TOPICS IN ORGANOMETALLIC CHEMISTRY 32

Volume Editors G. Jaouen · N. Metzler-Nolte

Medicinal Organometallic Chemistry

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Medicinal Organometallic Chemistry

Volume Editors: Gérard Jaouen and Nils Metzler-Nolte

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Aims and Scope

The series *Topics in Organometallic Chemistry* presents critical overviews of research results in organometallic chemistry. As our understanding of organometallic structures, properties and mechanisms grows, new paths are opened for the design of organometallic compounds and reactions tailored to the needs of such diverse areas as organic synthesis, medical research, biology and materials science. Thus the scope of coverage includes a broad range of topics of pure and applied organometallic chemistry, where new breakthroughs are being made that are of significance to a larger scientific audience.

The individual volumes of *Topics in Organometallic Chemistry* are thematic. Review articles are generally invited by the volume editors.

In references *Topics in Organometallic Chemistry* is abbreviated Top Organomet Chem and is cited as a journal.

Introduction

Interdisciplinary research is increasing, and often actively encouraged, across many disciplines in the sciences. While this does not relieve individual researchers from the obligation to strive for originality and a deep understanding of their particular field of research, innovative concepts may emerge by combining seemingly incompatible areas of research. One such area that seemed impossible to exist to many chemists only 20 years ago is *Bioorganometallic Chemistry*, i.e., the study of biological applications of organometallic compounds. An organometallic compound, by definition, contains at least one direct, covalent metal–carbon bond. As such, it was automatically felt to be sensitive to air and water and hence unsuitable for biological applications by the great majority of chemists. In 1985, an article appeared which used the term "bioorganometallic chemistry" in its introduction, and in 1986, the term was used in the title of a review article [1a, b], probably the first mentions of this term, probably the first mention of this term in a highly visible place. Admittedly, quite a few compounds and activities existed well before 1985, which we would, in hindsight, classify as bioorganometallic. Vitamin B_{12} (Fig. 1) and the whole family of cobalamins are arguably organometallic compounds with biological functions of highest importance, e.g., in amino acid and nucleotide biosynthesis. The body of a healthy human contains only a few mg of cobalamins, but even this small amount is absolutely necessary for the well-being and indeed survival of each of us. Dorothy Crowfoot Hodgkin, who solved the crystal structure of Vitamin B_{12}, or cyanocobalamin, was awarded a Nobel Prize in Chemistry for this hallmark in structural chemistry in 1964. Even though the term did not even exist during her lifetime, Dorothy Hodgkin could thus be counted as a grandmother of bioorganometallic chemistry. While most scientists would likely find some ridicule in such a postmortem assignment of scientific achievements, it is also undoubtedly evident that the creation of a common intellectual roof – visible by a term or a prominent founding father – will promote and even induce research in a particular area. This, in fact, is quite a common observation, and it was certainly a motivation for the editors to initiate the present volume on *medicinal organometallic chemistry*.

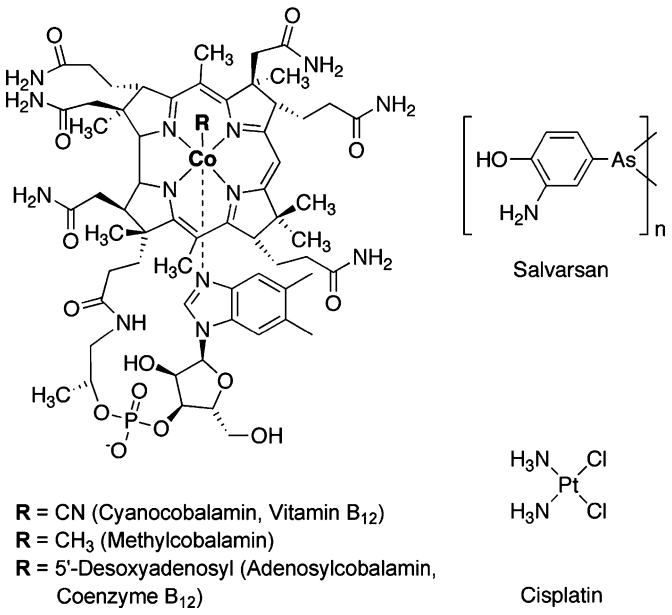

Fig. 1 Three metal compounds of medicinal importance: Cyanocobalamin, also known as Vitamin B_{12}, Ehrlich's compound no.606, or Salvarsan, and Cisplatin

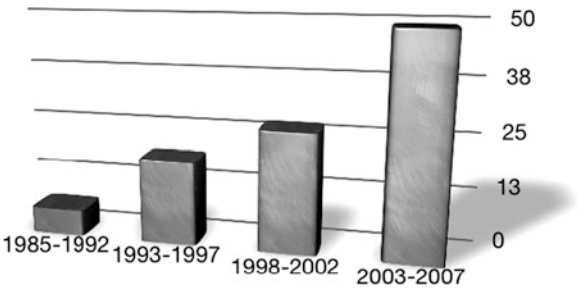

Fig. 2 Number of publication found in SciFinder by using the keyword "bioorganometallic chemistry" since 1985

Since this first mention of a definition and some biological applications of organometallic compounds, the field has grown exponentially, as shown in Fig. 2. A closer inspection of the publications listed under the keyword "bioorganometallic chemistry," in fact, reveals that quite a significant portion of those deal with medicinal applications of organometallic compounds. We can thus define the term *Medicinal Organometallic Chemistry* as dealing with the synthesis and

biological evaluation of organometallic compounds for diagnostic or therapeutic applications, as well as investigations into their mode of action.

Historically, it is interesting to note that the first medicinally important compound that was actually identified by a systematic screening effort was in fact a metal-containing compound. In 1909, Paul Ehrlich filed a patent for a compound (no. 606, later termed Salvarsan) that had been identified after a year-long systematic synthesis-and-evaluation effort in his laboratory. The compound contained arsenic bonded to a substituted aromatic ring, and thus in fact is an organometallic compound (Fig. 1). From his extensive synthesis efforts, Ehrlich was able to deduce what in modern terms would be described as structure–activity relationships, that is, to draw connections between the chemical structure of a compound and its biological activity. Salvarsan was active against syphilis, a bacterial infection for which at the time no adequate cure was available. The drug was in use until about the end of the World War II, when it was gradually replaced by modern antibiotics, primarily from the penicillin family. Interestingly from a medicinal chemistry point of view, penicillin was discovered by accident. Following the discovery of penicillin from a natural source, major efforts were initiated by pharmaceutical companies as well as by public institutions to identify new active entities by what became known as high-throughput screening. It is only in the last two decades or so that systematic drug design and structural modifications to improve biological activity – initially proposed by Ehrlich in the quest for his "magic bullets" against microbes – are reentering medicinal chemistry in the initial phases of drug discovery. It will become clear throughout this volume that medicinal organometallic chemistry is undergoing a similar transition from random screening of compounds to informed drug design and targeted investigations into their mode of action.

Salvarsan contains arsenic as its metallic element. Likewise, there are other important main group compounds with biological activity and indeed some drug candidates. Examples would comprise tin compounds, which are not only used as antifouling agents and antibacterials, but also have promising antiproliferative activity. Other examples for compounds with possible anticancer activity include the main group elements Ge and Ga. Boron compounds have been suggested for a long time in boron neutron-capture therapy (BNCT), and more recently applications for topical antifungal agents have been suggested. A rather comprehensive list of pharmaceutical applications of all the elements is found in a recent book by Gielen and Tiekink [2]. In the present book, we have included a chapter on Salvarsan and As compounds mainly for historical interest in the subject (chapter "Arsenic-Based Drugs: From Fowler's Solution to Modern Anticancer Chemotherapy" by Gibaud and Jaouen), but we will concentrate on medicinal applications of transition metal compounds otherwise.

One of the great successes of transition metal compounds is Cisplatin. This compound, with a very simple structure (and, incidentally, not even a single carbon atom!), is one of the most successful anticancer drugs [3]. After its serendipious discovery by B. Rosenberg, Cisplatin quickly gained clinical approval in 1978. It has inspired research efforts into literally thousands of Pt-containing compounds, from which two more have reached world-wide approval (Carboplatin and

Oxaliplatin), and a few others have obtained regional approval. Platinum compounds are today used in more than half of all anticancer treatment regimes, and they are among the three most-prescribed anticancer drugs. More than 60 years after the introduction of Salvarsan, the success of Cisplatin has renewed the interest of the scientific community in metal-based drugs. At about the same time as the discovery of Cisplatin, recognition rose of the importance of metal ions in many enzymes and biological processes. This produced the field of *Bioinorganic Chemistry* as an interdisciplinary research effort between inorganic, physical, and biochemists. Although *Bioinorganic Chemistry* has many more facettes [4], its main focus still is enzymology of metallo-enzymes and the synthesis and study of model complexes thereof [5, 6]. As most metallo-enzymes contain the elements N, O, and S as ligands to the metals, the notion arose that coordination compounds with these elements are almost exclusively reserved for biological applications – evidently proven for medicinal inorganic chemistry as well by the structure of Cisplatin. Cobalamin, although its structure was well known at the time, was seen as the odd boy on the block and investigations into its chemistry regarded as a slight curiosity at least by most main-stream bioinorganic chemists.

To be fair to all sides, we have to contrast the above-described developments with the route that organometallic chemistry took during the same times. The discovery and subsequent structure elucidation of ferrocene, probably the quintessential organometallic compound, by Fischer and Wilkinson in the early 1950s marks the beginning of modern organometallic chemistry. Both were awarded the Nobel Prize in 1973. Ten years before this, Ziegler and Natta were awarded the Nobel Prize for their discovery of the low-pressure polymerization of olefins by transition metal catalysts. In between these two Nobel Prizes, many new groups of organometallics were discovered, such as carbenes and carbynes, and whole new groups of ligands were uncovered in their properties and applications. Also, the discovery of Pd-catalyzed C–C bond forming reactions (collectively referred to as "Heck reactions") probably revolutionized organic synthesis in the late 1960s. Collectively, these developments gave an enormous impetus to the development of organometallics for applications in catalysis. Moreover, because Ziegler–Natta catalysis as well as most Heck reactions require oxygen-free, anhydrous conditions, thousands of researchers were trained to apply such conditions whenever using organometallic compounds. Consequently, the notion developed that this was a general feature of *all* organometallics – regardless of the fact that, for example, ferrocene itself is perfectly stable to air and water.

Also in the 1970s, a few seminal papers reported on the anticancer activity of organometallic transition metal compounds. Köpf et al. reported that titanocene dichloride, and several other bent metallocene dihalides to a varying degree, was able to inhibit tumor growth in mice in vivo [7]. Unsurprisingly, the idea for this investigation arose from the formal structural similarity of the metal *cis*-dihalide unit in the metallocenes and Cisplatin. Surprisingly, however, even the simple ferrocenium cation was subsequently shown to have at least some antiproliferative activity [8]. This fact could not be explained by the knowledge that existed at those times about the mode of action of metal-based drugs, and it has remained quite a

mystery for a rather long time. The intriguing activity of ferrocene derivatives, however, has inspired later work, and some of these more recent achievements will be covered in chapter "Ferrocene Functionalized Endocrine Modulators as Anticancer Agents" by Hillard et al. Titanocene-dichloride has entered clinical trials, but it finally failed in phase II trials, most likely due to problems with the stability of the preparations used and decomposition of the compound. A major lesson from this failure is that formulation of a compound into a usable drug is vitally important for its clinical success. This is nowadays a commonplace in medicinal chemistry in general, with the famous Lipinski "Rule of Five" being just one peculiar expression of this [9]. For researchers in inorganic and organometallic medicinal chemistry, however, the ramifications of this lession are still not universally recognized or accepted. Promising new titanocenes as well as their formulation as drugs are covered in chapter "Titanocenes: Cytotoxic and Anti-angiogenic Chemotherapy Against Advanced Renal-Cell Cancer," by Hogan and Tacke.

Detailed investigations were performed into the mechanism of action of the platinum anticancer drugs. A broad consensus has emerged that the active species interact with DNA as the final and lethal target, although protein interactions will certainly contribute to the overall efficacy of the drug in vivo. In fact, these numerous and very detailed investigations have led to the paradigm that metal-based drugs will almost inevitably interact with cellular DNA as their target. This assumption was certainly fruitful in initiating biological investigations and method development for the interaction of metal compounds with DNA in general. Some promising Ru anticancer drug candidates, for which DNA interaction is a likely mode of action, are covered by Pizarro et al. in chapter "Activation Mechanisms for Organometallic Anticancer Complexes" of this book.

However, as with all paradigms, new insight arises when the paradigm fails or is brought to fall. In the present case, the metals as well as their particular complexes have a diversity of chemical properties, so the notion that they should all come down on one and the same cellular target – DNA – seems almost ridiculously unlikely. At least four chapters in this book (chapters "Organometallics as Structural Scaffolds for Enzyme Inhibitor Design" by Mulcahy and Meggers, "Organometallic Antitumour Agents with Alternative Modes of Action" by Dyson et al., "Ferrocene Functionalized Endocrine Modulators as Anticancer Agents" by Hillard, Vessières and Jaouen "Bioorganometallic Chemistry and Malaria" by Biot and Dive) actually describe the thoughtful design and detailed biological investigations of organometallic drugs that will *not* interact with DNA as their main target but are designed to interact with protein targets (chapters by Meggers and Dyson) or yet other targets, some of which are not even fully uncovered. To assist in the study of cellular interaction of organometallic drugs, they can be covalently linked to biomolecules such as peptides. This may on the one hand make them targeted to certain cell types which possess receptors for the peptide in question, or may assist in intracellular delivery of the conjugate to a particular target, e.g., the nucleus, thereby contributing to studies into the mechanism of action of the conjugate as described in the chapter "Biomedical Applications of Organometal–Peptide Conjugates" by Metzler-Nolte.

A question one might ask is why *organometallic* compounds should be so special? Several reasons can be given, some of which relate to the process of drug development in medicinal chemistry in general. For one, organometallic compounds are frequently low-valent, and so are not harmful as oxidants themselves. Second, they are in many cases kinetically inert and will thus act as a whole compound, e.g., when inhibiting enzymes. This is most vividly shown by the now several crystal structures that have been obtained for the Cp-Ru kinase inhibitors in complex with their protein targets (chapter "Organometallics as Structural Scaffolds for Enzyme Inhibitor Design" by Mulcahy and Meggers). Moreover, and because the metal is usually relatively strongly bound to its carbon ligands, organic transformations on the periphery of the molecule are possible. This provides opportunities for rather extensive structural modifications while keeping the metal and its ligands intact. This approach is maybe best illustrated by the solid-phase synthesis of organometal-peptide conjugates in the chapter "Biomedical Applications of Organometal–Peptide Conjugates" by Metzler-Nolte. There is, however, finally the fact that a metal complex has more to offer in terms of medicinal applications than its organic ligands, i.e., metal-specific reactivity will be something that medicinal *inorganic* chemists will always look for, and in some cases this has indeed been confirmed and the metal complexes are thus active beyond their organic ligands, e.g., as described in chapters "Activation Mechanisms for Organometallic Anticancer Complexes," "Organometallic Antitumour Agents with Alternative Modes of Action," "Ferrocene Functionalized Endocrine Modulators as Anticancer Agents," and "Carbon Monoxide: An Essential Signalling Molecule."

This last consideration is most obvious for radioactive metals. In fact, one very successful low-valent organometallic agent that is used by the million every year in radio imaging is a compound called Cardiolite. This ^{99m}Tc(I) hexakis-isonitrile complex (Fig. 3) is used for radio imaging of the cardio-vascular system. More recently, the $Tc^{I}(CO)_3$ core has been amply used for imaging purposes in bioconjugates. This topic is covered in the chapter "Organometallic Radiopharmaceuticals" by Alberto. Indeed, a kit formulation for the easy preparation of the

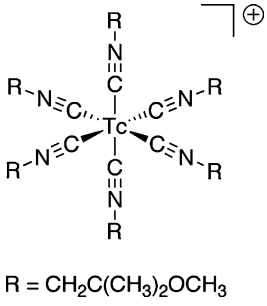

Fig. 3 Cardiolite, an organometallic ^{99m}Tc compound used for radio imaging

$[Tc(CO)_3(H_2O)_3]^+$ is commercially available from Mallinkrodt and is becoming popular at least for research purposes. In addition, other documented commercial interest exists in organometallic compounds for medicinal applications: Two compounds from Megger's group are commercially available for basic research as potent and selective kinase inhibitors. Several compounds are close to clinical trials, such as two Ru complexes from the groups of Sadler and Dyson, as well as the compound termed Ferrocifen from Jaouen's group. Most notably, the antimalarial compound Ferroquin from Brocard and Biot is developed by Sanofi-Aventis. It is at the end of clinical phase II trials and will soon enter phase III trials. To this end, it has already been synthesized on a >100 kg scale and hopefully, there will soon be another (i.e., nonplatinum) metal-based drug on the market.

Finally, it is one purpose of this book to document the advancement that medicinal chemistry with metal ions has made over the past few years. Apart from extensive biochemical work on the mode of action of Cisplatin, most investigations in pre-2000 have followed a rather crude synthesis-and-cytotoxicity-testing approach. Nowadays, we see quite a diverse and mechanism-oriented field of study. In aspiring to improve our understanding, synthetic chemists have learned to talk to, and collaborate with, biochemists and cell biologists, as well as medical doctors. Issues which are rather common in modern (organic) medicinal chemistry, such as target identification, lead design, structure–activity relationship, structural biology, and computational methods, are slowly but steadily becoming accepted and common tools in the medicinal chemistry of metal compounds as well. It is our belief that medicinal chemistry with *organometallic* compounds in particular has contributed in particular to this progress, and therefore rightly claims a place of its own in medicinal chemistry. We are aware of the fact that many problems still remain to be tackled, and that our understanding of the mode of action of many active organometallics is still rather crude, nor are our methods for discovery of new active compounds well developed. However, as stated in the outline of this introduction, clarity about the status of a research field, its methods and possibilities will increase visibility and hopefully attract new researchers with again more novel ideas and inspirations. We hope that the present book will serve its purpose to this end.

The editors realize that everyone involved in this project probably had many other important tasks to perform and deadlines to meet in parallel to this project. We therefore appreciate the energy, effort, but also the patience of all of you during the production process of this volume. We would like to thank first all our authors, as well as their coworkers for their contribution not only to this book, but also to the field as a whole. The publisher, namely Jörn Mohr and Jutta Lindenborn, is thanked for support of this project and professional management of the production process. To all readers, we hope that this book will inspire your chemical imagination and you will be enthusiastic to contribute your knowledge and expertise to this young but already blossoming field of interdisciplinary research. Enjoy the book!

Paris, France — Gérard Jaouen
Bochum, Germany — Nils Metzler-Nolte

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Arsenic-Based Drugs: From Fowler's Solution to Modern Anticancer Chemotherapy

Stéphane Gibaud and Gérard Jaouen

Abstract Although arsenic is a poison and has a predominantly unfavorable reputation, it has been used as pharmaceutical agent since the first century BC. In 1786, Thomas Fowler reported the effects of arsenic in the cure of agues, remittent fevers, and periodic headaches. From this time on and despite abusive use, some interesting indications began to appear for trypanosomiasis, syphilis, and blood diseases. The first significant organoarsenical drug (atoxyl) was synthesized by Pierre Antoine Béchamp in 1859 by chemically reacting arsenic acid with aniline but additional experimentations on the properties of arsenic led Paul Ehrlich, the founder of chemotherapy, to the discovery of salvarsan in 1910. From the Second World War, Ernst A.H. Friedheim greatly improved the treatment of trypanosomiasis by melaminophenyl arsenicals. Until the 1990s some organoarsenicals were used for intestinal parasite infections but carcinogenic effects were displayed and all the drugs have been withdrawn in USA, in Europe, and elsewhere. In 2003, arsenic trioxide (Trisenox®) was re-introduced for the treatment of very specific hematological malignancies.

Keywords Atoxyl · Leukemia · Melarsoprol · Salvarsan · Syphilis · Trypanosomiasis · Tryparsamide

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Contents

List of Abbreviations

APL	Acute promyelocytic leukemia
CML	Chronic myeloid leukemia
CNS	Central nervous system
HAT	Human African trypanosomiasis
HIV	Human Immunodeficiency Virus
Mel	Melarsen
Mel B	Melarsoprol
Mel W	Melarsonyl potassium

1 Introduction

Arsenic is a toxic metalloid that exists throughout nature in organic and inorganic forms. It is commonly present in soils at several mg/kg, in marine sediments. The basic element arsenic exists in either of three allotropic forms: yellow, black, or gray; the stable form is a silver-gray, brittle crystalline solid. It tarnishes rapidly in air [1]. It has very few uses. Arsenic can be found naturally on earth; it occurs naturally as microcrystalline masses, found in Siberia, Germany, France, Italy, Romania, and in the USA. Most arsenic is found in conjunction with sulfur in minerals such as arsenopyrite (AsFeS), realgar (As_4S_4, red arsenic, Fig. 1), orpiment (As_2S_3, yellow arsenic), and enargite (Cu_3AsS_4). White arsenic (As_2O_3, arsenic trioxide, Fig. 1) is produced by roasting arsenic ores (realgar) and purifying the smoke. In traditional Chinese Medicine, arsenic has been used therapeutically for more than 2,400 years as refined preparations of realgar (As_4S_4) or orpiment (As_2S_3) [2]. In ancient Greek and Roman civilization it has been used in medicine; Hippocrates, Aristotle, Dioscorides and Pliny the Elder are said to have used it [3]. Arsenic has been administered in a variety of formulations: topical pastes (used for

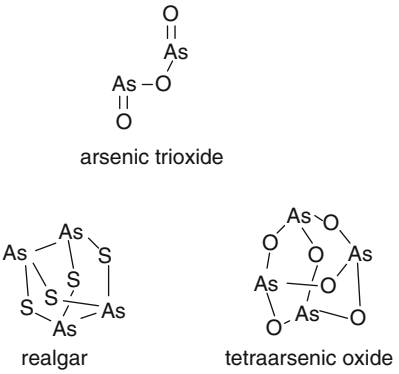


Fig. 1 Structure of inorganic arsenicals: arsenic trioxide, realgar, and tetraasrenic oxide

the treatment of skin cancers), vapor inhalations, intravenous injections, and oral solutions (usually in the form of arsenous acid) and even enemas [4]. As_2O_3 is an amphoteric oxide, which shows a marked preponderance for its acidic properties. It dissolves readily in alkaline solutions to give arsenites and is included in many medical tonics. For example, in 1887 Judie D. Lipscomb relates the effects of a medicine made of plant infusions (tulip-tree bark, willow bark, blue gentian, wild-cherry bark, dogwood bark, sassafras, and flowering almond), whisky, sodium chloride, quinine sulfate and Fowler's solution of arsenic [5]. Mary H. Ramsaur [6], proposed a new medicine, called "Docenella," for curing neuralgia, rheumatism, toothache, headache, sores of long standing, fevers, chills; it was said to remove eruptions of all kinds, beautify the complexion by purifying the blood, impart brilliancy to the eyes, make the old feel young again, etc. This medicine was prepared as follows: "to one pint of whiskey add one ounce of wild ratsbane, (wild arsenic, pipsissewa), ten grains cloves, one teaspoonful of ginger, one teaspoonful of cornmeal. Mix all this together and let it stand for 3 days. Take a wine glass full three times a day, and rub the parts affected with a flannel, saturated with the liquid, until the pain or disease is removed."

Progressively and despite abusive use, some interesting therapeutic applications began to appear in trypanosomiasis, syphilis, and blood diseases. This chapter focuses on the major applications of arsenic-based drugs from 1700 to the present day.

2 Malaria and Fevers

By the year 1700, arsenic was recommended in the treatment of malaria [7,8]. Rosinus Lentilus, who served as the personal doctor of the margrave of Bade-Durlach and later as the regular doctor of the Duke of Wurtemberg, made the first observations on the use of arsenic in the treatment of malaria. These observations were published in the "Ephémérides des curieux de la nature" [7]. Nevertheless,

other practitioners were convinced that arsenical treatment can "heal fevers by killing patients" [9]. On the contrary, Thomas Fowler was somewhat braving danger when he re-introduced arsenous acid. He was a physician to the general infirmary of the country of Stanford when he suggested a solution of potassium arsenite [10]. In his medical reports on the effects of arsenic in the cure of agues, remittent fevers, and periodic headaches (1786), he explained how he tried to imitate the "tasteless ague drop" that was prescribed in the Stanford hospital. The "solutio mineralis" became Fowler's solution and was used as a substitute for the ague drops and costly Peruvian bark (i.e., quinine). Physicians who used the drug had to prescribe only a few drops of Fowler's solution. One of the best supporters of arsenic was Thomas Hunt [11,12].

3 Trypanosomiasis

Human African trypanosomiasis (HAT), or sleeping sickness constitutes a major public health threat in Africa, particularly in Central Africa, with ~60 million people at risk of contracting the disease [13]. The parasite (*Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*) is inoculated by the bite of the tsetse fly and propagates through both the blood and lymph systems (stage 1). If untreated, the disease slowly overcomes the defences of the infected person with symptoms including anemia, cardiac, endocrine, and kidney disease and disorders. The parasite passes through the blood-brain barrier (stage 2, neurological phase) after a highly variable interval. The second stage of the disease is associated with neurological symptoms and is often difficult to diagnose in the absence of clinical signs.

In early Modern Times, the history of HAT was closely linked to the slave trade [14]. The trypanocidal activity of arsenic was discovered fortuitously by Sir David Linvingstone (1813–1873). He relates in "Arsenic as remedy for the tsetse bite" that the idea occurred in his mind about the year 1847–1848 [15]. Sir Livingstone tried the remedy on horses (two grains of arsenic) but 2 months after an apparent cure, the mare became like a skeleton and he saw her at last perish through sheer exhaustion. Subsequently, better results were obtained with organoarsenical drugs (Table 1).

3.1 Atoxyl

The first significant organoarsenical drug was synthesized by Pierre Antoine Béchamp (1816–1908) by chemically reacting arsenic acid with aniline [16,17]. Béchamp isolated a colorless compound that he regarded as an anilide of arsenic acid (Fig. 2a). Some 40 years after its initial synthesis, Wolferstan Thomas in Liverpool demonstrated the trypanocidal activity of atoxyl [18] and a few years later it was also used in the treatment of syphilis [19]. Hence, Béchamp's anilide began to be used to treat disease. At the same time, Ferdinand Blumenthal described

Table 1 Major trypanocidal organoarsenic drugs

Drug	Trade marks	Structure	First use
Atoxyl (Sodium arsanilate)	Trypoxyl® Protoxyl®	Pentavalent	1905
Tryparsamide (Glyphenarsine)	Tryponarsyl® Tryparsone® Novatoxyl® Trypotan® Tryponurile® = Tryponarsyl + hexamethylenetetramine	Pentavalent	1921
Orsanine (Isomer of Stovarsol)	–	Pentavalent	1925
Melarsen	–	Pentavalent	1939
Melarsen-oxide	–	Trivalent	1941
Melarsoprol	Arsobal®	Trivalent (dithiarsolane)	1949

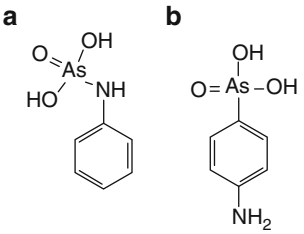


Fig. 2 Chemical structure of atoxyl (**a**) hypothesized by Béchamp (Béchamp's anilide) (**b**) real structure of atoxyl

his many experiments with rabbits [20], determining the toxicity of atoxyl compared to Fowler's solution. From these experiments it was discovered to be about 40 times less toxic than the As_2O_3 solution. This illustrated the point that organic arsenicals in general were much less toxic than inorganic arsenicals. Besides, Paul Ehrlich studied some of the chemical properties of this drug. From that time on there seemed to be no question that atoxyl was not an anilide but an amino arsenic acid (Fig. 2b). Paul Ehrlich and Alfred Bertheim published a paper to that effect in 1907 [21]. Nevertheless this compound had a treacherous toxic action in the organism, as indicated by experiments and clinical evidence. The most interesting and prominent effect of its toxic action is manifested by the production of amblyopia. Atoxyl amblyopia is the result of optic nerve atrophy, with primary involvement of the retina, though in certain cases lesions, shown experimentally and apparently clinically, are very suggestive [22,23].

3.2 Tryparsamide

Tryparsamide (*p*-glycineamidophenylarsonate) is considered as the first clinically effective arsenical for sleeping sickness. The drug was developed by Walter

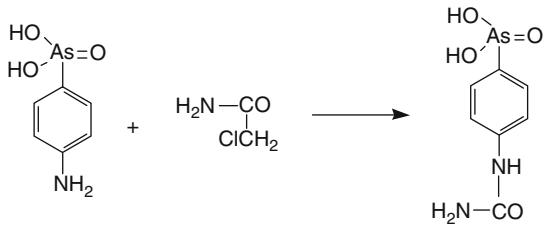


Fig. 3 Synthesis of tryparsamide

A. Jacobs and Michael Heidelberger at the Rockefeller University (Fig. 3) [24]. Tryparsamide possessed a marked trypanocidal activity in human trypanosomiasis caused by *Trypanosoma gambiense*, especially in the late stages of the infection [25]. Moreover, the drug was extremely soluble in H_2O and could be administered intramuscularly as well as intravenously [26]. From this time on, wide treatment programs were initiated (in 1923 in Belgian Congo, in 1926 in Cameroun and in 1935 in Central Africa). The treatment included arsenical drugs (tryparsamide) and non-arsenical drugs (suramin). Unfortunately, this extensive use led to the first cases of arseno-resistant *Trypanosoma,* being reported in the early 1930s. This phenomenon worsened in the 1940s [27] and the use of new compounds was necessary.

3.3 Melaminophenyl Arsenicals

Dr. Ernst A. H. Friedheim (1899–1989), a pathologist, microbiologist, and chemist developed new organoarsenical drugs in the 1940s. He was a native of Zurich and received several degrees; in medicine from the University of Zurich, in microbiology from the Pasteur Institute in Paris, in tropical medicine and hygiene from the University of Hamburg and in chemistry from the University of Geneva. He also studied at the Max Planck Institute in Berlin, the Institut d'Optique in Paris and Rockefeller University. He synthesized melarsen ("colorless pentavalent," Mel) in 1939 [28,29]. For several years, chemists have noticed that trypanocidal activity is partly related to amines or amides groups [30] (the haplophore). For example, 4-(4, 6-dichloro-1,3,5-triazin-2-ylamino) phenylarsonic acid, obtained readily from atoxyl and 2,4,6-trichloro-1,3,5-triazine (i.e., cyanuric chloride, Fig. 4a) [29], is a very toxic substance of no therapeutic value; however, it acquires considerable trypanocidal activity when at least one of its halogens is replaced by an amino group (Fig. 4b) [28]. In 1944, the process had been improved and consisted of a condensation of 6-chloro-1,3,5-triazine-2,4-diamine with atoxyl (Fig. 4c, melarsen) [31].

Melarsen was used orally or intravenously but doses were very inaccurate. In fact, it seemed to be less active than tryparsamide and numerous deaths were attributed to this drug [32]. It was abandoned in favor of melarsen oxide (Fig. 5) obtained by reduction of melarsen with sulfur dioxide in the presence of

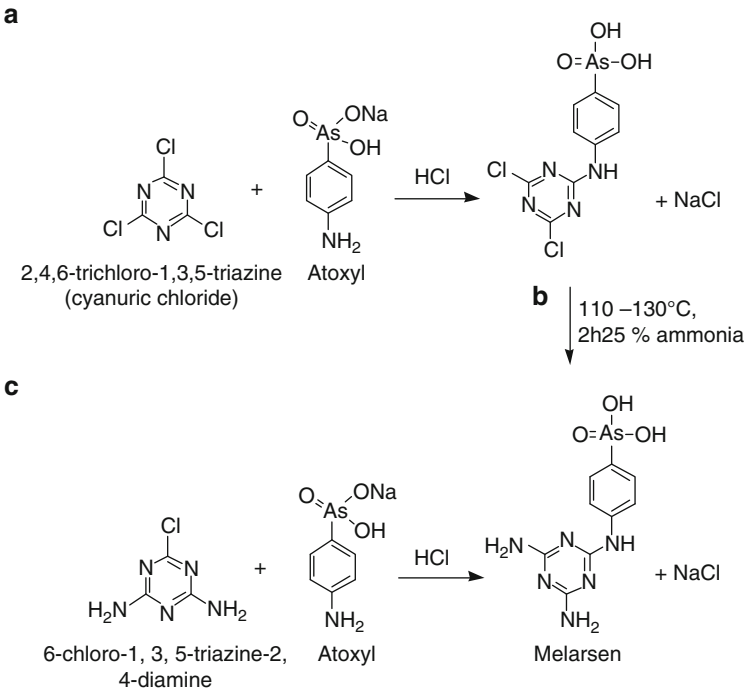
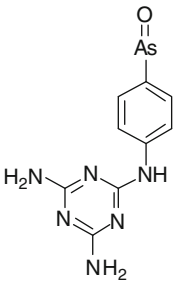


Fig. 4 Synthesis of melarsen

Fig. 5 Chemical structure of melarsen oxide



hydrochloric acid [33]. With this drug, Friedheim introduced the use of trivalent organoarsenicals for the treatment of trypanosomiasis. This new drug was more toxic than melarsen but was more active against trypanosomes [32]. He reported that the drug was effective against both stages of the disease: blood and nodes were cleared in the first 24 h, cerebrospinal fluid (CSF) was cleared of parasites after 1 week, and albuminorachia was back to normal after 3 or 4 months. In 1945 Peters and co-worker [34] published an article on the reversal of the toxic effects of the arsenic derivative war chemical weapon, lewisite, by dithiol compounds, which possess the property of forming a stable cyclic arsenical ring, (ditihiarsolane).

