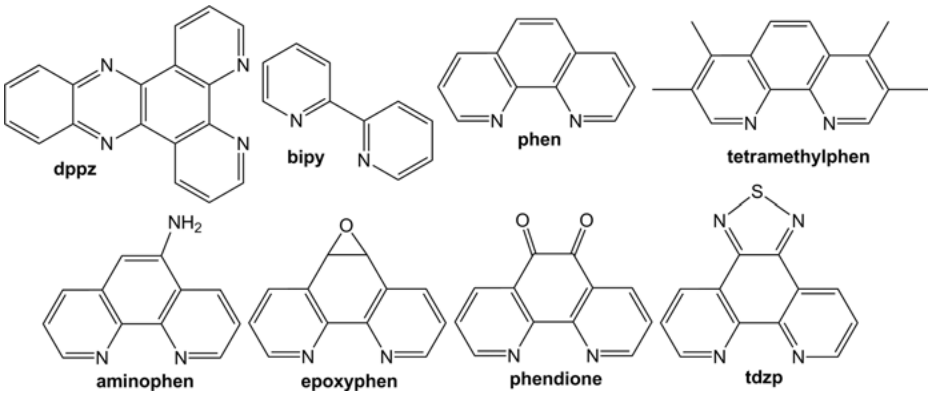

Figure 7. Potential DNA-intercalating ligands.

2.3. Selection of the Metal Center

The selection of the metal center for the design of prospective metal-based antiparasitic agents should be based not only in considerations related to its biological function but also in relation to the chemical nature of the corresponding ligands.

The choice between essential *versus* non-essential metal ions in the design of metal-based drugs is generally based on toxicity arguments. However, as is well known, toxicity is a dose-dependent matter. Essential metal ions can become toxic at high doses as well as the so called toxic ones can be well tolerated in low doses. In addition, the same element may be beneficial or noxious depending on speciation (the nature and stability of the whole metal compound) [51]. Despite this fact, as previously stated, a high percentage of the developed metal-based antiparasitic compounds include non-essential metal ions and, in particular, those that have proved to be useful in cancer therapy.

2.3.1. Essential Metal Ions

Previously described heteroleptic V^{IV}-O complexes with semicarbazones and DNA intercalating ligands showed good activities against trypanosomatid parasites and they were much more active than the polypyridyl compounds included in their coordination spheres. This fact evidenced that the presence of the V^{IV}-O core is determinant of the activity. The selection of vanadium as metal center was based on the potential of vanadium species to interact with ATPases relevant for calcium homestasis of the parasite, their ability of inducing reactive oxygen species (ROS), and of being subversive substrates of the specific parasitic enzyme trypanothione reductase [31]. In addition, from the chemical point of view, the selected ligands were appropriate for vanadium complexation.

Essential metal ions were also selected for the development of metal-based antiparasitic agents with commercial bisphosphonates as ligands. A significant clinical disadvantage of bisphosphonates is their poor oral bioavailability, due to the high ionization of phosphonate groups at physiological pH. This disadvantage could potentially be attenuated by coordination to a metal ion through the phosphonate groups. The selection of first-row transition metal ions in the $+2$ oxidation state was based on the chemical nature of these coordinating groups. As previously stated, nearly all obtained coordination compounds of these bisphosphonates were more active against *T. cruzi* amastigotes than the corresponding free ligands [43–45].

2.3.2. Other Pharmacologically Interesting Metal Ions

Based on the reported metabolic similarities between tumor cells and highly proliferative trypanosomatid parasites, metal ions that were successfully investigated for the development of antitumoral metal-based drugs were also selected for the search of antiparasitic metallodrugs. Platinum, palladium, ruthenium,

gold, and rhenium, among others, have been the most intensively studied. As it has been stated throughout this chapter, the biological activity of metal complexes is not only dependent on the nature of the metal ion but also on the ligands, co-ligands, and counter ions. Therefore, it is not possible to state which of these metal ions would be preferred for the development of antiparasitic metal-based drugs. Successful examples of all of them can be found in the literature [20, 21, 28–32]. However, ruthenium, which has demonstrated to be a promising alternative to platinum in cancer therapy, seems to have some advantages for the antiparasitic action as well. Similar to highly proliferative tumor cells, parasites need high concentrations of iron for carrying out essential biological functions and also for virulence. Thus, by mimicking iron, the uptake of ruthenium compounds into the parasite could be favored. In particular, the binding of ruthenium antiparasitic compounds to transferrin could be a suitable "trojan horse" for the entrance of ruthenium-based antiparasitic drugs into the parasite.

In addition, from a chemical point of view, ruthenium has two stable oxidation states, Ru(II) and Ru(III), both octahedral and accessible in physiological media and the redox potential of which can be finely tuned selecting the appropriate ligands leading to the possibility of *in vivo* interconversion. The chemical relative "softness" of both ruthenium oxidation states makes them avid for ligands with sulfur and nitrogen donors, which are ubiquitous in the major biomolecules, like DNA, serum and cellular proteins, and enzymes. Moreover, the rate of ligand exchange of ruthenium compounds is able to balance the inertness required for the compound to reach its target site (e.g., DNA), to minimize unnecessary side effects, and to allow the sometimes required activation necessary for binding to the targets. Finally, ruthenium has the possibility of generating both classical coordination compounds and organometallic ones [20].

2.3.3. Organometallic Cores

Bioorganometallic compounds have at least one σ metal–carbon bond and are relevant in a biological context. They have been mainly studied as prospective antitumor drugs leading to compounds that have entered or are entering clinical trials. Organometallic compounds have also been proposed as an alternative for the rational design of metal-based antiparasitic drugs offering many advantages with respect to classical coordination compounds. A high structural variety, the possibility of achieving kinetically stable compounds, the lipophilic nature that could favor the *in vivo* behavior, and an interesting redox chemistry can be emphasized [21, 52].

Many different organometallic cores have been assayed: ferrocenyl derivatives, Ru-arene, *fac*-$\{M(CO)_3\}^+$ with M = Re or Mn, M-COD (COD = 1,5 cyclooctadiene, M = Rh, Ru, Ir), cyclometallated M–C bonded ligand (M = Pd, Au) among others [21]. As previously stated for classic coordination complexes, biological properties of the developed organometallic compounds are not only dependent on the organometallic core. Therefore, it is difficult to compare its effectiveness in the generation of a prospective antiparasitic agent. However, results recently reviewed by us point to the Ru-arene moiety as the most promising scaffold for

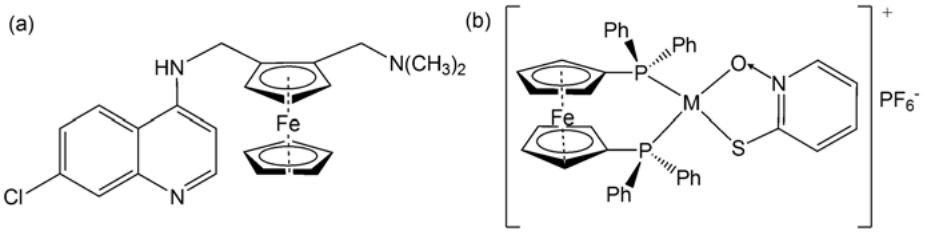

Figure 8. Structure of (**a**) ferroquine and (**b**) M-dppf-mpo complexes (M = Pd or Pt).

the development of prospective drugs against trypanosomatids, based on bioactive ligands. High activities and selectivities were obtained together with simultaneous good activities on more than one trypanosomatid parasite. Successful examples including Ru-arene moieties have been described in previous sections [21].

On the other hand, based on the success of ferroquine (Figure 8) for malaria treatment, ferrocenyl derivatives have been developed as prospective drugs against trypanosomatid parasites. Two main strategies have been assayed: covalent bonding of the ferrocenyl fragment to a pharmacologically interesting organic molecule (like in ferroquine) or its inclusion as part of a ligand (like dppf) in heterobimetallic compounds.

One successful example of the second strategy involves the Pd(II)-Fe(II) and Pt(II)-Fe(II) heterobimetallic compounds that include pyridine-2-thiolato-1-oxide (mpo) as bioactive ligand (Figure 8). Both $[M(dppf)(mpo)]PF_6$ (M = Pd or Pt) complexes showed activities against the epimastigote form of *T. cruzi* in the nanomolar range, about 10–20 times higher than the reference drug nifurtimox and two to five times higher than the free mpo ligand. Even though the anti-*T. cruzi* activity displayed by the new compounds was similar to that of the previously developed classical coordination compounds $[Pd(mpo)_2]$ and $[Pt(mpo)_2]$, the inclusion of the dppf ligand leads to a lower unspecific cytotoxicity and hence, higher selectivity to the parasites [53].

2.4. Selection of Co-ligands and Counterions

The possibility of including in a metal coordination sphere, together with the bioactive ligand, other co-ligands is one of the advantages of the rational design of metal-based prospective drugs. These co-ligands can be selected in order to fine-tune the chemical and physicochemical properties of the compounds like solubility, lipophilicity, kinetic and thermodynamic stability, and redox potential of the metal ion among many others. An adequate lipophilicity and water solubility are desired properties when developing a prospective drug. In this sense, different co-ligands have been included in the developed antiparasitic metal compounds in order to modulate these properties.

As previously discussed, the inclusion of an organometallic fragment like that present in dppf is a good strategy when intending to obtain lipophilic compounds. For this same purpose, triphenylphosphine (PPh_3) has also been used as co-ligand.

On the other hand, ligands like dimethylsulfoxide (DMSO), acetylacetonate (acac), ethylenediamine (en), and 1,3,5-triaza-7-phosphaadamantane (PTA) among others give rise to more hydrophilic and sometimes water-soluble compounds.

Based on this approach, four series of ruthenium complexes with the previously described 5-nitrofuryl-containing thiosemicarbazones (Figure 6) as bioactive ligands and different co-ligands were developed. Compounds of the formula $[Ru^{II}Cl_2(HTS)_2]$, $[Ru^{III}Cl_3(DMSO)(HTS)]$, $[Ru^{III}Cl(PPh_3)(TS)_2]$, $[Ru^{II}Cl_2(HTS)(HPTA)_2]Cl_2$ (TS = deprotonated form of the thiosemicarbazone ligand, HPTA = protonated form of PTA) were evaluated *in vitro* against *T. cruzi*. Complexes of the series $[Ru^{II}Cl_2(HTS)_2]$, $[Ru^{III}Cl_3(DMSO)(HTS)]$, and $[Ru^{III}Cl(PPh_3)(TS)_2]$ were extremely insoluble and poorly active whereas the inclusion of the hydrophilic phosphine PTA in the $[Ru^{II}Cl_2(HTS)(HPTA)_2]Cl_2$ complexes makes them, as expected, quite water-soluble but their biological activities did not increase. On the other hand, a good correlation between the obtained lipophilicity of the complexes and their *in vitro* activity could be observed [54]. A regular tendency is evidenced in each series. All complexes containing the *N*-phenyl-substituted thiosemicarbazone (HTS4, Figure 6) as ligand are the most lipophilic and also the most active. As expected, the inclusion of the bulky non polar co-ligand PPh_3 makes the $[Ru^{III}Cl(PPh_3)(TS)_2]$ series the most lipophilic one and again, the most active [54, 55].

The effect of the nature of the counterion on the biological activity of charged metal compounds is also relevant. Differences in solubility of the different salts could explain the differences in the activity results. For example, a complex of the formula $[Ru_2^{II}(p\text{-cymene})_2(TS2)_2]^{2+}$ was obtained both as hexafluorophosphate (PF_6^-) or chloride salt. The latter showed a 17-fold higher activity against *T. cruzi* than the less soluble compound $[Ru_2(p\text{-cymene})_2(TS2)_2](PF_6)_2$ [47, 48].

3. IDENTIFICATION AND VALIDATION OF PARASITE TARGETS

In general, two main approaches have been used for drug discovery against trypanosomatid parasites, a phenotypic and a target-based approach. Which of these approaches is more productive is still being debated by medicinal chemists. In a phenotypic approach, large series of compounds are screened *in vitro* against the parasite looking for a hit or lead compound whose chemical structure can be further modified to improve biological properties in the search for a drug that could enter clinical trials. On the other hand, in the target-based drug design a specific parasite target is identified and drugs are designed to affect this target specifically [18, 56]. In this sense, new parasite-specific drug targets should be identified and strategies to foresee how resistance to drugs against such targets may arise and may be avoided should be developed. The ideal situation is that specific parasite targets which are absent in the host cells are identified and that the compound under research does not affect the mammalian host cells. In the search for new drugs and drug targets, two complementary approaches were

described involving either a structure-based or a target-based approach. In this sense, based on the study of the biology and biochemistry of trypanosomatid parasites, several druggable targets have been identified. A druggable target is a molecule (such as a protein) that is predicted to bind with high affinity to a drug altering the function of the target with a therapeutic benefit to the patient [23, 57, 58]. For instance, potential targets in *T. cruzi* include on the one hand organelles that are absent or significantly different in the mammalian host cells and that are present in the clinically relevant forms of the parasite (trypomastigotes and amastigotes), i.e., mitochondrion, cytoskeletal-related structures, acidocalcisomes, and glycosomes. Most molecular targets are key enzymes involved in processes that are essential to parasite survival, such as sterol biosynthesis, antioxidant defences, and bioenergetic pathways [59].