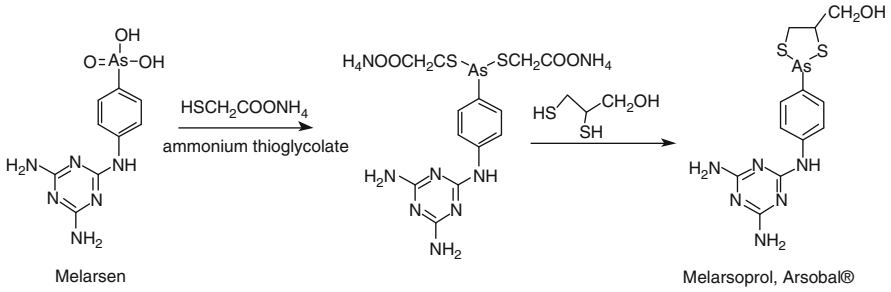


Fig. 6 Synthesis of melarsoprol

This work has been one of the great achievements in the treatment of certain war-gas casualties and gave Friedheim the idea of how to lower the toxicity of the derivatives of phenylarsonic acid. Applied to melarsen oxide, the reaction produced an interesting compound called melarsoprol (Mel B). The formation of the dithiarsolane ring with concomitant reduction of As^V to As^{III} can be performed by stirring one equivalent of the arsonic acid derivative (i.e., melarsen) with six equivalents of aqueous 5.5 M ammonium thioglycolate for 30 min at 50°C (Fig. 6) [35,36]. Although the compound had a lower toxicity, it retained its trypanocidal activity. One of its major drawbacks was a very poor solubility in water and many other solvents. Moreover the diathiarsolane ring was easily hydrolyzed in water. The only injectable solvent that provides stable solutions is propylene glycol [37]. Melarsen use is frequently associated with several serious side effects, particularly a potentially deadly arsenical encephalopathy, and the solvent is also a powerful irritant that often causes thrombophlebitis. Water-soluble arsenical agents such as melarsonyl potassium (Mel W, Trimelarsan®) have also been synthesized, but showed little improvement over Mel B [38–40]. Currently melarsoprol is still used in African countries and remains the first-line treatment for patients with *Trypanosoma brucei gambiense* trypanosomiasis who are in the late stages of the disease. Unfortunately, melarsoprol is highly toxic and treatment can result in the development of an extremely severe reactive arsenical encephalopathy (RAE) in about 10% of patients with a 50% mortality rate [41,42]. This RAE is characterized by a meningoencephalitis with diffuse astrocytosis and the presence of macrophages, lymphocytes, and plasma cells in cerebral white matter as well as perivascular cuffing [43,44]. Current treatment regimens were developed in the 1940s without any knowledge of the drugs characteristics [45,46]. These regimens generally comprise of two or three series of 3–4 daily i.v. injections. Graded dosing of melarsoprol was the standard approach for the treatment of *Trypanosome brucei rhodesiense* in the hope of reducing the risk of melarsoprol-induced encephalopathy. In fact, a continuous treatment schedule at 2.2 mg/kg/day for 10 days has been shown to be effective, but did not reduce the risk of encephalopathy [47,48]. Co-administration of prednisolone can reduce the risk of reactive encephalopathy [49]. The mechanism of action of the drug has also been partially clarified: dithiarsolanes (Mel B or Mel W) are hydrolyzed by water to melarsen oxide

which can react with many endogeneous compounds containing SH groups, especially trypanothione (primary target for arsenical drugs against African trypanosomes). On the other side of the molecule, the $NH_2-CH=N$ arrangement, is also related to the trypanocidal activity [50]. Indeed, the African trypanosome possesses at least two nucleoside transporter systems designated P1 (which also transports inosine) and P2 (which also transports both adenine and the melaminophenyl-containing arsenicals by structural analogy). Although this agent exhibits an excellent antitrypanocidal activity, its use has been limited to advanced stages of the disease because of severe side effects [48]. Among alternative treatments, eflornithine (DMFO, Ornidyl®), alone or combined with nifurtimox, is being increasingly used as first-line treatment [51]. However, the quantities of eflornithine required to achieve cures and the high cost of this compound may limit its use in human medicine [52]. Therefore, eflornithine monotherapy is not a long-term solution. Moreover, it is less effective in HIV endemic areas and could cause parasite resistance [53]. Consequently, melarsoprol is still necessary and despite its unacceptable toxicity it is unlikely that any new drugs will replace it within the next 5 years [54].

4 Syphilis

The first cases of syphilis were observed in Naples among the invading army of Charles VIII in 1495. When the army disbanded shortly after the campaign, soldiers returned to their homes and disseminated the disease across Europe. The facts occurred shortly after the return of Colombus and his men from the New World. Recently Harper et al. demonstrated that, among a collection of pathogenic *Treponema*, the closest relatives of syphilis-causing strains identified were found in South America, providing support for the Colombian theory of syphilis's origin [55]. A century ago, syphilis had become a major public health threat in Europe. In the 1900s, practitioners began to substitute mercury and potassium iodine with new organoarsenical compounds. For example, atoxyl exerted a favorable therapeutic action on all exanthema forms of syphilis, but its therapeutic value was greatly decreased by its toxicity. The small doses that were tolerated required a long time to exert their action. Moreover, it was abusively used in venereal infections; for example G. Scherber recommends 10% atoxyl plaster in cases of local luetic infections [19]. Nevertheless, the introduction of salvarsan and other organoarsenical drugs (Table 2) caused a revolution in the treatment of syphilis.

4.1 Arsphénamines

Paul Ehrlich (1854–1915, Nobel prize 1908), a physician with an exceptional understanding of organic chemistry, screened derivatives up to the 606th preparation [56]. He synthesized salvarsan (Ehrlich 606) by the reaction of

Table 2 Major organoarsenic drugs in the treatment of syphilis

Drug	Trade marks	Structure	First use
Atoxyl (Sodium arsanilate)	Trypoxyl® Protoxyl®	Pentavalent	1905
Salvarsan (Arsphenamine)	Salvarsan®	Trivalent	1910
Neosalvarsan (Neo-arsphenamine)	Neosalvarsan® Novarsenobenzol® Arsebenyl®	Trivalent	1912
Sulfarsphenamine	Sulfarsenol® Sulf-arsebenyl®	Trivalent	1923
Bismuth arsphenamine sulfonate	Bismarsen®	Trivalent	1924

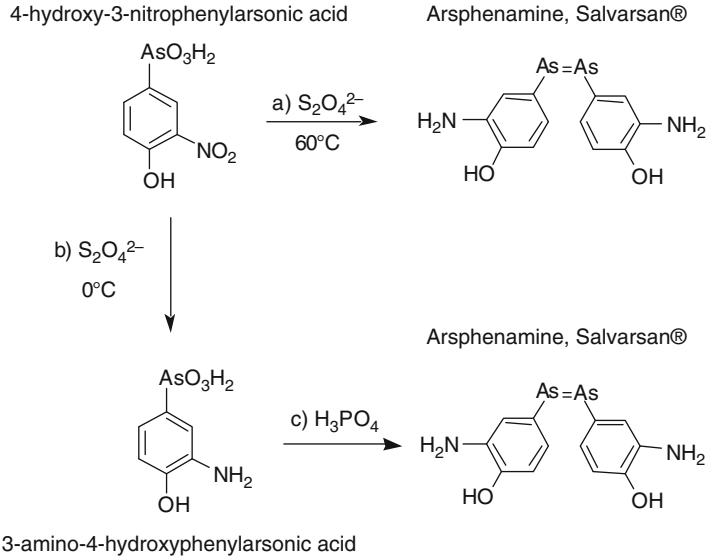


Fig. 7 Synthesis of salvarsan (**a**) initial route of Ehrlich and Bertheim (simultaneous reduction of the nitro group and As^V) (**b**, **c**) Christiansen's two-step process

4-hydroxy-3-nitrophenylarsonic acid with dithionite (Fig. 7a). This simultaneously reduced the NO_2 group to NH_2 and As^V to As^{III} to give a material of formula 3-H_2N-4-$HOC_6H_3As.HCl.H_2O$. The synthesis was not always reproducible and frequently contained sulfur impurities. A two-step process that involved the initial reduction of the NO_2 group by sodium dithionite followed by a reduction of As^V with hypophosphorous acid was proposed by Christiansen in 1920 (Fig. 7b and 7c) [57]. Interestingly, the true structure of salvarsan was recently clarified: in 2005, Nicholas C. Lyod et al. reported the first definitive evidence for the composition of salvarsan, based on electrospray ionization mass spectrometric data [58]. All the evidence indicates that salvarsan in solution consists of cyclic species (RAs)*n* with

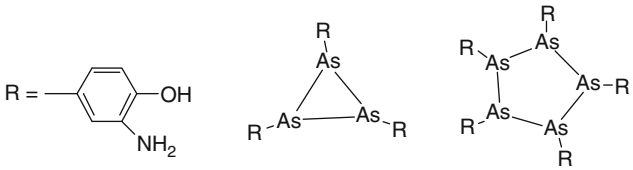


Fig. 8 Real composition of salvarsan: cyclic species $(RAs)n$ with $n = 3$ and $n = 5$

$n = 3$ and $n = 5$ (Fig. 8) as the preferred sizes. In essence, salvarsan appears to serve as a slow-release source of $RAs(OH)_2$ [58].

Although the structure was not known, Ehrlich achieved many successes with this drug. Ilya Mechnickov (1845–1916) helped him develop an animal model for syphilis that eliminated the need for human "volunteers" [59]. Hence, the compound 606 was tested against fowl spirochetes and provided a rapid cure, and so Ehrlich turned to his ultimate target: *Treponema pallidum*. The first clinical trial of 50 patients with late-stage syphilis using salvarsan was conducted in 1909 and ended with an impressive positive outcome. However, as salvarsan, became more widely used, side effects began to be reported more frequently [60], and Ehrlich suffered a period of humiliating accusations [61]. Undeterred, he investigated the origin of the side effects and found that the drug was rapidly degraded to toxic products when dissolved in impure water without basification or when the therapeutic solution was allowed to stand in air. Consequently, solutions should be prepared at the time of injection and "just alkaline," hyperalkaline ones exerting vascular sclerosis. Although the oral route was proposed [62], intravenous administration is the only route recommended.

Ehrlich had a close relationship with the Hoechst Company and licensed the patents of the drugs (Salvarsan®). This made the production of large quantities for clinical use possible. Thus, the transposition of his research into a marketed drug was rapidly achieved. During the First Word War, kits were specially made for the administration of the product. The doses were 0.4–0.6 g for a man and 0.3–0.45 g for a woman; the glass tube containing the powder was dissolved in 3 or 4 oz of warm physiological 0.9% saline, prepared with freshly distilled water, in a ten-once graduated glass measure. Then, 10 ml of sodium hydroxide 0.1 N was added, forming a new precipitate; this precipitate was dissolved by a further addition of sodium hydrate. To correct these shortcomings, Ehrlich proposed chemical modification of salvarsan to produce a water-soluble analog that did not require basification. In 1912, after another 300 derivatives had been made, compound 914 (Neosalvarsan®, neoarsphenamine) proved to be safe and active. The drug was not quite as effective as Salvarsan®, but it was easier to use and was less toxic. Moreover, intravenous injections could be given in the consulting room, and the patient was allowed to go home immediately afterwards. France began marketing the drug the next year under the name of Novarsenobenzol® and Meurice-UCB (Belgium) sold it under the name of Arsebenyl®. Salvarsan® was used in the first part of the twentieth century, until the end of the Second World War. Subsequently, the works of Sir Alexander

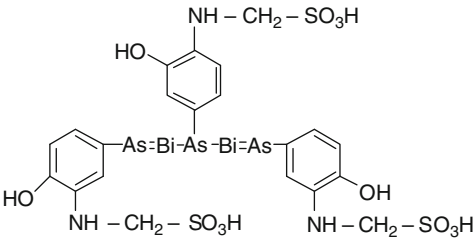


Fig. 9 Chemical structure of bismuth arsphenamine sulphonate (Bismarsen®)

Fleming [63], Chain [64,65], Florey [66,67] and Heatley [68] led to the discovery of penicillin, still the treatment of choice for syphilis after all these years.

4.2 Arseno-Metallic Compounds

In 1915, Ehrlich and Karrer published details of complex salts of arsenobenzene and the heavy metals characterized by great stability and deep color. Only silver arsphenamine and silver neoarsephenamine found a place therapeutically, [69].

Bismuth arsphenamine sulphonate (Bismarsen®, Fig. 9) a yellow powder readily soluble in water, was first prepared by Raiziss in 1924 [70]. Stokes and Chambers [71] were the first to use the drug clinically, giving two injections a week for 14 weeks. Four such courses separated by intervals of a fortnight were administered in all. Nevertheless the effects were slow compared with the arsphenamines, although the tonic effect was greater and the side effects less numerous. Relapses, particularly in the central nervous system (CNS) were more frequent. Consequently, Rayburn and Boyd emphasized the fact that some individuals with neurosyphilis who were intolerant to arsenic in any other form could nevertheless tolerate it in the form of Bismarsen® [72]. The low toxicity, the tonic effect, and the ease of administration were the chief advantages in favor of Bismarsen®. The other compounds of bismuth with arsenic were not extensively tested in humans.

5 Treatment of Amebiasis, Worms, Trichomonas Vaginalis, and Vincent's Angina

More recently, organoarsenical compounds were used in the treatment of amebiasis, worms, trichomonas vaginalis, Vincent's angina, and even other ORL infections (Table 3).

Amebiasis refers to the infection caused by *entamoeba histolytica* and is estimated to cause about 70,000 deaths per year worldwide [73]. It is a gastrointestinal infection that may or may not be symptomatic and can remain latent in an infected

Table 3 Organoarsenic drugs in the treatment of amebiasis, worms, trichomonas vaginalis, and Vincent's angina – Examples of trade marks

Drug	Trade marks (examples)	Structure
Stovarsol[a] (Acetarsol, acetarsone, acetphenarsone)	Stovarsol®[b] SVC®[c] Gynoplix®[c]	Pentavalent
Carbarsone	Amibiarson®[b] Leucarsone®[b]	Pentavalent
Diphetarsone	Bemarsal®[b]	Pentavalent
Arsthinol	Balarsen®[b]	Trivalent (dithiarsolane)
Neoarsphenamine	Collunovar®[d]	Trivalent
Sulfarsphenamine	Collusulfar®[d]	Trivalent

[a]More than 40 trademarks of (or with) arcetarsol were marketed worldwide
[b]Essentially in the treatment of amebiasis
[c]Vaginal tablets – trichomonas vaginalis infections
[d]Collutory: Vincent's angina and other ORL infections

person for several years. Symptoms can range from mild diarrhea to dysentery with blood and mucus in the stool. Since the beginning of the twentieth century, organoarsenical drugs were tested orally in the treatment of amebiasis. For example in 1918, Salvarsan®, combined with emetine provided a radical cure of the infection [74]. Studies undertaken by E. Fourneau at the Institut Pasteur de Paris on organoarsenical drugs [75] revealed some important aspects on the specificity of trivalent arsenic on protozoans. Nevertheless, it was vigorously denied that any other trivalent arsenicals were effective against these parasites. In fact, several pentavalent derivatives were also active and had a lower toxicity [76]. For example acetarsol (Stovarsol®, Goyl®) was used until the early 1980s and diphetarsone (Bemarsal®) was withdrawn only in 1996 in France (Fig. 10).

Carbarsone an antiprotozoal organoarsenical, was introduced in 1931 for the treatment of *Trichomonas vaginalis* and *Entamoeba histolytica* infections (Fig. 10) [77,78]. It was available for use in the USA as a drug for the treatment of amebiasis as late as 1991 (Amibiarson®). Thereafter, it remained available as an additive for turkey feed, for improving weight and controlling Blackhead disease [79]. Another pentavalent (diphetarsone, Bemarsal®) appeared in 1952 for the treatment of amebiasis [80,81] and offered some advantages over the other available drugs (Fig. 10). Applications were rapidly extended to the oxyuriasis and the trichocephaliasis [82]. Finally, in 1953, arsthinol (Barlarsen®, Fig. 10), a new dithiarsolane synthesized by E.A.H. Friedheim [83] was marketed by Endo Products [84]. This compound is a trivalent arsenical with applications somewhat similar to the pentavalent arsenicals previously available for oral use. Interestingly arsthinol is well tolerated and has recently showed good antileukemic activity [36]. In the 1990s many publications based principally on epidemiological findings, demonstrated that exposure to arsenic is associated with an increased risk of a variety of neoplastic diseases, including skin, lung, bladder, liver, and kidney cancers [85,86]. As a result the last intestinal arsenicals were withdrawn from the market.

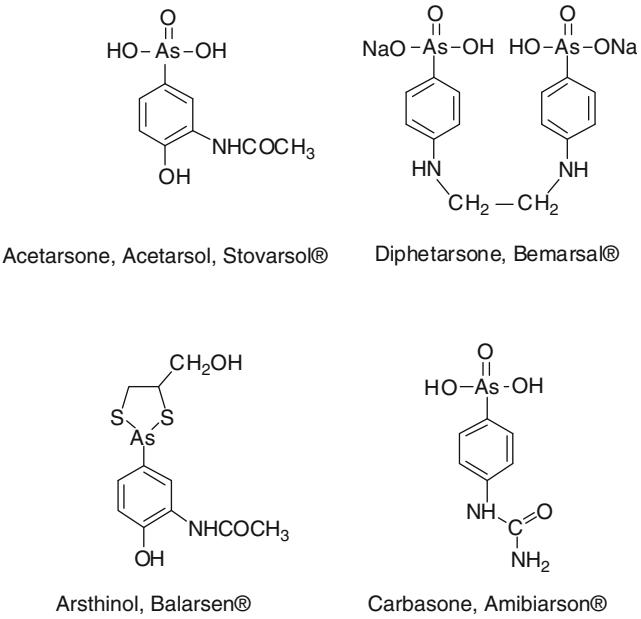


Fig. 10 Major organoasenicals used in the treatment of amebiasis

6 Blood Diseases and Disorders

In the latter part of the nineteenth century arsenic (Fowler solution) was the standard remedy for blood diseases. It was used to increase red blood cells and hemoglobin in certain anemias and to decrease white blood cells in leukemia. The antianemic effects observed in malaria or leukemia, were not as fully understood as they are now, this probably led to a dangerous extension of application. For example, chlorotic anemia, observed in young ladies was commonly treated with iron and arsenic. It was believed to increase and improve red blood cell formation. Culter and Bradford [87] were among the first to study quantitatively and scientifically the effects of arsenic on blood cells. In 1878 they published the results obtained from five cases studied at the Boston City Hospital. In simple anemia they found an initial increase in both red and white blood cells, but afterwards there was a steady elimination. Hence, arsenic was extensively used (Fowler's solution) for "the diseases of the blood." The experiments performed in 1878 by Culter and Bradford were also carried out on patients with hematologic diseases [87] and brought some interesting results. For example, in patients with myelogenous leukemia, erythrocytes were reduced from 3,064,000 to 1,841,500. Similar results were found by Stockman and Greig in rabbits and dogs in 1898 [88]. There are some other isolated reports of cases of leukemia being treated with a solution of potassium arsenite, in which blood counts were reported and in these the number of circulating white blood cells were temporarily greatly reduced;

the spleen became smaller and the patients showed improvement [89]. With the works of Röntgen on X-ray [90] and the demonstration by Senn of its effects on leukemia [91] arsenic fell into disrepute and was completely replaced by modern chemotherapy in the second part of the twentieth century. This was partly due to its low efficiency compared to modern chemo- and radiotherapy, but mostly because of the potential carcinogenicity. During this time, arsenic was still available in Chinese medicine preparations. In fact, two arsenic compounds have been utilized in traditional Chinese medicine for more than 500 years. The Pishang, or white arsenic, containing essentially white arsenic (arsenic trioxide, As_2O_3) was recorded in the Compendium of Materia Medical (edited by Li Shizhen, 1518–1593) [2]. It was still used in the treatment of certain skin diseases, asthma and to promote the healing of surgical wounds [92]. The second compound, Xiong-huang (realgar, As_4S_4) was more recently administered in the treatment Chronic Myeloid Leukemia (CML).

In the 1970s a group of researchers at the Harbin Medical Institute reported encouraging results in the treatment of malignant lymphoma (e.g., CML, Acute Promyelocytic Leukemia (APL)), lymphoma, and esophageal carcinoma. The administered drug (Ailing-1) contained a mixture of As_2O_3 with traces of mercury [93]. In 1996, these results were confirmed with pure As_2O_3 on APL [94]. Recently, arsenic has re-emerged in the West and the results of clinical trials using arsenic-based drugs in cancer have been extensively reviewed [95–98] Moreover numerous studies have tried to understand the mechanisms of action. As previously stated, arsenic has a high affinity for sulfur and hence, reactive sulfur-containing molecules such as reduced thiols with an available sulfur atom have a significant tendency for binding to arsenic. In vivo, arsenic can modulate the functions of various key proteins (enzymes, ions transporters, etc.). It can react with mono- and dithiols, particularly the latter when two thiols are located in close proximity, acting to cross-link the thiols together [1]. As_2O_3 appeared to have a particular selectivity for APL cell lines (e.g., NB4 cell line); this selectivity was first related to a downregulation of Bcl-2 expression and a modulation of PML-RARα/PML proteins [99]. In fact, several investigations have shown that As_2O_3 acts on numerous intracellular targets including several signal transduction pathways, which appears to be dependant or independent of PML-RARα. One of the key targets could be the intracellular glutathione redox system [100]. Compared with other leukemia cells that are less sensitive to As_2O_3, NB4 cells contain lower levels of glutathione peroxidase (GPx), glutathione-S-transferase and catalase and relatively higher levels of intracellular hydrogen peroxide (H_2O_2), suggesting that NB4 cells detoxify As_2O_3 and catabolize H_2O_2 less efficiently [101]. In 2003, As_2O_3 was marketed in some European countries and in the USA for the treatment of refractory APL (Trisenox®). In addition, trivalent arsenic bonded at a phenyl ring is able to form much more stable covalent cross-links to cysteine residues compared to arsenic in small molecules such as arsenous acid or arsenite [102]. As melarsoprol is still available for the treatment of trypanosomiasis, the aromatic drug As^{III} was studied in detail for the treatment of hematological diseases [103–105]. The results have actually demonstrated that melarsoprol was more

potent than As_2O_3 at equimolar concentrations but did not affect PML-RARα nuclear localization [104]. In comparative studies with arsenic trioxide, melarsoprol displayed a broad and potent antileukemic effect against both acute and chronic lymphoid and myeloid cell lines [36,106]. A Clinical trial attempted to confirm these interesting results on refractory leukemia in patients [96]. The dose and schedule was drawn from trypanosomiasis treatment. Unsurprisingly, excessive CNS toxicity was associated with the experiment.

7 Conclusion

Although they have very few uses in modern medicine, arsenic-based drugs have a prestigious history. Most of these drugs were marketed without serious clinical trials and without any knowledge of their mechanism of action. In the 1980s and in the 1990s, drug regulation organizations considered that the benefits/risk balance was unfavorable and all the pharmaceutical specialties were withdrawn in Europe and in USA. Nevertheless, a favorable benefits/risk balance may appear for cancer treatment and other serious diseases. Now the mechanism of action of As_2O_3 is partly known; this can probably explain the renewal of interest in arsenical drugs.

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Activation Mechanisms for Organometallic Anticancer Complexes

Ana M. Pizarro, Abraha Habtemariam, and Peter J. Sadler

Abstract Organometallic complexes offer potential for design as anticancer drugs. They can act as inert scaffolds and specifically inhibit enzymes such as kinases, or as pro-drugs which undergo activation by various mechanisms. The activation of metallocenes, arene, alkyl or aryl complexes by hydrolysis, and metal- or ligand-based redox reactions is discussed.

Keywords Medicinal Organometallics · Photoactivation · Hydrolysis · Anticancer · Titanium · Ruthenium · Osmium · Ferrocene · Tin

Contents

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1 Introduction

Metal-based pharmaceuticals offer unprecedented versatility in medicinal chemistry because of the different building blocks from which they can be constructed, the variety of available interactions (H-bond, π-stacking, coordinative bond, spatial recognition), the combination of rigidity around the metal and flexibility in the ligands, the kinetics of ligand substitution when coordinative bonds with biomolecules are formed and because of their redox properties.

Recently, the benefits of these properties in medicinal chemistry have begun to emerge. The misconception that organometallic complexes lack stability under conditions in which living organisms thrive, has been one reason why they have not been considered as suitable candidates as pharmaceuticals until quite recently. However, in the last decade there has been a flurry of activity with regard to the development of organometallics in a biological context [1]. Organometallic complexes which are stable in physiological environments are now being developed as, for example, anticancer agents, radiopharmaceuticals for diagnosis and therapy, and biosensor probes.

Achieving aqueous stability is a prerequisite for metal-based drugs, as lack of control of speciation makes pharmacokinetic studies difficult, thus hindering, or, even excluding, progress towards clinical development. Titanocene dichloride (**1**) was the first organometallic anticancer drug to reach phase II trials. Its withdrawal from clinical evaluation epitomises the stability challenge which is inherent in organometallic complexes. The complicated hydrolysis of titanocene dichloride at physiological pH has made characterisation of the active species responsible for antitumour activity difficult. At pH values above 4, loss of the Cp ligands results in the formation of insoluble Ti oxo species [2, 3]. From this point of view, the development of organometallic drugs which can be activated in a controlled manner towards specific targets, with well characterised speciation behaviour, is of paramount importance.

In this chapter, we classify organometallic complexes with cytotoxic activity against cancer cells with regard to the chemical-physical processes that activate them towards interactions with biological targets. As we have noted previously in a classification of the biological activity of metal complexes [4], the activity can depend intimately on the metal and some or all of its ligands as an intact complex or fragment, on the metal itself and on the ligands themselves. This classification has recently been elaborated upon by Alessio and co-workers for both organometallic and coordination complexes [5].

In the emerging field of medicinal organometallic chemistry, various strategies for the activation of complexes have been employed. These can facilitate interactions with biomolecules either through the incorporation within the structure of a known pharmacophore, thus altering the reactivity profile of the organometallic fragment and thereby delivering enhanced cytotoxicity, or through the inclusion of labile groups with favourable ligand substitution characteristics. For example, for

complexes containing a labile group prone to substitution, such as the Ru arene piano stool complexes of the type $[Ru(\eta^6\text{-arene})(XY)(Z)]^{n+}$ (where XY = chelating ligand, and Z = labile halide), the activation can occur through hydrolysis of the Ru–Z bond, in a similar manner to cisplatin. Hydrolysis of the Pt–Cl bond in cisplatin inside the cells, where the chloride concentration drops from ca. 100 mM to ca. 23 and 4 mM in the cytoplasm and nucleus, respectively, is believed to generate active aqua species. The aqua adduct interacts with the biological target, DNA, and the drug exerts its cytotoxic effect.

Other pathways for activating organometallic complexes towards substitution reactions may be triggered inside the cells by internal sources such as oxidation of a less labile ligand, by chelate ring-opening, and even by an external source such as light of a certain wavelength.

The strategy used by, for example, Jaouen, Meggers and Metzler-Nolte, involves enhancing the biological activity of an already-active molecule such as tamoxifen, staurosporine or aspirin by incorporating an organometallic fragment into the structure. In the case of ferrocifens, however, the metal atom has been found not to be a mere spectator, but is involved in the anticancer activity of the drug through redox activity. Finally, there are studies which use organometallic cages as delivery vehicles.

These different modes of activation of organometallic complexes for anticancer activity are now discussed in more detail.

2 Activation Through Cleavage of M–X Bonds

In this section we review organometallic complexes in which the metal plays a functional role, i.e. the cytotoxic activity is believed to derive from direct binding of a fragment of the metal complex to the biological target. For this binding to occur, one or more of the original ligands on the metal must undergo a substitution reaction. Since the biological environment of the cell is aqueous, many of these substitutions occur through hydrolysis in first place, and a second substitution replaces the bound aqua ligand by the target biomolecule. Control of drug activity and refinement of the structures for these types of complexes requires that such substitution reactions proceed in a predictable way.

2.1 Titanocenes

Titanocene dichloride, $[Ti^{IV}(\eta^5\text{-}C_5H_5)_2Cl_2]$, will be remembered in the history of metal-based drugs as a pioneer in the field of organometallic complexes with anticancer activity.

In the late 1970s, Köpf-Maier et al. investigated the antitumour activity of a series of metallocenes, and in 1979 reported in detail the antitumour activity of titanocene dichloride (**1**) [6]. This complex is active against a diverse range of human carcinomas, including gastrointestinal and breast [7]. It progressed successfully through phase I clinical trials [8, 9] into phase II.

The Ti–Cl bond in titanocene dichloride undergoes fast hydrolysis. The hydrolysed forms of the organometallic complex appear to interact with DNA [10–17], with most of the biological studies suggesting that nuclear DNA is a potential intracellular target of $[Ti(Cp)_2Cl_2]$. Moreover, Ti^{IV} binds strongly to human serum transferrin and this protein could be involved in the delivery of titanium(IV) into cancer cells [18]. The mechanism of transferrin-mediated uptake of the drug may involve cleavage of the Ti–Cp bonds (in addition to the hydrolysis of the Ti–Cl bonds) and binding of the Ti^{IV} to the specific iron sites of human transferrin (hTF), yielding the adduct Ti_2–hTF. Titanium(IV) binds very strongly to hTF, but ATP facilitates the release of Ti^{IV} from transferrin adducts (as might happen inside cells) [19].

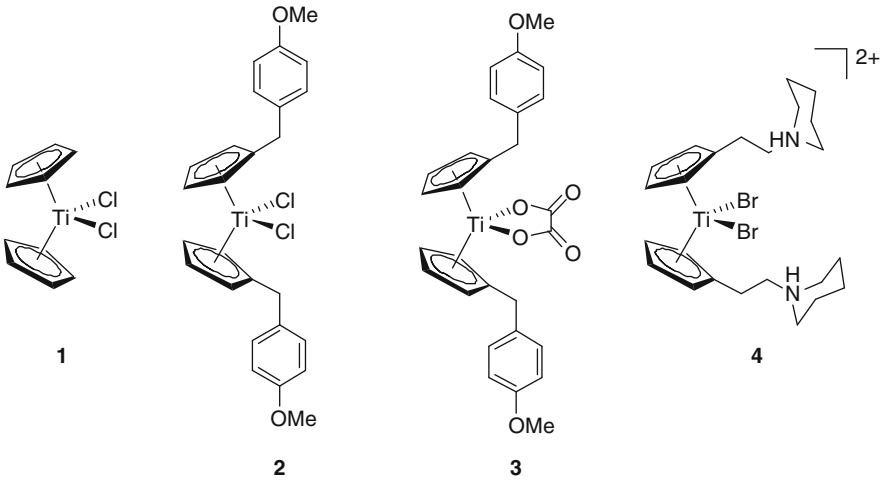
Ultimately, formulation problems as a result of rapid hydrolysis, i.e. instability, halted further evaluation of the drug [2, 20]. In addition, the efficacy rates of titanocene dichloride in Phase II clinical trials in patients with metastatic renal-cell carcinoma and metastatic breast cancer were too low to support further development [21, 22].

Although the progress of titanocene into the clinic has been hampered by the complicated characterisation of its metabolites [17, 20], the discovery of its cytotoxic activity has triggered the search for titanocene derivatives and other metallocenes ($[M(Cp)_2Cl_2]$, where M is, e.g. V, Mo) that show similar or better antineoplastic activity [23–26] whilst controlling aqueous activity [3, 20, 27].

In the search for an improved aqueous stability, Tacke et al. have prepared titanocene derivatives with bridged-cyclopentadienyls, the so-called *ansa*-titanocenes. The same group has successfully optimised the substitution pattern on the Cp ring of titanocene dichloride derivatives developing the un-bridged organotitanium titanocene Y (**2**) [28, 29]. Compound **2** showed in vivo activity against A431 and PC-3 xenografts, models for epidermoid and human prostate cancer, respectively, similar to cisplatin [30, 31]. The authors have recently optimised the labile ligands, exchanging the chlorides for more suitable anions [32] and found that an oxalato derivative, oxali-titanocence Y (**3**), exhibits cytotoxicity in LLC-PK cell line (mimic of kidney carcinoma) with a potency similar to that of cisplatin. In vivo studies in CAKI-1 (renal cancer) tumour-bearing mice have shown a statistically-significant tumour growth reduction with respect to the control group [33].

Similarly, McGowan et al. have synthesised a number of new ionic titanocene organometallic complexes (e.g. **4**), which exhibit cytotoxicity against different human tumour cell lines including a cisplatin-resistant cell line [34].