On the other hand, many researchers are investigating new enzyme targets in *T. brucei*, searching for more efficient and selective inhibitors that are capable to cause the parasite's death with less toxicity to the host. Trypanothione reductase, farnesyl diphosphate synthase, 6-phospho-gluconate dehydrogenase, UDP 4′-galactose epimerase, and other enzymes are some of the studied targets [12].

In the metal-based drugs field, many of the publications do not identify potential targets for metal complexes although promising compounds that show acceptable to good IC_{50} values and selectivity indexes are included. However, metal complexes with compounds bearing antiparasitic activity as ligands, are expected to maintain the target of the bioactive compound. For example, FPPS has shown to be the target of copper, nickel, and manganese complexes with the bioactive ligand risedronate ([1-hydroxy-1-phosphono-2-(pyridin-3-yl)ethyl]phosphonate). This enzyme is involved in the biosynthesis of polyisoprenoids and sterols in the parasite and it is the main target of bisphosphonates. The improved antiproliferative effect against intracellular amastigotes of *T. cruzi* of the complexes with respect to the free ligand could be related to the high inhibition of the farnesyl diphosphate synthase enzyme [43]. On the other hand, the effect on parasitic redox metabolism has been described as the mode of action of previously mentioned 5-nitrofuryl-containing thiosemicarbazones and their Pd(II), Pt(II), and Ru(II) complexes. Trypanosomatid parasites are very sensible to free radicals having little defenses against them. Through the 5-nitrofuryl pharmacophore, compounds are able to generate toxic free radicals, mainly ROS. This mechanism is retained in the corresponding metal complexes and, in some cases, the concentration of these free radicals correlated with the antiparasitic activity of the compounds [47, 49, 60].

In addition, other general targets could be affected by a prospective metal-based antiparasitic drug due to the presence of a metal in its scaffold, like DNA and parasite-relevant enzymes. Both types of biomolecules show suitable donor atoms that could act as metal binding sites, i.e., nitrogens of the nucleobases of DNA and sulfur, nitrogen, and oxygen donor atoms of the amino acid residues of enzymes. Although DNA is not a specific parasite target, it could be an interesting one, similarly as it is in the field of antitumoral compounds. Being quickly dividing cells, parasite and tumor cells share this target and a mutual action of anticancer and antiparasitic drugs has been commonly observed for organic as well as for metallodrugs, as previously commented [27]. DNA interaction studies have been

performed for some metal compounds bearing antiparasitic activity. For example, previously described palladium and platinum complexes with bioactive 5-nitrofuryl-containing thiosemicarbazones have proved to interact with DNA and the mechanism of such an interaction has been studied by different methods such as gel electrophoresis, circular dichroism, viscosity measurements, atomic force microscopy, and ethidium bromide competition studies, among others [61].

On the other hand, as stated, other metal complexes have been designed intending to target DNA. Most members of a family of structurally related [VO(L-2H)NN] compounds, where L is a tridentate salicylaldehyde semicarbazone derivative and NN is a bidentate 1,10-phenanthroline derivative showing DNA-intercalating ability, have shown activity on *T. cruzi* and a remarkable interaction with DNA [62]. In addition to DNA, different metal complexes have shown inhibitory effects on cruzipain, a parasite cysteine protease involved in many fundamental parasite processes. In this case, the ligands act as chaperones delivering a metal fragment to the enzymatic target [29].

Identification and validation of putative drug targets could be traditionally supported by genomics and proteomics. Nevertheless, this approach is only very recently used for antiparasitic drug development since the proteome and genome of the selected parasite were previously needed. For instance, the genome of *T. cruzi*, *T. brucei*, and *L. major*, which are mainly responsible for the three diseases of interest in this chapter, have only been decoded in recent years [4].

4. METALLOMICS IN PARASITES

Metallomics is a growing field in Inorganic Medicinal Chemistry that in general supports target validation by leading to understand metal uptake, trafficking, function, and excretion in biological systems and the interaction with the genome, proteome, transcriptome, and metabolome [19, 63–65]. Drug uptake is usually determined by measuring the metal amount entering the target cell by atomic absorption spectroscopy or inductively coupled plasma mass spectrometry.

Some infective parasite stages invade host cells, like the amastigote form of *T. cruzi* that invades muscle cells or *Leishmania* trypomastigotes that invade macrophages. Therefore, it is extremely important to assess drug uptake by the parasite in this situation. Once inside the parasite, quantification of drug distribution in it is also relevant. This information could be a guide to understand the mechanism of action and to identify parasite processes affected by the drug. Usually, studying the distribution of the drug in different cell fractions includes measurements by the mentioned techniques of capture by mitochondria, nucleus, DNA, RNA, and soluble and insoluble proteins as a first stage. Again, no such studies are reported for antitrypanosomatid metal-based agents. Only recently, some of these studies have been initiated by our group on an oxidovanadium(IV) heteroleptic compound (unpublished results) and on a platinum organometallic compound, both identified as hit compounds against *T. cruzi* (Figure 9) [53, 62, 66]. The proposed study involved the analysis of the drug uptake and subcellular localization, as well as the evaluation of the proteomic and transcriptomic changes induced in the parasite by the compounds.

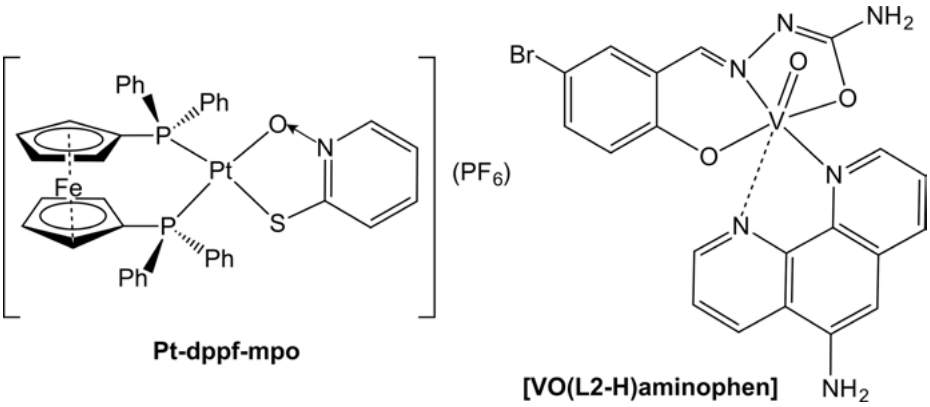

Figure 9. Structure of 1,1′-bis(diphenylphosphino)ferrocene pyridine-2-thiolate-1-oxide Pt(II) hexafluorophosphate, Pt-dppf-mpo, and of [VO(L2-H)aminophen], where L2 is 5-bromosalicylaldehyde semicarbazone and aminophen is 5-amino-1,10-phenanthroline.

For instance, Pt-dppf-mpo showed a good uptake percentage for parasites treated with a concentration of 1× (75 %) and 10× (19 %) the calculated IC_{50} value, respectively. This uptake is excellent in comparison to metallic antiproliferative compounds like cisplatin, oxaliplatin or pyrodach-2 whose uptake percentages in tumor cells were calculated as 3, 1, and 0.1 %, respectively [67, 68]. The uptake rates expressed as pmol of platinum per hour per parasite were in the same order of magnitude than the ones estimated for other platinum-based compounds tested as antitumorals [69]. The ferrocene moiety was included in the structure as co-ligand during the rational design of Pt-dppf-mpo to increase the lipophilicity of the compound. The resulting increased permeability could be related with the inclusion of this ferrocene moiety. After 24 h of incubation, Pt-dppf-mpo induced necrosis in parasites, preceded by depolarization of the mitochondrial membrane potential. Cell vitality assays showed high esterase activity in treated parasites. However, despite this increase in metabolic activity, treated epimastigotes showed rounded morphology and loss of flagellum accompanied with a reduction in mobility when increasing compound concentration and/or time of incubation. The parasite mitochondrion seems to be a probable target of the compound. Proteomic and transcriptomic analysis is currently in progress to assess if processes associated with mitochondria could be involved in the mechanism of action.

5. PRECLINICAL STUDIES

Many natural and synthetic compounds have been assayed for activity against *T. cruzi*, but only a few compounds have advanced to clinical trials. None of them is metal-based. As previously discussed, the development of new drugs requires the identification and validation of parasite targets, the screening of the new

compounds against the targets or the whole parasite and standardized procedures to advance leading compounds to clinical trials. In the case of *T. cruzi* some general procedures and criteria were established by different health organizations [70–72]. The disappointing results obtained in the last decade with azole organic derivatives which were expected to be the next commercially available drugs for the treatment of Chagas disease, evidenced a lack of correlation between preclinical and clinical development and the need of standardized protocols to carry prospective drugs from preclinical research into clinical trials. In this sense, PIDC/Fiocruz (Program for Research and Technological Development on Chagas Disease) and the non-profit institution Drugs for Neglected Diseases Initiative (DNDi) proposed a sequential methodology for testing *in vitro* and *in vivo* new drug candidates for the treatment of Chagas disease that could allow a quick and standard evaluation of them and their entrance in clinical trials. Minimum steps and requirements for the determination of efficacy were established and a standard *in vitro* and *in vivo* flowchart for screening potential drugs was presented. The protocol includes the determination of the *in vitro* effect on trypomastigotes and intracellular amastigotes, selectivity index, acute toxicity in mice, parasitemia reduction, and cure in acute infection. According to these guidelines, the selectivity index *in vitro* should be ≥ 50 to move a compound forward to further studies [70].

Those compounds accomplishing regulatory rules could be tested *in vivo* in animal models like mice. Initial studies involve acute infection development in the selected animal model, treatment with a selected dose of the prospective drug, and follow up of parasitemia with time. Successful results at this preclinical stage could enable the new drug candidate to move to further usual preclinical studies in animals involving pharmacology, toxicology, and drug formulation. Common drug clinical trials would follow.

More recently, the Japanese Global Health Innovative Technology Fund convened with experts from the DNDi disease-specific criteria for hits and leads for visceral leishmaniasis and Chagas disease, among other infectious diseases, for their advancement to clinical studies to expedite the discovery of new drugs. Several general criteria like confirmation of the chemical structure of the hit and an acceptable *in vitro* response (a sigmoidal concentration-growth inhibition curve reaching a maximal 100 % efficacy) were established for both diseases. A hit should not have highly reactive or unstable moieties in the pharmacophore and it should be amenable to structural variation by chemical synthesis. In addition, it should show a greater than 10-fold selectivity using a mammalian cell line for testing cytotoxicity (for example, HepG2 or Vero cells). Specific criteria for hits against *T. cruzi* and *Leishmania* were also established including minimum *in vitro* IC_{50} values ($< 10\ \mu M$) and minimum expected effects on *in vivo* models of the diseases at selected specific doses [72].

Similarly, the Special Programme for Research and Training in Tropical Diseases, which is leading research on discovery against neglected diseases, has recently defined as criteria to consider a compound as a drug hit for African trypanosomiasis to show an IC_{50} of $\leq 0.2\ \mu g/mL$ against the whole organism and a selectivity index $SI > 100$ [73].

No metallo compounds designed for the treatment of diseases caused by trypanosomatid parasites have entered clinical trials yet. However, ferroquine (Figure 8), a ferrocenyl derivative of the antimalarial drug chloroquine has reached phases I and II clinical trials with promising results [74].

6. CONCLUDING REMARKS AND PERSPECTIVES

Several classical coordination and bioorganometallic compounds have shown *in vitro* activity on trypanosomatid parasites and have been identified as prospective antiparasitic drugs. Although some chemical scaffolds were identified that could lead to metal-based drugs, this Inorganic Medicinal Chemistry field is currently underdeveloped. It provides high potential for the development of novel prospective drugs against trypanosomatid parasites due to the large diversity of bonding modes and structures and the high versatility of design it offers. In particular, compounds which include ligands bearing activity against parasites led to quite successful initial results. Nevertheless, some general failures could be detected in the research performed up to the moment in order to discover new drugs against trypanosomatid parasites and are detailed as follows [20, 21].

1. Most of the reported studies were performed on the forms of the life cycles of the parasite that are not completely relevant for the *in vivo* effects, although it is well known that the susceptibility to drugs of the different stages of the parasitic life cycle could be significantly different. Only few studies were performed on the biologically relevant forms like trypomastigote and amastigote forms of *T. cruzi*. This failure is usually a consequence of deficient biological laboratory facilities due to the high costs of facilities with adequate security levels to work with the relevant forms.
2. Further development of structurally related series of compounds would get insight into quantitative structure-activity relationships that are still lacking in this field and that would help in the process of drug development by identifying hit and lead compounds.
3. Metal compounds could suffer different chemical processes in biological medium due to the exposure to different bioligands, like proteins, peptides, organic and inorganic acids, chloride, and water. In general, detailed studies in solution of the compounds are lacking. The identification of the active species in a biological medium would be relevant in the way to the clinic. None of these speciation studies had been performed for antiparasitic compounds until recently when solution chemistry studies of bioactive essential metal complexes of the bisphosphonate ibandronate were reported by our group [45].
4. Studies on the potential mechanism of action of the new compounds in the parasite are usually lacking in most of the reported studies. The identification of the affected parasite targets is essential to understand the activity, unspecific cytotoxicity, and selectivity towards the parasites. Metabolomic, proteomic, and transcriptomic studies would surely help in getting

insight into parasite uptake and distribution and into the mechanism of action of the new compounds by identifying parasite enzymatic processes and metabolic routes affected by the prospective metal drugs.

5. Only few of the reported compounds have moved forward to animal *in vivo* studies and some of them do not fulfill the guidelines of the World Health Organization (WHO) and previously described guidelines of different related institutions for doing so. As described in this chapter, several studies should be systematically performed to decide if a specific compound merits animal trials. In particular, compounds should fulfill guidelines including acceptable values of selectivity towards the parasite.
6. Nanotechnology and the use of delivery systems should also be studied to avoid usual toxicity and solubility problems of metal-based drugs and to improve bioavailability, as it has been explored in recent years in the development of metal-based antitumoral drugs [75, 76].
7. Another approach not particularly developed yet is the use of combination therapy; that means the simultaneous administration or association of new metal-based drugs with current organic commercial drugs searching for synergistic effects or potentiation of their actions. Polypharmacology is one of the strategies currently suggested by the WHO for the treatment of some infectious diseases like tuberculosis [77]. This approach tries to overcome low efficacy, low drug tolerance, and drug resistance development typical of monopharmacology. Concerning the treatment of Chagas disease, research evidenced favorable effects on efficacy and tolerance of the use of combinations of organic compounds bearing different chemotypes. The studies focused on the reduction of drug doses to overcome drug toxicity and on the diminution of the duration of treatments [78–82].

In spite of the mentioned general failures, the development of prospective antitrypanosomatid drugs is an area of increasing interest in the academia, and it will surely lead to the development of very interesting chemistry and medicinal chemistry advances and new metal-bioactive products. Sustained collaborative drug discovery efforts are needed to succeed in developing new metal-based drugs for the treatment of diseases by trypanosomatid parasites.

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ABBREVIATIONS

acac	acetylacetonate
ALE	alendronate
ATPases	adenosine triphosphate hydrolytic enzymes

bpy	bipyridine
Bz	benznidazole, *N*-benzyl-2-(2-nitro-1H-imidazol-1-yl)acetamide
COD	1,5-cyclooctadiene
Cp	cyclopentadienyl
CTZ	clotrimazole
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNDi	Drugs for Neglected Diseases Initiative
dppf	1,1′-bis(diphenylphosphino)ferrocene
en	ethylenediamine
FPPS	farnesyl diphosphate synthase
GHIT	Global Health Innovative Technology
HAT	human African trypanosomiasis
HIV	human immunodeficiency virus
IBA	ibandronate
IC_{50}	half maximal inhibitory concentration
IFN	interferon
KTZ	ketoconazole
L. major	*Leishmania major*
mpo	pyridine-2-thiolato-1-oxide
NBPs	nitrogen-containing bisphosphonates
NTDs	neglected tropical diseases
PAM	pamidronate
PIDC	Program for Research and Technological Development on Chagas Disease
PPh_3	triphenylphosphine
PTA	1,3,5-triaza-7-phosphaadamantane
QSAR	quantitative structure-activity relationship
RIS	risedronate
ROS	reactive oxygen species
SI	selectivity index
T. brucei	*Trypanosoma brucei*
T. cruzi	*Trypanosoma cruzi*
TDR	Research and Training in Tropical Diseases
TFN-α	tumor necrosis factor alpha
UDP	uridine diphosphate
WHO	World Health Organization

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14

Chemical and Clinical Aspects of Metal-Containing Antidotes for Poisoning by Cyanide

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Abstract: Physiological metabolism of cyanide takes place by a single major pathway that forms non-toxic thiocyanate that is subsequently excreted. Rhodanese is the primary enzyme to execute metabolism of cyanide with minor pathways from other sulfurtransferases *in vivo*. The rhodanese enzyme depends on sulfur donor availability to metabolize cyanide and poisoning occurs at elevated cyanide concentrations *in vivo*. Cyanide interacts with over 40 metalloenzymes, but its lethal action is non-competitive inhibition of cytochrome *c* oxidase, halting cellular respiration and causing hypoxic anoxia. Only a handful of antidotes for treatment of cyanide poisoning are known; they are primarily inorganic compounds and metal complexes which are intended to intercept cyanide before it inhibits cellular respiration. The inorganic compounds manipulate hemoglobin, forming methemoglobin, or supply sulfur for the rhodanese enzyme. The metal complexes intercept the cyanide and bind it before reaching its target. Cobalt complexes of corrins and vitamin B_{12} derivatives are the state-of-the-art agents, while the longest employed complex, Co_2EDTA, is designed to deliver "free" cobalt for binding of cyanide. Compounds that are in development are discussed from the point of how they are designed to intercept cyanide. The challenge of reversing the cyanide inhibition of cytochrome *c* oxidase is based on the catalytic active site structure and reactivity. General information about history and occurrence of poisoning and clinical symptoms is discussed and the challenges related to analytical methods available to analyze blood cyanide levels and to confirm the presence of cyanide poisoning.

Keywords: cyanide · antidotes · cobalt · molybdenum · cystic fibrosis · anoxia · inhalation injuries · cytochrome *c* oxidase · methemoglobin · poisoning treatments

1. INTRODUCTION

Cyanide is an important endogenous molecule that is metabolized by sulfurtransferase enzymes to form thiocyanate. Its biological function is not completely understood, and it is usually present in low concentrations in blood; *in vivo* it is excreted at a rate sufficiently rapid to prevent buildup [1]. However, situations can arise in which biological metabolism is not sufficiently rapid to prevent cyanide toxicity. The cyanide anion hydrolyzes *in vivo* to form hydrogen cyanide (HCN). Cyanide is an excellent π-acid and a good ligand for most transition metal ions. It interacts with many metalloenzymes, including hemoglobin, catalases, cytochromes, peroxidases, and oxidases with an accessible ligand position [2]. Hemoglobin and cytochrome *c* oxidase (CcO) play critical roles in the function of oxygen transport and ATP production; inhibition of these enzymes results in life-threatening situations. Commercially available countermeasures for cyanide toxicity employ inorganic compounds and specifically cobalt complexes have been successful as antidotes. Four key commercial antidotes are Cyanokit™, Kelocyanor™, thiosulfate, and CAK™ (Cyanide Antidote Kit) of which Cyanokit™ and Kelocyanor™ employ cobalt complexes as the active ingredient.

Mentions of poisoning by cyanide often invoke thoughts of news reports of suicides, histories of mass murders, or even as the dramatic end of the antagonist

in fictional writing. In modern society, poisoning by cyanide of multiple victims takes place in everyday situations and its occurrence is considered underestimated by clinical physicians [3, 4]. Cyanide salts are commonly used in industry for chemical synthesis and for purification of metals; worldwide usage in industry was about 1,400,000 tons in 2004 and is predicted to grow by 1 % annually [5]. In this context, accidents are the most common and most likely source of cyanide poisoning. These accidents include industrial accidents, and fires in residential, forest, business, or industrial settings. Industrial fires are potential sources of large-scale cyanide inhalation injuries.

Although large amounts of cyanide are mostly encountered in industry or fires, there are naturally occurring sources of cyanide. Cyanide is an endogenous molecule formed in many biological pathways, for example, during regeneration of nerve cells [6]. It is a toxin, metabolized by rhodanese. Consumption of foods containing cyanogenic glycosides, such as cassava, almonds, and fruit kernels may lead to release of cyanide upon digestion. The body uses the rhodanese enzyme to process small amounts of cyanide and therefore, cyanide toxicity does not occur via consumption of properly handled and processed foods.

Fire smoke presents a less obvious source of cyanide poisoning. Currently our residences contain more synthetic materials compared to the middle of last century, and according to fire smoke toxicity studies, more HCN is formed in fires today than 70 years ago, because the synthetic materials burn both faster and hotter than the natural fibers in wool and silk [7]. Documented cases of cyanide poisoning worldwide include numerous residential fires, as recently as in the residential Grenfell Tower fire in London in 2017 where a fire started by a refrigerator spread to ignite the building insulation which did not meet fire standards [8]. Nightclubs have drawn the attention of scientists studying cyanide's contribution to inhalation injuries because they are often highly decorated with synthetic materials, and have few windows and doors [9, 10].

Physical properties of hydrogen cyanide make it an evasive compound to study *in vivo* and *in vitro*. Its reported toxic levels are broad and there are no unique biomarkers or symptoms that specifically indicate cyanide poisoning. It is therefore challenging to confirm cyanide poisoning in the clinic, especially for moderate poisoning cases. Spectroscopic analytical methods that quantify cyanide [11] are time-consuming – albeit necessary – to complete prior to treatment with chelating antidotes. Treatment for cyanide poisoning becomes more complicated if concurrent carbon monoxide poisoning is present. Studies show that survival is reduced in fire victims that have both carboxyhemoglobin (COHb) and HCN present in their blood [12].

There are very few commercial treatments available to mitigate cyanide poisoning. They are mostly inorganic compounds: nitrites, thiosulfate, and cobalt complexes [13]. Organic compounds are also used, although only dimethylaminophenol (4-DMAP) has been successful commercially [13]. Metal complexes are the most efficacious commercially available antidotes to treat cyanide poisoning [13]. The current state-of-the-art product is a cobalt derivative of vitamin B_{12}. The organic compounds and nitrates belong to a class of compounds called methemoglobin (MetHb) formers [13]. The current available arsenal of antidotes

has limitations as mass casualty treatments because of their routes of administration and the necessary doses required [14]. The clinical use of cyanide poisoning antidotes is practiced with careful consideration of their efficacy and side effects.

2. NATURALLY OCCURING CYANIDE

Cyanide is formed as a metabolite from cyanogenic glycosides present in many edible plants and plant products. It is believed to have important biological functions that are not completely understood. This section discusses the fate of cyanide obtained from naturally occurring food and endogenous sources.

2.1. Physiological Metabolism

Cyanide can be produced by different endogenous intracellular metabolisms and is present in human blood at approximately 3 μM [15]. Therefore, it is not surprising that there are biochemical pathways in the body for detoxification of cyanide. Cyanide is metabolized in the body via one major and several minor routes. The major route of detoxification is by conversion to the less toxic thiocyanate, which is then excreted in urine. This reaction is catalyzed by sulfurtransferases: Enzymes that catalyze the formation, interconversion, and reactions of compounds containing sulfur atoms. About 80 % of cyanide is metabolized to thiocyanate by this reaction [16, 17]. Rhodanese is the longest known and best-understood sulfurtransferase [18]. Two other less studied enzymes that can participate in cyanide detoxification include 3-mercaptopyruvate (3-MP) and serum albumin sulfurtransferases. It has been hypothesized that 3-mercaptopyruvate sulfurtransferase (3-MST) is primarily responsible for supplying available sulfur in the liver [18]. This sulfur is the primary substrate for the thiocyanate formation by rhodanese. Serum albumin sulfurtransferase is considered responsible for the transport of the sulfur from the liver to other organs needing cyanide detoxification [18]. Minor pathways for cyanide detoxification include the reaction of cyanide with endogenous hydroxocobalamin (CobOH) to form the non-toxic cyanocobalamin (Section 3.2), and the reaction of cyanide with cystine to form aminothiazoline- and iminothiazoline-carboxylic acids that are excreted in urine [16, 17]. Small amounts of cyanide are excreted unchanged via the lungs, urine, sweat, and saliva [16]. The bitter almond smell from HCN may be detected in exhaled air, and it may be respirated as β-thiocyanoalanine in saliva and sweat [16, 17].

Rhodanese was first described in 1933 as thiosulfate:cyanide sulfurtransferase. It reacts with sulfane sulfur to form a persulfide (-SSH) at the cysteine residue Cys-247 [19]. Rhodanese is a mitochondrial and a double displacement enzyme that catalyzes the abstraction of sulfane sulfur from thiosulfate or other sulfur-containing substrates from the sulfane reserves of the body, and transfers it to the cyanide ion to form thiocyanate (Figure 1). A sulfur donor substrate (thiosulfate, $S_2O_3^{2-}$) is cleaved by rhodanese to form a covalently substituted sulfur-

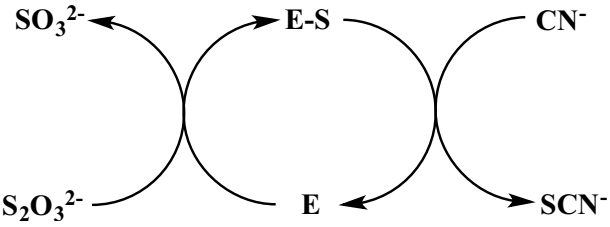

Figure 1. Schematic detoxification of cyanide by rhodanese.

enzyme (E-S). The enzyme-sulfur complex is then attacked by the sulfur acceptor or substrate (cyanide, CN^-) to produce the final product (thiocyanate, SCN^-) and regenerate the free enzyme (E) [17].