Although hydrolysis may play an important role in the activation of titanocene derivatives as tumour inhibitors, few studies as yet support this postulate.



2.2 *Ruthenium and Osmium Arenes*

In the 1970s, Clarke et al. reported $[Ru(NH_3)_5(purine)]^{3+}$ complexes capable of inhibiting DNA and protein synthesis in human nasopharyngeal carcinoma cells in vitro [35] and subsequently initiated interest in ruthenium complexes as potential anticancer pharmaceuticals [36]. During the following decade, Mestroni et al. developed hexacoordinated Ru^{II} complexes with dimethylsulfoxide and chloride ligands, particularly, *cis*- and *trans*-$[RuCl_2(dimethylsulfoxide)_4]$, which exhibited anticancer activity and were shown to interact both in vitro and in vivo with DNA, their most likely target [37]. Today, there are two Ru-based anticancer drugs in clinical trials: NAMI-A [38–41], developed in Trieste by Mestroni, Alessio, and co-workers, and KP1019, developed by Keppler and co-workers in Vienna (Chart 1) [42–45].

Octahedral ruthenium(III) complexes are relatively inert towards ligand substitution. The reduction from ruthenium(III) to ruthenium(II) as an activation process prior to DNA binding was first suggested in the late 1970s by Clarke and co-workers [35, 46–50].

Consequently, organometallic ruthenium(II) and osmium(II) arene complexes have recently attracted interest as anticancer agents [51]. The presence of a π-bonded arene in Ru^{II} (and Os^{II}) complexes can have a dramatic influence on their chemical reactivity. There is a delicate balance between electron donation from the arene into the empty Ru 4d orbitals and back-donation from the filled $4d^6$ orbitals into vacant arene orbitals. This is influenced by the donor–acceptor power of the arene (e.g. hexamethylbenzene as a strong donor, in contrast to biphenyl which may act as acceptor) and by the other ligands on Ru^{II} which can influence the

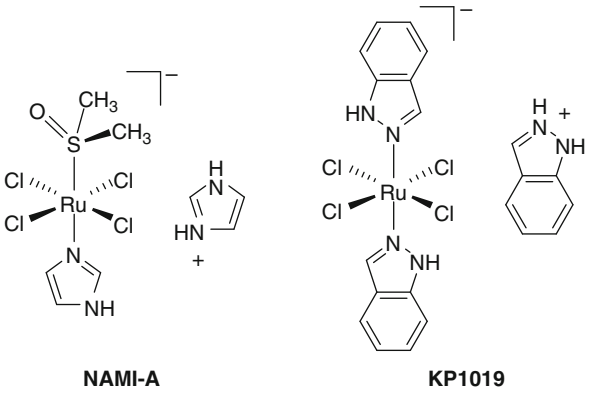


Chart 1 Structures of Ru^{III} complexes NAMI-A and KP1019

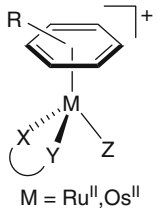


Chart 2 General structure of Ru^{II} and Os^{II} 'piano-stool' arene complexes

availability of the Ru $4d^6$ electrons, e.g. presence of strong π-acceptor chelating ligands such as bipyridine and azopyridine [52], or donor strength of mondentate ligands (e.g. iodide vs. chloride) [53].

The potential of Ru^{II} and Os^{II} η^6-bound arene complexes of the type $[M^{II}(\eta^6$-arene)(X)(Y)(Z)] as antitumour drugs has been explored [54–57]. These complexes possess characteristic 'piano-stool' structures (Chart 2, where XY is a neutral chelating ligand, and Z a monoanionic ligand). In these complexes, the metals are already in their lower oxidation state, which may be important for the cytotoxicity of the drug in vivo [58]. The arene ligand, binding as an η^6-electron donor and a π-acceptor, confers stability to the +2 oxidation state. The presence of a chelating ligand, XY, provides additional stability to the whole structure and the capability of tuning the electronic properties of the metal centre. The monodentate ligand Z allows activation of the molecule: if labile, such as halide, it can provide a vacant coordination site for biomolecules. Small variations of the arene and the 'legs of the stool' confer versatility to the molecule and the capability of fine-tuning their pharmacological properties [53, 59].

In general, Ru^{II} and Os^{II} arene complexes show promising cytotoxic activity against human ovarian cancer cell lines, some of them as potent as cisplatin and carboplatin. Some structure–activity relationships have been established

[51–55, 59–66]. As an example, when the chelating ligand is ethylenediamine and the leaving group is chloride, the cytotoxicity against A2780 human ovarian cancer cells increases with increasing size of the coordinated arene [60]. However, substitution of chloride by bromide or iodide has only a marginal effect on the cytotoxicity of ethylenediamine complexes [53]. Additionally, substitution of the chelating ligand by relatively labile monodentate ligands leads to less cytotoxic complexes [54]. The importance of the chelating ligand for moderating activity is evident when complexes such as [Ru(η^6-*p*-cymene)Cl($CH_3CN)_2$]PF_6, and [Ru(η^6-*p*-cymene)Cl_2(isonicotinamide)] are compared to [Ru(η^6-*p*-cymene)Cl(en)]PF_6. The Ru arene complexes with monodentate ligands show low or no activity (IC_{50} values > 150 μM) while the chelated ligand-containing complex possesses high activity (9 μM, compared to 0.5 μM for cisplatin in these tests) [54]. A possible explanation for this lack of activity might be their high reactivity: they may be inactivated before reaching their target. Another example of the relevance of the ethylenediamine chelating ligand comes from a recent comparison of the activity of [Ru(η^6-*p*-cymene)Cl(en)]PF_6 with its non-chelated analogue [Ru(η^6-*p*-cymene)Cl($NH_3)_2$]PF_6. The latter showed only modest cytotoxicity in the three cell lines A549 (non-small cell lung carcinoma), CH1 (ovarian carcinoma), and SW480 (colon carcinoma), with IC_{50} values 293–542 μM, compared to 3.5–7.1 μM for the former in the same cell lines [67]. This lack of activity may be related to rapid hydrolysis of both Cl^- and NH_3 ligands and subsequent formation of hydroxy-bridged dimers, as found in the solid state. The X-ray structure of the Os dimer: [{Os(η^6-*p*-cymene)}$_2$(μ-$OCH_3)_3$]PF_6 has been reported [67].

The effect of changing the three building blocks, i.e. η^6-arene, chelating ligand and monodentate group, in the activation of organometallic Ru^{II} and Os^{II} drugs is explored in more detail below.

2.2.1 Hydrolysis of the Ru–Z Bond

Activation through hydrolysis of the Ru–Z bond may be important for the mechanism of action of this class of drugs and their chemical behaviour in aqueous media has been extensively investigated [52, 53, 68–70]. Hydrolysis of [Ru(η^6-arene)Cl(en)]$^+$ is suppressed in the presence of 100 mM chloride ions [68]. The intracellular chloride concentration is significantly lower than the extracellular concentration (ca. 4 and 23 mM in nucleus and cytoplasm, respectively, vs. 103 mM outside the cell) [71]. Thus, although the chlorido form (Ru–Cl) of the complex probably resists aquation in the extracellular medium, hydrolysed products (Ru–OH_2/OH species) are likely to predominate inside cells under physiological conditions. In general, ruthenium arene complexes that hydrolyse also exhibit cancer cell cytotoxicity, whereas those which do not undergo aquation exhibit little or no activity [53]. This observation supports the assumption that this type of complex binds to biomolecules coordinatively to exert their cytotoxic activity.

The rate and extent of hydrolysis of the Ru–Z bond are highly dependent on the nature of Z, more labile leaving groups giving rise to faster hydrolysis [53].

For example, the differences between Cl^- and Br^- in complexes of the type [Ru(η^6-arene)(en)Z]$^+$, where arene is biphenyl, indane or benzene, is not dramatic, however, when Z = I^- the hydrolysis is slower (3- to 7-fold). Other leaving groups such as pyridine or pyridine derivatives can slow down the hydrolysis further and even block it almost completely on biologically-relevant time scales. Such complexes are not cytotoxic towards cancer cells within 24-h drug exposures. There are a few exceptions such as [Ru(η^6-hexamethylbenzene)(en)(SPh)]PF_6, where the monodentate ligand is thiophenolate. This complex does not hydrolyse, but intriguingly is active. The mechanism of activation of this complex is different (vide infra).

Changing the arene can also have a significant effect on the rate and extent of hydrolysis. For example, there is a 2-fold difference in the hydrolysis rate between the biphenyl complex [Ru(η^6-biphenyl)Cl(en)]PF_6, and the tetrahydroanthracene complex [Ru(η^6-5,8,9,10-tetrahydroanthracene)Cl(en)]PF_6 or the dihydroanthracene complex [Ru(η^6-9,10-dihydroanthracene)Cl(en)]PF_6, demonstrating that variations in the steric and electronic effects of arene ligands can modulate the aquation rate [68].

The chelating ligand can also exert a strong effect on the hydrolysis rate and equilibrium constant, attributable not only to electronic (strength of π interactions) but also steric effects (competitive binding of Z and water). Density functional calculations have suggested that aquation occurs by a concerted ligand exchange mechanism (more associative character in the substitution of Z by H_2O) [53]. An example of the effect of the chelating ligand on the rate of hydrolysis is represented by the series [Ru(η^6-indan)Cl(*N*,*N*)]PF_6 where *N*,*N* = 2,2′-bipyridine, 4,4′-dimethyl-2,2′-bipyridine, and phenanthroline, with values for the pseudo first-order rate constants of 121.0 ± 1.0, 113.0 ± 2.0, and 75.1 ± 1.2 ($\times 10^3$ min^{-1}) (determined by UV–vis spectroscopy for 50 μM solutions in 95% H_2O, 5% MeOH) [61]. In general, the reported half-lives for aquation of the chlorido ethylenediamine Ru^{II} arene complexes are shorter than those for bipyridine analogues [68].

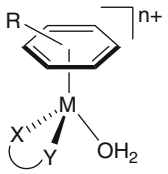
As important as the rate of hydrolysis for drug activation is the pK_a of the coordinated water molecule of the activated aqua adduct. The pK_a value determines whether the active species containing the more labile Ru–OH_2 bond or the less reactive deprotonated form Ru–OH prevails in solution at a given pH [72]. pK_a values for the coordinated water ligand in most of Ru arene complexes are around 8, therefore, at pH 7 aqua adducts should predominate. It has also been observed that control over the pK_a of the bound water molecule has an impact on the reactivity of the complex, with a pK_a higher than 7 leading to an active complex, while a pK_a lower than the physiological pH (ca. 7) resulting in an inactive species, since the hydroxo form would dominate rendering the complexes non-cytotoxic [68]. It has been found that the pK_a of the aqua adduct is as tuneable as the hydrolysis rate. It can be affected by the nature of the arene (8.3 for [Ru(η^6-*p*-cym)Cl(en)]PF_6, 7.9 for [Ru(η^6-bz)Cl(en)]PF_6 and 7.7 for [Ru(η^6-biph)Cl(en)]PF_6, for example) and the chelating ligand, since more electron withdrawing groups (better π-acceptors arenes) will divert the electron density away from the metal centre leaving it more

depleted of negative charge and making the bound water molecule more acidic (lower pK_a; Table 1).

The nature of the chelating ligand (*N,N* or *N,O* or *O,O*) can have a major influence on the pK_a values of the Ru−OH_2 group (Table 1). Replacement of ethylenediamine by anionic acetylacetonate (acac) as the chelating ligand, raises the pK_a of the aqua adduct [69]. A neutral ethylenediamine ligand, however, increases the acidity of a bound water molecule. This reasoning also explains the relatively higher basicity of the bound water molecule of the glycinate aqua adduct in comparison to the ethylenediamine aqua adduct. Within a series of *O,O*- negatively charged ligands with different substituents and aromaticities, the trend becomes more complicated.

Ruthenium(II) arene complexes of general formula [Ru(η^6-arene)Cl_2(PTA)] (PTA = 1,3,5-triaza-7-phosphatricyclo[3.3.1.1]decane) which possess antitumour activity have been reported by Dyson and co-workers (**5**, RAPTA-C) [74–76]. The RAPTA compounds showed pH-dependent DNA-damaging properties. These complexes were originally believed to be activated by the protonation of the PTA ligand at pH values close to physiological pH [77]. However, further studies rationalised the pH-dependent DNA binding by the aqua/hydroxo equilibrium for [Ru(η^6-arene)Cl(OH_2)(PTA)]$^+$ [78], and the activation of the organometallic complex is

Table 1 pK_a data for Ru and Os organometallic complexes with different arenes and chelating ligands



Arene	Metal (M)	Chelating ligand (XY)	pK_a	Reference
p-cym	Ru	acac	9.4	[69]
p-cym	Ru	maltol	9.2	[73]
p-cym	Ru	trop	9.1	[70]
p-cym	Ru	gly	8.7	[60]
p-cym	Ru	en	8.2	[72]
bz	Ru	en	7.9	[59]
biph	Ru	en	7.7	[68]
p-cym	Os	maltol	7.6	[73]
p-cym	Os	acac	7.6	[59]
bz	Os	acac	7.3	[59]
biph	Os	acac	7.1	[59]
bz	Os	en	6.3	[59]
biph	Os	en	6.3	[59]
p-cym	Os	bipy	5.8	[63]
p-cym	Os	phen	5.8	[63]

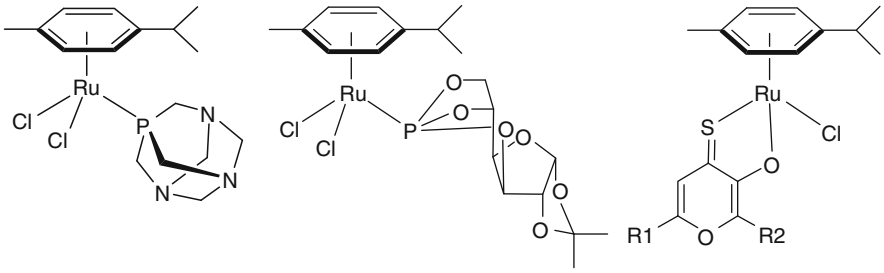
p-cym para-cymene; *bz* benzene; *biph* biphenyl; *acac* acetylacetonate; *maltol* maltolate; *trop* tropolonate; *gly* glycinate; *en* ethylenediamine; *bipy* bipyridine; *phen* phenanthroline

therefore dependent on hydrolysis of one of the Ru–Cl bonds. Related phosphine complexes have also been investigated in which the labile chlorides are replaced by *O,O*-chelating ligands which are highly soluble and kinetically more stable. Complexes [Ru(η^6-*p*-cymene)(C_2O_4)(PTA)] and [Ru(η^6-*p*-cymene)($C_6H_6O_4$)(PTA)] resist hydrolysis but maintain the cytotoxic properties towards cancer cells in a similar way as their precursors containing two chlorides in place of the carboxylate ligands [79]. Moreover, when PTA is replaced by 3,5,6-bicyclophosphite-α-D-glucofuranoside ligands the ruthenium complex (e.g. **6**) undergoes pH-dependent DNA binding but have complicated hydrolysis equilibria. The species which interacts with model base guanine and proteins in vitro is found to be the dimer, a product of further hydrolysis of the P–O bonds in the phosphite ligand [80].

In aqueous solution ruthenium complexes with aminomethyl-substituted 3-hydroxy-2-pyridone ligands exist as stable trimeric metalla-macrocycles and are converted into the reactive monomeric *O,O*-chelate species at low pH, thus delivering the organometallic complex to the cancer cells without undergoing deactivation [81].

Organometallic ruthenium-arene compounds bearing a maltolate ligand have been shown to be nearly inactive in cytotoxicity assays. However, dinuclear Ru-arene derivatives were found to show cytotoxic activity in cancer cells, which increases with the spacer length between the metal centres [82].

Moreover, ruthenium(II) arene complexes with pyrone-derived ligands became cytotoxic upon replacement of the coordinated *O,O*-donor with an *S,O*-donor. The thiopyrone complexes (**7**) are at least by an order of magnitude (in IC_{50}) more active than their pyrone analogues [83, 84].



RAPTA-C

5

6

R1 = H, R2 = CH_3
R1 = CH_3, R2 = H

7

2.2.2 Hydrolysis of the Os–Z Bond

A good example of how to tune a family of oganometallic complexes all the way from a lack of cytotoxic activity to IC_{50} values comparable to those of cisplatin and carboplatin is the newly developed series of osmium arene organometallic complexes.

When comparing two transition metals from the same group in the periodic table but from different rows, much lower ligand exchange rates are often associated with the heavier congener (osmium complexes in comparison with those of ruthenium). The hydrolysis of chlorido Os^{II}-(η^6-arene) complexes containing ethylenediamine as the chelating ligand is very slow (ca. 100× slower than that of the analogous active ruthenium complexes) [59] (Fig. 1). Additionally, the pK_a of the water molecule bound to Os^{II}-en complexes is ca. 1.5 units lower, i.e. more acidic, than for the Ru^{II} complexes (Table 1). Therefore at physiological pH the osmium complexes can exist largely in the less reactive Os–OH form [63]. Changing the chelating ligand from en to acac in Os^{II} arene complexes has a marked effect on increasing the rate and extent of hydrolysis [69], becoming so fast for complexes such as [Os(η^6-arene)(acac)Cl], where the arene is *p*-cymene, benzene or biphenyl, that the rate constant cannot be determined by NMR (hydrolysis occurring in less than 10 min) [59]. Moreover, replacement of en by acac has the expected effect on the acidity of the coordinated water, raising the pK_a by ca. one unit, attributable to the increased electron-density on the metal centre. However, dissociation of oxygen-chelating ligands from osmium at micromolar concentrations deactivates the complexes under biological testing conditions [59]. The acac complexes were unstable in aqueous solution due to the formation of inert hydroxo-bridged species, which could explain their lack of cytotoxicity.

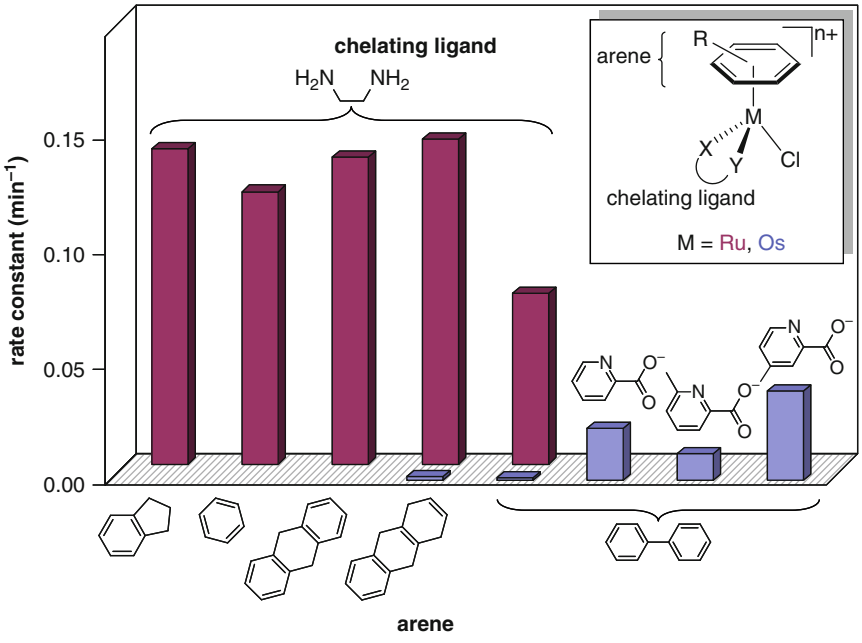
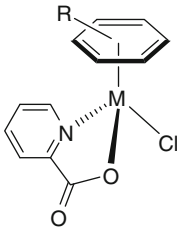


Fig. 1 Hydrolysis data for Ru and Os organometallic complexes with different arenes and chelating ligands. Data from [53, 63, 64, 66]

The inert hydroxo-bridged species were also a product of (very fast) hydrolysis of *p*-cymene osmium complexes with glycinate, L-alaninate, α-aminobutyrate and β-alaninate. However, complexes with picolinate as the chelating ligand, [Os(η^6-*p*-cym)Cl(pic)] **8** and [Os(η^6-biph)Cl(pic)] **9**, with pyridine as *N*-donor and carboxylate as *O*-donor, hydrolyzed with half-lives of 0.20 and 0.52 h (298 K), and aqua adduct pK_a* values (pK_a value for solutions in D_2O) of 6.67 and 6.33, respectively. Complexes **8** and **9** were cytotoxic towards A2780 human ovarian cancer cells, with IC_{50} values of 8 and 4.2 μM, respectively [64].



8, arene = p-cymene
9, arene = biphenyl

Novel picolinate derivatives have been synthesised that show hydrolysis rates intermediate between the slow values for the complexes with *N,N*-chelating ligands, and the rapidly-hydrolysing complexes with *O,O*- or *N,O*- amino acid-like chelating ligands. For these organometallic osmium complexes, the hydrolysis rates fall into the range of the 'active' ruthenium-arene relatives (Fig.1) [66].

Organometallic osmium-carbonyl clusters such as $[Os_3(CO)_{10}(NCCH_3)_2]$ are apoptosis-inducing agents with anticancer therapeutic potential. The mechanism appears to involve the loss of the labile acetonitrile ligand [85].

Once the ruthenium or osmium arene organometallic complex is activated with the formation of the aqua species, [Ru/Os(η^6-arene)(OH_2)(X)(Y)] (Chart 3), the metal becomes a potential centre for nucleophilic attack by biomolecules. The binding of Ru^{II}/Os^{II} arene complexes to nucleobases is of special interest, since DNA could be the ultimate target for this class of organometallic complexes. A number of studies have confirmed this postulate [86, 87] and investigated in detail such interactions [53, 54, 72, 88–93]. DNA interactions of Ru and Os arene complexes have recently been reviewed [94].

Ruthenium arene en complexes interact preferentially to guanine residues in double helical DNA. In addition to coordination to guanine, non-covalent,

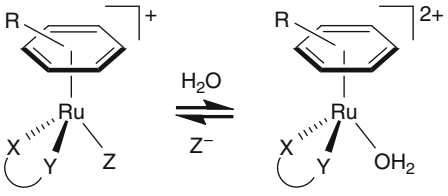


Chart 3 Hydrolysis of Ru^{II} arene complexes.

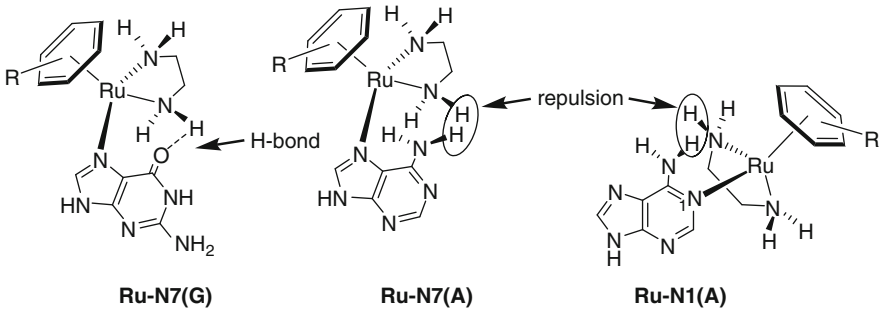


Chart 4 Interactions within guanine (G) and adenine (A) adducts of arene Ru-en anticancer complexes

hydrophobic interactions between the arene ligand and the DNA bases, such as π-stacking, arene intercalation and minor groove binding can occur [86]. The above observations clearly suggest a mechanism of action of Ru^{II} arene complexes different from that of cisplatin [87]. This is also true for osmium arene complexes [65].

The interaction between $[Ru(\eta^6\text{-arene})Cl(en)]^+$ complexes and guanine, thymine, cytosine and adenine derivatives has been investigated in aqueous solution [72]. The reactivity of the various binding sites of nucleobases towards Ru^{II} at neutral pH decreases in the order G(N7) > T(N3) > C(N3) > A(N7), A(N1) (with binding to cytosine being very weak and almost none to adenine derivatives). This base-selectivity appears to be enhanced by the ethylenediamine NH_2 groups, which form hydrogen bonds with exocyclic oxygens (e.g. C6O of G) but are non-bonding and repulsive towards exocyclic amino groups of the nucleobases (e.g. $C6NH_2$ of A) (Chart 4) [72]. Density Functional Theory (DFT) calculations on these complexes with isolated nucleobases show good agreement with experimental data, which indicated preference for binding to guanine over any other base (ca. 100 kJ/mol) [95].

Nucleoside selectivity is altered by exchanging the H-bond donor *N,N*-chelating ligand, such as ethylenediamine, for an H-bond acceptor *O,O*-chelating ligand, such as acetylacetonate. The acac-type complexes show similar affinity for adenosine (N1- and N7-bound) and for guanosine and there is little binding to cytidine or thymidine [69, 70]. This work demonstrates how the more versatile octahedral organometallic Ru^{II} complexes can acquire different biomolecular recognition features in comparison with square-planar complexes such as cisplatin. Recently, Liu et al. studied the interaction of the monofunctional fragment $\{Ru(\eta^6\text{-biphenyl})(en)\}^{2+}$ with the 14-mer d(ATACATGGTACATA)·d($TAT^{17}G^{18}$TACCATGTAT) by HPLC-ESI-MS and 2D NOESY NMR [92]. When the duplex is annealed at high temperature, ruthenation occurs at N7 of every guanine residue of the oligonucleotide, and at one site (G^{18}), two different conformers were identified involving intercalation of the arene between G^{18} and adjacent T^{17} in one, and in the second the arene is non-intercalated but stacked on a tilted adjacent thymine lying on the surface of the major groove (Fig. 2).

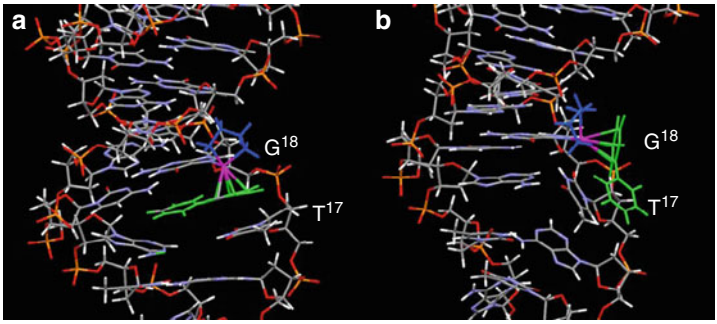


Fig. 2 Molecular models of two conformers of the 14-mer duplex d(ATACATGGTACATA) · d (TAT^{17}G^{18}TACCATGTAT) ruthenated at N7 of G^{18} with monofunctional fragment {Ru(η^6-biphenyl)(en)}$^{2+}$: (**a**) showing the intercalation of the arene between G^{18} and T^{17}; (**b**) the arene is non-intercalated but stacked on a tilted T^{17}. Colour code: Ru *purple*; en *blue*; biphenyl *green*. Adapted from [92]

2.2.3 Sulfur Oxidation

Redox reaction pathways can activate Ru^{II} arene thiolate complexes towards biomolecules. In unbuffered solutions (pH 3), the reaction of the complex [Ru(η^6-biph)Cl(en)]$^+$ with the tripeptide glutathione (γ-L-Glu-L-Cys-Gly, GSH) results in the loss of the chelating ligand (en), and the formation of the dinuclear sulphur-bridged dimer [{Ru(η^6-biph)}$_2$(GS-μ-*S*)$_3$]$^{2-}$ as the major product. However, in phosphate buffer (pH 7) and under physiologically-relevant conditions, the thiolato complex [Ru(η^6-biph)(en)(GS-*S*)] is formed which subsequently undergoes oxidation to give the sulfenato complex [Ru(η^6-biph)(en)(GS(O)-*S*)] quite readily. In the presence of cGMP (guanosine-3′,5′-cyclic monophosphate), the cGMP adduct [Ru(η^6-biph)(en)(cGMP-*N*7)]$^+$ is formed as the major product even in the presence of a large molar excess of GSH [96]. These results demonstrate that oxidation of coordinated glutathione in the thiolato complex [Ru(η^6-biph)(en)(GS-*S*)] to the sulfenate [Ru(η^6-biph)(en)(GS(O)-*S*)] provides a facile route for the displacement of S-bound glutathione by guanine N7, a route for RNA and DNA ruthenation (Chart 5). FT-ICR MS studies with ^{18}O-labelled water as solvent have suggest that oxygen atoms in sulfenate and sulfinate products may originate from water [97].

These ruthenium arene complexes can also induce oxygenation of cysteine residues in proteins. For example, when complexes [Ru(η^6-*p*-cym)Cl(en)]$^+$ and [Ru(η^6-biph)Cl(en)]$^+$ react with human serum albumin both bind to surface histidine (His128, His247, His510) and methionine (Met298) residues, but only the *p*-cymene complex gains entry to the cleft containing the free cysteine thiolate (Cys34) and induces oxidation to the sulfinate [98], indicating the critical role the arene plays in these interactions. Oxidation of the cysteine results in the weakening of the Ru–S bond and can trigger transfer of ruthenium to other protein sites or nucleobases.

There is a wider general interest in understanding the oxidation of cysteine thiolates in proteins since they are involved in redox-sensing reactions [99]. Therefore, such oxidation reactions of thiols induced by Ru coordination may also play a more general role in the pharmacological activity of Ru-arene complexes by coupling Ru coordinative binding to redox processes both outside and inside cells.

The oxidation of the non-chelated thiolato group to a sulfenato group in the complex $[Ru(\eta^6\text{-arene})(en)SR]^+$ (arene = hexamethylbenzene, or *p*-cymene and $R = {}^{i}Pr$, Ph) has been reported by Holm et al. [100]. The arene and the hydrogen donor ethylenediamine play a significant role in controlling the stability and reactivity of the Ru-bound sulfenate. Moreover, the sulfenato complexes are readily protonated with pK_a values of 3.37 and 3.61, respectively. The protonated complexes can easily hydrolyse to form reactive aqua or chlorido species (Chart 6). This is in contrast to the thiolato complex which does not hydrolyse. Thus the oxidation of the thiolato to the sulfenato group by the Ru arene complexes generates a labile leaving group, paving the way for interactions with biomolecules [100].

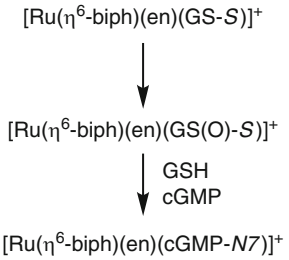


Chart 5 Activation of a thiolato ruthenium complex by ligand oxidation

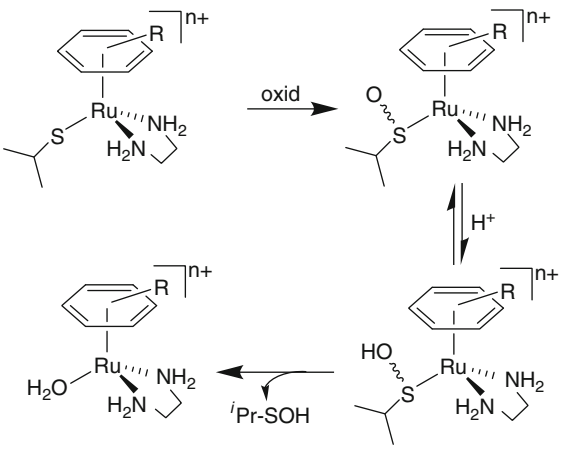


Chart 6 Activation of ruthenium thiolato complexes by oxidation followed by hydrolysis

It is interesting to note that cysteine sulfenates are found in catalytic centres of enzymes [101] and are of much interest in biological signalling processes [102–107]. An important feature of the sulfenato group compared to the thiolates and sulfinates is that it can exert high trans effects [101, 108, 109] and can exist in either oxidised [110] or reduced [111, 112] forms, in addition sulfenates have a negatively charged oxygen that forms strong hydrogen bonds [113, 114].

The thiolato complex $[Ru(\eta^6\text{-hmb})(en)SR]^+$ (hmb = hexamethylbenzene, $R = {}^{i}Pr$) can also undergo activation by ligand-based redox reactions involving a remarkably efficient oxygen-GSH couple under physiological conditions [115]. In these reactions GSH is almost completely oxidised to form GSSG and the thiolato complex is oxidised to the sulfenato complex $[Ru(\eta^6\text{-hmb})(en)S(O)R]^+$.

A combined XAS and DFT study of the complexes $[Ru(\eta^6\text{-arene})(en)SR]^+$, where arene is *p*-cymene or hexamethylbenzene and R is isopropyl or phenyl, has shown that the Ru–S bond is susceptible to ligand oxygenation and the influence of substituents on the sulfur and arene [116]. Sulfur K-edge XAS indicates that S_{3p} donation into the Ru_{4d} manifold depends strongly on the oxidation state of the sulfur atom, whereas Ru K-edge data suggest little change at the metal centre. DFT results are in agreement with the experimental data. Oxygenation of the thioate sulfur has little effect on the strength of the Ru–S/Ru–SO/$RuSO_2$ bond. In sulfenato complexes, the terminal oxo group makes a large contribution to charge donation making it more susceptible to ligand exchange, but only if protonation of the terminal oxo group occurs. These results are consistent with the observed hydrolysis of the sulfenato complexes under acidic conditions [100], and further indicate that modifications to the arene group and the thiolato group have a measurable impact on the Ru–S bond.