The rate-limiting step in cyanide metabolism is governed by the concentration of available sulfur substrates in the body – mainly from thiosulfate, but also from cystine and cysteine. The tissue distribution of rhodanese is highly variable; although it is located in the mitochondria of all tissues, the highest concentrations are found in the liver, kidney, brain, and muscle. The rate of natural detoxification of cyanide by rhodanese *in vivo* is about 1 μg/kg body weight per minute [15].

In cases of acute poisoning, high doses of cyanide quickly deplete the endogenous supply of CobOH [17], and sulfur substrates for the rhodanese enzyme catalysis that is neither sufficiently rapid nor highly bioavailable to handle emergencies [16, 17]. Under these conditions, assistance from antidotes is necessary [17].

2.2. Formation of Endogenous Cyanide

Cyanide is known to initiate the elevation of intracellular Ca^{2+} that results in activation of Ca^{2+}-sensitive enzymes and cellular processes, such as protein kinase C, phospholipase A_2, and neurotransmitter secretion [15]. Cyanide is generated in both white blood cells [20] and neural tissue; it may function as a neuromodulator in the latter [6, 15]. It has been suggested that leukocytes and neural cells generate cyanide for different purposes [21].

Cyanide is generated in leukocytes during phagocytosis [20]. Addition of glycine enhances cyanide production and the reaction is catalyzed by the enzyme myeloperoxidase (MPO) [22]. MPO is an iron heme metalloenzyme contained in azurophil granules in the cytoplasm of resting leukocytes, but it is released during phagocytosis into the phagocytic vacuole where it catalyzes the reaction of chloride and peroxide to form hypochlorous acid and can N-chlorinate amino acids [21]. The N-chlorination of glycine catalyzed by MPO yields cyanide and cyanogen chloride. The enzymic chlorination of glycine converts it to N-monochloroglycine, which can undergo an acid-catalyzed dismutation to N-dichloroglycine as shown in the following equations [22].

$$2\,ClHN\text{–}CH_2\text{–}COOH + H^+ \longrightarrow {}^+H_3N\text{–}CH_2\text{–}COOH + Cl_2N\text{–}CH_2\text{–}COOH \quad (1)$$

N-dichloroamino acids are unstable and decompose to the corresponding nitriles. N-dichloroglycine forms HCN upon decomposition [22]:

$$Cl_2N\overset{\frown}{}COOH \xrightarrow{-2\,HCl} N\equiv C-COOH \longrightarrow HCN + CO_2 \quad (2)$$

Cyanogen chloride is also formed, through chlorination of HCN by either N-monochloroglycine or by the myeloperoxidase enzyme itself [22].

The mechanism of cyanide generation in neural tissue demonstrates that nerve cells generate cyanide in an oxidative process similarly to the leukocytes. Glycine addition was found to enhance cyanide production in rat pheochromocytoma (adrenal gland tissue tumor) cells [21]. A myeloperoxidase inhibitor was found to effectively decrease cyanide release in neural tissue, showing that the process of cyanide generation involves a peroxidase enzyme [21]. Therefore, an oxidative mechanism catalyzed by a peroxidase enzyme can generate cyanide in neural cells as well as in leukocytes [21].

The benefit of cyanide generation in nerve cells is not yet clear but it may be important in certain physiological and pathological processes. It has been proposed that cyanide is an important gaseous neuromodulator similar to nitric oxide, carbon monoxide, and hydrogen sulfide [15]. It is known to potentiate exocytotic release of neurotransmitters and to modify the responsiveness of N-methyl-D-aspartate (NMDA) receptors [6]. The NMDA receptor is an ion channel protein in nerve cells that is activated by glutamate or glycine to allow positive ions to flow through [6].

HCN is formed in the brain, can mediate a number of biological actions, and is metabolized by specific brain enzymes [15]. Quantification of cyanide in rat brain tissues revealed that cyanide levels are higher in the hypothalamus than in the cortex, cerebellum, or medulla. The extent to which cyanide modulates brain function may vary in different brain regions [15]. The pattern of distribution of rhodanese is similar to that of cyanide, as its levels are higher in hypothalamus than in cortex, cerebellum, or medulla. The similarities of cyanide and rhodanese levels may have a functional significance [6].

Pseudomonas aeruginosa is the most important pathogen infecting the lungs of patients with cystic fibrosis (CF), as its presence leads to higher rates of morbidity and mortality [23]. *P. aeruginosa* demonstrates extensive resistance to antibiotics, and it is one of a limited number of bacteria that can synthesize hydrogen cyanide. The presence of *P. aeruginosa* in the lungs of CF and non-CF bronchiectasis patients is associated with accumulation of cyanide in sputum. The mean concentration of 72 μM cyanide in sputum of *P. aeruginosa*-positive CF and non-CF bronchiectasis patients has potential clinical significance compared to toxic or lethal blood cyanide levels of 40 μM. At this average concentration, cyanide is expected to inhibit cellular respiration in the local environment of the CF lung [23].

2.3. Formation of Cyanide through Food Consumption

Cyanide occurs naturally as cyanogenic glycosides in at least 2000 plants, and humans can be exposed to cyanide by eating cyanogenic foods. The cyanogenic glycoside amygdalin (Figure 2) is present in about 1000 plant species, e.g., cassa-

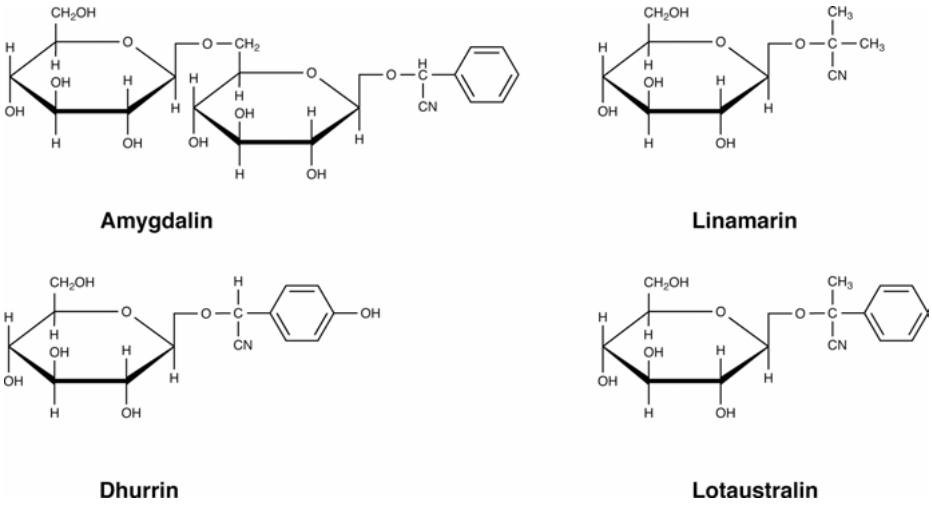

Figure 2. Structures of cyanogenic glycosides.

va, bitter almonds, lima beans, pits of stone fruits (cherries, peaches, apricots), apple seeds, sweet potato, corn, cabbage, linseed, millet, and bamboo. Linamarin and lotaustralin are cyanogenic glycosides found in cassava, linseed, beans and peas, and the cyanogenic glycoside dhurrin is found in sorghum, millet, tropical grass, and maize [15].

Upon hydrolysis of cyanogenic glycosides, cyanide, a sugar, and a ketone or aldehyde are formed. As an example, hydrolysis of amygdalin forms glucose and benzaldehyde, releasing hydrogen cyanide (Figure 3.) [2]. Glycosidases of the intestinal microflora and, to a lesser extent, glucosides of the liver and other tissues release cyanide after ingestion [16]. Hydrolysis may also occur during food preparation involving heat and water [16]. The concentration of cyanogenic glycoside can vary widely, depending on genetic and environmental factors, location, season, and soil types [16]. Examples of edible plants containing cyanogenic glycosides and their cyanide concentration are shown in Table 1 [16].

Most of the cyanogenic plants and their products are staple foods in tropical countries [16, 24]. Cyanide poisonings after ingestion of cyanogenic foods are reported more frequently in countries where these foods are major components of the diet. When these foods are ingested in large quantities or without adequate preparation, they can cause acute cyanide toxicity. Cassava is estimated to be a staple food for 500 million people. Cassava can be eaten raw, cooked, or grated and roasted into flour, known as gari. Frequent, long-term ingestion of cyanogenic foods, particularly in the presence of protein-calorie malnutrition, is associated with several different diseases affecting mainly the nervous system, such as tropical ataxic neuropathy and spastic paraparesis (also known as mantakassa in Mozambique or konzo in Congo) [16, 24].

Outbreaks of spastic paraparesis have occurred in different countries, especially during droughts and dry seasons where the affected populations rely mostly

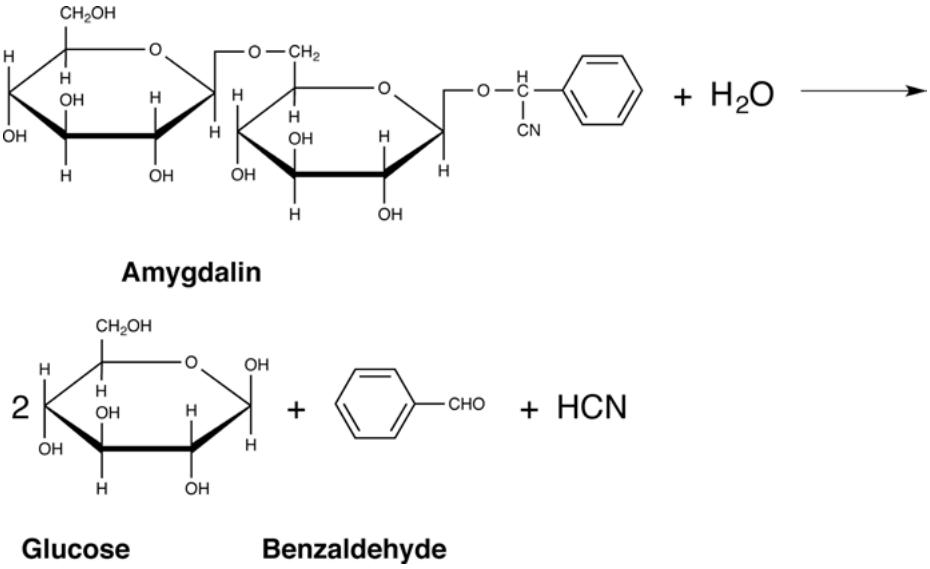

Figure 3. Hydrolysis of amygdalin releases cyanide.

Table 1. Concentration of cyanide in various edible plants.

Type of plant	Cyanide concentration (mg/kg)*
Cassava (bitter) / dried root cortex	2360
Cassava (bitter) / whole tubers	380
Cassava (sweet) / whole tubers	445
Gari flour (Nigeria)	10.6–22.1
Sorghum / whole immature plant	2400
Bamboo / immature shoot tip	7700
Lima beans from Java (colored)	3000
Lima beans from Puerto Rico (black)	2900
Lima beans from Burma (white)	2000

* Weights are based on description, e.g., cassava is dried, or tubers that are either sweet or bitter and not dried specifically. Values in beans are based on dry weight.

or almost exclusively on cassava as staple foods [16]. In 1981–1982, an epidemic of spastic paraparesis was reported in a drought-stricken area of Mozambique that relies on cassava as food source, where 1102 cases were documented. The daily intake of cassava resulted in an estimated daily cyanide consumption between 14–30 mg per person. The onset of spastic paraparesis was acute with symptoms including fever, pain (especially in the legs), paresthesia, headache, dizziness, vomiting, weakness in arms, and difficulty with speech and vision [16].

Tropical ataxic neuropathy is an upper motor neuron disease characterized by irreversible paraparesis [16]. It is a chronic poisoning syndrome due to long-term

frequent ingestion of cassava, particularly in parallel with protein malnutrition. The disease rarely occurs in children under the age of 10 years, with most incidences among adults in the age groups from 40 to 60. Patients with the disease usually have a history of almost total dependence on a monotonous diet of cassava derivatives [16].

Acute cyanide poisoning by cyanogenic foods has been reported, although much more literature is available on the effects of long-term exposure to cyanogenic foods [16]. Accidental poisonings include ingestion of choke cherries, apricot kernels, seeds, or candy made from apricot kernels [16]. Acute cyanide poisoning after cassava ingestion has been reported, where it has led to the death of entire families following consumption of inadequately cooked cassava [16, 24].

3. POISONING BY CYANIDE

Cyanide poisoning results from exposure to salts of cyanide, hydrogen cyanide or cyanogenic compounds. Cyanide has a hydrolysis constant pK_{Hyd} of 4.8 that results in protonation at physiological conditions (pH 7.4) forming HCN. Reactions of hydrogen cyanide with metalloproteins such as hemoglobin and cytochromes are facile because cyanide is a strong ligand for many metal centers. Cyanide enters the bloodstream quickly and dissipates easily into tissue [1]. As a solid salt it is absorbed through skin, or immediately forming hydrogen cyanide upon ingestion [1]. Its toxic dose depends on the route of poisoning, and because of its reactivity, the recorded values vary. Although a well-known poison since the early Roman Empire when cherry laurel water was used to poison enemies [25], study of its chemistry *in vivo* has not focused on its endogenous formation and biochemistry although it has been long known that blood contains low concentrations of cyanide [26], and it is found in exhaled air [27]. Recent research efforts have been directed towards investigating its endogenous formation and biological reactions [15, 21, 28].

3.1. Occurrences of Poisoning

Exposure to hydrogen cyanide is more common than generally believed, although lethal doses are rare [29]. Cyanide intoxication is infrequently lethal in everyday life because of its volatility and reactivity. Specific incidents of murders by cyanide, suicides, and various incidents of cyanide poisoning were reviewed by D. J. Gee in 1987 [30].

The largest risk of cyanide poisoning for the general public is exposure to smoke inhalation from residential, industrial, and forest fires. In recent years increased attention has been paid to the toxicity of fire smoke, its composition and the effects of the compounds formed in the smoke on human health [31]. Long term effects on human health after non-fatal cyanide exposure are not well known; this topic is gaining interest because of increased awareness of exposure

and concurrent smoke inhalation injuries [29]. In this context the presence of cyanide in fire smoke has become a public health concern because HCN is formed in an incomplete combustion of organic materials containing nitrogen. This applies to natural materials such as silk and wool as well as synthetic materials such as acrylonitriles and polyurethanes [32]. Other potential sources are accidental volatilization of HCN in industry accidents, chemical warfare, and by insecticides [32]. Since cyanide salts are used in large volumes as starting materials in the chemical industry for chemical synthesis and are the key ingredient in metal processing, large quantities of cyanide salts are used daily worldwide. If these salts come into contact with moisture they instantaneously form hydrogen cyanide and volatilize [33], harming anyone in its vicinity. For the same reason, aqueous solutions used for metal processing need to be stored properly. Industrial cyanide poisoning incidents are most likely to occur in metal processing or chemical storage facilities, and chemical synthesis plants.

3.1.1. Accidental Exposure

The Swedish chemist Carl Wilhelm Scheele, who isolated hydrogen cyanide from Prussian blue was also the first reported victim of accidental cyanide poisoning in 1786 following a handling mishap in his laboratory [34]. There are perhaps three key sources of accidental exposure to cyanide: industrial accidents, diet, and fire smoke. Other documented accidental sources are from drug metabolism or tobacco smoke [29].

Carbon monoxide poisoning is the most recognized contributor in smoke inhalation injuries in fire victims [31]. Standard practices in the clinic do not presume cyanide poisoning to be present in mild smoke inhalation injuries. The implication is that victims with smoke inhalation injuries are not routinely treated for cyanide poisoning but only for carbon monoxide poisoning. Investigation of the blood cyanide levels of victims of fire smoke in residential fires and in nightclub fires concluded that about 35 % of all fire victims have significant levels of cyanide in their blood [14, 35, 36]. These studies show that concurrent CO and HCN poisoning causes hypoxia by different mechanisms and the combined toxicity is exacerbated.

In August of 2015, 173 individuals died and 797 non-fatal injuries were recorded following an explosion at the port of Tianjin in China which resulted in formation of thick smog and fires that burned for 2 to 3 days. Additionally, several police officers and 95 firefighters died, which is the largest first responder casualty reported in China. The reporting did not include contribution of cyanide poisoning to the non-fatal injuries although its large number was attributed to a high level of air pollution. The explosion was caused by overheated dry nitrocellulose that subsequently ignited other chemicals stored in the vicinity, including large amounts of cyanide salts. In addition, ammonium nitrate and other potentially toxic and/or explosive compounds were stored in the port, enhancing the intensity of the explosion and contributing to the resulting smog and air pollution. The storage facility also contained 700 tons of sodium cyanide, some of which leaked into the sewer system. HCN formed from two different sources in

this accident: first, when cyanide salt came in contact with water, it volatilized at 26 °C which is the average Tianjin summer temperature, and secondly from the burning nitrogen-containing nitrocellulose [37]. Fires are a good example of an incomplete combustion resulting in other products, such as carbon monoxide, HCN, nitric oxide, and others.

Smoke inhalation from tobacco contains sufficient cyanide to raise the cyanide blood levels of cigarette smokers compared to non-smokers [38, 39]. It is debatable if this is intentional or accidental exposure, but tobacco amblyopia or vision impairment can be found in heavy smokers and is characterized by visual field defects and hindered central vision. It is also associated with vitamin B_{12} deficiency [38, 39].

Case studies of cyanide poisoning were compiled for fires that occurred in nightclubs around the world in the years from 2003 to 2013 [9, 10]. In 2013, 237 deaths were confirmed from asphyxiation (mixture of carbon monoxide and hydrogen cyanide) after a fire in a nightclub in Brazil. The fire started from a spark that ignited decorations in the club ceiling. Victims collapsed before fire fighters arrived and blocked the doors delaying rescue. Reports are available from similar incidents in Argentina, Russia, Thailand, China and USA, reporting deaths of 15 to 194 victims per case [9, 10].

Most residential fires produce cyanide gas in sufficient quantity to cause poisoning; as was the case in the Grenfell Tower fire in London in 2017 [8]. Several people were treated for cyanide poisoning at emergency rooms afterwards.

3.1.2. Intentional Exposure

Intentional use of cyanide is best known in the context of suicides or warfare. It is well documented that toxic gases were tested as chemical warfare agents (CWAs) during World War I (WWI) [40, 41]. Although attempted, hydrogen cyanide was not used successfully because of its volatility, low density, and reactivity that made its controlled use challenging. During WWI, the heavier gases such as cyanogen chloride, chlorine and phosgene proved easier to control. After WWI, the Treaty of Versailles banned Germans from producing and using chemical weapons, and in the Geneva Protocol of 1925, sixteen nations pledged never to use gas as a weapon of warfare [40]. Despite these agreements, the definition of CWAs was unclear and there were no restrictions regarding their development and stockpiling. It is believed historically that because of the heavy casualties in WWI and added burden on medics on battlefields, CWAs were minimally employed during WWII [40]. The USA ratified the Geneva Protocol in 1975 [42]. Currently, 98 % of the global population is represented by the Organization for the Prohibition of Chemical Weapons (OPCW) [43], limiting themselves in production and use of chemical weapons; however, terrorist and non-state organizations are not bound by these treaties.

The largest known scale of cyanide use in warfare was the use of Zyklon B in WWII. Zyklon B is HCN combined with an eye irritant and other substances. Although initially developed as a pesticide, it was used in gas chambers in Nazi death camps. HCN was volatilized in lethal doses to exterminate approximately

one million people [44]. In 1978 in Jonestown, Guyana, 909 individuals died after ingesting a lethal mixture of cyanide and fruit juice. The Jonestown massacre remains the largest loss of lives from intentional cyanide poisoning since WWII. Many children died in this incident, which is debated to be a mass suicide or a mass murder [45, 46]. The illicit addition of a cyanide salt to Tylenol that was discovered in drug stores in Chicago in 1982 is another well-known case of intentional poisoning that resulted in seven deaths [47]. Many more deaths were prevented by rapidly alerting the public and recalling the product. More recent intentional use of cyanide was alleged during the Iran-Iraq war during the time period of 1980 to 1988 [48]. This war has been compared to WWI in terms of heavy casualties and the use of chemical weapons. It was suggested that HCN was employed in combination with the sarin attack in the Tokyo subway terrorist attack in 1995 because precursor compounds for HCN release were found in subway and railway restrooms [49]. There are also indications that it was used in combination with the explosives used in the first attack on the World Trade Center in 2001 [50].

3.2. Clinical Aspects

The lethality of cyanide is dependent on the dose and route of ingress. The maximum safe inhalation concentration for humans is generally accepted as 11 mg/m^3 [4] and concentrations higher than 20 mg/m^3 are expected to exhibit at least slight effects. Reported averages based on various studies show the dose-effect curve is very steep, and instant death takes place at LC_{100} of 300 mg/m^3 [16]. Concentrations between 70 mg/m^3 to 300 mg/m^3 are also fatal in 30 minutes or less. Inhalation toxicity for airborne toxins is often denoted C_t, where LC_{t50} is the lethal dose for 50 % of the population and is reported in concentration (mg/m^3) times exposure time (min). The LC_{t50} for HCN is generally reported as 2,500 to 5,000 mg $\times$ min/m^3 which translates to an LC_{50} of 83 mg/m^3 to 167 mg/m^3 for 30 minutes [4]. This is a broad dose range but is consistent with data from many sources [16]. In comparison, lethal doses via ingestion of potassium cyanide (KCN) are reported to be as little as 150 mg to as much as 3.0 g [13]. The amount per body weight varies from 0.7 mg/kg to 3.5 mg/kg [51]. The broad dose variation observed is consistent with results from animal studies demonstrating that variation in the pH of the stomach affects absorption, with the least acidic pH delaying absorption which normally takes about 2 to 3 hours [52]. Ingestion is accompanied by rapid onset of symptoms because cyanide dissipates rapidly, although dose-related death may be delayed. Solutions of cyanide contain both hydrogen cyanide and its conjugate cyanide anion and both are absorbed very rapidly through skin. The lethal dose via skin absorption is reported as 100 mg/kg [16]. Treatment for cyanide poisoning becomes complicated if concurrent carbon monoxide poisoning is present. The lethal toxicity of HCN and its salts is species-dependent and may be correlated to the abundance of the rhodanese enzyme in their bodies [4]; as a result, dogs tolerate lower HCN levels than rats or mice although they are larger animals.

Theoretically, poisoning by cyanide is expected to have two characteristic symptoms; cherry-red skin accompanied by the odor of bitter almonds from the victim's breath. The odor of bitter almonds is believed to be a confirmation of the presence of HCN; consequently, the assumption follows that the smell of the exhaled air from a potential victim is a good indication of cyanide intoxication [53]. This is not a generally valid assumption, because 40 to 60 % of the population is unable to detect the odor [53]. Cyanide inhibition of CcO blocks tissue uptake of oxygen by cells and the expected result is an increase in oxygenation of the venous blood and a victim who is red in the face with flushed skin is an indication of poor tissue oxygenation [29]. Although expected, this sign is seldom described in case reports.

Cyanide poisoning may be difficult to confirm because of a general lack of analytical markers, and is not easily treatable outside of a hospital or emergency room setting. Therefore, it is not standard clinical practice to treat victims of fire smoke inhalation injuries for cyanide poisoning. The large range of symptoms and lack of a specific biomarker complicates the diagnosis of cyanide poisoning. Clinical manifestations of cyanide poisoning vary widely, depending on the dose and route of exposure, and may range from minor upper airway irritation to coma, cardiovascular collapse, and death within minutes [54]. Indicative early signs and symptoms of cyanide poisoning include headache, vertigo, confusion, general symptoms of central nervous system (CNS) excitement such as anxiety, personality changes and agitation, progressing to seizures, hypotension, and increased respiration (hyperpnea, dyspnea, bradycardia) [55]. Diaphoresis, flushing, and weakness may also be present. Latent-appearing indications of CNS depression, such as coma and dilated, unresponsive pupils, complex arrhythmias, pulmonary edema, and death are prominent signs of cyanide intoxication [56, 57]. These signs and symptoms are not exclusively characteristic of cyanide poisoning, which makes the distinction from other types of poisoning very difficult without a history of exposure.

The symptoms of a non-fatal inhalation injury from HCN are milder, and limited data is available regarding sublethal toxicity and its symptoms [6]. Fire toxicity studies, including concurrent carbon monoxide and cyanide toxicity, confirm that Parkinsonian symptoms (Chapter 4 in this book, Section 2.1) are traceable to untreated HCN exposure [6]. Patients suffering from inhalation injuries from residential fires, who receive both treatment for carbon monoxide and cyanide poisoning recover faster and better than those who are only treated for carbon monoxide poisoning [58].

Smoke inhalation patients with a relatively normal oxygen saturation and concurrent hypotension, altered mental state and elevated plasma lactate concentrations are likely poisoned by cyanide. Whole blood CN^- measurements may not be available at the admitting treatment center and in a review of clinical signs of cyanide poisoning [59] it was suggested if a very high blood lactate concentration is found in combination with signs of neurological incapacitation, and/or soot in the mouth, the attending physician should suspect poisoning by cyanide. Metabolic acidosis with a blood lactate concentration above 8 mmol/L is considered a certain sign of poisoning by cyanide [59]. The lactate and cyanide concen-

trations increase proportionally. Generally, a lactate concentration of 10 mmol/L is considered as a sensitive and specific indicator of CN^- poisoning [12]. Fire victims' blood is typically analyzed to determine the exact cause of death. The cause of death is generally accepted as being from carbon monoxide poisoning if COHb levels are over 50 % of the blood hemoglobin [60, 61]. In victims of modern fires, hydrogen cyanide is an increasing contributor to the cause of death and it is often indicated by lower COHb levels than is accepted for CO poisoning as a cause of death.