2.2.4 Bifunctional Ru Arenes

In an attempt to achieve a stable bifunctional ruthenium arene complex Melchart et al. prepared the tethered complexes $[Ru(\eta^6{:}\eta^1\text{-arene})Cl_2]$, where two of the three legs of the piano-stool are chlorides and the third is a NH_2 group from a tethered arm pendant from the π-bonded arene [117]. This complex contains two *cis* reactive Ru–Cl bonds, as does cisplatin which is known to form intrastrand crosslinks in DNA as a major lesion leading to cell death, and also maintains the presence of an H-bond donor, directed towards nucleobase interaction [89, 118]. However, these complexes hydrolyse very rapidly yielding mainly mono-aqua mono-chlorido species. In spite of the apparent formation of bi-functional adducts with model nucleobases such as 9-ethylguanine, they failed to give evidence of cross-linking with calf thymus DNA. The second G is bound only weakly, which together with the rapid hydrolysis, might explain their lack of cytotoxic activity.

2.2.5 Chelate Ring Redox

The diamine complex [Ru(η^6-*p*-cym)Cl(*o*-pda)]PF_6 (*o*-pda = *o*-phenylenediamine), undergoes concomitant ligand-based oxidation and hydrolysis (38% after 24 h) in water [119]. The oxidation also occurs in methanol. The iodido diimine complex [Ru(η^6-*p*-cym)I(*o*-bqdi)]I (*o*-bqdi = *o*-benzoquinonediimine) does not undergo hydrolysis, whereas the chlorido complex hydrolyses relatively rapidly ($t_{1/2}$ = 7.5 min). Loss of cytotoxic activity was observed upon oxidation of the amine ligand to an imine (e.g. IC_{50} = 11 μM for [Ru(η^6-*p*-cym)Cl(*o*-pda)]PF_6 vs. IC_{50} > 100 μM for [Ru(η^6-hmb)Cl(*o*-bqdi)]Cl against A2780 ovarian cancer cells). This oxidation can be controlled through changes in electronic properties of the other ligands (arene and monodentate ligands) in the complex. The loss of activity in this series of complexes may be related to the absence of hydrolysis as well as to the formation of less stable adducts with guanine, which would lead to weaker binding to DNA. Interestingly the diimine (*o*-bqdi) complexes can be reduced by the tripeptide glutathione but readily undergoes re-oxidation in air.

2.2.6 Irradiation

Photodynamic therapy is currently used in the clinic as a method for treating cancer. Irradiation of a photosensitizer, such as a porphyrin, with light leads to conversion of ground-state triplet oxygen into excited state and highly reactive singlet oxygen which damages DNA and kills the cells. However tumours are often deficient in oxygen and therefore the prospect of using excited state transition metal complexes that could kill cancer cells by an oxygen-independent mechanism is an attractive one. For example, some organometallic ruthenium arene complexes are capable of undergoing photo-induced reactions with DNA bases [120].

UV or visible irradiation of [{Ru(η^6-indane)Cl}$_2$(μ-2,3-dpp)]$(PF_6)_2$ (2,3-dpp = 2,3-bis(2-pyridyl)pyrazine) in aqueous (or methanolic) solution leads to arene loss, and the DNA binding of this organometallic ruthenium dimer increases after irradiation. The non-irradiated dinuclear complex forms DNA adducts that only weakly block RNA polymerase, while irradiation transforms adducts into stronger blocks for RNA polymerase. Irradiation of [{Ru(η^6-indane)Cl}$_2$(μ-2,3-dpp)]$(PF_6)_2$ in the presence of DNA leads to an increased frequency of cross-linking. In addition, there is a 40-fold increase in fluorescence of the unbound compared to bound arene. These results show that photoactivation of dinuclear Ru^{II} arene complexes can simultaneously produce a highly reactive ruthenium species that can bind to DNA and a fluorescent marker (the free arene). Importantly, the mechanism of photoreactivity is also independent of oxygen. These complexes, therefore, have the potential to combine both photo-induced cell death and fluorescence imaging of the location and efficiency of the photoactivation process [121].

An example of a piano-stool Ru^{II} arene complex, [Ru(η^6-*p*-cym)(bpm)(py)]$(PF_6)_2$ (where bpm = 2,2′-bipyrimidine and py = pyridine) that can be activated

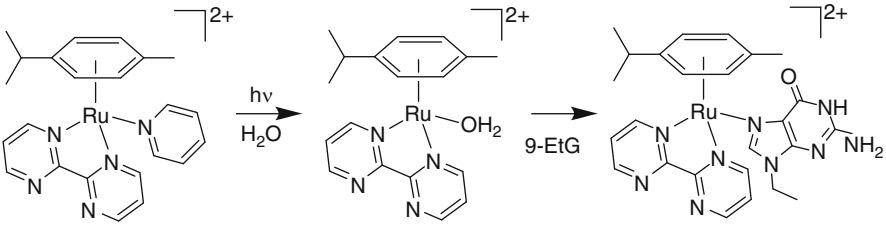


Chart 7 Photoactivation of a ruthenium arene complex

by visible light to photo-dissociate selectively a monodentate ligand (py), conventionally non-labile (on a timescale and under conditions relevant for biological reactivity) has been reported recently (Chart 7). Irradiation generates a reactive aqua spcies, able to bind to a DNA base. Such behaviour creates a platform for control of the hydrolysis reaction of the complex and phototriggers binding of an anticancer pro-drug to biomolecules [122].

2.3 Other Transition Metal Complexes

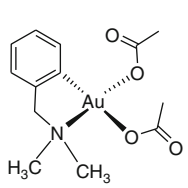
Gold(III) organometallics are isoelectronic with cisplatin-like Pt^{II} complexes. For example, complex **10** hydrolyses in water [123] and has shown activity in human tumour xenograft models [124]. However, its mechanism of action is different from that of cisplatin [124, 125]. The role of hydrolysis as an activation step for this class of compounds is not yet clear.

Other examples of organogolds with cytotoxic activity are complexes such as **11**, [Au(bipy^{c}-H)(OH)]PF_6 [126], and analogues in which the OH has been substituted by bulkier ligands such as: 2,6-xylidine-H and *p*-toluidine-H [127]. For these analogues, hydrolysis may represent an activation step.

Very recently, the mitochondria-targeted antitumour agents gold(I) *N*-heterocyclic carbenes (NHC) have been reported that combine both selective mitochondria targeting and selective thioredoxin reductase inhibition properties [128]. The lipophilic cationic organogold(I) complex [Au((^{i}Pr)$_2$Im)$_2$]Cl (**12**) induces caspase-9 and caspase-3 activation in human breast adenocarcinoma cells suggesting that this class of complexes causes selective cancer cell death through a mitochondrial apoptotic pathway. Additionally, promising gold NHC complexes such as the gold(I) cysteine derivatives **13** and **14** have been recently reported that exert antiproliferative activity in cancer cell lines comparable to that of cisplatin [129].

Moreno et al. have developed highly active platinum(II) and palladium(II) organometallic complexes derived from the *N*,*C*-chelating ligand 2-(dimethylaminomethyl)phenyl (dmba) (e.g. **15**) or pentafluorophenyl ligands and studied reactions with representative model nucleobases, namely, 1-methylthymine, 1-methyluracil, and 1-methylcytosine [130–133]. The complexes interact strongly with DNA. Values of IC_{50} were also determined for these new organometallic platinum complexes against the tumour cell lines (HL-60, A2780, A2780cisR,

NCI-H460 and T47D), and all were more active than cisplatin. Given the success enjoyed by cisplatin-like platinum coordination complexes, this appears to be a promising area of research.



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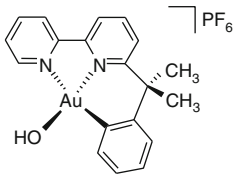

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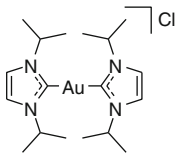

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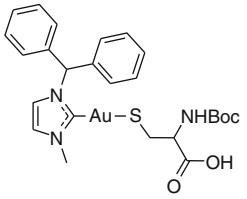

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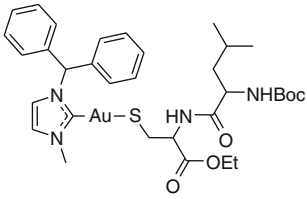

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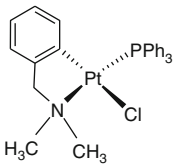

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3 Metal Complexes as Catalytic Drugs

Here we describe an example of metal-based catalysis is described followed by an example of ligand-based catalysis.

Ruthenium(II) arene anticancer complexes, for example $[Ru(\eta^6\text{-}C_6Me_6)(H_2O)(en)]^{2+}$, can catalyse regioselective reduction of NAD^+ in the presence of formate as hydride donor in vitro [134], although the reaction rates are ca. 50× slower than those catalysed by rhodium(III) pentamethylcyclopentadienyl complexes [135]. Interestingly A549 human lung cancer cells appear to tolerate high (millimolar) levels of formate well and so the possibility arises of exploring the catalytic activity of organometallic metal complexes in cells. Recent work has shown that the efficiency of such catalysts can be greatly improved by appropriate choice of the chelated *N,N*-ligand [136].

The presence of a π-bonded arene ligand together with a strongly chelated σ-donor/π-acceptor azopyridine ligand and iodide as a strong monodentate ligand, produces Ru^{II} arene complexes $[Ru(\eta^6\text{-arene})(azpy)I]^+$ (where arene = *p*-cymene or biphenyl, and azpy = *N,N*-dimethylphenyl- or hydroxyphenyl-azopyridine) which are relatively inert toward activation by hydrolysis in aqueous solution. Surprisingly, despite this inertness, these complexes are highly cytotoxic to human ovarian A2780 and human lung A549 cancer cells. Fluorescence trapping experiments in A549 cells suggest that the cytotoxicity arises from an increase in reactive oxygen species. These azopyridine complexes undergo activation by reduction of the ligand. While

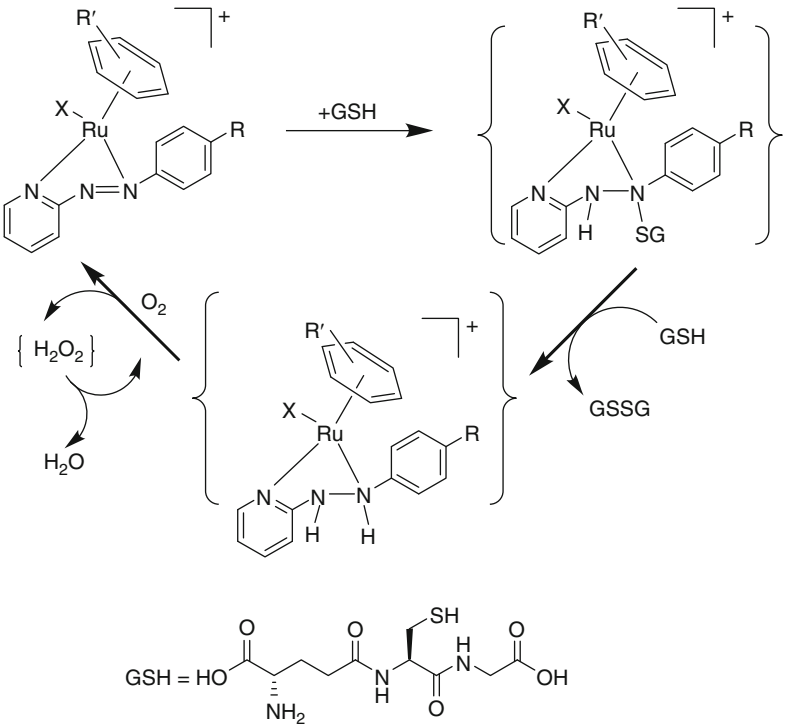


Chart 8 Possible scheme for catalytic activity of ruthenium azopyridine complexes

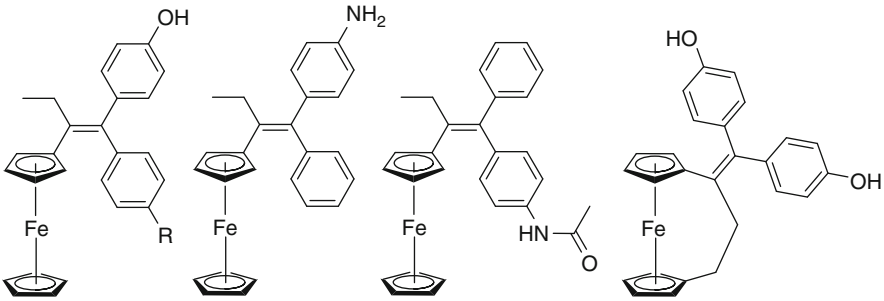
azopyridine ligands alone are difficult to reduce, the reduction potentials are biologically accessible when the azopyridine is coordinated to Ru^{II} [137]. Intriguingly the ruthenium complex appears to act as a catalyst in reactions with GSH; millimolar amounts of GSH can be oxidised to GSSG in the presence of micromolar ruthenium concentrations in a cycle which appears to involve ligand-centred reduction of the azo bond by GSH forming a hydrazo intermediate, followed by formation of GSSG and regeneration of the azo bond (Chart 8). Such ligand-based redox reactions provide new concepts for the design of catalytic drugs.

4 Structural Scaffolds

4.1 Ferrocenes and Ferrocenyl Derivatives

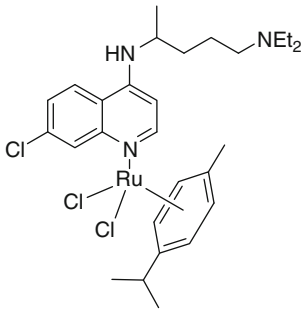
Depending on the presence or absence of the oestrogen receptor in the cells, breast cancer is often treated by endocrine therapy (tamoxifen) or chemotherapy, respectively. Organometallic derivatives of hydroxytamoxifen (e.g. ferrocifen, **16**, and its

derivatives) have been explored as a potential therapy for breast cancer and other cancers by Jaouen and co-workers. The presence of an organometallic fragment (ferrocene), and a pharmacophore (hydroxytamoxifen), generates compounds which display a new therapeutic spectrum consisting of anti-oestrogenicity and cytotoxicity. A structure-activity relationship (SAR) study has shown that a ferrocene group linked to a *para*-phenol group by a conjugated spacer, is a necessary motif for strong cytotoxic effects against both the hormone-dependent MCF-7 and hormone-independent MDA-MB-231 breast cancer cell lines, involving oxidative quinone methide formation [138]. Ferrocenyl quinine methides, potentially cytotoxic species, are formed by metabolic and chemical oxidation of ferrocenyl phenols [139]. The binding of ferrocifen to the oestrogen receptor is believed to produce an enhanced antiproliferative effect together with additional cytotoxicity induced by the redox properties of ferrocene [138]. It is interesting to note that the isostructural ruthenocene derivative is also active in hormone-dependent breast cancer cells but lacks the antiproliferative effect of ferrocifen against hormone-independent cells [140]. Electrochemical experiments suggest that on replacement of the phenol group by analogous amine and acetamide complexes **17** and **18**, both complexes can be transformed to oxidised quinoid-type species, analogous to those observed previously for the ferrocene phenols [141]. New ferrocenophane compounds have been reported recently, one of which (**19**) has an anti-proliferate effect seven times greater than the corresponding noncyclic species, with IC_{50} values of 90 and 94 nM towards hormone-independent MDA-MB-231 breast and PC-3 prostate cancer cell lines [142].



16 **17** **18** **19**

In a strategy which involves incorporation of a biologically active compound into a metal fragment, to achieve enhanced activity and possibly with a different mode of action, Sanchez-Delgado et al. have synthesised complexes containing a Ru^{II} arene fragment and the anti-malarial drug chloroquine (**20**) and chloroquine diphosphate (**21**). These complexes display activity against chloroquine-resistant parasites and also inhibit the growth of colon cancer cells, especially **21**, for which an IC_{50} value of 8 μM was observed. Chloroquine binds to ruthenium preferentially through the quinoline nitrogen atom, whereas chloroquine diphosphate is π-bonded through the carbocyclic ring [143].



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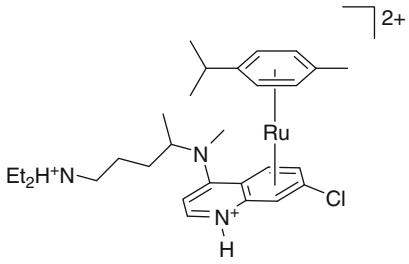

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4.2 Glutathione-S-Transferase Inhibitors

Organometallic ruthenium-arene complexes conjugated to ethacrynic acid have been prepared as part of a strategy to develop novel glutathione-S-transferase (GST) inhibitors with alternate modes of activity through the organometallic fragment (binding at Cys47 and to a lesser extent Cys101) and hence potentially enhanced antiproliferative activity.

The dichloro-Ru^{II}-arene complex conjugated to ethacrynic acid [Ru(η^6-*p*-cymene) Cl_2(EA–imidazole)] more rapidly inactivated GST P1-1 than the carboxylato analogues, suggesting that the ruthenium centre plays a role in the inactivation of the enzyme [144].

4.3 Kinase Inhibitors

Meggers and co-workers have pioneered the use of inert organometallic Ru^{II} and Os^{II} complexes as scaffolds bearing organic compounds with biological activity, such as staurosporine (Chart 9), a known kinase inhibitor [145–147]. The creation of unique and defined molecular structures based on metal scaffolds tailored to fit (and inhibit) the active sites of kinase enzymes is an active area of research. Kinases are important therapeutic targets since their mutations and deregulation are linked to diseases. They have a highly conserved ATP binding site for which the design of organic inhibitors is challenging [148].

This strategy has resulted in the discovery of nanomolar and even picomolar ATP-competitive ruthenium-based inhibitors for different protein kinases [146]. In particular one of these complexes DW1/2 (Chart 9) has shown remarkable anticancer properties in several cancer cell lines (300 nM of the complex maximally inhibited cell growth in most of the melanoma cell lines tested) [149].

Recent structural studies confirm that these complexes bind to the ATP-binding site of protein kinase [150]. Superimposition of the co-crystal structures of Pim-1

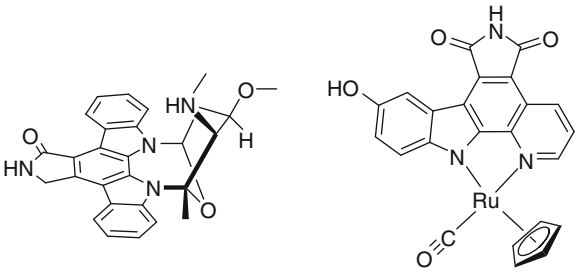


Chart 9 The kinase inhibitor staurosporine and the ruthenium complex DW1/2

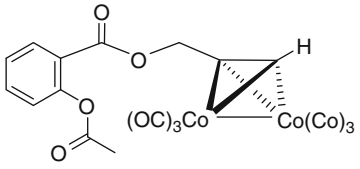
with (S)-Os and (S)-Ru (PDB: 3BWF and 2BZI, respectively) showed virtually identical structures [151]. Unlike ruthenium and osmium-arene relatives, where substitution reactions appear to have an essential role in their cytotoxic activity, it was found that the isostructural Ru^{II} and Os^{II} complexes display almost indistinguishable biological activities and identical modes of action as protein kinase inhibitors.

The targeting of proteins with metal complexes, both organometallic and coordinative metal-based compounds, has been recently reviewed by Meggers [148].

Keppler et al. have synthesised Ru^{II} and Os^{II} arene complexes with paullones as ligands to confer solubility on these otherwise insoluble cyclin-dependent kinase (CDK) inhibitors [152]. No dramatic differences between the ruthenium and the osmium complexes were found in the IC_{50} values against A549, CH1 and SW480 cancer cell lines.

4.4 COX Inhibitors

The cobalt-alkyne analogue **22** of the non-steroidal anti-inflammatory drug aspirin (acetylsalicylic acid) exhibits antiproliferative activity against human breast cancer cell lines [153]. The cytotoxicity is related to cyclooxygenase (COX) inhibition, which retards the growth of established tumours and enhances their response to conventional therapies [154]. COX is involved in eicosanoid metabolism and targeting this pathway is a viable strategy for the development of new anticancer agents. It has been speculated that **22** binds to cyclooxygenase in a fashion similar to aspirin followed by an acetylation of a surface residue.



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5 Metal as a Carrier for Active Ligands

In order to target drug delivery, it is very important to study the distribution of the drug within the body. For example radiolabelling of the anticancer complex [Ru(η^6-fluorene)Cl(en)]PF_6 with the β-emitter ^{106}Ru (half-life = 1.01 y) has allowed its biodistribution to be studied; 0.25 h post i.v. injection at a dose of 25 mg kg^{-1}, ^{106}Ru is well distributed throughout the tissues of a rat [155].

Various other strategies for investigating biodistributions of metallodrugs are being developed by different groups.

5.1 Side-Chain Hydrolysis

A fluorescent complex [Ru(η^6-*p*-cym)Cl(L)]Cl (L = 2-[(2-aminoethyl)amino] ethyl-2-(methylamino)benzoate) has been synthesised by tagging a small fluorogenic reporter onto the chelating ligand. The interaction of this complex with porcine liver esterase (PLE) showed that esterase-catalysed hydrolysis reactions can liberate methylisatoic acid (MIAH) from the ruthenium complex suggesting a possible use of similar derivatives in esterase-activated Ru-based prodrug delivery systems. The hydrolysis reaction appears to be slow [156].

5.2 Ruthenium Cages

Stable ruthenium arene cages consisting of a cationic hexanuclear metalla-prism $[Ru_6(p\text{-}^{i}PrC_6H_4Me)_6(tpt)_2\text{-}(dhbq)_3]^{6+}$ (which incorporates *p*-cymene ruthenium building blocks and is bridged by 2,5-dihydroxy-1,4-benzoquinonato (dhbq) ligands and connected by two tpt subunits (tpt = trigonal 2,4,6-tris(pyridin-4-yl)-1,3,5-triazine)) have been synthesised. The cages can encapsulate and deliver $Pd^{II}(acac)_2$ or $Pt^{II}(acac)_2$ complexes in a Trojan-horse manner. The encapsulated Pd and Pt complexes are insoluble in water and therefore inactive. The capsule itself showed cytotoxicity which was remarkably enhanced by encapsulating the acac complexes (IC_{50} of 23 μM decreased to 12 and 1 μM, respectively) [157]. A review article on the synthetic strategies to obtain metalla-rectangles, triangular metalla-prisms and metalla-boxes with arene ruthenium complexes has been recently published [158].

6 Photoactivation and Photosensitizers

Therrien et al. have developed organometallic ruthenium porphyrin compounds for anticancer photodynamic therapy. These combine synergistically the photodynamic action of porphyrins with the DNA damage-related cytotoxicity of Ru^{II}-arene

complexes. Porphyrin arene-ruthenium(II) and osmium derivatives and cyclopentadienyl-iridium and -rhodium analogues have been prepared as potential photosensitising chemotherapeutic agents. The ruthenium complexes showed good photo-toxicities toward melanoma cells when exposed to red laser light at 652 nm [159].

The organometallic ruthenium-arene fragments improved the aqueous solubility of the otherwise insoluble porphyrins without modifying the photophysical properties of the photosensitizer. Additionally, the presence of the ruthenium on the porphyrins facilitated uptake into melanoma cells [160].

7 Organotins

Since the early finding in 1972 that triphenyltin acetate (but not the corresponding chloride) retards tumour growth in mice [161], a number of organotin derivatives have been prepared and tested in vitro and in vivo [162–166].

It has been suggested that, in solution, triorganotin compounds may undergo spontaneous disproportionation into the corresponding diorganotin and tetraorganotin derivatives [167] while, in vivo, the loss of one alkyl or aryl group may occur through the intervention of enzymes such as aromatase [168].

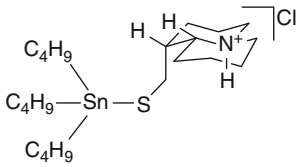
On the basis of the above consideration, diorganotin compounds might be considered to be the ultimate cytotoxic agents and the frequently observed higher activity of triorganotins may be related to their pharmacokinetic behaviour.

Inhibition of macromolecular synthesis, mitochondrial energy metabolism, and reduction of DNA synthesis, as well as direct interaction with the cell membrane (increase in cytosolic Ca^{2+} concentration), have been implicated in organotin-induced cytotoxicity [169, 170]. Promotion of oxidative and DNA damage in vivo has been detected [171]. Oxidative damage and increased concentration of intracellular calcium ions seem to be the major factors contributing to triorganotin-induced apoptosis in many cell lines [172].

Sn^{IV} organometallic compounds can induce apoptosis [163, 173, 174]. However, the exact mechanism of anti-tumour action of organotin(IV) compounds remains unknown. Although the mechanism of this antiproliferative activity is not well established, it has been suggested [175] that organotin(IV) compounds wield antiproliferative effects through binding to thiol groups of the proteins hence differing from the behaviour of several cytotoxic complexes of other metals.

A comprehensive review of recent advances on organotin anticancer therapeutics has been recently published [172].

Interesting are recent findings for the tributyl complex tri-*n*-butyltin(IV) lupinylsulfide hydrogen fumarate (IST-FS 35, **23**) which inhibitis both the P388 myelomonocytic leukaemia and the B16-F10 melanoma, implanted subcutaneously in BDF1 mice. The mechanism is not understood [166, 176].



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Triorganotin carboxylates may exist in monomeric or polymeric forms, while diorganotin derivatives may exist as true dicarboxylates or as distannooxane salts $[(R_2SnO\text{-}COR')_2O]$ and may further aggregate in a number of ways that influence both solubility and bioavailability. More work on the molecular basis for the activity of tin complexes is needed.

8 The Future for Medicinal Organometallics

Organometallic complexes provide versatile platforms for drug design. Metal–carbon bonds can exert major electronic and steric effects, which in turn can be used to control their biological activity. In particular such complexes offer the possibility of novel mechanisms of action compared to purely organic drugs and have the potential for combating drug resistance as well as treating currently intractable conditions.

Some organometallic complexes are inert enough to act as scaffolds which can be designed to interact sterospecifically with biomolecular targets and act, for example, as enzyme inhibitors. In general though, most are 'pro-drugs' which can undergo ligand exchange and/or redox reactions before they reach the target site, it is important to demonstrate that such reactions can be controlled so as to maximise activity and minimise unwanted side-effects.

In this chapter, we have illustrated how π-bonded metal arene complexes can undergo activation by hydrolysis, by metal-based or ligand-based redox reactions, or by irradiation with light. The choice of not only the arene (benzene and cyclopentadienyl derivatives) but also the other ligands in the complex is crucial for determining both thermodynamic and kinetic stability and biological activity. This is well illustrated by the first organometallic complex to enter clinical trials as an anticancer agent titanocene dichloride [177]. Variations to both the Cp substituents and to the monodentate ligands can change the aqueous reactivity and perhaps help prevent the complicated hydrolysis reactions which made clinical formulation of this compound difficult.

Some organometallic complexes are well known to possess catalytic activity and it is interesting to consider the possibility that catalytic drugs could be developed. The problem of catalyst poisoning may be difficult to overcome in a biological medium but perhaps this can be used to switch off the activity when required.

There are major challenges to be tackled relating to the chemical and biochemical mechanisms of action of organometallic complexes, for example involving the mechanism of displacement and release of coordinated arenes and cyclopentadienyl ligands which can expose several reactive coordination positions on a metal ion. Also the metabolism of coordinated arene and Cp ligands (e.g. modification by P450 enzymes in the liver) may have a major effect on the biodistribution and excretion of complexes.

We can expect to see new organometallic complexes become candidates for clinical trials as anticancer drugs in the near future and they will also find applications in other fields of therapy. Medicinal organometallic chemistry is in its infancy but shows much promise for future developments.

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Organometallic Antitumour Agents with Alternative Modes of Action

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Abstract The therapeutic index of drugs that target DNA, a ubiquitous target present in nearly all cells, is low. Nevertheless, DNA has remained the primary target for medicinal chemists developing metal-based anticancer drugs, although DNA has been essentially abandoned in favour of non-genomic targets by medicinal chemists developing organic drugs. A number of organometallic drugs that target proteins/enzymes have been developed and these compounds, based on ruthenium, osmium and gold, are described in this chapter. Targets include cathepsin B, thioredoxin reductases, multidrug resistance protein (Pgp), glutathione S-transferases and kinases. It is found that compounds that inhibit these various targets are active against metastatic tumours, or tumours that are resistant to classical DNA damaging agents such as cisplatin, and therefore offer considerable potential in clinical applications.

Keywords Bioorganometallic chemistry · Cancer chemotherapy · Mode of action · Organometallic compounds · Protein binding · Targeted drugs

Contents

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1 Introduction

The application of cisplatin and other platinum compounds in the clinic for the treatment of a range of cancers has transformed medical practises [1]. Combination therapies and highly evolved dosing protocols have led to the extensive use of platinum compounds with estimates that more than 50% of all patients with cancer receive them – often in advance of surgery [2]. Despite the considerable success of platinum compounds, there remains a need for new drugs that operate via different mechanisms so that they are effective on platinum-resistant tumours. Moreover, the therapeutic index of platinum drugs is very low since they target DNA, which is a ubiquitous target present in all cells, and better tolerated compounds are desirable.

While DNA has remained as the primary target for researchers developing metal-based drugs, medicinal chemists developing novel organic compounds for the treatment of cancer have abandoned DNA in favour of non-genomic targets that regulate the cell cycle or inhibit specific over-expressed enzymes present in the cancer cells. Such a targeted approach offers certain advantages over non-targeted approaches, notably that side effects are reduced, although such compounds tend to be effective on a narrower range of tumours. Although somewhat non-rational in their design, at least in the early stages of their development, certain gold drug candidates were shown to operate via a targeted mode. Other targeted organometallic compounds based on rational design include a series of tamoxifen-analogues derivatised with various organometallic units described in Chapter X, and a class of highly potent kinase inhibitors in which an organometallic unit acts as a scaffold, described in Chapter X. In addition to these classes of compounds a number of organoruthenium and organogold compounds display novel modes of action that appear to be significantly different from classical platinum drugs and their effects are increasingly linked to protein targets. In this chapter the chemical and biological properties of these two main classes of non-classicial anticancer compounds are described.

2 DNA as a Target

It is well established that the major cytotoxic effect of platinum-based anticancer drugs stems from binding of the platinum centre to DNA. The coordination of platinum(II) centres to DNA causes structural modifications which ultimately lead to the induction of apoptosis. Ruthenium compounds were developed as alternatives to platinum compounds and initially their binding to DNA, similarly to platinum compounds, was considered to be central for their anticancer effect. The ruthenium

compound indazolium trans-[tetrachlorobis(1*H*-indazole)ruthenate(III)], KP1019, was found to interact with DNA, but compared to cisplatin, exhibited lower interstrand cross-linking and only a moderate capacity of introducing secondary structural changes, and the formed adducts were less effective in terminating transcription [3,4]. Nevertheless, many ruthenium species, both coordination complexes and organometallic compounds, have been shown to be capable of binding to DNA or model compounds [5–8]. In the case of Ru(III) compounds, reduction to Ru(II) in the tumour, due to the reductive environment characteristic of tumour tissue, is thought to activate the compounds for biomolecule binding (including DNA) [6]. Reduction by glutathione was shown to influence the reactivity of KP1019 towards guanine 5′-monophosphate and, as for platinum compounds, the *N*7 of guanine was found to be the preferred binding site [9]. However, more recent studies have revealed that DNA is not necessarily the primary target of ruthenium anticancer compounds and a higher affinity to proteins relative to DNA for an organometallic ruthenium(II)-arene compound has even been observed [10]. Another compound, imidazolium *trans*-[tetrachloro(dimethylsulfoxide)(1*H*-imidazole)ruthenate(III) (NAMI-A) inhibited metastatic tumours and both *in vitro* and *in vivo* data appears to exclude DNA as the primary target, in line with the observation that the binding of NAMI-A to DNA is much weaker than that of platinum complexes [11] (Fig. 1).

Many gold compounds that are highly cytotoxic *in vitro* against representative human tumour cell lines, with IC_{50} values often falling in the low micromolar or even nanomolar range, do not induce evident signs of apoptosis in treated human tumour cells via the formation of DNA interactions, but their actual modes of action have not been univocally identified [12]. A strong association of gold with DNA has been demonstrated only in a few cases, in particular those where the interaction is mainly dependent on the nature of the ligand [e.g. for gold porphyrin [13] and gold terpyridine [14] complexes]. Indeed, as gold compounds manifest, on the whole, a lower affinity for DNA than platinum(II) compounds [15–17], it appears that apoptotic cell death is a result of DNA-independent processes. Accordingly, cytotoxic gold compounds, like ruthenium compounds, are often found to overcome resistance to cisplatin confirming the occurrence of a substantially different mode of action.