3.2.1. Cyanide Pharmacokinetics and Pharmacodynamics

Cyanide is a small molecule with good solubility properties in both aqueous and organic media. It diffuses quickly within the body and it is completely distributed five minutes after absorption [62], displaying first-order kinetics during the period of initial toxicity [4] with a half-life in plasma of about 14 minutes [56] and an elimination half-life of one hour from whole blood *in vivo* [59]. The ratio of cyanide in red blood cells (RBCs) to plasma is at least 100 : 1 [1]. Thus, cyanide is found in RBC rather than in the plasma and analysis of whole blood needs to be facile to quantify cyanide in blood. Cyanide tissue levels show variation in organ distribution depending on the route of exposure. If inhaled, cyanide is found mainly in the lung, heart, and brain [4, 63]. However, ingestion of cyanide results in much higher levels in the liver comparatively, although it is also found in the stomach wall and kidneys [64]. Cyanide has wide-ranging cardiovascular effects most of which are poorly understood [54, 65]. Animal studies and clinical reports agree that the heart, lung, and brain are important organs in the study of cyanide toxicity.

Data from studies with rats and mice suggest that a single, acute administration of a cyanide salt leads either to death or complete recovery. However, data from HCN inhalation studies in dogs, rabbits, monkeys, and humans suggest that death may be delayed for up to eight days [66, 67]. The neurological sequelae of cyanide intoxication were reported as being delayed for up to a year [25]. These delayed changes in regional sensitivities of the brain are thought to be due to hypoxic stress and are analogous to those seen following sublethal CO poisoning.

4. METAL-CONTAINING ANTIDOTES

4.1. General Information

The first report of cyanide's ability to inhibit tissue oxidation reactions was published as early as 1876 [68]. Early experiments with cyanide poisoning used sodium nitrite as an antidote. The first proposed mechanism for cyanide detoxification was described in the early 1930's [69, 70]. Thiosulfate was proposed as a sulfur donor for rhodanese to form thiocyanate. In the experiments, a combination of sodium nitrite, amyl nitrite, and thiosulfate served as the antidotal combi-

Table 2. Antidotes used in acute cyanide poisoning.

Treatment	Pros	Cons	Administration route	Single dose* (adult)
Nitrites	Potent	Impair tissue delivery of oxygen	Inhalation	Determined based on cyanide poisoning levels
Thiosulfate	Efficient Safe	Slow onset of action	Intravenous	12 g
Dicobalt EDTA	Potent Quick acting Effective for late treatments	Numerous negative side effects	Intravenous	First dose 300 mg, may be repeated. A too large dose leads to cobalt toxicity
Hydroxocobalamin	Potent Safe for smoke inhalation injuries	Red coloration of skin and urine Expensive	Intravenous	5–15 g

* Doses are not based on body weight

nation and is still used for treatment of poisoning by cyanide. Cobalt compounds were introduced as early as 1894 as potential antagonists for cyanide in a single report [71] but continued research is documented since 1952 when synthesis of cyanocobalamin was reported from hydroxocobalamin (now known commercially as vitamin B_{12}) [72]. Soon thereafter, dicobalt ethylenediaminetetraacetate (EDTA) was reported as more efficacious than the nitrite-thiosulfate combination [73]. Since 1960, the nitrates and the cobalt compounds have been developed into commercial products for the treatment of poisoning by cyanide. After the September 11, 2001 attack in New York City, interest in development of chemical countermeasures to protect the general public against potential CWAs was renewed.

Currently there are four types of antidotes commercially available to treat acute cyanide poisoning in humans [59]: MetHb-forming antidotes (amyl nitrite and sodium nitrite), sodium thiosulfate, Cyanokit™ (hydroxocobalamin), and Kelocyanor™ (dicobalt EDTA). They were mostly designed to prevent the cyanide from attaining its physiological target and were reviewed in 2007 [74]. Since then, additional antidotes have been reported at different stages of development [75–77]. Table 2 summarizes basic information regarding the antidotes.

Clinical use of these antidotes requires consideration of both efficacy of the antidotes and the safety of their use with respect to the source of poisoning in

each case [17]. MetHb-forming agents are potent but impair tissue delivery of oxygen and therefore should not be used in smoke inhalation victims. Sodium thiosulfate is both efficient and safe but has a slow onset of action and is usually not successful by itself for acute poisoning cases. Dicobalt EDTA and hydroxocobalamin are efficient and act immediately, and although they have been used successfully for decades, they have numerous side effects limiting its use. CobOH has less side effects and appears to be safe to use for smoke inhalation victims [17].

New antidotes in development are included in the discussion that follows. They are not proven antidotes but deserve mentioning specifically from the clinical point of view and future endeavors.

Until 2006, the "Cyanide Antidote Kit" (CAK) was the only commercially available antidote approved by the FDA for treatment of cyanide poisoning in the USA. The kit contains amyl nitrite pearls, sodium nitrite solution, and thiosulfate [14]. The amyl nitrite pearls are crushed and administered via inhalation. This acts as a temporizing measure before sodium nitrite is administered intravenously. Both sodium nitrite and amyl nitrite are MetHb formers, with the sodium nitrite inducing rapid formation of MetHb in red blood cells [14]. Preferential binding of cyanide to MetHb to form cyanomethemoglobin is an approach to mitigate cyanide poisoning by competing with CcO for cyanide binding [29]. The oxidized form of heme iron (Fe^{3+}) in MetHb has a higher binding affinity for cyanide than does CcO. As hemoglobin is abundant in blood, intravenous administration of a methemoglobin former neutralizes cyanide quickly. Sodium thiosulfate is additionally administered intravenously to react with cyanide when it is released from MetHb.

The formation of MetHb is a practical strategy for trapping cyanide and preventing it from reaching CcO. Fire victims with carbon monoxide poisoning may have high levels of COHb, which compromises oxygen transport *in vivo*. The combination of methemoglobinemia and carboxyhemoglobinemia reduces the oxygen-carrying capability of the blood below life-sustaining levels [14]. Although methemoglobin formers are potent treatments, caution is required because of potential severe side effects. Vasodilation and hypotension are strong risk factors during treatment, and a large dose can be lethal [29, 78].

Sodium thiosulfate was first suggested in 1895 by S. Lang as a potential treatment for poisoning by cyanide [79, 80]. It is a natural substrate of the enzyme rhodanese and acts by donating a sulfur atom to the enzyme's catalytic site. The sulfur atom forms a very reactive persulfide that instantaneously reacts with cyanide to form thiocyanate. The crystal structure of the enzyme is metal-free [81], although several early studies suggested that the catalytic site undergoes a metal-assisted conformational change during the persulfide formation. Zinc was suggested as the most likely metal to act as a volunteer to aid in the conformational change [82]. The thiocyanate is excreted in urine (Section 2.1) [83, 84]. Rhodanese bioavailability is tissue-specific and it is localized in mitochondria [17]. Thiosulfate is a large divalent anion, and it is thought to penetrate cell membranes slowly leading to a slow onset of action and is therefore a sufficient antidote only in mild to moderate cases of toxicity [83]. It is often co-administered with other antidotes, e.g., as in the Cyanide Antidote Kit™ [14]. Thiosul-

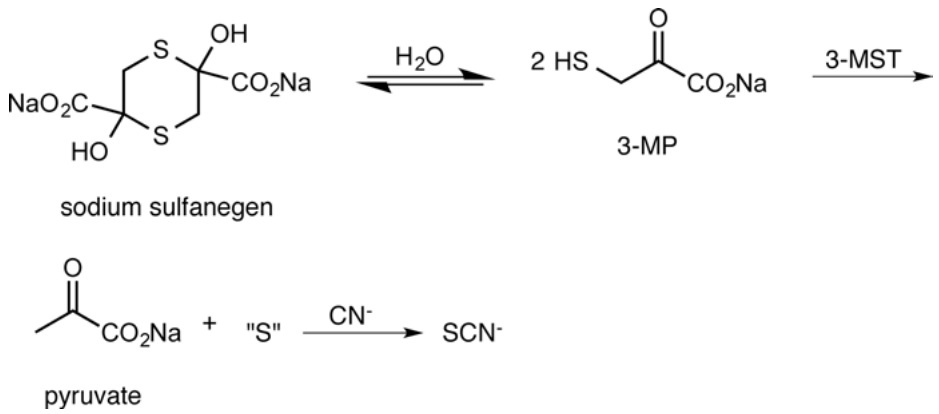

Figure 4. Schematic conversion of sulfanegen to thiocyanate.

fate was used *off-label* worldwide until 2012, when the United States Food and Drug Administration (US FDA) approved it as a drug [85]. It is administered intravenously in large doses of about 12 g over a period of ten minutes [12]. Thiosulfate has been used to prevent cyanide toxification from the administration of the vasodilator sodium nitroprusside [83]. Cyanide forms as a byproduct of sodium nitroprusside metabolism, and several cases of toxicity and death due to high doses have been reported [70]. A constant infusion of thiosulfate is administered along with the treatment of sodium nitroprusside, and is effective in preventing cyanide lethality [83].

Cyanide is also converted to thiocyanate by other sulfurtransferases such as 3-MST (Section 2.1) which catalyzes the transfer of sulfur to cyanide by abstracting the sulfur from the cysteine catabolite 3-MP (Figure 4). Drugs that mimic this process are in development. Poor stability and bioavailability of 3-MP render it a suboptimal drug candidate and have encouraged development of prodrugs of 3-MP. One of these is called sodium sulfanegen (Figure 4) which has been tested in animal models [75].

Sodium sulfanegen proved highly effective in the rescue of pigs from a lethal dose of cyanide via intravenous administration. Sulfanegen has parallels with thiosulfate as an approach because it is based on providing a sulfur donor for the enzymatic reaction. The main advantage over thiosulfate is an expected increased bioavailability of the sulfur donor at the enzyme catalytic site that could enhance the drug efficacy and reduce the necessary quantity of treatment. Sulfanegen is suitable for intravenous administration and efforts are underway to improve its suitability for intramuscular administration [75].

4.2. Cobalt-Containing Antidotes

Cobalt complexes have long been known to be effective antidotes for cyanide toxicity, and they have been studied for therapeutic uses (see Chapter 11 of this

book). The drawback of using them as emergency treatments for acute cyanide poisoning is the potential toxicity of many cobalt complexes [71, 86]. Dicobalt EDTA and hydroxocobalamin are used as emergency treatments for acute cyanide poisoning. While the use of CobOH is considered safe, the numerous side effects of dicobalt EDTA limit its use to confirmed cases of cyanide poisoning [69]. The approach is to intercept the cyanide and neutralize it without compromising oxygen transport.

4.2.1. Kelocyanor™ (Dicobalt EDTA)

Kelocyanor™ is commercially available in Europe, but not in the USA [71]. It is the preferred treatment for cyanide poisoning cases in the United Kingdom, provided that cyanide toxicity is definitely present [24]. Kelocyanor™ is the cobalt salt of EDTA, Co_2EDTA [87]. Dicobalt EDTA has been crystallized as $[Co^{II}(H_2O)_4Co^{II}EDTA]\times 2H_2O$ (Figure 5) [88].

Although both cobalt metal centers are octahedral, they have very different coordination environments. One cobalt metal center is in a closed coordination geometry formed by two nitrogen atoms and four carboxylate oxygen atoms of the EDTA, while the other cobalt contains four water molecules in its coordination sphere and two carboxylate oxygen atoms of the $EDTA^{4-}$ ion bridge both cobalt centers. The crystal structure was published long after the antidote was developed and commercialized [88]. It shows two Co(II) metal centers where one Co(II) is in a closed coordination geometry, and the other one is similar to an aquated Co(II) ion. The solution structure of the antidote has neither been explained nor studied in detail and the behavior and chemistry of this antidote *in vivo* is not fully known. It may be assumed that it dissociates to $[Co(EDTA)]^{2-}$ and $[Co(II)(H_2O)_n]^{2+}$ in aqueous solution, although the level of aquation has not been verified. The aqueous Co(II) may represent the "free" cobalt ions

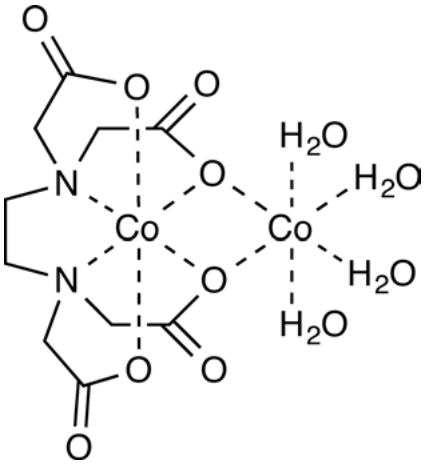

Figure 5. Structure of $[Co^{II}(H_2O)_4Co^{II}EDTA]\times 2H_2O$.

available to bind cyanide and the $[Co(EDTA)]^{2-}$ is excreted unchanged. This is an efficient antidote because it has a relatively low molecular weight and binds (theoretically) up to six moles of cyanide per mole of dicobalt EDTA. Dicobalt EDTA has a high affinity for CN^- and reacts rapidly with cyanide to form the much less toxic cobalticyanide and monocobalt edetate, both of which are excreted in urine within 24 h of administration [16]. Sources disagree on the exact nature of the cyano complex formed, both $[Co(CN)_6]^{3-}$ [86] and $[Co(CN)_6]^{4-}$ [87] have been reported. *In vivo* studies of the stoichiometry of the reaction of dicobalt EDTA and cyanide reveal the antidotal effects of dicobalt EDTA possess efficacy at molar ratios of two cyanides per mol of cobalt [89]. Some free cobalt ions are always present in a solution of dicobalt EDTA. These cobalt ions induce toxicity and the use of dicobalt EDTA in the absence of sufficient levels of cyanide *in vivo* will lead to serious cobalt toxicity [24]. Numerous side effects have been reported, including vomiting, tachypnea, chest pain, hypotension, ventricular arrhythmias, seizures, urticaria, facial, laryngeal or neck edema, and anaphylactic shock [17]. These side effects are enhanced if the patient does not have cyanide poisoning; therefore, this treatment is reserved for cases in which the diagnosis is certain and/or patients do not respond to other treatments. Animal data suggest glucose has a protective role against cobalt toxicity, and it has been recommended to co-administer glucose [24, 59]. Administration of glucose by itself does not show any efficacy and its use has no reported rationale. In the clinic, a 300 mg dose of dicobalt EDTA is infused over one minute and an additional 300 mg dose may be repeated five minutes later if no improvement is observed. Each injection of dicobalt EDTA must be followed immediately by 50 mL units of 50 % glucose (v/v) [17]. Animal studies confirmed that dicobalt EDTA improves survival, even when administered rather late in the classical sequence of events of cyanide poisoning [77]. The high mole per mole ratio of cyanide to metal center may contribute to that effect.

4.2.2. Cyanokit® (Hydroxocobalamin)

Cyanokit® employs hydroxocobalamin (Figure 6) as the antidotal compound. It has been used as a treatment option for cyanide poisoning in France since the 1980s and was formally licensed there in 1996 [90]. It was approved by the US FDA in 2006, for the treatment of known or suspected cyanide poisoning [91].

Hydroxocobalamin is a metal complex based on corrin, a tetrapyrrole ring. The central metal ion is a six-coordinated cobalt(II) positioned in the center of the corrin ring. Four of the coordination sites are occupied by the corrin ring, the fifth by an α-axial dimethylbenzimidazole group, and the sixth by a β-axial hydroxo group [92].

CobOH is an analog of vitamin B_{12} – sometimes denoted vitamin B_{12a}. While hydroxocobalamin has a hydroxo group in the β-axial position, cyanocobalamin (vitamin B_{12}) bears a cyano group [88, 92]. The reaction of CobOH with cyanide is a ligand exchange reaction between the hydroxy group and the cyanide in the β-axial position. The antidote reaction operates on an equimolar basis to form the non-toxic cyanocobalamin, which is subsequently excreted in urine [14].

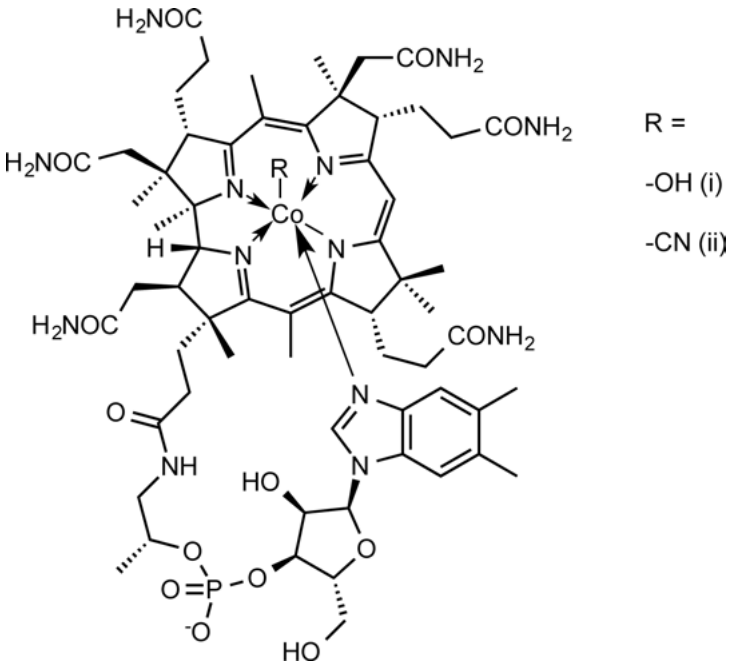

Figure 6. Structures of (i) hydroxocobalamin and (ii) cyanocobalamin.

CobOH has a rapid onset of action because it enters into the different tissue compartments almost immediately following intravenous administration. It distributes to the erythrocytes and plasma cells and reaches the cerebrospinal fluid within 30 minutes. This approach is safe because it employs a biologically relevant compound bearing high biological availability and an optimal environment for cyanide coordination. It bypasses both CcO and rhodanese, differentiating itself because it neither improves the sulfur substrate efficacy (thiosulfate and sulfanegen) nor intervenes in oxygen transport as do the methemoglobin formers (nitrates). CobOH has exhibited improved hemodynamic stability in both human and animal studies [59].

In the clinic, doses of 5 g of CobOH per person will effectively treat intoxicated patients with up to 40 μmol/L blood cyanide levels without the need for an additional dose [14]. A 5 g dose administered intravenously is generally well tolerated experimentally and clinically in healthy volunteers [12]. A prospective study on 69 patients treated at the scene for suspected smoke inhalation-associated cyanide poisoning revealed no serious side effects attributed to CobOH after intravenous infusions of 5 g (maximum 15 g). Out of the 42 patients that were confirmed to have cyanide poisoning, 67 % survived after treatment with CobOH [12]. The most common adverse effects were chromaturia, pink or red discoloration of the skin, hypertension, headaches, nausea, and increased blood pressure. CobOH appears to be safe for non-clinical treatment of fire victims with or without cyanide poisoning [93].

4.2.3. Cobinamide

Cobinamide (Figure 7) is the penultimate compound in the biosynthesis of cobalamin. It is similar in structure to cobalamin, except it lacks the dimethylbenzimidazole nucleotide tail coordinated to the cobalt atom in the α-axial position of cobalamin. Cobinamide is reported as a compound in development as an emergency treatment for cyanide poisoning in smoke inhalation victims and other mass casualty cases [94].

Cobalamin only has the β-axial binding site available for cyanide binding, while cobinamide has both α-axial and β-axial ligand binding sites, binding two cyanide moles per mole. The dimethylbenzimidazole group of cobalamin has a negative *trans* effect on the α-axial binding site, reducing cobalamin's affinity for axial ligands. The absence of these *trans* effects leaves cobinamide with much greater affinity ($K_A \approx 10^{14}$ M^{-1}) for cyanide ions than cobalamin (10^{12} M^{-1}) [93, 94]. This suggests that cobinamide could become a better option as an emergency treatment for cyanide poisoning than Cyanokit®. Studies indicate cobinamide is several-fold more effective than cobalamin in rescuing mammalian cells and *Drosophila melanogaster* from cyanide toxicity [94]. Studies in New Zealand white rabbits in a sublethal cyanide exposure animal study showed that intramuscularly administrated cobinamide rapidly and effectively reversed the physiological effects of cyanide poisoning [76]. Experiments with cobinamide showed promising results when administered intramuscularly [94].

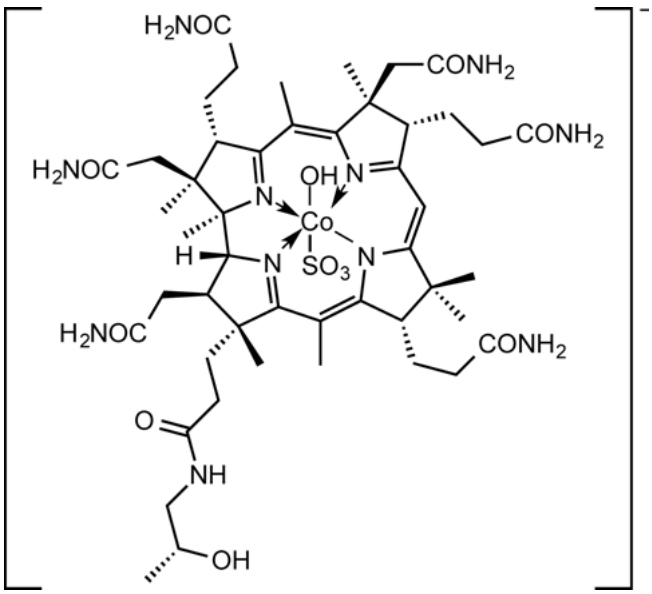

Figure 7. Structure of cobinamide.

4.3. Molybdenum-Containing Antidote

The currently available cyanide poisoning treatments have many positive features. Thiosulfate and sulfanegen address the importance of substrate availability for the enzyme to operate at its peak efficiency. The cobalt complexes demonstrate efficacy and ability to compete for binding of cyanide with the CcO target. The dicobalt EDTA binds many molecules of cyanide per mole of complex, and hydroxocobalamin is the safest treatment available. Cobinamide is still in the early stage of development and may outperform hydroxocobalamin in terms of efficacy and safety. An ideal antidote for cyanide poisoning is an antidote that may be used without confirmation of cyanide poisoning. Ideally, such treatment has an appropriately facile and rapid mode of administration at the accident site and has rapid onset of action without impairment of oxygen-carrying ability of the blood.

One more approach in development uses molybdenum which has not been previously employed for cyanide poisoning treatments.

4.3.1. Molybdenum Sulfur Compounds

Many molybdenum compounds are non-toxic and tetrathiomolybdate reached phase 2 clinical trials as a treatment for Wilson's disease [95] (see also Chapter 12 in this book). The approach used for the molybdenum complex adapts the utility of a catalytic drug. By offering a catalyst that is biocompatible with thiosulfate in terms of solubility and bioavailability, the sulfur substrate access to a catalytic site that forms thiocyanate is increased. With the above criteria in mind, dinuclear molybdenum-sulfur complexes were developed to catalyze the reaction of thiosulfate and cyanide *in vivo* and to serve as potential emergency treatment for cyanide poisoning [96].

The reaction of thiosulfate and cyanide is catalyzed by rhodanese (see Section 2.1). Water-soluble molybdenum sulfur complexes are efficient catalysts for the reaction of thiosulfate and cyanide to form thiocyanate and sulfite *in vitro* [97]. The catalytic reaction is facile in aqueous solution and efficiently converts all available cyanide to thiocyanate. The overall reaction is shown in Figure 8. The cyanide reacts with the disulfide of the molybdenum complexes forming thiocyanate and a sulfido complex. The resulting sulfido complex reacts with thiosulfate, regenerating the disulfide complex and forming sulfite. The overall reaction is comparable to the enzymatic reaction with thiosulfate as substrate. However, increased bioavailability is intended to result in a more efficient detoxification.

One criterion for a suitable cyanide poisoning treatment is that the antidote should have a high benefit-to-risk ratio. The molybdenum sulfur complexes exhibit low cytotoxicity and potentially very high aqueous solubility [97]. High water solubility is an important factor for the intramuscular administration route. Preliminary experiments in an animal model indicate some of the molybdenum sulfur complexes are non-toxic at concentrations that correspond to lethal cyanide concentration and may be good candidates as an emergency treatment [97].

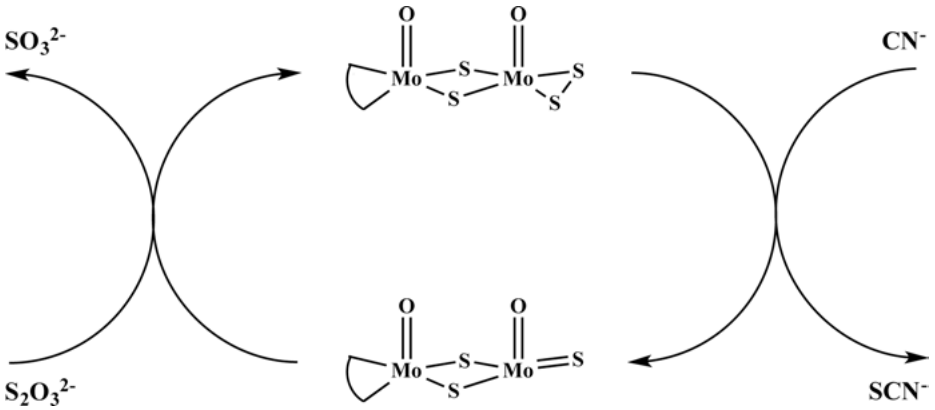

Figure 8. Proposed detoxification of cyanide by a molybdenum sulfur compound.

The prospect of a catalytic antidote *in vivo* is attractive as it implies that a smaller quantity of antidote would be required, whereas a large dose per treatment is time-consuming to administer and impractical in emergency situations.

4.5. Chemical Aspects

Cyanide is commonly found as HCN or as a salt with various counter cations. The cyanide anion is usually the toxic agent in common commercial cyanide salts. HCN is a volatile clear or pale blue liquid with a boiling point of 26 °C. Its pK_a is 9.2 and therefore, at physiological pH of 7.4 it is mostly found as HCN. For handling purposes it is normally prepared in basic solutions to completely deprotonate HCN thus preventing volatilization.