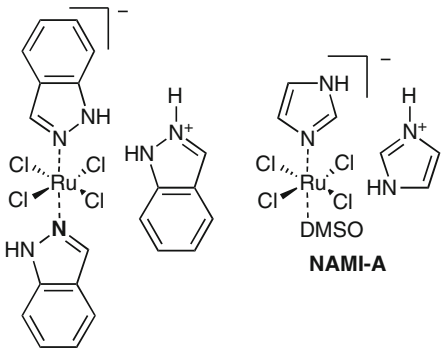

Fig. 1 Structures of KP1019 and NAMI-A

While DNA does not appear to be the primary target for ruthenium and gold drugs (see below), the mechanism by which a drug exerts its effect is extremely complex, and it is not unreasonable to state that, while much has been learnt regarding metallodrug targets, the complete mapping of the molecular mechanism of any metal-based drug has not been accomplished. Thus, while we believe the research described in this chapter is important, the studies are essentially the start of a shift in the field of metal-based drugs, of which organometallic compounds represent an increasingly important sub-section. Hopefully this Chapter will stimulate a greater interest in (protein) targeted metal-based chemotherapies.

3 Organoruthenium Compounds

As far as we are aware the first example of a ruthenium(II)-arene compound to be evaluated for anticancer activity was described by Tocher and co-workers in 1992 [18]. The field remained essentially dormant until the explosion in interest led largely by our group and that of Sadler, who has extensively studied ruthenium(II)-arene complexes with ethylenediamine and related ligands, and describes these compounds in the Chapter X in this book. It should be noted that other groups have also contributed to the field as will become clear in the course of this Chapter.

4 Ruthenium-Arene PTA (RAPTA) Compounds

Relatively simple ruthenium-arene compounds of the general formula [Ru(η^6-arene)Cl_2(pta)], (pta = 1,3,5-triaza-7-phosphatricyclo-[3.3.1.1]decane), termed RAPTA compounds (see Fig. 2 for some examples) were first reported in 2001 [19].

RAPTA-C has been the subject of detailed reactivity, computational, *in vitro* and *in vivo* studies. In aqueous solution, one chloride ligand is usually hydrolysed, the extent of hydrolysis depending upon the concentration of the complex in solution, the concentration of chloride, the pH and temperature [20]. Once hydrolysed, RAPTA compounds can rapidly react with potential biomolecular targets such as DNA and proteins. However, hydrolysis does not appear to be a prerequisite for reaction with biomolecules as in blood plasma, where the chloride concentration is high enough to

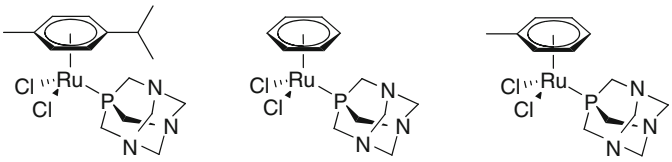

Fig. 2 Structures of prototypically RAPTA compounds

suppress hydrolysis, binding to serum proteins is still observed. Although the nature of these RAPTA-protein adducts has yet to be identified, model studies indicate that both chloro ligands and eventually also the pta ligand are lost upon protein binding.

In vitro cytotoxicity studies of simple RAPTA compounds show that they are considerably less cytotoxic than platinum drugs. Indeed, many cannot even be classed as cytotoxic, in that while many only show limited cytotoxicity towards cancer cells they are non-toxic towards model healthy cells. This observation correlates well with *in vivo* studies that show RAPTA compounds to be non-toxic at high doses to healthy animals [21].

Indeed, the *in vivo* effect of RAPTA compounds is strikingly different to most other anticancer compounds, both metal-based and organic drugs. RAPTA-C and RAPTA-T inhibit lung metastases in CBA mice bearing the MCa mammary carcinoma, reducing their weight and number, with only mild effects on the primary tumour being observed. As far as we are aware the only other drug that displays this type of selectivity is NAMI-A [22].

Several experiments to delineate the biological mechanism by which RAPTA-T selectively reduces the number and weight of metastatic tumours have been undertaken on a series of breast (cancer) cells. It was found that RAPTA-T inhibits some steps of the metastatic process including detachment of cells from the primary tumour, migration, and the re-adhesion of cells to a new growth substrate. These effects were all increased with cells grown on components of the extra-cellular matrix such as collagen IV and fibronectin. It is also interesting to note that the effects of RAPTA-T were more pronounced when these experiments, used to model metastatic progression, were performed with the highly invasive MDA-MD-231 breast cancer cells compared with the non-invasive MCF-7 or the non-tumourigenic HBL-100 derived from breast tissue.

Furthermore, RAPTA-C derivatives in which the chlorides have been replaced by chelating dicarboxylate ligands that resist hydrolysis display very similar *in vitro* activity to RAPTA-C [23]. Interestingly, RAPTA-C and carboplatin induce similar effects on DNA and yet their *in vivo* activities are markedly different [24]. This disparity may be attributed to targets other than DNA being relevant to provide their therapeutic effect.

5 RAPTA Targets

While the effect on tests requiring the interaction of the tumour cells with extra cellular matrix components suggest that the interaction of RAPTA compounds with cell surface molecules may be responsible for part of their activity, the compounds also accumulate within cancer cells and interact to a significant extent with proteins in the cytoplasm. Adduct formation of RAPTA compounds with proteins has been studied using mass spectrometric methods [10, 25, 26]. In general, rapid and irreversible binding has been observed. In a recent study the reactivity of cisplatin and RAPTA-C with a mixture of proteins was probed without using any

chromatographic separation prior to analysis by electrospray ionization mass spectrometry [27]. It was found that cisplatin was moderately reactive towards the proteins without any discrimination/selectivity, whereas RAPTA-C was considerably more reactive and could also bind preferentially to specific proteins. The ability of RAPTA-C to bind selectively to proteins has important implications for its mode of action and presumably also to possible toxic side-effects.

In the search for possible targets the inhibition activity of a range of RAPTA compounds was studied for two specific enzymes – namely thioredoxin reductases (TrxR) and cathepsin B (cat B), important targets in cancer chemotherapy [28]. TrxR was chosen as it is highly relevant with respect to gold-based drugs (see below) and cat B is implicated in various stages of metastasis which correlate to the observed *in vitro* effects of RAPTA-T [29]. It turned out that while the selected compounds (Fig. 3) are not inhibitors of TrxR with the exception of carboRAPTA-C, they are active inhibitors of cat B [28].

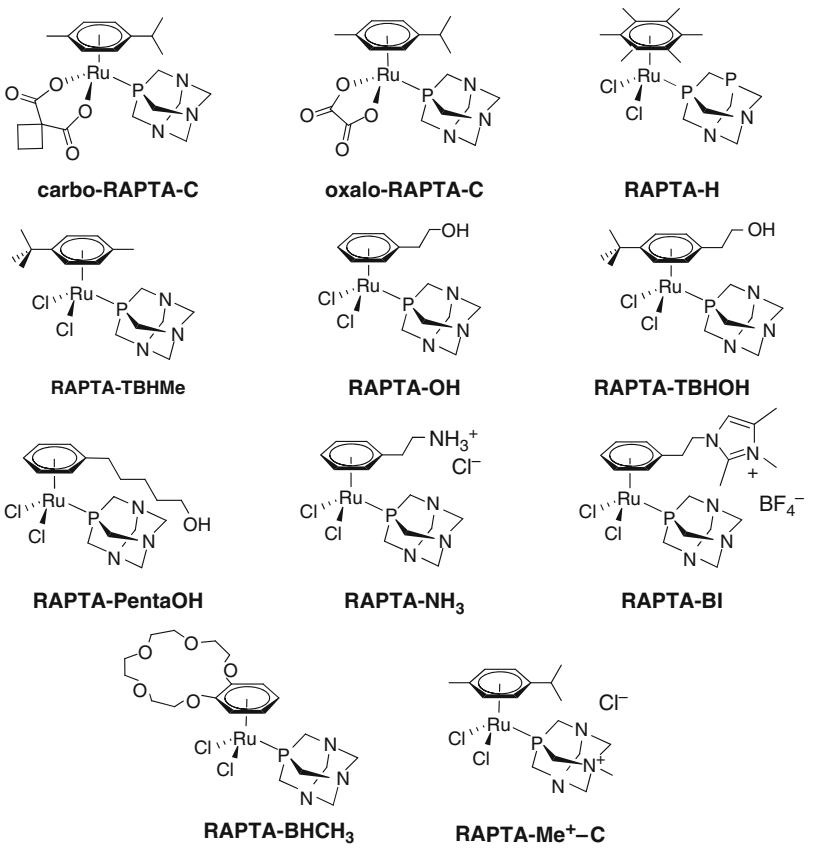

Fig. 3 Structures of RAPTA compounds screened for TrxR and cat B inhibition (see Fig. 2 for RAPTA-C and RAPTA-T)

Cat B is an abundant and ubiquitously expressed cysteine peptidase of the papain family and makes up a major fraction of lysosomal enzymes that is capable of degrading components of the extracellular matrix in various diseases [30–32]. Cat B is also a prognostic marker for several types of cancer [33], and increased expression and secretion of cat B has been shown to be involved in the migration and invasion of various tumours [34–36]. The precise role of cat B in solid tumours is not fully understood, but it has been proposed to participate, along with other cysteine cathepsins, in metastasis, angiogenesis, and tumour progression [37]. Indeed, cat B inhibitors reduce both tumour cell motility and invasiveness *in vitro* [38]. Recently, metal complexes based on rhenium, gold and palladium were shown to be effective inhibitors of cat B [39–44].

The IC_{50} values for the RAPTA compounds screened for cat B inhibition are in the low μM range, indicating that RAPTA compounds might be effective inhibitors of cat B at physiologically attainable concentrations. It is noteworthy that this data matches *in vitro* data well, which shows that RAPTA-T, for example, prevents detachment and migration of aggressive tumour cells.

Docking studies were undertaken to establish how RAPTA compounds might interact with the active site of the enzyme [28]. Cat B consists of a light chain (Lys^{1}–Arg^{49}) and a heavy chain (Val^{50}–Thr^{253}) [45] and has seven disulfide bridges [46]; the active site of cat B is formed by an activated Cys and by a His and an Asn residue (Fig. 4). There are two main interaction site pockets; a large hydrophobic one and a smaller one that is more accessible to the solvent and is located near the active site.

Inhibition of cat B appears to be related to coordination of the ruthenium(II) centre to the active site-cysteine residue. The docking studies also show that the nature of the arene ligand influences the binding affinity. Overall, a reasonable agreement was found between the cat B inhibitory activity of the RAPTA compounds and the computationally derived stability of the cat B-RAPTA adducts. Figure 4 shows the docking of RAPTA-pentaOH with the Ru-Cys29 interaction in the active site, and close interactions with residues Gly72 (hydrogen bonding to a nitrogen atom of the pta ligand), and His108 and His109 (hydrogen bonding to the hydroxyl group on the arene ligand). In addition, the remaining chloride ligand interacts with the backbone amide nitrogens of Cys29, in which the side chain sulphur atom coordinates to the ruthenium centre and the alkyl and aryl portions of the coordinated ligands find favourable interactions with hydrophobic sites of the protein. Also, the pta ligand sits in a pocket occupied by a conserved water molecule in the native protein, forming interactions with Gly72.

6 Ruthenium-Arene Targeted Drugs

Based on the encouraging *in vivo* antitumour properties of RAPTA compounds a number of derivatives have been prepared and studied in which the basic RAPTA framework has been modified in a rational way to overcome specific problems in

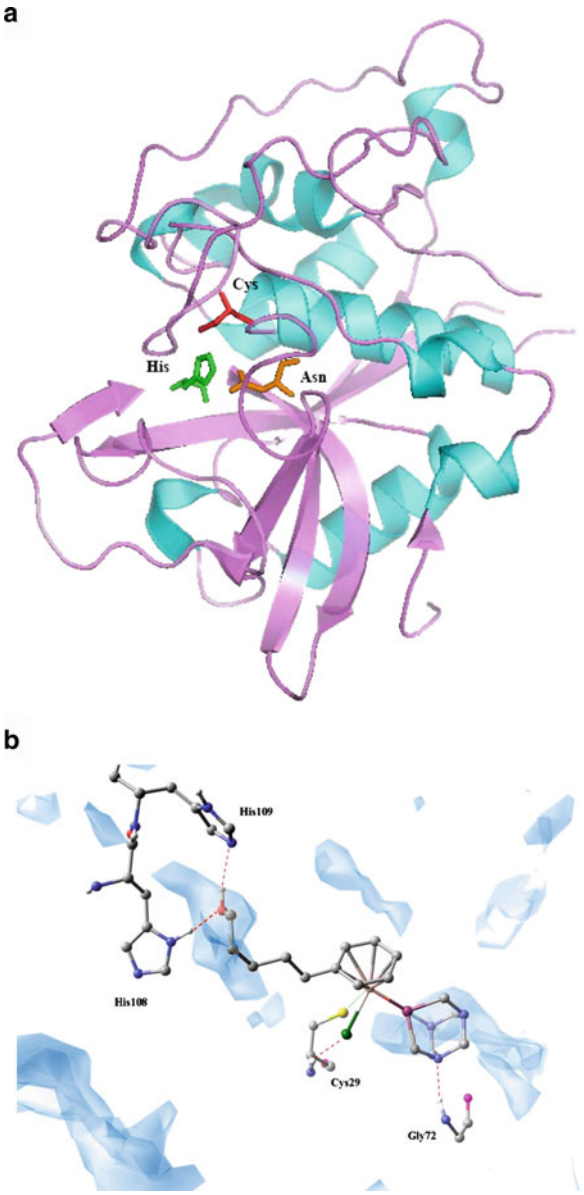

Fig. 4 (a) The structure of bovine spleen procathepsin B [47] is shown with the active site Cys, Asn and His residues. The PDB coordinates 1QDQ for bovine cath B were obtained from the Structure Database, NCBI. (b) Docking geometry of RAPTA-pentaOH to cat B illustrating the main interactions of the ligand with the residues flanking the active site (reproduced, with modifications, from ref. [28]. The contour map for the hydrophobic field calculated with the sidemap module implemented in Maestro is also shown (transparent).

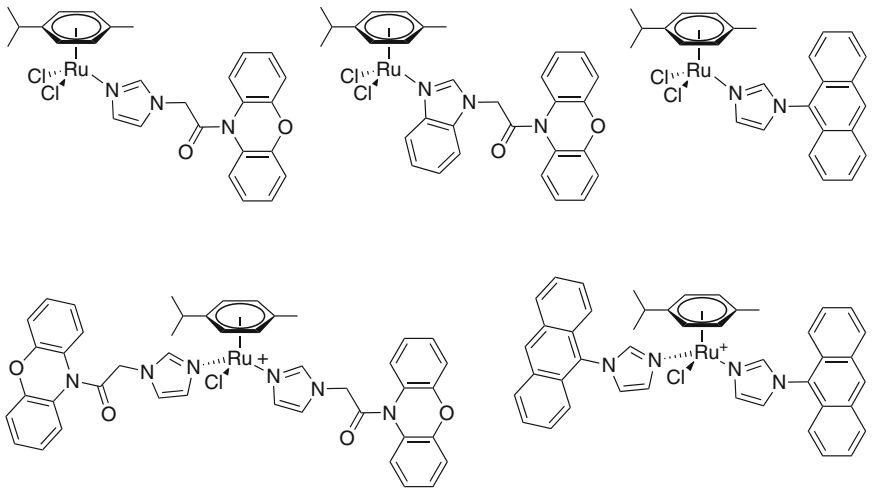

Fig. 5 Structures of ruthenium(II)-arene compounds incorporating modified Pgp reversal agents as ligands

oncology and to improve drug efficacy. Within this context, it has been shown that if ligands that endow specific biological function are incorporated into the ruthenium–arene sub-structure it is possible to produce organometallic compounds that offer advantages over existing drugs. For example, a range of phenoxazine-type inhibitors of P-glycoprotein (Pgp), one of the key proteins involved in multidrug resistance [48], have been modified with an imidazole group that was subsequently used to tether the inhibitor to a ruthenium(II)-arene (Fig. 5). The Pgp inhibition activity of the compounds correlates well with their ability to inhibit cell growth. Of the various compounds prepared, an inactive anthracene structural analogue became highly effective (to a greater extent than the known inhibitors) once coordinated to the ruthenium(II) centre. Due to the presence of the anthracene group, the complex is fluorescent and its uptake into cells was therefore studied. In comparison to the free anthracene-based ligand, the uptake of the ruthenium(II)-arene complex is accelerated and accumulation of the complex in the cell nucleus was observed. Accumulation of ruthenium in cell nuclei has already been described for the phase II clinical trial drug KP1019 [49]. Nevertheless, the fact that coordination of the Pgp inhibitor to the ruthenium(II) centre leads to a strong increase in cytotoxicity implies that the organometallic compound not only inhibits Pgp but also induces cell death via a second mechanism, and can therefore be considered as bi-functional.

RAPTA complexes designed to inhibit Glutathione-*S*-transferases (GST), a cytosolic detoxification enzyme associated with drug resistance, have been developed (Fig. 6) [50–52]. The strategy essentially involves combining an established organic inhibitor with the ruthenium(II)-arene unit. The most effective inhibitor that is active against a broad range of GSTs, particularly the P1-1 isoform that is

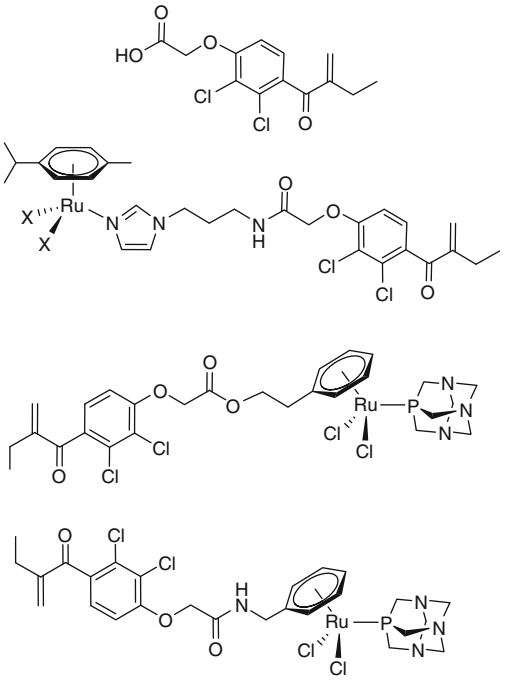

Fig. 6 Structures of ethacrynic acid and some Ru(II)-arene derivatives (X = Cl, oxalate, 1,1-cyclobutyldicarboxylate)

over-expressed in drug-resistant tumours, is ethacrynic acid. Ethacrynic acid has been tested in combination with a range of alkylating agents against drug-resistant cancers as adjuvant therapeutics.

Ethacrynic acid has been linked to the ruthenium(II)-arene frame via two different approaches. In one approach the acid is connected via an imidazole which coordinates to the ruthenium(II) centre, in place of the pta ligand in RAPTA-type compounds. In the second approach the ethacrynic acid is attached to the arene, via either an amide or ester linker, in such a way that it should be cleaved enzymatically once inside the active site of GST. The ability of the ruthenium complexes to inhibit GST P1-1 activity was comparable or better than free ethacrynic acid whereas RAPTA-C, employed as a control, exhibited no inhibitory effect on GST P1-1, even at high concentrations.

There are two major families of soluble GSTs in humans which are located in the cytosol and mitochondria. These enzymes are dimeric proteins that can be grouped into several gene-independent classes on the basis of their primary structure [53]. Their three-dimensional structures do not differ significantly [54], although the different classes display a range of functions [55]. The close similarity in the GST structure between the classes extends to the active site which contains a very similar binding site for GSH (G-site), but with differences in the hydrophobic co-substrate binding site (H-site), leading to a degree of substrate selectivity. GST P1-1 possesses two solvent accessible cysteine residues that affect catalytic activity when

modified: Cys47 located near the G-site is critical for maintaining the conformation and stability of the G-site and Cys101 located at the dimer interface, which can form a disulfide bridge with Cys47 [56]. Enzyme kinetics and electrospray mass spectrometry experiments using GST P1-1 and its cysteine-modifed mutants revealed that the organometallic complexes are effective enzyme inhibitors, rapidly inactivating the enzyme via coordination to Cys47, and to a lesser extent Cys101. Moreover, the compounds are highly effective against the GST P1-1 positive A2780 and A2780cisR ovarian carcinoma cell lines – amongst the most active ruthenium complexes known.

In a related strategy to those described above, but one not connected with drug resistance, paullone-type cyclin dependent kinase (CDK) inhibitors based on the 7,12-dihydroindolo[3,2-*d*]benzazepin-6(5*H*)-one fragment [57] were modified to act as bidentate ligands. These bidentate ligands were coordinated to ruthenium(II)-arene and osmium(II)-arene fragments (Fig. 7) which resulted in compounds with nearly identical IC_{50} values in the low micromolar and high nanomolar range. While the compounds have not been screened for CDK inhibition, they exhibit very different reactivity to DNA model compounds, which suggests a DNA-independent mode of action [58].

In addition to targeting enzymes within cancer cells, certain functionalities can be used to transport drugs to tumours following their administration, by exploiting delivery pathways within the body. In this context it is known that porphyrin derivatives concentrate in cancer cells [59, 60], and moreover, they are also efficient photosensitizing agents [61]. Recently, 5,10,15,20-tetra(4-pyridyl)porphyrin (TPP) ruthenium(II)-arene and osmium(II)-arene derivatives and iridium and rhodium analogues with the pentamethylcyclopentadienyl ligand were explored as anticancer agents for photodynamic therapy (Fig. 8) [62]. It was found that all of the compounds, with the exception of the rhodium complex, were taken up into cancer cells. Moreover, these complexes were moderately cytotoxic. Notably, the ruthenium complexes presented good phototoxic activities at 10 μM, being considerably more active on irradiation with light. Localization of one of the ruthenium

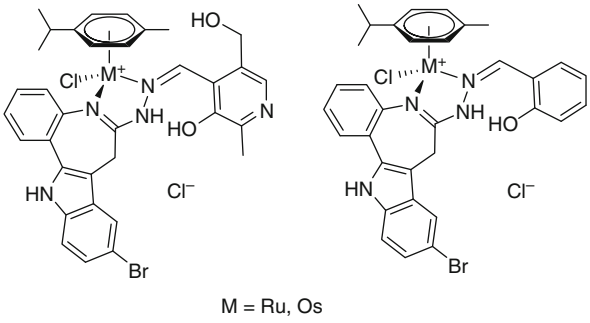

Fig. 7 Examples of ruthenium(II)-arene and osmium(II)-arene paullone complexes with high *in vitro* anticancer activity

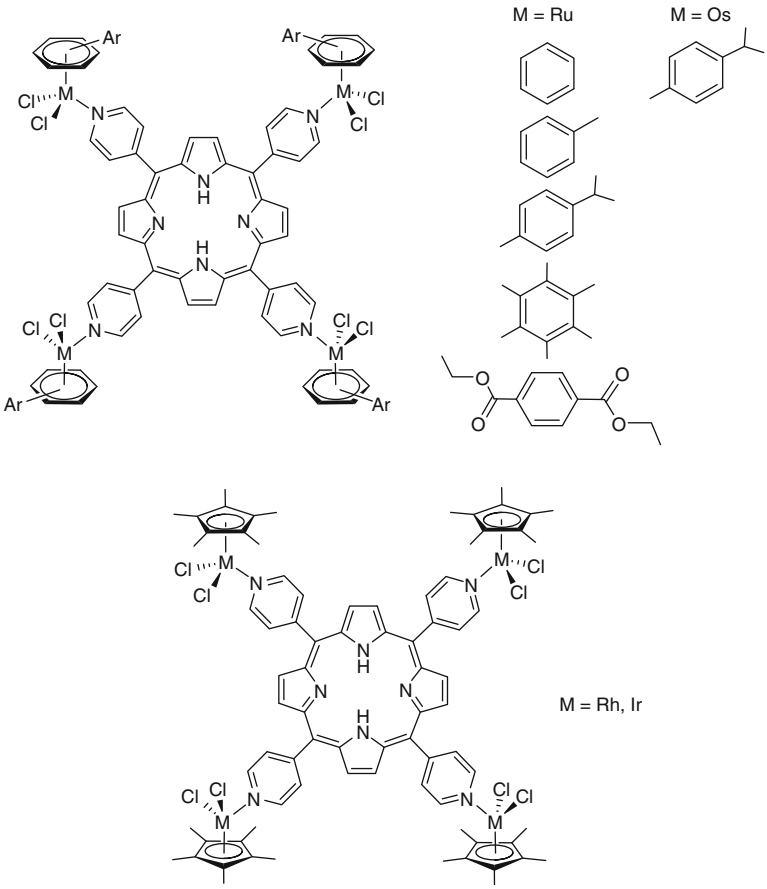

Fig. 8 Structures of organometallic porphyrin derivatives evaluated as photosensitisers for photodynamic therapy

compounds in cells using fluorescence microscopy showed that it did not accumulate in the nucleus or mitochondria implying a non-DNA mode of action.

Certain sugars accumulate in tumour cells and organometallic ruthenium (II)-arene complexes with phosphite-carbohydrate ligands (Fig. 9) have been shown to be moderately cytotoxic against different human tumour cell lines. Similar activity, with regard to the IC_{50} value, was observed in A2780 cells and a cisplatin-resistant phenotype (A2780cisR), suggesting a different mode of action to cisplatin. In addition, only low activity was found against a non-tumoural endothelial cell line, indicating selectivity for cancer cells [63].

Dinuclear Ru-arene, complexes based on maltol-derived ligands with a varying spacer length (Fig. 9) were shown to exhibit high *in vitro* anticancer activity [64, 65], whereas the mononuclear analogue was inactive [66]. In another approach, two organometallic Ru-arene moieties were linked via a ferrocene unit (Fig. 10)

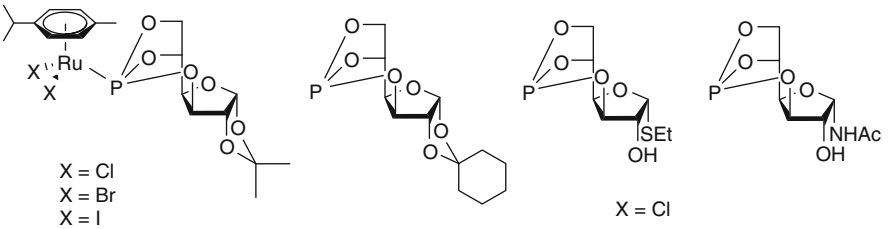

Fig. 9 Examples of P-sugar-ruthenium(II)-arene complexes

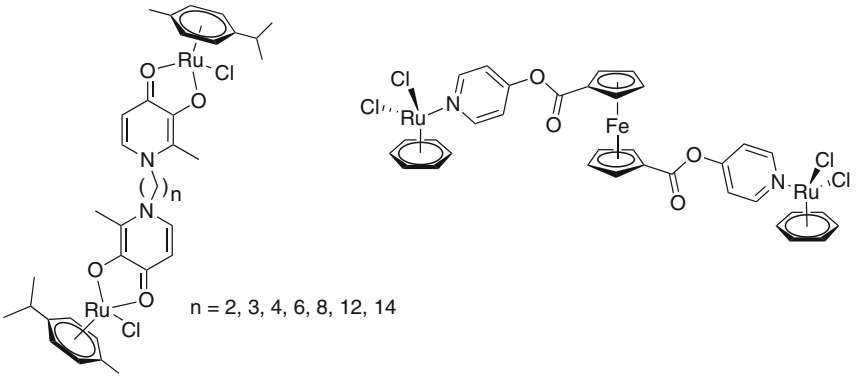

Fig. 10 Examples of dinuclear Ru(II)-arene complexes

increasing the cytotoxicity against the A2780 and A2780cisR human ovarian carcinoma cell lines relative to their monoruthenium analogues. The altered redox potential of the ferrocene unit in the diruthenium complexes may contribute to the increased cytotoxicity. Similar activity was obtained in A2780 and A2780cisR cells which indicate a mechanism of action different to cisplatin, although the actual target has not been identified [67].

7 Organogold Compounds

Inspired by the discovery of the anticancer properties of cisplatin, square planar d^8 Au(III) complexes, that are isoelectronic and isostructural with Pt(II) complexes, were soon evaluated for their anticancer properties. However, compared to platinum(II) compounds, the gold(III) analogues were found to be kinetically more labile than the corresponding platinum(II) compounds, light-sensitive and easily reduced to metallic gold. As a result of these problems, and also the observation of systemic toxicity during the first animal studies, gold(III) compounds were abandoned. However, in the last 15 years renewed interest in gold(III)-based anticancer therapies has evolved as gold(III) complexes exhibiting improved stabilities, lower toxicities and favourable *in vitro* pharmacological properties were discovered [68].

Notably, a series of organogold(III) DAMP compounds (DAMP = 2-[[(dimethylamino)methyl]phenyl]) (Fig. 11) exhibited encouraging anticancer activity *in vitro* [69,70]. Moreover, these studies also demonstrated that the organometallic compounds possess moderate pharmacological activity on animal models.

A series of gold(III) complexes with bipyridyl ligands have been shown to be cytotoxic [71]. The compounds [Au(bipy^{c}-H)(OH)]PF_6 (Aubipy^{c}) (bipy^{c} = 6-(1,1-dimethylbenzyl)-2,2′-bipyridine) and [Au(bipy)$(OH)_2$]PF_6 (Aubipy) (bipy = bipyridine) (Fig. 12) were particularly active [72], the former being an organogold(III) complex [73].

Other examples of organogold complexes evaluated *in vitro* include [Au(bipy^{dmb}-H)(2,6-xylidine-H)]PF_6 (Auxyl) [(bipy^{dmb} = 6-(1,1-dimethylbenzyl)-2,2′-bipyridine)] and [Au(py^{dmb}-H)$(AcO)_2$] (Aupy) (py^{dmb} = 2-(1,1-dimethylbenzyl)-pyridine) (Fig. 11) [74].

Aubipy^{c} is moderately water soluble and gives rise to intense LMCT bands in the range 300–370 nm, diagnostic of gold in the +3 oxidation state, and thus has been used to probe reactions with potential biomolecular targets. Notably, Aubipy^{c} does not react with biological reducing agents, such as ascorbate, whereas Aubipy is readily reduced [75].

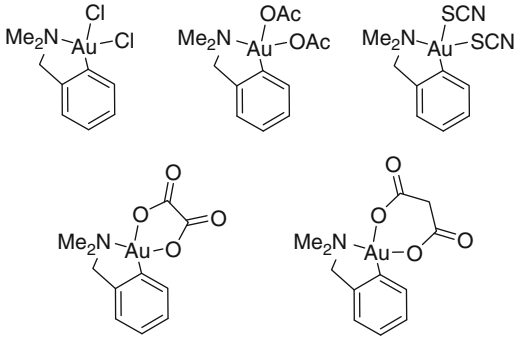

Fig. 11 Structures of organogold(III)-DAMP compounds (DAMP = 2-[[(dimethylamino)methyl]phenyl]).

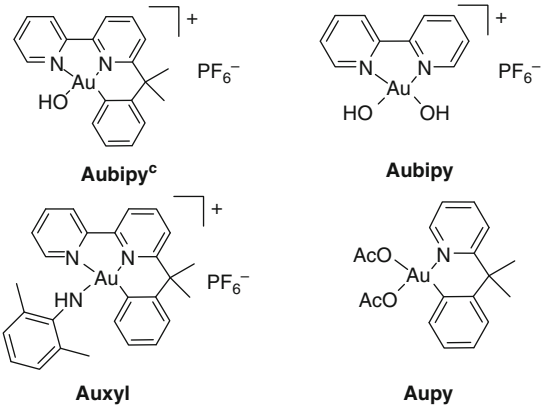

Fig. 12 Structures of [Au(bipy^{c}-H)(OH)]PF_6 (Aubipy^{c}), [Au(bipy)$(OH)_2$]PF_6 (Aubipy), [Au(bipy^{dmb}-H)(2,6-xylidine-H)]PF_6 (Auxyl) and [Au(py^{dmb}-H)$(AcO)_2$] (Aupy) [(bipy^{dmb} = 6-(1,1-dimethylbenzyl)-2,2-bipyridine); py^{dmb} = 2-(1,1-dimethylbenzyl)-pyridine)]

The *in vitro* cytotoxicity of the bipyridyl gold(III) complexes was established on the human ovarian carcinoma cell line A2780 and the cisplatin-resistant variant. The IC_{50} values are in the low micromolar range and Aubipy^c is the most active with a two-fold higher activity than cisplatin in the cisplatin-resistant cell line. Activities in other cisplatin resistant cell lines suggest that the biomolecular mechanism of these gold(III) complexes is not the same as those of cisplatin. Indeed, the interactions of these complexes with nucleic acids were found to be weak and that stronger adducts are formed with proteins [16].

8 Proposed Targets

The possible mechanism of action of organogold complexes remains largely unknown; however, some potential targets have been identified. The few gold(III) compounds on which advanced pharmacological testing was performed suggest a mechanism that is distinct from classical platinum(II) compounds. Even though some evidence for direct DNA damage has been obtained in a few cases, effects on nucleic acids and on the cell cycle appear to be very modest, so that it is unlikely that DNA is the primary target [76]. It is more likely that alternative biochemical mechanisms are based on the modification of relevant proteins. Gold(I) and gold(III) compounds are known to target, rather strongly and selectively, thiol and imidazole groups of proteins (and also selenol groups) [77], and the formation of adducts with these groups may represent the molecular basis for their biological actions.