Cyanide has a very strong affinity for metal ions with high oxidation states. It has strong affinity for both Fe^{3+} and Fe^{2+} hemoproteins leading to low-spin states [98] where k_{diss} for ferroheme has been published as 1.0 M, and 10^{-4} M for ferrihemes [46]. Methemoglobin is the Fe(III) version of normal hemoglobin and its ability to preferentially bind cyanide compared to normal Fe(II) hemoglobin is exploited in the use of "methemoglobin former" antidotes (see Section 4.1). Similarly, cyanide has strong affinity towards both Co^{2+} and Co^{3+} metalloenzymes such as vitamin B_{12} [99]. Hydrogen cyanide reacts *in vivo* in a facile reaction to form the cyanide anion that can interact with metalloenzymes bearing accessible coordination sites based on its property as a strong π-acid. About 40 different enzymes are potential targets. These enzymes include hemoglobin, cytochromes, catalases, peroxidases, carbonic anhydrase, and oxidases. Despite its reactivity, cyanide is thought to exert its ultimate lethal effect of histotoxic anoxia by binding to the active site of cytochrome *c* oxidase.

Bovine heart cytochrome *c* oxidase has been crystallized and analyzed at a 2.8 Å resolution [100]. The structure is made up of 13 subunits and it was crystallized as a dimer in its oxidized state. It has three metal centers: Cu_A, heme *a*,

and heme *a3*/Cu_B. The metal binding sites are described here briefly while its structure and functions are described well elsewhere [101]. The enzyme (Complex IV) is a membrane-bound electron transfer protein and CcO is the last in a sequence of such proteins in the cellular respiratory chain. The reaction reduces oxygen to water and protons. The protons are pumped across the mitochondrial membrane barrier from the matrix side into the cytosol. The overall reaction catalyzed by CcO may be written as:

$$O_2 + 4e^- + 8\,H^+ \text{ (in)} \longrightarrow 2\,H_2O + 4\,H^+ \text{ (out)} \quad (3)$$

The electron transfer takes place by a route where incoming electrons from cytochrome *c* are transferred first to the Cu_A site that is a bimetallic copper site. The Cu atoms are 2.38 Å apart and are held in overall tetrahedral coordination geometry by three pairs of cysteine, histidine and glutamate sidechains. The electrons are then transferred from Cu_A to the heme *a* site. Heme *a* is an iron cofactor with an octahedral low-spin Fe^{2+} that is axially coordinated by histidine residues. In the CcO structure, heme *a* and heme *a3* are connected by a tripeptide linkage. Reduced heme *a3* has a five-coordinate high-spin Fe^{2+} (S = 2) center with one axially ligated histidine. The Cu_B metal center faces the iron heme *a3* center at about 4.70 Å distance. The copper atoms are positioned in a trigonal planar coordination geometry ligated by three histidine residues. Oxygen molecules entering at the heme *a3*/Cu_B catalytic site are reduced with the electrons transported from heme *a*. In the crystal structure of the oxidized enzyme, the oxygen molecule was found as a peroxo group bridging the Fe^{3+} and Cu^{2+} metal centers [102]. The oxidized state of the catalytic site is EPR-silent with the Cu and Fe antiferromagnetically coupled (Fe^{3+}, S = 5/2, Cu^{2+}, S = 1/2); titration studies confirmed six electron equivalents are needed to bring the enzyme back to the reduced state [103]. Magnesium and zinc ions present in the crystal structure are believed to have roles related to water transport from the hydrophobic catalytic site.

The active form of CcO is the fully reduced state. The generally accepted mechanism involves binding of oxygen to the reduced state with Fe^{2+} and Cu^+; after O–O bond cleavage, the metal centers are in Fe(IV)=O and Cu^{2+} oxidation states and a tyrosyl radical has formed on a tyrosine residue near the copper center. Computational studies indicate several steps take place to form two water molecules and the reduced enzyme state [104].

The reaction mechanism of cyanide with CcO has been studied in the fully oxidized and in the reduced state [105]. Cyanide binds to the iron heme *a3* in CcO preventing the oxygen reduction [106]. The reaction of CN^- with the oxidized enzyme forms a low-spin cyanoferric cytochrome oxidase complex (CcO-CN) [107]. The CcO-CN complex formation is dependent on the cyanide concentration behaving as a non-competitive inhibitor at low cyanide concentrations but the reaction rate becomes first-order at elevated cyanide concentrations. The stoichiometric reaction of CcO with CN^- *in vitro* is completed after 10 minutes of incubation time in the reduced state compared to 18 hours in the oxidized state [105]. The observed reactivity difference was assumed to arise from a differ-

ent and less accessible conformation of heme *a3* in the oxidized form leading to a dependency of the formation constant of cyanide with CcO on the redox state of the enzyme [105].

The lethality of cyanide is primarily related to the difficulty in reversing its binding to CcO. The dissociation of cyanide is very sluggish, although it is facilitated *in vitro* with reducing agents such as dithionite. The current antidotes and treatments for cyanide poisoning do not include competitive antagonists for the CcO-CN complex, although it has been suggested this may be possible in studies where diaminomaleonitrile was examined as a potential antagonist to cyanide inhibition of CcO [108].

Recent evidence suggests CN^- bound to CcO in the mixed valence state is stabilized as Fe^{3+}-bound cyanide and reduction of the cyanide-bound enzyme leads primarily to reduction of metal centers other than heme *a3* [109]. This is in agreement with previous studies where the dissociation of cyanide was postulated as a requirement for reduction of the heme *a3* iron and dissociation of cyanide [107]. The final form of the inhibited enzyme is conformationally different than a "normal CcO reduced state" and it has the heme *a3* in the ferric state [110].

In cases of concurrent poisoning by CO and HCN, it is well established that CO is a competitive inhibitor of O_2 binding at the Fe^{2+} metal center of the heme *a3* in the reduced CcO. Oxygen administration is the primary clinical treatment for CO poisoning and the recovery of the oxidation-reduction state of the mitochondria is delayed during reoxygenation [111]. The effects observed with CO were demonstrated in rat brain cortices by differential spectroscopy *in vivo* [112].

Efforts in developing antidotes have focused on preventing the formation of the CcO-CN complex. Generally, ligand exchange between CN^- and NO in the axial position on heme should be possible. However, *in vitro* studies using NO generated conflicting results whether NO is able to reactivate the enzyme; although it is a substrate for CcO, it binds to the Cu_B metal center and forms nitrite through a different mechanism [113, 114].

4.6. Analytical Aspects

Analysis of cyanide or hydrogen cyanide in biological fluids is challenging. Determination of CN^- in blood requires rapid sampling and processing because cyanide is not stable in blood samples [3]. Collection of samples, storage, and analysis needs to be performed with care, and samples need to be analyzed as soon as possible. Biological samples such as tissue and fluids have multiple potential interferences. Analytical methods detailing sampling and analysis of blood and tissues for the presence of cyanide are well documented [11]. Cyanides in aqueous samples or drinking water are quantified by analysis of total cyanides with a semi-automated colorimetric method as well as non-automated colorimetric methods, distillation, electrochemical, or UV analysis [11]. A special class of aqueous cyanide samples includes metal complexes that act as weak acids and are mostly applicable to wastewater analysis from mining of precious metals [16, 115].

Analysis in cases of suspected cyanide poisoning is mostly focused on thiocyanate and cyanide quantification or qualitative confirmation. Thiocyanate is mostly found in plasma. As blood cyanide is mostly found in erythrocytes, analysis of whole blood is required to determine blood cyanide concentrations [116], although quantification from whole blood samples is likely to be unreliable in patients with respect to the concentration of exposure because of its short half-life in blood. Still, estimates of tissue levels are necessary concurrent studies in forensic cases [35].

The time involved in the analysis of blood samples in the laboratory is a bottleneck when treatment against cyanide intoxication depends on confirmation of poisoning. In recent years there has been considerable interest in the development of more rapid and reliable analytical methods suitable for detection of cyanide in blood and its quantification [117, 118]. Spectrophotometric methods have attracted much attention [119–121] although many of the organic reaction-based colorimetric methods are limited to aqueous sample processing and are not useful for biological matrices [122]. A few techniques are commonly used to perform analysis of whole blood cyanide. These methods comprise Conway microdiffusion, isotope-dilution gas chromatography-mass spectrometry (ID GC-MS), or fluorescence detection for trace amounts of cyanide. The former two methods are more suitable for clinical confirmation of cyanide blood levels. The ID GC-MS method is an automated procedure capable of using whole blood samples, and provides complete analysis within two hours [123]. Given these available methods for the analysis of CN^- blood concentrations, the decision to begin treatment for cyanide poisoning is often a clinical one [124]. Documentation of blood cyanide levels is still useful in confirming the clinical diagnosis and in subsequent follow-up investigations.

The Conway microdiffusion method is based on liberation of HCN from the blood into the gas phase. A common treatment of the liberated HCN is to convert it into the anion with sodium hydroxide and employ a colorimetric quantification. Colorimetric reagents for quantification of aqueous samples of cyanide are organic-based sensors like picric acid [125] or a combination of chloramine-T and isonicotinic acid/barbituric acid (König reaction) [126]. Results using this colorimetric method are available within three hours. Disadvantages of colorimetric methods include their relatively long sample processing time and toxicity of the organic reagents [126]. The liberated HCN may alternatively bind to water-soluble metal complexes with high affinity towards cyanide.

Metal complexes such as metalloporphyrins and corrins have absorption spectra that are strongly dependent on the nature of their axial ligands [127–130] and may be utilized as analytical sensors if the ligand concentration and absorbance show a linear relationship [131, 132]. Two such complexes are hydroxocobalamin forming cyanocobalamin [131] or methemoglobin to form cyanomethemoglobin [133]. Use of water-soluble metal complexes with high affinity towards cyanide is the most recent addition to the colorimetric analytical methods. Two of these involve use of cobalt-based corrins; either cobinamide-hydrolyzed vitamin B_{12} derivative − or a cobyrinic acid derivative. The cobinamide has a linear response at a very low concentration range [134]. The cobyrinic acid derivative was reported as an effec-

tive, water-soluble compound for spectroscopic analysis. Both compounds are capable of detecting levels of parts per million of cyanide [131]. Interestingly, the methodology for the latter compound was developed in a crude biological matrix and qualitative confirmation of the presence of cyanide may be obtained using a colored chart to match the cyanide concentration. This method may be ideal for field detection of cyanide and possibly cyanide poisoning.

Interest in qualitative methods to detect biological cyanide is of general importance for populations that consume cyanogenic plants as a food staple [135]. Cyanogenic glycosides present in foods can cause severe chronic and significant acute public health problems [136, 137]. Cyanogenic glycosides hydrolyze to form glucose, HCN, and alcohol (see Figure 3). The newer analytical methods developed either use an indirect method by quantifying the glycosides themselves, the glucose product, or HCN directly. These methods include direct estimation of cyanogenic glycosides by gas chromatography [138], and detection of either glucose or HCN using potentiometric [139, 140], amperometric [141], fluorometric [142] or enzymatic techniques [142–144].

Renewed interest in the development of treatments against cyanide poisoning has led to a parallel interest in developing rapid and reliable methods to confirm the presence of cyanide in blood and aqueous samples. Progress has been made in the last ten years, and in a few years there may be a rapid method available for use in the field or in the clinic to confirm the presence of cyanide and assist determining treatment.

5. CONCLUDING REMARKS

Despite its toxicity, accidental death from cyanide poisoning is not common. This is mostly due to its reactivity and volatility. Results from various researchers indicate that it may present long-term health issues after non-lethal exposure and treatment after non-lethal exposure may serve as preventive care.

The currently available commercial emergency treatments are all post-exposure products. They were developed using one of three approaches: (i) intercepting the cyanide quickly and neutralizing it with methemoglobin, (ii) increasing the sulfur substrate availability for the biological cyanide detoxification pathway, or (iii) by chelating the cyanide with a metal complex without affecting oxygen transport. These approaches all have their merits and downsides. An approach that combines interception and rapid detoxification without affecting other biological functions may be beneficial.

Cyanide is an endogenous molecule with unexplained biological roles. While it may still be true that it is only a toxin the body has to dispose of, there is much that is unknown or unclear about its function. It is an interesting molecule to study and many questions are left to answer.

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ABBREVIATIONS AND DEFINITIONS

CAK	Cyanide Antidote Kit
CcO	cytochrome *c* oxidase
CcO-CN	cyano-cytochrome *c* oxidase
CF	cystic fibrosis
CNS	central nervous system
COHb	carboxyhemoglobin
CobCN	cyanohydroxocobalamin
CobOH	hydroxocobalamin
CWA	chemical warfare agent
4-DMAP	dimethylaminophenol
EDTA	ethylenediamine-N,N,N′,N′-tetraacetic acid
EPR	electron paramagnetic resonance
US FDA	United States Food and Drug Administration
HCN	hydrogen cyanide, prussic acid
ID GC-MS	isotope-dilution gas chromatography mass spectrometry
LC	ethal concentration
LC_{t50}	lethal concentration in fifty minutes
LC_{50}	50 % lethal concentration
MetHb	methemoglobin
3-MP	3-mercaptopyruvate
MPO	myeloperoxidase
3-MST	3-mercaptopyruvate sulfurtransferase
NMDA	N-methyl-D-aspartate
OPCW	Organization for the Prohibition of Chemical Weapons
RBC	red blood cells
UV	ultraviolet
WHO	World Health Organization
WWI	World War I
WWII	World War II

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