In this context, a recent hypothesis is that gold compounds may exert their relevant cytotoxic effects through direct antimitochondrial action; a hypothesis originally developed for classical antiarthritic (but also cytotoxic) gold(I) compounds such as [(2,3,4,6-tetra-*O*-acetyl-1-(thio-κ*S*)-β-d-glucopyranosato)(triethylphosphine)gold(I)] (auranofin) and aurothiomalate [78]. Evidence suggests that inhibition of TrxR, a selenoenzyme critically involved in the regulation of the intracellular redox state and of mitochondrial functions [79], could be highly relevant. Inhibition of TrxR results in the opening of the mitochondrial pore leading to the release of cytochrome c and ultimately inducing apoptosis.

TrxRs are homodimeric flavoproteins [80] that catalyze the NADPH-dependent reduction of thioredoxin (Trx), a ubiquitous 12 kDa protein that is the major protein disulfide reductase in cells [81], and belongs to the pyridine nucleotide-disulfide oxidoreductase family [82]. Each monomer includes an FAD prosthetic group, a NADPH binding site and an active site containing a redox-active selenol group. Electrons are transferred from NADPH via FAD to the active-site selenol of TrxR, which then reduces the substrate Trx [83]. The crystal structure of TrxR is shown in Fig. 13 [84].

Structural studies suggest that when the active site selenocysteine group is in the reduced form TrxR displays a high reactivity toward metal ions [85, 86]. It is possible that gold(III) compounds such as auranofin exert their cytotoxic effects by causing direct mitochondrial damage through selective modification of the selenol

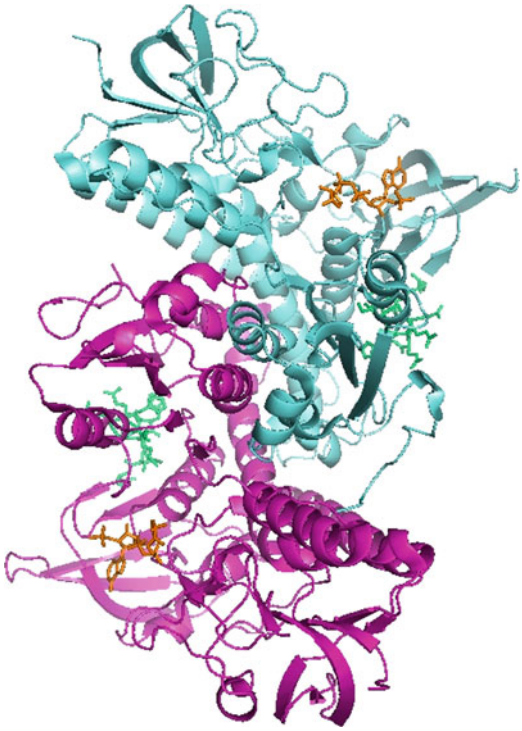

Fig. 13 Ribbon representation of the dimer of human TrxR (Sec→Cys) (PDB entry 2J3N). Bound FAD and $NADP^+$ are shown as ball-and-stick models

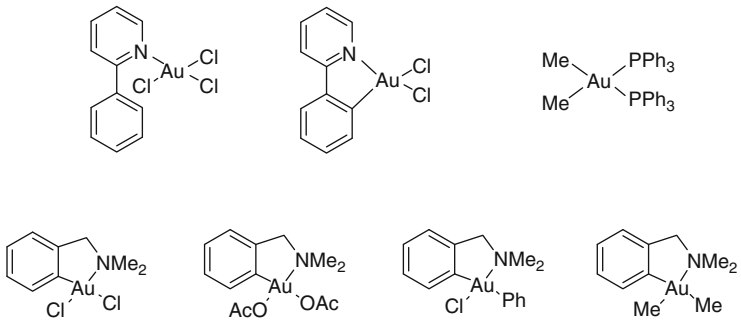

Fig. 14 Structures of the organogold(III) compounds that inhibit human TrxR1

active site in TrxR [87–91]. The organometallic compounds Aubipy^c and Auxyl inhibit mitochondrial TrxR2 and disrupt mitochondrial function [87, 88], triggering mitochondrial swelling, although to a lesser extent than auranofin [92]. Other gold (III) compounds (Fig. 14) have been shown to inhibit human TrxR [93]. The compound with two gold-carbon bonds, Au(DAMP)(phenyl)Cl, is the most potent inhibitor of TrxR ($IC_{50} = 2.2$ nmol/l) although it did not show any anticancer activity against MCF-7 breast cancer and HT-29 colon cancer xenografts.

9 Protein-Mediated Tumour Targeting

The role of potential protein interactions in blood serum with respect to drug delivery, storage and toxicity should not be overlooked. The low toxicity of KP1019 is attributed, at least in part, to transferrin-mediated drug transport [94], with KP1019 binding strongly to transferrin in the iron-binding pockets [4, 49, 95–100] . Indeed, its imidazolium analogue, which binds more weakly to transferrin, is less cytotoxic *in vitro* [101].

The coordination of indazolium ligands, as present in KP1019, appears beneficial for transferrin binding, compared to analogous complexes with imidazole or triazole ligands [102], as these ligands are better accommodated into the iron binding sites [97].

Human serum albumin (HSA) adducts might also contribute to the cytotoxic effect of metallodrugs by acting as potential storage vectors. However, the exact mode of action, especially intracellular pathways, is not well understood. Notably, HSA is known to accumulate in tumours and has been exploited as a carrier for various organic anticancer drugs such as chlorambucil, doxorubicin and paclitaxel [103]. Chlorambucil and paclitaxel conjugated to HSA exhibit comparable cytotoxicity to the parent drugs *in vitro*, but are less toxic *in vivo*. In addition, a doxorubicin prodrug that exploits endogenous serum albumin as a drug carrier also showed superior antitumour activity on murine renal cell carcinoma *in vivo* [104, 105] Thus, many metal drugs bind to HSA in blood serum and could be targeted to cancer cells. Indeed, a RAPTA-type compound attached to HSA via a pH switchable linker (Fig. 15) proved to be far more cytotoxic than the free RAPTA complex [50, 51].

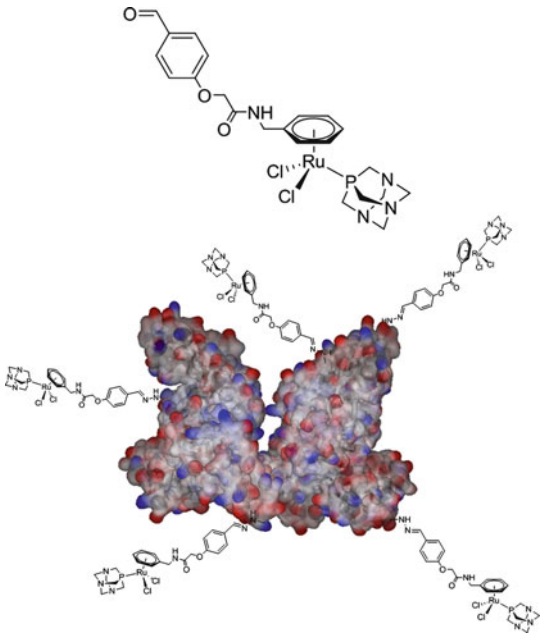

Fig. 15 (*top*) The functionalised RAPTA complex that is readily tethered to HSA, (*bottom*) a representation of the HSA-RAPTA drug [50, 51]

10 Concluding Remarks

The last decade has witnessed many promising developments in the field of medicinal chemistry, with organometallic compounds displaying anticancer and antimetastasis activity, and finding applications as antibiotics, antiviral and anti-parasitic agents [106–108].

Although the unique properties of organometallic compounds that make them so promising in a range of medicinal applications are not easily quantified, it appears that the increased stability that many exhibit over conventional coordination compounds is advantageous. Organometallic compounds also offer advantages over organic molecules, with, for example, increased drug uptake being a key factor, and the potential for the comparatively facile design of multi-functional compounds. Thus, it appears that some organometallic compounds incorporate the best features of inorganic and organic compounds, although much remains to be learnt in this respect.

Evidence is mounting to suggest that non-DNA based targets are important for the mechanism of a number of organometallic anticancer drugs, although partial DNA damage cannot be excluded and is probably partly important. A drug that is active against several different targets is less prone to drug resistance which is highly advantageous. Nevertheless, to fully take advantage of non-DNA targets, increasing efforts must be devoted to rational drug design based on a detailed understanding of the nature of the active sites of relevant drug targets. By steering away from DNA as the sole target it is likely that many new and exciting developments lie ahead in the rapidly developing field of organometallic medicinal chemistry.

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Ferrocene Functionalized Endocrine Modulators as Anticancer Agents

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Abstract We present here some of our studies on the synthesis and behaviour of ferrocenyl selective endocrine receptor modulators against cancer cells, particularly breast and prostate cancers. The proliferative/anti-proliferative effects of compounds based on steroidal and non-steroidal endocrine modulators have been extensively explored in vitro. Structure–activity relationship studies of such molecules, particularly the hydroxyferrocifens and ferrocene phenols, have shown the effect of (1) the presence and the length of the *N*,*N*-dimethylamino side chain, (2) the presence and position of the phenol group, (3) the role of the ferrocenyl moiety, (4) that of conjugation, (5) phenyl functionalisation and (6) the placement of the phenyl group. Compounds possessing a ferrocene moiety linked to a *p*-phenol by a conjugated π-system are among the most potent of the series, with IC_{50} values ranging from 0.090 to 0.6 μM on hormone independent breast cancer cells. Based on the SAR data and electrochemical studies, we have proposed an original mechanism to explain the unusual behaviour of these bioorganometallic species and coin the term "kronatropic" to qualify this effect, involving ROS production and bio-oxidation. In addition, the importance of formulation is underlined. We also discuss the behaviour of ferrocenyl androgens and anti-androgens for possible use against prostate cancers. In sum, ferrocene has proven to be a fascinating substituent due to its vast potential for oncology.

Keywords Anti-androgen · Anti-oestrogen/Anti-oestrogenic · Breast cancer · Cytotoxic/Cytotoxicity · Electrochemistry/Electrochemical · Oestradiol · Ferricenium · Ferrocene · Ferrocenophane · Hydroxyferrocifens · Hydroxytamoxifen (OH-Tam) · Oestrogen receptor (ER) · Oestrogen/Oestrogenic · Phenol · Prostate cancer · Quinone · Raloxifen · RBA · ROS · Steroid · Tamoxifen · Testosterone · Vectorisation

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Contents

1 Ferrocene and Medicinal Chemistry

The simultaneous discovery of the structure of ferrocene by Wilkinson [1] and Fischer [2] in 1952 was one of the most important advances in the history of chemistry and led to the birth of modern organometallic complexes. Ferrocene remains exceptional among metal-containing molecules: it has an external surface that resembles that of an aromatic nucleus, can be easily functionalized, is stable in non-oxidising environments and is therefore a suitable substituent in medicinal chemistry. It is lipophilic and compact, and thus does not greatly modify the pharmacological properties of molecules where it replaces a phenyl ring, for example. It is not particularly toxic, with oral and interperitoneal LD_{50} values of 1,320 and 500 mg kg^{-1} for rat and 832 and 335 mg kg^{-1} for mouse. Dogs fed daily with 300 mg kg^{-1} of ferrocene for 6 months were diagnosed with massive iron overload, but no deaths were observed and the dogs recovered afterwards [3]. Due to its lipophilicity, ferrocene has been used as an alternative to the use of iron salts for administration of Fe(II); "ferrocerone", the sodium salt of *o*-carboxylbenzoyl ferrocene was patented by Nesmeyanov in 1971 and used to treat iron-deficiency anaemia [4]. This is the only ferrocenyl derivative to reach clinical use so far.

Ferrocene has been used to replace functional groups, particularly phenyl rings, in existing drugs to give new compounds, but with mixed results. Some of these, such as the ferrocenyl derivatives of amphetamine and phenytoin, Chart 1, completely lacked the activity of the parent compounds [5]. Other ferrocenyl compounds, however, showed an improved therapeutic spectrum, such as the

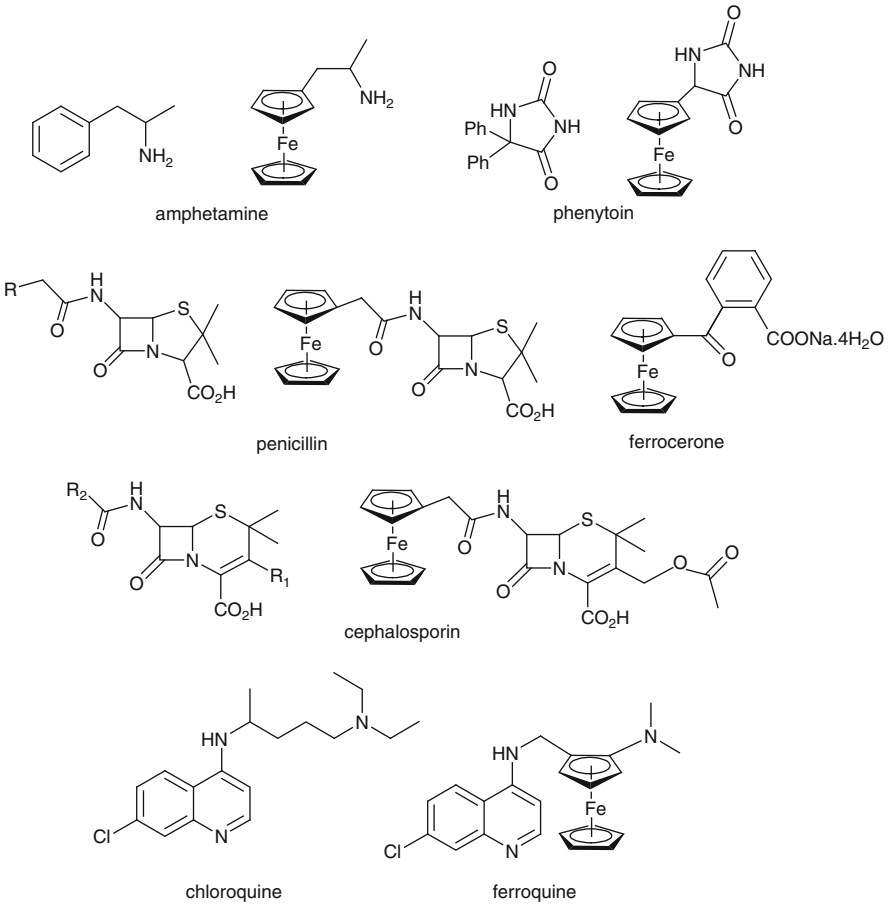


Chart 1

ferrocenyl penicillin and cephalosporin derivatives, which showed activity against drug-resistant bacteria and low toxicity in vivo [6, 7]. Brocard et al. introduced ferroquine, the ferrocenyl analogue of chloroquine, a standard anti-malarial drug, in 1997 [8]. Ferroquine is not only active against chloroquine-sensitive *Plasmodium* parasites, but also against chloroquine-resistant strains, and is now in phase II clinical development by Sanofi-Aventis [180]. Anti-malarial activity has also been displayed by some ferrocene chalcones [9], while ferrocene peptide conjugates have shown anti-bacterial activity [10, 11].

The anti-cancer properties of ferrocene-containing molecules first gained attention in the 1970s [12–14] and this field greatly benefited from the discovery of the anti-proliferative properties of simple ferricinium salts on Ehrlich ascite tumours in

1984 [15, 16]. This effect is only observed at high concentrations (optimum dose of around 250 mg kg^{-1} for implanted mice) and ferrocene itself showed no effect. This was followed by the proposal that ferrocenyl compounds could be oxidised in vivo, and that both ferricenium- and ferrocene-containing compounds could give rise to cytotoxic effects [17]. To this end ferrocene has been incorporated into water-soluble polymers [18–21], tethered to a DNA intercalator, [22] phosphino compounds, [23–26] peptides, [27] vitamin B1 [28] and other biomolecules [9, 29–31]. Diferrocene compounds [32] and ferrocene-bearing transition metal ligands [33–35] and a variety of other small ferrocenyl molecules [36–40] have also been investigated for anti-cancer activity. Cytotoxic pathways involving free radical production and DNA damage have been suggested for the activity of ferricenium compounds [41–43]. Three reviews and two book chapters concerning the medicinal chemistry of ferrocene have been recently published [44–48].

2 Breast and Prostate Cancer

Some forms of cancer are associated with natural hormones and the over-expression of hormone receptors. These include hormone-dependent breast and prostate cancers [49], which account for approximately two-thirds of breast and prostate cancer cases. Their proliferation is promoted by the naturally occurring hormones oestradiol (E_2) and testosterone (as the active metabolite dihydrotestosterone, DHT), respectively, although synthetic xenobiotics such as bisphenol A have also been implicated. For patients diagnosed with such cancers, an anti-oestrogen [50] or anti-androgen [51, 52] treatment is prescribed, commonly the non-steroidal selective estrogen receptor modulator (SERM) tamoxifen [53] for ER+ breast cancer, and nilutamide [54, 55], flutamide [56–58] or bicalutamide [59] for AR+ prostate cancer. These agents are susceptible to acquired resistance, and a favourable response to this treatment is obtained in only one-third of patients over the long term (5+ years). Tamoxifen is furthermore associated with side effects such as the increased risk of endometrial uterine cancer and blood clotting in the lungs. So while the prognosis of hormone-dependent breast and prostate cancers is still better than that of hormone-independent cancers, the curative end-point for the former is still reached in only a minority of cases.

3 Hydroxyferrocifens

In 1996, Jaouen et al. first coupled ferrocene to the active metabolite of tamoxifen, OH-Tam, Chart 2 [60, 61]. The resulting "hydroxyferrocifens" (with various lengths of the *N*,*N*-dimethylamino chain, $n = 2$–5, 8) were designed to combine the anti-oestrogenic properties of tamoxifen with potentially cytotoxic properties of ferrocene [62–64, 177]. In replacing the phenyl group of OH-Tam by the aromatic

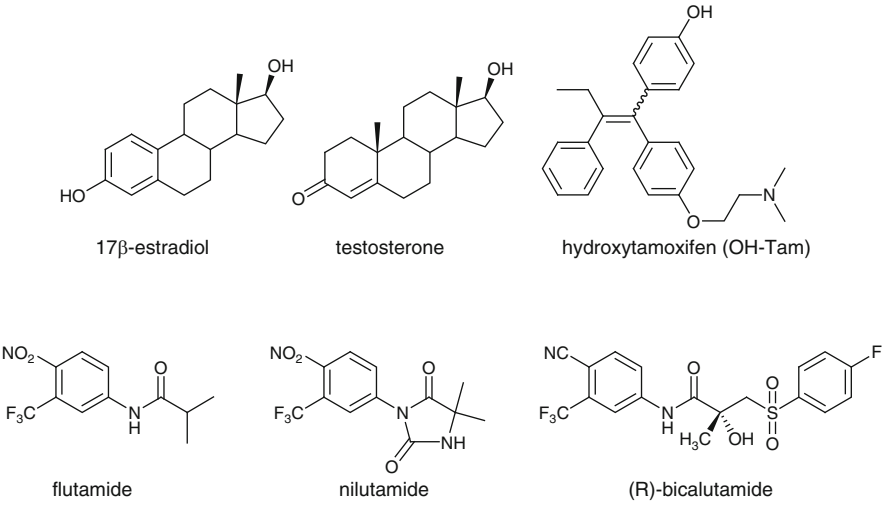
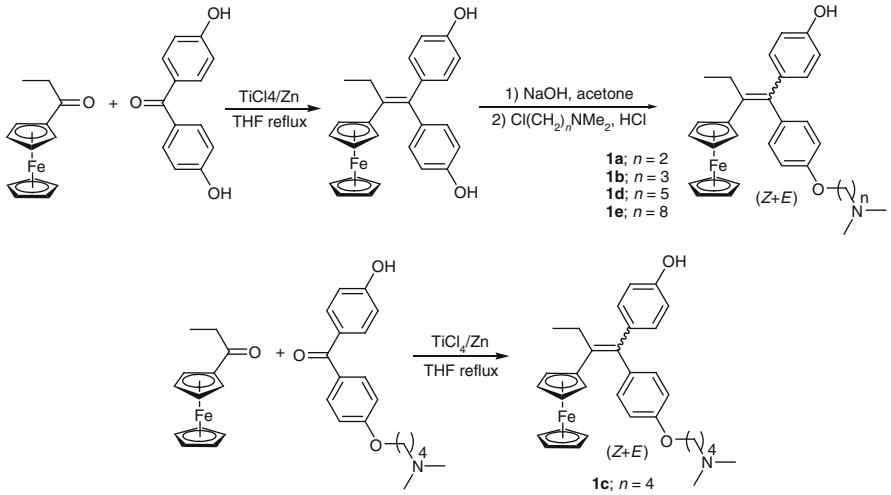


Chart 2

ferrocene unit, it was hoped that the new molecule would retain sufficient recognition by the oestrogen receptor to preserve the anti-oestrogenic effects of tamoxifen, while simultaneously targeting the ferrocene moiety to the receptor, and thus the DNA. Although the first synthetic pathway reported was multi-step and suffered from a low overall yield, [60] a more efficient path via a McMurry cross-coupling reaction was quickly implemented, Scheme 1 [61]. In some cases, the *Z* and *E* isomers could be separated by fractional crystallisation ($n = 2$) or plate chromatography ($n = 4$).

Isomerisation between *E* and *Z* isomers is well known for such substituted alkenes as diethylstilbestrol [65] and hydroxytamoxifen [66]. Of the solvents studied, the isomerisation of hydroxyferrocifen **1b** was most rapid in chloroform, with a complete isomerisation of the *Z* isomer to a 50:50 mixture occurring in 1 h. No isomerisation was observed after 1 h in acetone, DMSO or benzene, and the extent of isomerisation over longer periods correlates with the acidity of the solvent [64]. Therefore, complete isomerisation of these molecules in the EtOH:H_2O or DMSO:H_2O (0.5%:95.5%) binary mixtures used in our 5 d cellular proliferation study protocol is to be expected, and such studies are therefore usually carried out on mixtures of isomers. In the few cases where individual isomers were introduced, no difference in activity could be ascertained, evidently due to rapid isomerisation when protic phenyl substituents were present [67]. However, under the conditions of the relative binding affinity (RBA) competition studies with ERα and ERβ (4°C, 3 h incubation), the *Z* isomer was clearly better recognised than its *E* counterpart, as previously observed for some triphenylethylenes [68–70]. The hydroxyferrocifens showed less affinity for ERα than OH-Tam (38.5%), but the values are nonetheless satisfactory, ranging between 2.3 and 14.6%, and decrease with increasing chain length. The hydroxyl group is essential for strong receptor binding, with RBA values for the non-hydroxylated ferrocifens with $n = 2$ of 0.9 (*Z*) and 0.014% (*E*) [64].



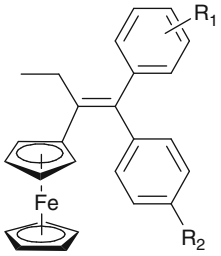
Scheme 1 Synthesis of hydroxyferrocifens

On MCF-7 breast cancer cells, those having a significant concentration of ERα, and thus classed as a hormone-dependent cell line [71, 72], the anti-proliferative effects of the hydroxyferrocifens are primarily anti-oestrogenic. At a concentration of 0.1 μM, the effects observed with **1a–c** are similar to that of OH-Tam. When the chain was lengthened, as for **1d** and **1e**, the anti-proliferative effect was weakened, and ferrocene alone had no effect. Molecular modelling of **1b** ($n = 3$), the most potent of the series, shows that the molecule can be accommodated by the binding site of ERα in its antagonist configuration. The interaction between Asp 351 and the nitrogen of the amino side-chain, important for the anti-proliferative activity of OH-Tam, provides the correct positioning of the organometallic molecule. This interaction supports the observation that an anti-oestrogenic effect comparable to that of OH-Tam is retained in the hydroxyferrocifen series.

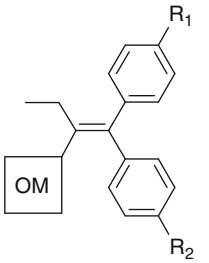
Interestingly, the antiproliferative effects of the hydroxyferrocifens **1b** ($n = 3$) and **1d** ($n = 5$) are definitely superior to OH-Tam at 1 μM; at this concentration, the contribution of a non-receptor-mediated and a dose-dependent cytotoxic component, which is not reversed upon the addition of oestradiol, begins to appear [64]. On MDA-MB-231 cells, possessing undetectable amounts of ERα and low amounts of ERβ, and thus classified as hormone-independent, these compounds have IC_{50} values as low as 0.5 μM (Table 1), while OH-Tam was completely inactive on these cells. Consequently, two kinds of behaviour can be observed: one which is similar to the anti-oestrogenic role of OH-Tam on the ER, and a cytotoxic effect that involves the ferrocene moiety.

Whereas neither ferrocene nor OH-Tam had any effect on the ER-negative cells at 1 μM, the combination of the two motifs yielded highly toxic molecules. With the notable exception of a few ruthenium complexes [73, 74], the anti-cancer properties

Table 1 Collected biological data for compounds **1**, **3**–**37**



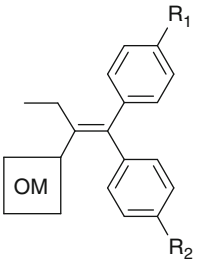
Compound[a]	R1	R2	RBA[b]		Cell viability at 1 μM (%)[c] or IC_{50}	
			ERα	ERβ	MCF-7	MDA-MB-231
1a[(148)]	*p*-OH	$O(CH_2)_2N(CH_3)_2$	14.6	10	29[d]	48
1b[(148)]	*p*-OH	$O(CH_2)_3N(CH_3)_2$	11.5	12	14	0.5 μM
1c[(146)]	*p*-OH	$O(CH_2)_4N(CH_3)_2$	12	–	37[d]	–
1d[(148)]	*p*-OH	$O(CH_2)_5N(CH_3)_2$	5	6	19	20
1e[(148)]	*p*-OH	$O(CH_2)_8N(CH_3)_2$	2.3	–	47	85
4[(50)]	*p*-OH	H	4.6	11	79	1.13 μM
5[(155)]	*p*-OH	OH	9.6	16.3	34	0.44 μM
7[(50)]	H	H	0.9	0.28	172	93
8[(50)]	*m*-OH	H	3.6	0.53	158	2.7 μM
9[(50)]	*m*-OH	OH	5.4	2.4	105	1.03 μM
(*Z*)-**21**[(170)]	*p*-Cl	H	0.29	0.36	152	83*
(*Z*)-**22**[(170)]	*p*-Br	H	0.26	0.55	153	86*
(*E*)-**23**[(170)]	*p*-CF_3	H	0.05	0.04	127	97*
(*Z*)-**24**[(170)]	*p*-CN	H	0.41	0.31	133	11 μM
(*E*)-**24**[(170)]			0.20	0.03	148	60 μM
25[(109)]	*p*-NH_2	H	2.8	1.08	180	0.8 μM
(*Z*)-**26**[(109)]	*p*-$NHC(O)CH_3$	H	3.5	0.18	112	0.65 μM
27[(48)]	*p*-O–$C(O)CH_3$	O–$C(O)CH_3$	6.5	0.8	0.8 μM	0.64 μM
28[(48)]	*p*-O–CH_3	O–CH_3	0.2	0.26	151	101
29[(48)]	*p*-S–$C(O)CH_3$	S–$C(O)CH_3$	0.27	0.03	187	99
30[(48)]	*p*-S–CH_3	S–CH_3	0.04	0.037	170	99



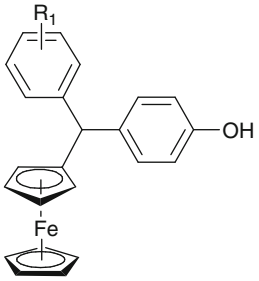
Compound[a]	OM	R1	R2	RBA[b]		Cell viability at 1 μM (%)[c] or IC_{50}	
				ERα	ERβ	MCF-7	MDA-MB-231
12[(142)]	Cp_2TiCl_2	H	$O(CH_2)_3N(CH_3)_2HCl$	–	–	202	–
13a[(149)]	$CpRe(CO)_3$	OH	$O(CH_2)_2N(CH_3)_2$	6.4	9	–	–

(*continued*)

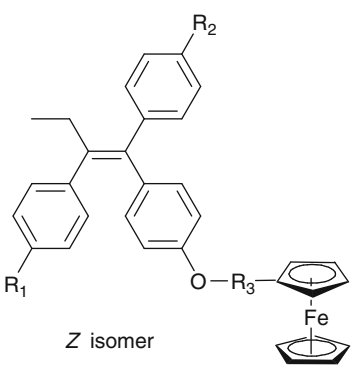
Table 1 (continued)



Compound[a]	OM	R1	R2	RBA[b]		Cell viability at 1 μM (%)[c] or IC_{50}	
				ERα	ERβ	MCF-7	MDA-MB-231
(*Z*)-**13b**[149]	$CpRe(CO)_3$	OH	$O(CH_2)_3N(CH_3)_2$	12.3	16.8	56	91
(*E*)-**13b**[149]				10.6	10.4	61	91
13c [62,149]	$CpRe(CO)_3$	OH	$O(CH_2)_4N(CH_3)_2$	8.5	6	37	–
(*Z*)-**13d**[149]	$CpRe(CO)_3$	OH	$O(CH_2)_5N(CH_3)_2$	1.14	12	59	86
(*E*)-**13d**[149]				1.13	6.7	65	88
13e[149]	$CpRe(CO)_3$	OH	$O(CH_2)_8N(CH_3)_2$	1	1.6	–	–
14a[108]	Cp_2Ru	OH	$O(CH_2)_2N(CH_3)_2$	85	19	84[c]	99
14b[108]	Cp_2Ru	OH	$O(CH_2)_3N(CH_3)_2$	53	13	66[c]	101
14c[108]	Cp_2Ru	OH	$O(CH_2)_4N(CH_3)_2$	20.6	10.9	61[c]	103
14d[108]	Cp_2Ru	OH	$O(CH_2)_5N(CH_3)_2$	14.5	13	63[c]	98
15[53]	CpCp*Fe	OH	OH	0.55	8.9	102	100
16[53]	$Cp(CH_3)_2$ PhospholylFe	OH	OH	1.5	10.8	139	97
17[53]	Cp_2Ru	OH	OH	10.8	17.1	123	94
18[53]	$CpRe(CO)_3$	OH	OH	1.9	11.1	123	97
19[53]	$CpMn(CO)_3$	OH	OH	2.4	13.0	126	98



Compound[a]	R1	RBA[b]		Cell viability at 1 μM (%)[c] or IC_{50}	
		ERα	ERβ	MCF-7	MDA-MB-231
20a[51]	*o*-OH	2.1	7.5	75	2.8 μM
20b[51]	*m*-OH	4.6	15.5	82	4.1 μM
20c[51]	*p*-OH	18.2	28	84	3.5 μM



Compound[a]	R_1	R_2	R_3	RBA[b]		Cell viability at 1 μM (%)[c] or IC_{50}	
				ERα	ERβ	MCF-7	PC3
31	OH	OH	CH_2	11.9	16.4	181	12 μM
32	OH	OH	$(CH_2)_2$	0.9	2.8	173	9.8 μM
33	OH	OH	$(CH_2)_3$	0.45	1.8	118	10.2 μM
34	OH	OH	$(CH_2)_4$	0.24	1.26	107	12 μM
32a	H	OH	$(CH_2)_2$	0.16	0.83	166	90*
32b	OH	H	$(CH_2)_2$	0.13*	0.18	192	84*
(*Z*)-**35**	OH	OH	$CH_2C(O)$	14	12	54*	7.8 μM
(*E*)-**35**				1.19	1.59	62*	8.3 μM
35a	H	OH	$CH_2C(O)$	4.1	6	108	83*
(*Z*)-**35b**	OH	H	$CH_2C(O)$	2.3	1.6	178	102*
(*E*)-**35b**				4.6	6	164	96*

Miscellaneous compounds

Compound[a]	RBA[b]		Cell viability at 1 μM (%)[c] or IC_{50}	
	ERα	ERβ	MCF-7	MDA-MB-231
3a[(153)]	12.5	–	193	100
3b[(153)]	28.1	–	155	13.4 μM
3c[(153)]	12	–	176	18.8 μM
3d [(153)]	43	–	178	93
6[(155)]	5.4	31	106	6 μM
10[(50)]	0.24	0.06	135	2.8 μM
11[(50)]	0.15	0.01	116	3.5 μM
36[(111)]	7.2	4.8	88	0.09 μM
37[(111)]	7.6	15.4	53	0.96 M

[a]All the complexes are a mixture of *Z* and *E* isomers when they exist, except when specified
[b]Mean of two experiments ± range, except where an asterisk appears
[c]Cell viability, measured as the percentage of proteins vs. the control set at 100% (except for **1a–e**, **12** and **13c** where the percentage of DNA is used), after 5 days of incubation at 1 μM or 10 μM (values marked with an asterisk); mean of two experiments ± range
[d]Percentage of proteins vs. the control with OH-Tam on MCF-7 at 83%

of most metal-containing drugs or drug leads are attributable to their direct interaction with DNA, such as observed in the cisplatin series [75]. The hydroxyferrocifens, however, seem to engage in a non-genomic pathway that could have

implications for presently incurable cancers and drug resistance problems. The origin of this cytotoxicity has been the subject of a structure–activity relationship study, where the stepwise modification of one aspect of the hydroxyferrocifen molecule has produced several analogues, to be discussed below.

4 Ferrocenyl Raloxifen Derivatives

As previously mentioned, despite the benefits of tamoxifen in the treatment of breast cancer, it is known to increase the risk of endometrial cancer and the formation of blood clots in women. Raloxifen, Chart 3, is a benzothiophene selective oestrogen receptor modulator, which, like tamoxifen, acts as an oestrogen antagonist in the breast. Formally known as LY 156,758 or keoxifen, it was first developed in the early 1980s as a candidate for the treatment of breast cancer [76]. However, it performed poorly against tamoxifen in laboratory models, and no further development followed until its ability to maintain bone density in post-menopausal women was recognised. It was approved under the new name of raloxifen for the treatment and prevention of osteoporosis (Evista®) [77, 78].

The benzo[*b*]thiophene ferrocene derivatives **2a**–**e** were investigated for their anti-proliferative properties against a variety of cell lines [79]. These compounds were designed in such a way as to maintain the tertiary amino chain so as to retain affinity for the oestrogen receptor. A key step in the synthesis is the acid catalysed cyclization of the thioether to obtain regioselectivity in the benzo[*b*]thiophene unit. All compounds showed IC_{50} values in the low micromolar range for ovarian (2008, C13*, RH4), cervical (A431), lung (A549), colon (HCT-15) and breast (MCF-7, MCF-7 ADR) cancer cell lines.

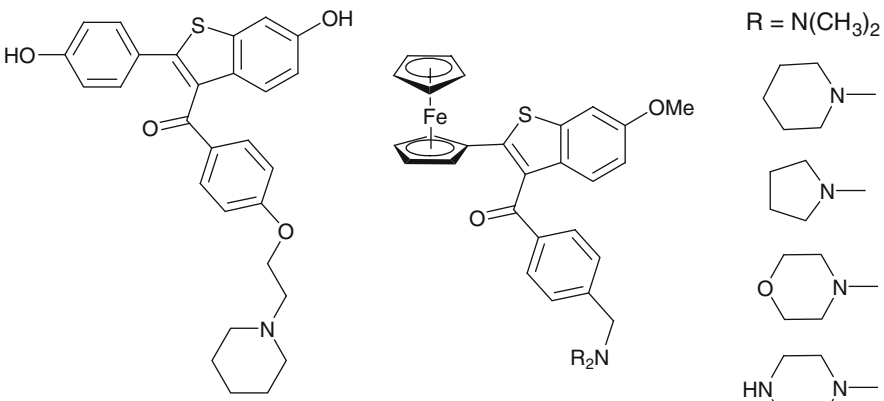


Chart 3

The piperazinyl derivative showed the greatest in vitro activity, with a mean IC_{50} value of 1.64 μM over all cell lines, that is, tenfold lower than cisplatin. It was particularly active against the hormone-dependent breast, cervix and ovarian cells, implicating an oestrogenic/anti-oestrogenic effect. This compound also activates caspase-3 in ovarian cells, suggesting that the mechanism of cell death is apoptosis rather than necrosis.

5 Ferrocenyl Oestradiol Derivatives

Before exploring the results of the SAR study on the hydroxyferrocifen-type molecules, it is worthwhile to address the utility of vectorisation. The above experiments with the hydroxyferrocifens showed that the vectorisation of ferrocene with the SERM tamoxifen yields a molecule exhibiting both anti-oestrogenic and cytotoxic properties. Because the ferricenium cation has been shown to damage DNA via reactive oxygen species (ROS) production [80], one could imagine that merely transporting the ferrocene entity into the nucleus via the ER-bioligand complex would be sufficient to produce a cytotoxic effect. To investigate this hypothesis, ferrocene was grafted onto the 17α-position of the oestradiol skeleton directly (**3a**) or via an alkyne linker (**3b**) and to the 7α position via a sulphur bridge (**3c**), Chart 4 [80–82]. At a concentration of 1 μM, these complexes, including the organic analogue **3d**, have a strong proliferative, oestrogenic effect on hormone-dependent cells (comparable to that of oestradiol) and no effect on

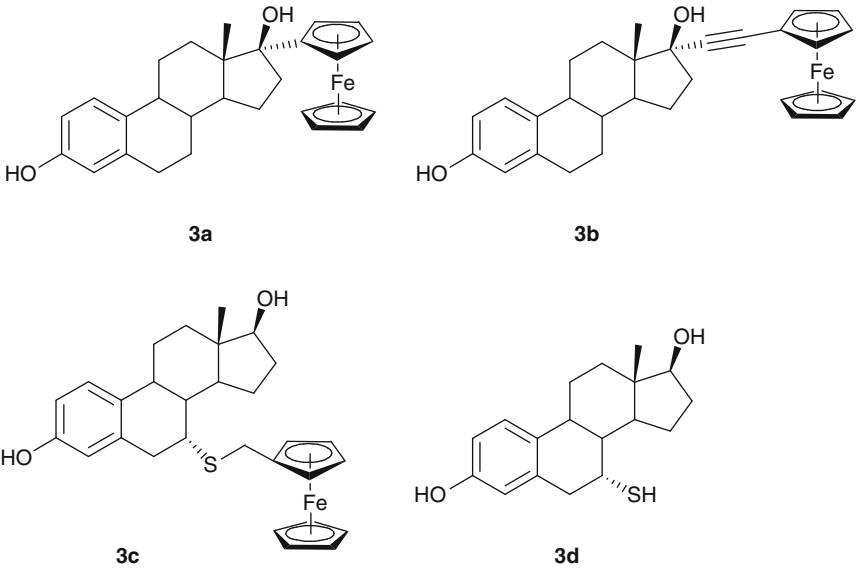


Chart 4

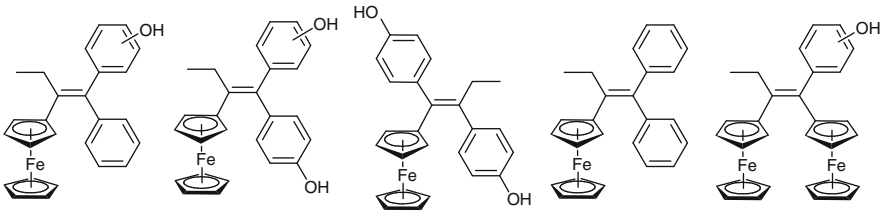
hormone-independent cells. The behaviour of the two complexes deviates at higher concentration; **3b** and **3c** become active on MDA-MB-231 cells at high concentrations, with modest IC_{50} values of 13.4 and 18.8 μM respectively, while **3a** is still not toxic at a concentration of 25 μM, the limit of its solubility. Thus, it is clear that the mere presence of a ferrocenyl entity in the interior of the nucleus is not, in itself, sufficient to cause significant cell death.

It should be mentioned that 17β-derivatives of oestradiol bearing $Co_2(CO)_6$ [83, 84], $CpRe(CO)_3$ [85–88], $CpMn(CO)_3$ [89] and $(C_6H_6)Cr(CO)_3$ [90] have also been synthesised.

6 Anti-cancer Structure–Activity Relationship Studies of Hydroxyferrocifens

6.1 N, N-*dimethylamino Side Chain*

The *N*,*N*-dimethylamino side chain is known to be essential for the anti-oestrogenic effects of OH-Tam in the breast, due to the deformation that it creates in the ligand binding domain of the ER [68, 69, 91–95]. Removal of this functionality from the hydroxyferrocifen skeleton yielded the monophenol **4**, and replacement by a *p*-hydroxyl group led to the diphenol **5** (Chart 5). Its regioisomer **6**, obtained by exchanging the phenol and ferrocenyl substituents, was also studied. Even lacking the anti-oestrogenic side chain, **4** and **5** are anti-proliferative on MCF-7 cells, with IC_{50} values of 4.9 and 0.7 μM [96], respectively. The simple position change in **6** yielded completely different behaviour, in fact, a slightly *proliferative* effect was observed at 1 μM against the ER+ cell line, likely due to an oestrogenic interaction with the ER. It should be emphasised that the anti-proliferative effect found for **4** and **5** can only be attributed to a concentration-dependent cytotoxic effect, as these compounds, lacking the side chain, are expected to be oestrogenic, as observed for compounds such as resveratrol [97–99], diethylstilbestrol [100] and the organic analogue of **5**, 1,1-bis(4′-hydroxyphenyl)-2-phenylbut-1-ene [101–103]. Therefore, the results on this cell line should be considered as a combination of oestrogenic and



4; para
8; meta

5; para
9; meta

6

7

10; para
11; meta

Chart 5

cytotoxic effects; for **4** and **5**, the cytotoxic effects are more strongly expressed at 1 μM to give an overall anti-proliferative effect; while for **6**, the oestrogenic effect can still be observed at this concentration and the compound is slightly proliferative.

This receptor-independent cytotoxicity is more clearly shown on the ER-negative MDA-MB-231 cells, for which the results are not confounded with oestrogenicity or anti-oestrogenicity. Again, the diphenol **5** is strongly cytotoxic, with an IC_{50} value of 0.4–0.6 μM, similar to that of the hydroxyferrocifen with a three-carbon atom chain (0.5 μM) [104], while the corresponding monophenol **4** is about half as toxic (1.13 μM) [105, 106]. On the other hand, **6** is considerably less toxic with an IC_{50} value of 6 μM (Table 1). While these results clearly show the importance of the position of the ferrocenyl group, the *N*,*N*-dimethylamino side chain, although necessary for anti-oestrogenicity, does not seem in this context to be an important factor in cytotoxicity. Recently, however, a Japanese group reported the synthesis of a tamoxifen derivative, bis(*N*,*N*-dimethylamino-phenetole), where the hydroxyl group in tamoxifen has been replaced by a second *N*,*N*-dimethylamino chain [107]. This compound showed remarkably high cytotoxicity on two leukaemia cell lines. Unpublished studies on the organic molecule where the side chains possess three carbons also indicate strong toxicity, with an IC_{50} value of 0.34 μM on ER-negative MDA-MB-231 breast cancer cells.

6.2 Presence and Position of the Phenol Group

While the disposition of the phenol group(s) around the ethylene core appears to influence the toxicity of hydroxyferrocifen analogues, is the phenol group itself crucial? The unsubstituted analogue, 2-ferrocenyl-1,1-diphenyl-but-1-ene (**7**, Chart 5), was accordingly synthesised by the standard McMurry cross-coupling pathway. It showed surprising non-zero RBA values for ERα and ERβ, a clear oestrogenic effect on the MCF-7 ER+ cell line, and is not cytotoxic at a concentration of 1 μM against the MDA-MB-231 cell line [105, 106].

The impact of the position of the hydroxyl group was studied via a series of *para*- and *meta*-substituted mono- and di-phenols, possessing one or two ferrocene groups, Chart 5 [105, 106]. Complexes **8**, **10** and **11** show the weakest anti-proliferative effects with IC_{50} values between 2.7 and 3.5 μM (Table 1); compounds **4** and **9**, possessing one ferrocene group and one *p*-phenol, are more cytotoxic (IC_{50} values around 1 μM); while **5**, possessing two *p*-phenols, is the most cytotoxic of the series (0.6 μM). These results show that the repositioning of one OH group from the *para*- to the *meta*-position reduces the cytotoxicity of the complex by a factor of approximately 2. The presence of a second ferrocenyl unit also decreases the cytotoxicity of the complexes, suggesting a steric component; here also the complex with an *m*-OH group is less cytotoxic than that with a *p*-OH.

The effect observed on ER+ breast cancer cells is again the result of the oestrogenic effect expected for these compounds, which all show an affinity for

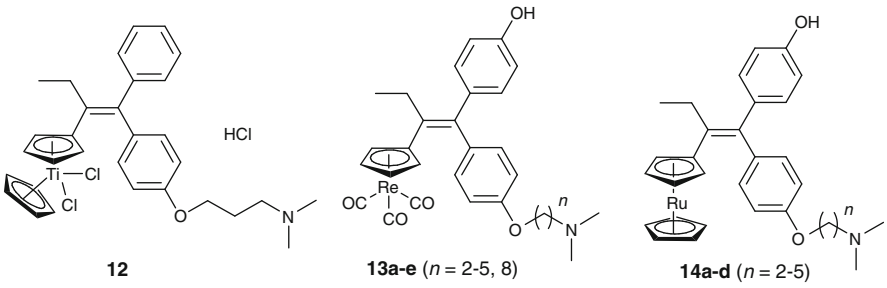
ERα, minus the cytotoxic component observed on the ER-negative cancer cells. At a concentration of 1 μM, only the most cytotoxic complexes **4** and **5** are able to overpower the oestrogenic effect of these compounds. Complexes **7** and **8**, which are the least cytotoxic on the MDA-MB-231 cells, show a clear proliferative effect. Compound **9** strikes a perfect balance between oestrogenic and cytotoxic effects and shows no overall effect on the MCF-7 cells at 1 μM.

6.3 *Role of the Ferrocene Moiety*

Hydroxytamoxifen and tamoxifen show anti-proliferative effects on MDA-MB-231 cells only at high concentration, with IC_{50} values of 29 and 34 μM, respectively, after an 18 h incubation [108], underlining the role of the ferrocene moiety in the cytotoxic effects of the hydroxyferrocifens. Tamoxifen and/or compound **5** derivatives have also been synthesised using other organometallic moieties such as Cp_2TiCl (**12**), $CpMn(CO)_3$ (**19**), $CpRe(CO)_3$ (**13, 18**) and Cp_2Ru (**14**, **17**), Charts 6 and 7.

Like the ferricenium cation, Cp_2TiCl_2 has also shown promising results against a wide range of cancers [109–111], although formulation problems halted its clinical trials [112, 113]. Photochemical decomplexation of the manganese moiety of the $CpMn(CO)_3$ hydroxytamoxifen analogue gave **12** in 98% yield [86–88]. On MCF-7 cells, **12** had a proliferative effect almost as strong as oestradiol itself, highly unusual for a complex possessing the anti-oestrogenic dimethyl amino side chain. Cp_2TiCl_2 alone gave rise to an even higher proliferative effect, suggesting that this entity, or one of its hydrolysis products (such as Ti^{4+}) [110] is responsible for the oestrogen-like behaviour and that **12** is likely unstable under cell culture conditions.

Group VII possesses two radioisomers, ^{99m}Tc and ^{188}Re, which could be incorporated into anti-oestrogenic radiopharmaceuticals to be used in imaging or therapy [114, 115]. A series of $CpRe(CO)_3$ tamoxifen derivatives were therefore synthesised via the McMurry coupling reaction with $n = 2$–5 and 8 (**13a–e)** [116, 117]. Contrary to the ferrocene series, the separable *Z* and *E* "hydroxyrhenocifen" isomers did not undergo interconversion in protic media. The RBA values were



12 **13a-e** (*n* = 2-5, 8) **14a-d** (*n* = 2-5)

Chart 6

satisfactory but variable, ranging from 0.7 (**13e**) to 12.3 (**13c**). Compounds **13b** and **13d** were tested on the MCF-7 and MDA-MB-231 cell lines. On MCF-7, these compounds were only slightly superior to OH-TAM at 1 μM, with the *Z* isomer a bit more active than the *E* isomer, consistent with the RBA values. With the MDA-MB-231 cells, only a very minor anti-proliferative effect was observed for both isomers of both molecules at 1 μM [116]. Although no unusual anti-proliferative effects were noted for these compounds, the fact that they behave similarly to OH-TAM suggests that they can be functionalised for radio imaging or therapy with no adverse effects. An important objective is the preparation of a hydroxytamoxifen complex of $Cp^{99m}Tc(CO)_3$ or $Cp^{188}Re(CO)_3$. In particular, the use of Alberto's reagents [118, 119] $[(H_2O)_3Re(CO)_3]^+$, $[(H_2O)_3Tc(CO)_3]^+$ or the exchange reaction between $[Re(CO)_6]^+$ and the ferrocene derivatives [86–88, 120] are possible pathways to reach this objective, and these studies are currently underway.

The higher congeners of the hydroxyferrocifens, the "hydroxyruthenocifens", were also prepared [121]. Several ruthenium complexes show anti-cancer and anti-metastatic activity [122–126], and this element can also exist as one of two γ-emitting isotopes, ^{97}Ru and ^{103}Ru, which may be useful in the radio imaging of cancer tumours [127]. The McMurry coupling reaction furnished mixtures of *Z* and *E* isomers of the *p*-bromo derivatives, which were then converted to the amino compounds **14a–d** ($n = 2$–5). Unlike the hydroxyferrocifens, the hydroxyruthenocifen isomers interconvert too rapidly to be separated by HPLC. The RBA values obtained on ERα were remarkably high for **14a** and **14b**, higher than that found for OH-TAM (39%), which is unprecedented for organometallic SERMs. On the MCF-7 breast cancer cell line, the hydroxyruthenocifen complexes show the expected anti-proliferative effect, similar or stronger to that observed with OH-TAM. However, these compounds have no cytotoxic effect on the MDA-MB-231 cell line at 1 μM. Therefore, the ruthenocene complexes of hydroxytamoxifen seem to act only as anti-oestrogens, similar to OH-TAM, and the organometallic moiety imparts no advantage.

Concerning analogues of the diphenol **5**, compounds functionalized with the organometallic fragments CpCp*Fe (**15**); Cp[$(CH_3)_2$phospholyl]Fe (**16**), Cp_2Ru (**17**), $CpRe(CO)_3$ (**18**) and $CpMn(CO)_3$ (**19**) have been studied ([105, 106], Chart 7). The nature of the organometallic moiety had a strong influence on ERα recognition, with RBA values ranging from 0.55% for the bulky CpCp*Fe derivative to 10.8% for the Cp_2Ru derivative. ERβ was better able to accommodate these compounds,

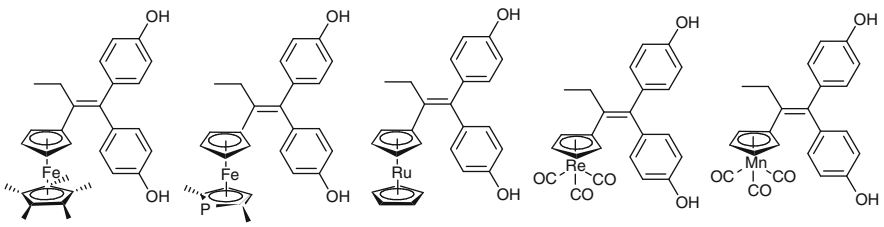


Chart 7

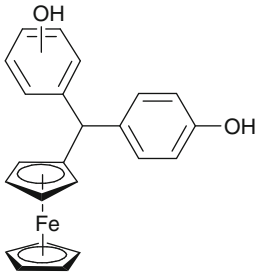
with RBA values ranging from 8.9% (CpCp*Fe) to 17.1% (Cp_2Ru). A study on the MCF-7 cell line yielded only proliferative, oestrogenic effects (**16**–**19**) or no effect (**15**), and none of the compounds showed any effect on the MDA-MB-231 cells at a concentration of 1 μM. Therefore, for both the hydroxytamoxifen and diphenol motif, the ferrocene moiety is crucial for cytotoxic activity.

6.4 Conjugation

To determine the role of the conjugated π-system linking the ferrocene and phenol groups in active compounds such as **1**, **4** and **5**, compounds linking the ferrocene moiety to the phenol group(s) by a sp^3 carbon instead of an ethylene group (**20a**–**c**, Chart 8) were synthesised and tested against the ER+ and ER− breast cancer cell lines [128, 129]. For each of these three complexes, one hydroxyl group remains in the *para* position, while the position of the second hydroxyl group varies between *ortho*, *meta* and *para* positions. The RBA values were variable, but were consistently higher for ERβ. On MCF-7 cells, at a concentration of 1 μM, these complexes are proliferative, and on MDA-MB-231 cells, a slight anti-proliferative effect is observed; substantially less important than that found for compound **5**. These complexes become toxic at higher concentrations, as shown by their IC_{50} values, respectively 2.8, 4.1 and 3.5 μM – approximately five times higher than the IC_{50} value found for **5**. Thus it appears, all other elements being equal, the compound possessing a π-system considerably outperforms its tetrahedral analogue.

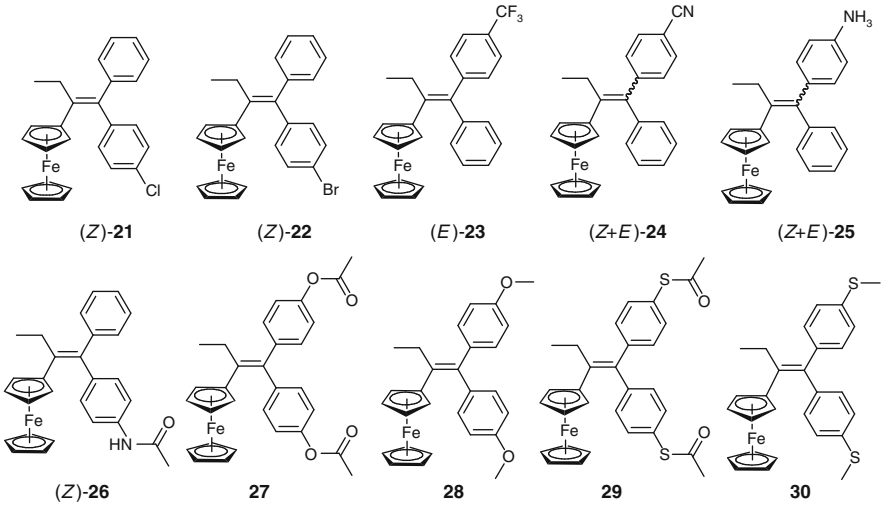
6.5 Phenyl Functionalisation

The influence of other phenyl substituents on anti-proliferative effects has also been studied. In one study, a series of halogen or pseudo-halogen *para*-substituted compounds with X = Cl, (*Z*)-**21**; Br, (*Z*)-**22**; CF_3, (*E*)-**23**; and CN, (*E*)-**24** and



20a; *o*-phenol
20b; *m*-phenol
20c; *p*-phenol

Chart 8



(*Z*)-**21** (*Z*)-**22** (*E*)-**23** (*Z*+*E*)-**24** (*Z*+*E*)-**25**

(*Z*)-**26** **27** **28** **29** **30**

Chart 9

(*Z*)-**24**, Chart 9, was prepared [130]. The pure isomers were isolated by fractional crystallisation, and their identity determined by X-ray crystallography. (*Z*+*E*)-**24** was obtained by heating (*Z*+*E*)-**22** with CuCN in DMF and the separation of the *Z* and *E* isomers was accomplished by HPLC, and likewise identified by X-ray diffraction. No isomerisation of the new complexes was observed by NMR spectroscopy in $CDCl_3$ after a 3-day period, contrary to what has been observed previously with the ferrocenyl phenol complexes. These latter compounds possess a labile proton and can isomerise readily, depending on the solvent, consistent with the mechanism proposed for diethylstilbestrol [65].

Lacking protic hydrogen bonding groups, the compounds have low, but non-zero, relative binding affinity values for the ERs (RBA $\leq$0.55%) as well as mildly exothermic ligand binding in in silico ER docking experiments. Compounds **21**, **22** and **24** have RBA values for the ERs on the same order of magnitude, while **23**, with the sterically demanding CF_3 substituent, has a considerably poorer affinity, and all compounds show oestrogenic activity on the MCF-7 cell line. On MDA-MB-231 cells, only the cyano complex (*Z*)-**24** showed any cytotoxic effect (IC_{50} = 10.9 μM), its isomer (*E*)-**24** is only very slightly cytotoxic (IC_{50} = 60 μM), while the Cl, Br and CF_3 derivatives had little effect even at 10 μM. This is the first time that a difference in cytotoxicity has been observed between *E* and *Z* isomers of such molecules, presumably because the protic molecules isomerize in situ and the resulting effects arise due to the mixture of isomers.

The analogous amine and acetamide compounds, (*E*+*Z*)-**25** and (*Z*)-**26**, respectively, as well as their corresponding organic molecules have also been studied ([131], Chart 9). All of the compounds have adequate RBA values for the ER, between 2.8 and 5.7% for ERα, and between 0.18 and 15.5% for ERβ, and exothermic ligand binding in in silico ER docking experiments. Compounds **25**

and **26** show dual oestrogenic/cytotoxic activity on the MCF-7 cell line; they are proliferative at low concentrations (0.1 μM) and anti-proliferative at high concentrations (10 μM). On the MDA-MB-231 cell line, the ferrocenyl complexes are strongly anti-proliferative with IC_{50} values of 0.8 μM for **25** and 0.65 μM for **26**, while the purely organic molecules show no effect.

The corresponding thiol compounds were also of interest, as the oxidation of aromatic thiols has been shown to generate H_2O_2 and provoke oxidative stress in cells [132, 133]. Although **29** was cleanly obtained via acetylation of the corresponding t-butyl thiol compound (itself obtained via a McMurry reaction), deprotection of the thiol with NH_4OH was not successful [134]. Likewise, deprotection of **30**, also obtained via the McMurry reaction, with $HNET_2$/NaH/HMPT did not yield the desired product. The ER affinities of the protected thiol compounds, as well as the methoxy analogue **28**, were quite low (<0.27 for ERα and <0.26 for ERβ), as opposed to that of the acetoxy **27** (6.5 for ERα). On MDA-MB-231 cells, only **27** showed any anti-proliferative activity, one which is comparable to that of the diphenol **5**. This strongly suggests that enzymes in the cells can hydrolyse the ester function to generate **5** in situ, as observed in other systems [135–138], and that **27** should be considered as a "prodrug". As thioesterases are also known to exist in breast cancer cells [139], one might expect a similar toxicity for the sulphur compound **29**. The inactivity of **29**, therefore, indicates a mechanism which is not operative when oxygen is replaced by sulphur. The lack of toxicity for ethers **28** and **30**, on the other hand, can be attributed to lack of hydrolysis in situ.

6.6 *Placement of the Ferrocene Group*

Due to the importance of the *N*,*N*-dimethylamino group for anti-oestrogenic effects, its replacement with various ethers has been widely investigated [68, 69, 91–95, 140–144], including the case where a ferrocenyl group is situated at the terminus of the side chain [145, 146], Chart 10. Compounds **31**–**34** showed an RBA-dependant proliferative effect on MCF-7 cells and a moderate anti-proliferative effect on androgen receptor-independent PC-3 prostate cancer cells with IC_{50} values around 10 μM. Compounds **32a**, **32b**, **35a** and **35b** also had a proliferative effect on MCF-7 cells, but only a modest effect on PC-3 cells. Only compounds (*Z*)- and (*E*)-**35** showed significant anti-proliferative effects on both MCF-7 and PC-3 cells (Table 1). MCF-7 cells were incubated with 10 μM of **35** in the presence and absence of 1 nM E_2; the addition of E_2 did not reverse the anti-proliferative effect of (*Z*)- or (*E*)-**35**, which indicated that the anti-proliferative effect is cytotoxic and not anti-oestrogenic. Oestrogenic properties of a compound are known to be expressed at low concentrations (10^{-8}–10^{-10} M), and (*Z*)- and (*E*)-**35** are indeed strongly oestrogenic at 10^{-9} M. Thus, once again, the activity of **35** on the proliferation of MCF-7 cells seems to be a combination of an oestrogenic character and a cytotoxic component; at high concentrations (>1 μM), cytotoxicity is dominant, and at low

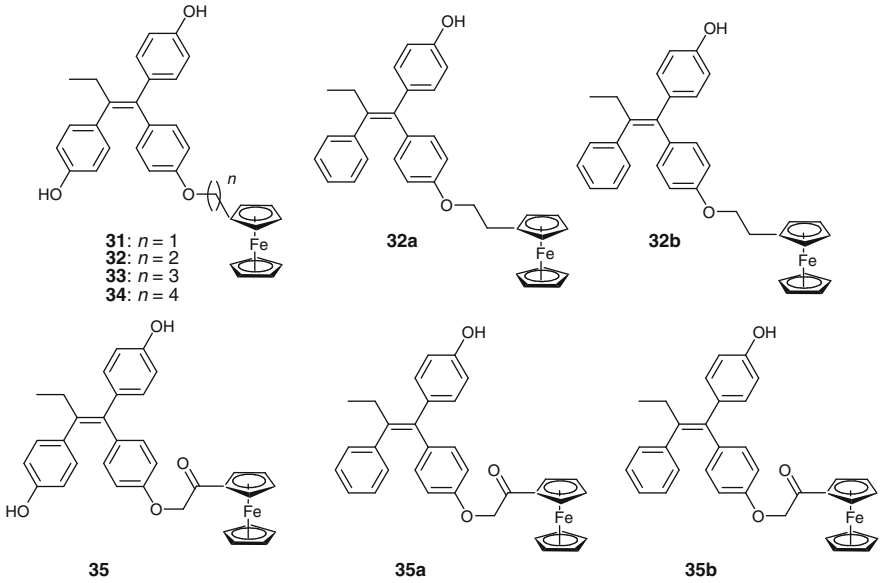


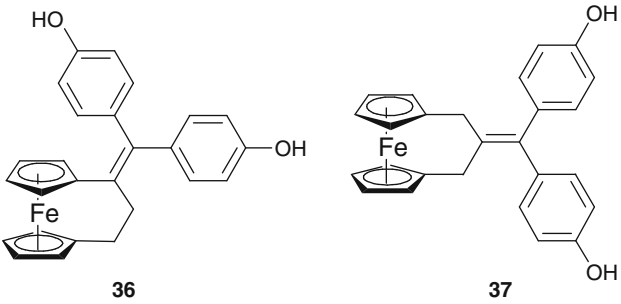
Chart 10

concentrations, the oestrogenic effect is more strongly expressed. Thus, despite the bulky side chain, **35** acts like the ferrocenyl phenols previously described, but is considerably less toxic.

6.7 Cyclic Compounds

Given the correct geometry, rigid molecules can more strongly bind to receptors than their flexible analogues. Accordingly, molecules **36** and **37**, possessing a cycle supported in the 1,1′ positions on each of the cyclopentadienyl rings of the [3] ferrocenophane, were prepared and studied, Chart 11 [147].

Both compounds show similar affinities for ERα (around 7%), while the RBA values for ERβ are more variable. Despite the similarity in ERα affinity, IC_{50} values of 4 μM for **36** and of 1 μM for **37** can be estimated for MCF-7 cells. It is clear that there is a competition on this cell line between the oestrogenic (positive) and cytotoxic (negative) effects, and it seems that **36** is more oestrogenic than **37**. Compound **36** showed an exceptional anti-proliferative effect on MDA-MB-231 and PC-3 cells, with an unprecedented IC_{50} value of 0.09 μM, an order of magnitude more cytotoxic than **37**, which lacks conjugation between the phenol and the ferrocene groups. The cytotoxic effect of compound **5**, lacking the ferrocenophane structure, but possessing a conjugated system, falls intermediate between **36** and **37**.



36 **37**

Chart 11

7 Mechanism

Two mechanisms accounting for the cytotoxicity for ferrocene complexes have been proposed, (1) ROS production, and (2) production of electrophilic species. In two papers, Osella and coworkers used ESR evidence to link the cytotoxicity of ferrocenium cations to the production of hydroxyl radical. This work also confirmed previous work demonstrating that the reduced ferrocene was not toxic [42, 43]. Similarly, Miwa et al. showed that the cytotoxicity of the ferrocenium cation arises from DNA damage due to hydroxyl radical production [41]. A study by Wlassoff et al. on the hydroxyferrocifen **1b** showed that the incidence of apoptosis in MCF-7 cells treated with **1b** was greater than that for cells incubated with ferrocene or tamoxifen separately, and that apoptosis was correlated with the concentration of ROS produced by the cell. The authors proposed that the overproduction of hydrogen peroxide in cancer cells provokes ROS via Fenton chemistry upon the interaction of iron, and that tamoxifen serves as a vector to bring the ferrocene into the cancer cell [148]. Osella and coworkers found, however, that **1b** showed negligible oxidative DNA damage as compared with tamoxifen or hydroxytamoxifen [149]. Another study on compounds **1b** and **5** showed that their cytotoxicity (high for **1b**, moderate for **5**) on melanoma cells cannot be linked with ROS production, although this may be a special case for melanoma cells, which possess a sophisticated anti-oxidant system [150]. Nonetheless, the role of these compounds on the regulation of ROS deserves more study.

A report that hydroxytamoxifen can form quinone methides (QM) as a result of bio-oxidation [151] inspired an electrochemical study of some of the compounds discussed in the structure–activity relationship study ([128, 129], reviewed in [152]). In MeOH medium alone (these compounds are only sparingly soluble in water), the cyclic voltammograms (CV) of most of the compounds exhibited the expected reversible Fc/Fc^{+} redox couple, often followed by that of the phenolic moiety (where appropriate). However, when an organic base such as pyridine was added, two distinct types of electrochemical behaviour were observed. In the cases of the compounds which showed low or no cytotoxic effects in vitro, very little change in the CV was observed upon the addition of base. However, for the most

biologically active compounds (viz. **1a–d**, **4**, **5**, **10**, **25**, **26**, **36** and **37**), the addition of pyridine caused two major modifications: loss of reversibility of the ferrocene/ferricenium couple and an increase of the ferrocene oxidation wave. An example of this phenomenon for **5** is shown in Fig. 1.

Therefore, the role that the ferrocenium group plays in the in vitro cytotoxicity appears to be that of an intramolecular electron acceptor. The inertness of the non-phenolic compound **7** to pyridine in this model system shows that a phenolic group is necessary for the reaction to take place. Likewise, for the unconjugated **20a–c**, chemical reduction of the Fe(III) atoms was not observed suggesting that the electron transfer process occurs through a coupling in the molecular π-system. Thus, as soon as an adequate base is available, a substantially fast intramolecular electron transfer may occur, thereby leading to the oxidation of the phenolic moiety made easier because of its displacement by the reaction of the phenoxy cation with the pyridine base [153–155].

Given the role of the ferrocene moiety as an intramolecular hole-reservoir, the conjugated π-system and the basic action of pyridine, the mechanism given in Scheme 2 for the generation of quinone methide species was proposed. The ferrocene moiety is oxidised, and the electron may be to a small extent delocalized

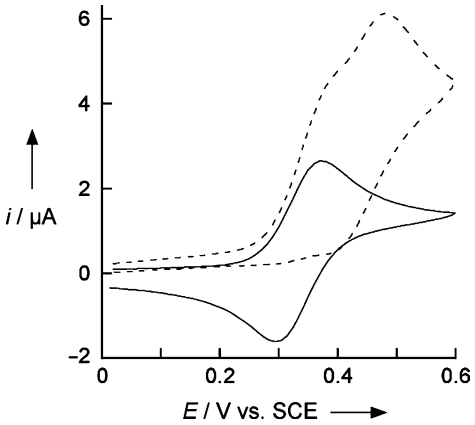
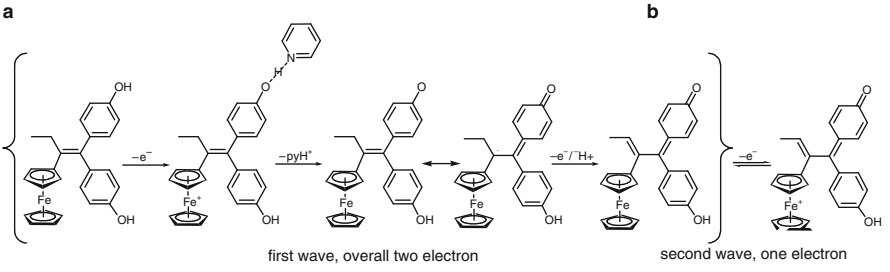


Fig. 1 Cyclic voltammograms of **5**, 2 mM in 0.1 M Bu_4NBF_4/MeOH in absence (*solid line*) and presence (*dashed line*) of pyridine in 1:6 volume ratio. Scan rate 0.5 Vs^{-1}. Pt electrode of 0.5 mm diameter. The phenol oxidations in MeOH occur at 0.879 and 1.17 V/SCE (not shown)



Scheme 2 PCET mechanism in the formation of quinone methides from ferrocenyl phenols

over the π-system. This imparts a partial positive charge to the hydroxyl group, thus acidifying the proton, which may then be easily abstracted by pyridine. The resulting phenoxy radical species can be described by many mesomeric structures, one of the most stable being the quinoid in which the radical is positioned on the α-carbon with respect to the ferrocene. This species may then be oxidised at the first oxidation wave, and indeed, the first wave tends towards a two electron process at the limit of infinite [base] and 1/scan rate. This second oxidation is then followed by another proton abstraction from the ethyl group, resulting in a QM structure, the ferrocene of which is oxidised on the level of the second wave.

Potential cell damaging pathways for tamoxifen include metabolism to electrophilic *o*-quinones, QM or carbocations, which can form adducts with DNA, GSH or proteins [151, 156–160]. It is presumed that the biologically active ferrocene compounds undergo similar oxidative metabolism, which is enhanced by the easier oxidation of ferrocene in comparison to phenol. Thus, it is postulated that the ferrocene moiety may be oxidised far from the biological target and may thus serve as an intramolecular carrier of the hole while the activated drug finds its way to its target. In this way, the ferrocene acts as a kind of intramolecular oxidation "antenna" and may oxidise the phenol group by intramolecular means, thus producing cytotoxic species in milder oxidising conditions.

This mechanism of activation has been supported by the observation that **5** provokes DNA damage and p53 upregulation, consistent with the intracellular generation of the alkylating quinone methide metabolite [161]. Compounds **1c** and **4** can also be chemically (Ag_2O) or enzymatically (cytochrome P450) oxidised to the QM [162]. The 3,5-dimethyl derivative of **4** has been crystallographically characterised in its phenolic and quinone methide form.

A few compounds deserve further comments. For compounds lacking phenol groups, the CVs for the amine and acetamide **25** and **26** also showed the typical intramolecular electron transfer signature. Furthermore, the covalent attachment of **25** to a variety of electrodes was electrochemically achieved either by direct oxidation of the amino group at 0.70 V/SCE or indirectly via the oxidation of the ferrocene moiety at 0.40 V/SCE [163]. Although imine methides are known and have indeed been implicated in cytotoxic processes [164–170], acetimine methides are rare and short lived [171]. Unstabilized (by metals, electron donating or withdrawing groups, etc.) thio-quinone methides, however, have never been isolated to our knowledge, which may account for the inertness of compound the acetylthio compound **29**.

In the case of the ferrocenophanes **36** and **37**, the CVs also suggest a similar mechanism to that shown in Scheme 2. Although the ferrocene moiety remains conjugated to the molecule in **36**, **37** possesses a methylene spacer which breaks the conjugation. In the former case, the intramolecular proton coupled electron transfer from the phenol to the ferricenium can be explained by the classical π-delocalized mechanism [128, 129]. For the unconjugated **37**, one must consider either that the electron transfer proceeds "through space" or via the formation of an intermediate α-methylene radical (such as α-methylene ferricenium molecules possess acidic

protons [172]), which can delocalize over the π system and undergo an additional oxidation step to yield the QM.

It is also interesting to note that for all of the mono-substituted compounds studied up to now, their cytotoxic effects at 10 μM loosely correlate with the resonance donating parameter of the *para*-substituent. The most active compounds are those with the strongest resonance donating substituents (OH, NH_2, $NHCOCH_3$); less active compounds (Br, Cl, CN) have weakly donating or weakly withdrawing character. Finally, the compound (CF_3) with the strongest resonance withdrawing character shows the lowest toxicity [130].

8 Formulation Studies

Some of the compounds previously discussed have been used in formulation studies with the eventual aim of enhancing the bioavailability and activity in vivo. The ferrocenyl compounds discussed here are quite hydrophobic, necessitating a drug delivery system. Compounds **4**, **5**, **7** and **36** were found to create 1:1 water soluble inclusion complexes with methylated β cyclodextrin (CD) after stirring overnight. Electrochemical experiments showed that, in methanol, they exhibited a weak interaction with the CD cavity, and that these interactions became stronger as the amount of added water increased, as expected.

The structure of the hydroxyferrocifen compounds affects both their solubility and their complexation dynamics. Investigations in the presence of pyridine show that the base favours the dissociation of the **5**-CD complex during the electron transfer step, but does not affect the follow-up reactivity of the electrogenerated ferricenium cation, which leads eventually to the corresponding QM, as reported in the absence of CD. The cytotoxicity of these CD-encapsulated organometallic complexes in MDA-MB-231 breast cancer cells was similar to those obtained in the absence of the solubilising agent, suggesting that CD could be an important component in further formulation studies [147, 163].

Compound **5** has been encapsulated in lipid nanocapsules (LNC) and swollen micelles and tested against 9L glioma cells in vitro and on a subcutaneous 9L-rat glioma model [173]. The loaded LNC were rapidly taken up by the glioma cells, while only a small percentage of the loaded micelles were able to enter the cells. Compound **5** and the **5**-LNC complex showed similar and strong activities (IC_{50} = 0.5 μM), while hydroxytamoxifen was much less toxic (IC_{50} = 35 μM) and ferrocene itself was non-toxic at the concentration of up to 100 μM. In vivo, the **5**-LNC complex significantly inhibited tumour growth; rats treated with a single injection of LNC at a dose of 2.5 mg kg^{-1} presented tumours three times smaller than the ones treated by blank LNC (700 mm^3). In contrast, rats treated by micelles loaded with **5** at the same dose had tumours that grew very quickly and reached 1,600 mm^3 (LNC control = 2,000 mm^3) by the end of the study.

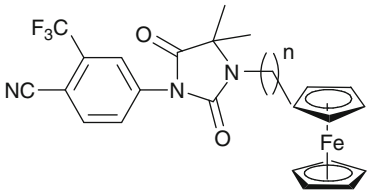
In another study, compounds **5** and **35** were incorporated in PEG/PLA nanospheres (NS) and nanocapsules (NC) [146]. In vitro drug release after high dilution of loaded

NPs was measured by E_2 binding competition in MELN cells. Notwithstanding potential drug adsorption at the NP surface, **5** and **35** were incorporated efficiently in NC and NS, which slowly released both compounds. When incubated with MCF-7 cells, they arrested the cell cycle in the S-phase and induced apoptosis, and these effects were enhanced when the compounds were encapsulated in NS. A decrease in their anti-proliferative activity in the presence of the anti-oxidant-tocopherol indicated that the production of ROS may be involved in the cytotoxic mechanism.

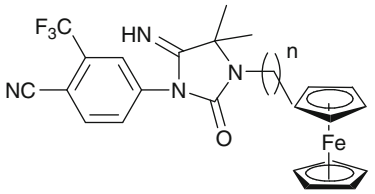
9 Ferrocenyl Androgens and Anti-androgens

Prostate cancer is the most prevalent cancer and the third leading cause of cancer death in American men [174]. Testosterone, the endogenous hormone, and dihydrotestosterone (DHT), its active metabolite, can promote the malignant growth of the prostate gland, and most prostate cancers are androgen-dependent [49, 51, 52, 175]. Therapeutic agents include the non-steroidal anti-androgens nilutamide, flutamide and bicalutamide, although, as with breast cancer, acquired and intrinsic drug resistance reduces the efficacy of such treatments. It was therefore postulated that the addition of ferrocene to nilutamide could likewise lead to molecules active on both hormone-dependent and -independent prostate cancers. The ferrocenyl group was attached to the free NH function of nilutamide derivatives to create compounds **38**–**41**, **C**hart 12. A second approach involved the introduction of the ferrocenyl moiety at C-5 of the hydantoin ring to obtain **42**, a disubstituted C-5 derivative bearing a 4-cyano-3-trifluoromethylphenyl moiety instead of a simple hydrogen atom. In addition, molecule **43**, a purely organic molecule related to **42**, but with a *p*-anisyl group instead of ferrocenyl, was prepared for comparison [179].

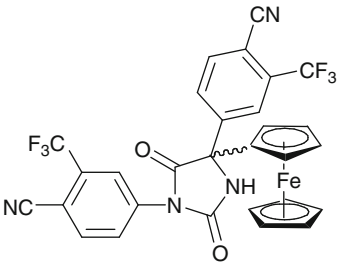
All of these compounds show some affinity for AR, although the RBA values found are low, i.e. less than 1%, for both the ferrocenyl complexes and nilutamide and bicalutamide. It is interesting to note that **38** and **39** have higher RBA values than those of nilutamide, while the values obtained for compounds **42** and **43** are lower. The effect of the newly synthesised molecules was then studied on both hormone-independent (PC-3) and hormone-dependent prostate cancer cells (LNCaP). At a concentration of 10 μM, complexes **40** and **41** show almost no anti-proliferative effect (less than 10% cell death), bicalutamide has a weak anti-proliferative effect (15%), the two N-substituted ferrocenyl complexes **38** and **39** have a significant anti-proliferative effect (37–34%, respectively), and the two bulky C-5-substituted compounds, i.e. the ferrocenyl complex **42** and the organic compound **43**, display similar, strong anti-proliferative effects. The IC_{50} values (μM) on this cell line are as follows: 68 (**38**), 69 (**39**), 5.4 (**42**) and 5.6 (**43**). On LNCaP, bicalutamide shows a rather weak anti-proliferative effect, nilutamide has an unexpected but clear and reproducible *proliferative* effect, while **42** and **43** have an almost identical strong anti-proliferative effect. The addition of DHT reverses the anti-proliferative effect of bicalutamide, but not the effect observed with **42** and **43**, suggesting that this is a non-receptor-mediated cytotoxic effect.



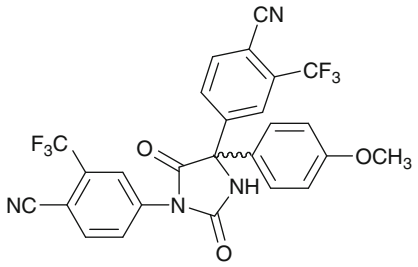
38: n = 1
39: n = 2



40: n = 1
41: n = 2



42



43

Chart 12

The synthesis and the structure–activity relationship of a series of organometallic complexes of the steroidal androgens testosterone and DHT have also been carried out [176]. These molecules were substituted at the C-17 position of the steroid with an ethynyl substituent grafted with various organometallic units, Chart 13. 3β-androstanediol was also substituted at C-16 and C-17, respectively, by an ethynyl and vinyl ferrocenyl unit. The ethynyltestosterone derivatives were obtained via a Stille coupling reaction between the appropriate iodo-organometallics and 17β-ethynyltestosterone stannyl derivatives. The ethynyl-DHT derivatives were synthesised by addition of the corresponding acetylide to the C-17 carbonyl of the steroid. The affinity for the androgen receptor is low, with RBA values ranging from 0.020 (**51**) to 0.57 (**46**), likely because the AR does not tolerate substitution in the 17 position.

The ferrocenyl derivatives **46**, **47**, **50** and **51** were tested on PC-3 hormone-independent cells and showed an anti-proliferative effect with IC_{50} values ranging from 4.7 to 12.2 μM respectively. These values are very similar, for **47**, or slightly better, for **46**, than those found for the most active ferrocenyl derivative of the non-steroidal anti-androgen nilutamide (see above). These values are lower than that found for the corresponding 17β-ferrocenyl-oestradiol derivative **3b** (IC_{50} = 13.4 μM) on MDA-MB-231 cells [81, 82]. Thus, the ferrocenyl–testosterone complex **46** is the most cytotoxic organometallic complex found so far in the steroidal/non-steroidal androgen series.

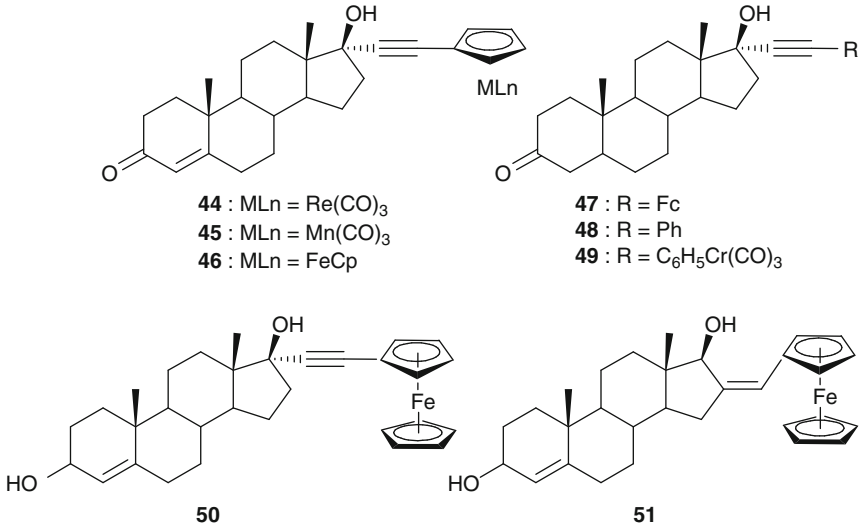


Chart 13

10 Summary

The inspiration for this work came from the recognition that the toxic molecules used in chemotherapy are often undermined by their non-specific character which can harm both healthy tissue and cancerous target cells. Therefore, the creation of organometallic steroidal and non-steroidal estrogen and androgen receptor modulators was investigated. The rationale was to vectorise a potentially cytotoxic organometallic moiety to facilitate its path to the target, originally envisioned to be DNA. The prototypical molecule of this class is the ferrocene-tamoxifen derivative, which shows dual anti-oestrogenic and cytotoxic effects, while tamoxifen itself is anti-oestrogenic but not cytotoxic. However, it was quickly recognised that the cytotoxic effects were not a result of the ferrocene molecule acting alone, but rather in concert with the organic part of the molecule. Through the study of a variety of hydroxyferrocifen analogues, it was discovered that the molecules of this series which possess a particular structural pattern including a conjugated system linking a ferrocenyl with a phenol, aniline or acetanilide group show the strongest cytotoxic effects. Electrochemical experiments strongly suggest that the toxic agent in the cell takes the form of an oxidised quinone methide, the formation of which is facilitated by the redox properties of ferrocene. This study has therefore provided us with a group of ferrocenyl complexes at our disposal with a range of IC_{50} values between 0.09 and 13 μM. The most powerful molecule to date, compound **36**, possesses a [3]ferrocenophane group conjugated to the phenol group; this pharmacophore and other cyclic species are currently under study. Exciting preliminary

results on a variety of cancer cell lines suggest a promising future for this new lead compound.

In this regard, the mechanism shown here to take into account the suprising activity of the ferrocene phenols such as **5** adds a particular originality to the therapeutic approach for organometallic drugs. It is likely that compound **5** follows the example of similar phenolic compounds, such as oestradiol, caffeic and ferulic acids, which produce ROS, such as H_2O_2 within cancer calls such as MDA-MB-231. However, **5** is different from the above-mentioned organic molecules, due to its redox-active ferrocene moiety, which can be reversibly transformed into ferricenium by oxidising agents. We have proposed that this oxidation event can, in the right conditions, launch a sequence of events leading to an active metabolite. The quinone methide can undergo Michael addition of known intracellular nucleophiles, such as glutathione, leading to redox imbalance and death of the cancer cell. So far our studies indicate that this mechanism does not seem to work in healthy tissue or reducing tissue at low concentration, suggesting a potential for a selectivity with these types of molecules. This would address one of the difficulties of the use of such drugs such as cisplatin, with its several side effects due to its primary action of intercalating DNA.

We propose here the term "kronatropic" to describe this novel effect. This adjective is formed from Kronos, the Greek deity who devoured his offspring, and Atropos (the eldest of the three Fates), who cut the thread of life. These molecules produce ROS "children" in the form of H_2O_2, which is then consumed by the oxidisable molecule to form the toxic quinone methide species. This original behaviour is based on the unusual reactivity of bioorganometallic species, which may provide a new line of attack for problems of high social importance.

The use of organometallic steroids or non-steroidal anti-androgens to target prostate cancer cells has given more mixed results. On the one hand, although compound **42** has an excellent IC_{50} value against AR-prostate cancer cells, it was shown that the organic analogue is as toxic. However, it was shown that the addition of ferrocene to the testosterone and dihydrotestosterone can produce cytotoxic molecules with low IC_{50} values.

Clearly, ferrocene is an interesting substituent, due to its vast potential for use in biology. It can act by means of specific particularities, whether it be its compact, aromatic, lipophilic character or its ability, in favourable cases, to act as a stable redox agent in biological media. These properties are beginning to be well identified and should find new fields of application in biology in the future.

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Titanocenes: Cytotoxic and Anti-angiogenic Chemotherapy Against Advanced Renal-Cell Cancer

Megan Hogan and Matthias Tacke

Abstract 6-Substituted fulvenes are interesting and easily accessible starting materials for the synthesis of novel-substituted titanocenes via reductive dimerisation, carbolithiation or hydridolithiation reactions, which are followed by a transmetallation reaction with titanium tetrachloride in the latter two cases. Depending on the substitution pattern, these titanocenes prove to be bioorganometallic anticancer drugs, which have significant potential against advanced or metastatic renal-cell cancer. Patients bearing these stages of kidney cancer have a poor prognosis so far and therefore real progress in the area of metal-based anticancer drugs may come from this simple and effective synthetic approach.

Keywords Anticancer drugs · Carbolithiation · Cisplatin · Fulvene · Hydridolithiation · LLC-PK · Titanocene

Contents

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1 Introduction

Beyond the field of platinum anticancer drugs, there is significant unexplored space for further metal-based drugs targeting cancer. Titanium-based reagents have significant potential against solid tumours. Budotitane [*cis*-diethoxybis(1-phenylbutane-1,3-dionato)titanium (IV)] looked very promising during its pre-clinical evaluation, but did not go beyond Phase I clinical trials, although a Cremophor EL®-based formulation was found for this rapidly hydrolysing molecule [1]. Much more robust in this aspect of hydrolysis is titanocene dichloride (Cp_2TiCl_2), which shows medium anti-proliferative activity *in vitro* but promising results *in vivo* [2–4]. Titanocene dichloride reached clinical trials, but the efficacy of Cp_2TiCl_2 in Phase II clinical trials in patients with metastatic renal-cell carcinoma [5] or metastatic breast cancer [6] was too low to be pursued. The structures of Budotitane and titanocene dichloride are shown in Fig. 1.

The field got renewed interest with P. McGowan's elegant synthesis of ring-substituted cationic titanocene dichloride derivatives, which are water-soluble and show significant activity against ovarian cancer [7]. More recently, novel methods starting from fulvenes and other precursors [8, 9] allow direct access to substituted titanocenes via reductive dimerisation with titanium dichloride, carbolithiation or hydridolithiation of the fulvene followed by transmetallation with titanium tetrachloride in the last two cases. Within this article, we will concentrate on the following four classes of substituted titanocenes, which can be synthesised using the above mentioned novel methods: *Ansa*-titanocenes, benzyl-substituted titanocenes, chiral 6-*N*,*N*-dimethylamino-functionalised titanocenes and achiral 6-bis-*N*,*N*-dimethylamino-functionalised titanocenes.

All four compound classes have the common feature that they can be synthesised using fulvenes as starting material. Fulvenes are easily accessible through the Knoevenagel condensation reaction of the accordingly substituted benzaldehyde and freshly cracked cyclopentadiene in methanol in the presence of catalytic amounts of pyrrolidine as seen in Scheme 1. 6-Arylfulvenes can be obtained as orange solids, in rare cases as oils in yields up to 95% [10] and their use is an elegant approach to introduce biologically active groups to the titanocene dichloride moiety.

Substituted titanocene dichlorides have potential application as metal-based anticancer drugs, and therefore, the cytotoxic activity of members of all four

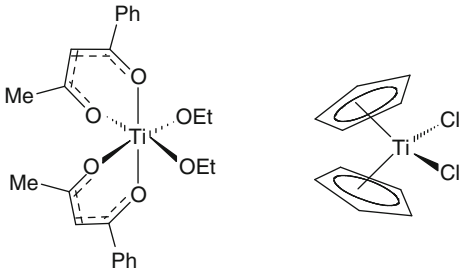
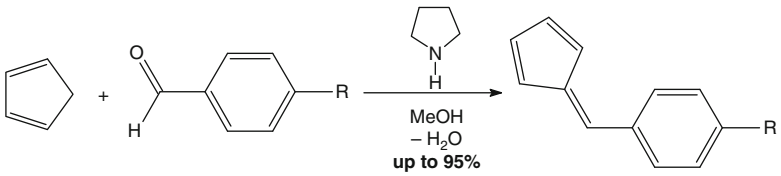


Fig. 1 Structures of the two titanium-containing anticancer drug candidates budotitane and titanocene dichloride



Scheme 1 Synthesis of 6-aryl fulvenes

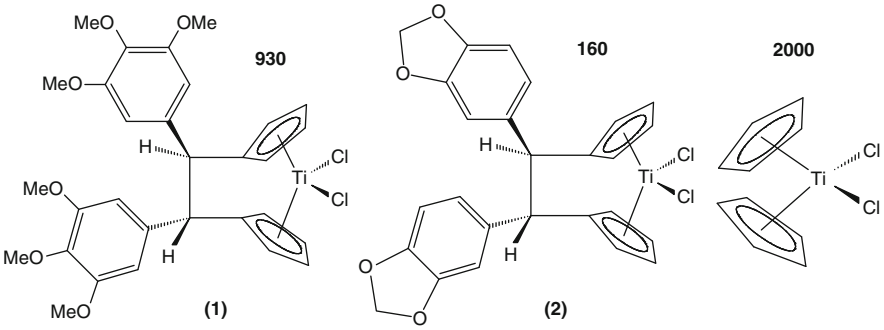


Fig. 2 IC50 values [μM] showing the effect of two *ansa*-titanocenes and titanocene dichloride on the viability of LLC-PK cells

titanocene classes has been determined and compared. The cytotoxicity was consistently determined on the pig kidney cell line LLC-PK using the so-called MTT-based assay [11], in order to establish structure–activity relationships. The LLC-PK cell line was chosen in accordance to the Phase I/II studies of Cp_2TiCl_2, where renal-cell cancer was identified as one of the main targets. For comparison we want to mention that the unsubstituted Cp_2TiCl_2 has an IC50 value of 2,000 μM and cisplatin, the most widely used metal-based anticancer drug, has an IC50 value of 3.3 μM when tested on the same cell line.

2 Benzyl-Substituted Titanocenes via Hydridolithiation

Aryl-substituted fulvenes can be dimerised with titanium dichloride in THF; the resulting mixtures of chiral *ansa*-titanocenes (see Fig. 2) can be synthesised in yields of around 50% and show low anti-proliferative potential with IC50 values of between 930 in **(1)** and 160 μM in **(2)** [12]. This is moderate improvement with respect to titanocene dichloride, which exhibits an IC50 value of only 2,000 μM against LLC-PK and is therefore not cytotoxic at all against this kidney cell line.

In order to avoid stereo centres and to increase the cytotoxicity, a novel class of substituted titanocene dichloride derivatives, the so-called "benzyl-substituted

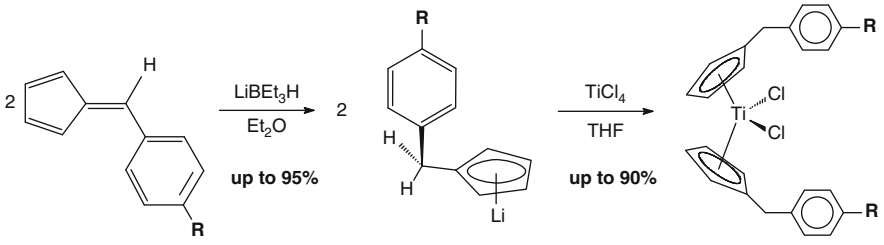
titanocenes", was developed and tested for their potential application as anticancer drugs.

Benzyl-substituted titanocenes do not have stereo centres and therefore stereoisomers do not exist, unlike their *ansa* analogues. In terms of *in vivo* and *in vitro* cell testing, this is advantageous. Previously, the presence of unseparated stereoisomers means that the issue of whether the compounds' cytotoxicities are related to specific isomers was not addressed. This is not of concern in the achiral benzyl-substituted titanocenes presented here.

As starting materials phenyl-substituted fulvenes are chosen, which can be synthesised from cyclopentadiene and benzaldehyde derivatives in high yield of up to 95%. The novel hydridolithiation reaction of Super Hydride ($LiBEt_3H$) and phenyl-substituted fulvenes results in the formation of lithium cyclopentadienide intermediates in almost quantitative yield. This is in general an interesting and applicable method towards the synthesis of a wide range of new benzyl-substituted metallocenes. By employing titanium tetrachloride, a wide variety of benzyl-substituted titanocene dichlorides have been synthesised [13, 14], including bis (*p*-methoxybenzyl cyclopentadienyl) titanocene dichloride (**3**), which was named Titanocene Y.

The nucleophilic addition of a hydride to the exocyclic double bond of fulvenes, using $LiBEt_3H$ as the hydride transfer reagent, resulted in the formation of the appropriately substituted lithium cyclopentadienide intermediates, which is insoluble under the reaction conditions chosen and can be isolated for purification purposes. Two equivalents of the substituted lithium cyclopentadienide undergo a transmetallation reaction when reacted with 1 mol equivalent of titanium tetrachloride in THF under reflux to give the appropriate non-bridged substituted titanocene dichloride in overall yields of up to 77% as seen in Scheme 2.

Super Hydride is one of the most powerful nucleophilic reducing agents available, capable of reducing many functional groups. It is also highly selective. The exocyclic double bond in aryl-substituted fulvenes has an increased polarity, due to the inductive effects of their respective aryl groups. This increased polarity allows for selective nucleophilic attack at this double bond and not at the diene component of the fulvenes. Other examples of the nucleophilic addition of hydrides to substituted fulvenes (albeit with alkyl or unsubstituted phenyl group functionality)



Scheme 2 Synthesis of benzyl-substituted titanocenes through the hydridolithiation reaction of aryl-substituted fulvenes using superhydride

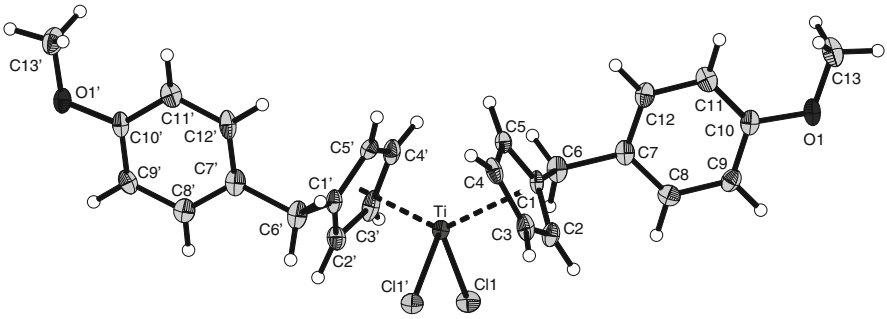


Fig. 3 Molecular structure of **3** (Titanocene Y); thermal ellipsoids are drawn on the 50% probability level (after Fig. 3 from [13] with permission of Elsevier. © 2005 Elsevier)

include the use of lithium aluminium hydride and the use of alkyl lithium species as highly reactive-hydride transfer reagents [15, 16].

The benzyl-substituted titanocene dichloride derivatives are well suited for X-ray diffraction studies; Titanocene Y was found to crystallise in the orthorhombic space group Pbca (#61) with eight molecules in the unit cell [13] as seen in Fig. 3. The molecules show a distorted tetrahedral coordination around the central titanium atom with a Ti–Cl bond length of 237 pm, while the Ti–Cp(centroid) length is found to be 206 pm. The Cl–Ti–Cl angle of the distorted tetrahedron is to be only 95.9°, while the Cp(centroid)–Ti–Cp(centroid) angle is widened to 130.7° due to bulkiness of the cyclopentadienyl group.

In the unit cell, the almost rod-like molecules are found to be arranged like match sticks in a matchbox and the packing efficiency is high resulting in no cavities in the structure, which could be occupied by solvent molecules. In addition, benzyl-substituted titanocene dichloride derivatives resemble to a certain degree fatty acid molecules due to their shape and lipophilicity, which will become important in the albumin binding part later on.

The *in vitro* cytotoxicity of the benzyl-substituted titanocenes was determined using a standard MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based assay: The benzyl-substituted titanocene dichloride derivatives are dissolved in DMSO, diluted with saline and exposed to the cell line LLC-PK (long-lasting cells-pig kidney) for 48 h. After washing, the cells are allowed to recover (or die) for 24 h, before MTT is added and the MTT reduction to a formazan (performed by living cells only) is measured photometrically. These cytotoxicity studies were undertaken to the same protocol used for the studies with *ansa*-titanocene dichloride derivatives, and therefore, the results are directly comparable. **3** showed an impressive cytotoxic activity with IC50 values of 21 μM [13], which represents a tenfold increase in cytotoxicity compared with a typical *ansa*-analogue and an impressive 100-fold improvement compared with the unsubstituted Cp_2TiCl_2. Further derivatives carrying a *p*-dimethylamino group **(4)** or its hydrochloride **(5)** exhibited less impressive IC50 values of 120 or 90 μM.

In analogy to the *ansa*-titanocenes, a series of unbridged heteroaryl-substituted titanocene dichlorides was synthesised [14]. Therefore, the furyl, thiophenyl and *N*-methyl pyrrolyl-substituted fulvenes were reacted with $LiBEt_3H$ and the resulting lithium intermediates were successfully transmetallated with titanium tetrachloride. The resulting heteroaryl-substituted titanocenes dichlorides **(6–8)** were obtained in yields of 24–63%. When tested for cytotoxicity on LLC-PK, the IC50 values were in the lower 10^{-4} and upper 10^{-5} mol/l range. This represents an increase in cytotoxicity when compared with the unsubstituted Cp_2TiCl_2 and a significant increase when compared with the analogous *ansa*-bridged compounds. In comparison to their *ansa*-analogues, they show a similar series of increasing cytotoxicity, depending on the heteroaryl substituent. This would indicate that *N*-methyl pyrrole substitution increases cytotoxicity more than thiophenyl or furyl substitution on the cyclopentadienyl rings, the latter two being very similar for both the *ansa* and unbridged analogues. The structures and IC50 values are presented in Fig. 4.

In order to improve the bioavailability of the benzyl-substituted titanocenes, different substitution patterns known for their biological activity, such as the benzo [1,3]dioxole, methoxyethoxy phenyl and the morpholino group, were introduced into the molecules. The unbridged benzo[1,3]dioxole-substituted titanocene dichloride (**9**) was obtained in a yield of 59% as an orange solid and when tested on the LLC-PK cell line a disappointing IC50 value of 280 μM was determined [17]. The *p*-methoxyethoxy-substituted titanocene dichloride (**10**) was obtained in a yield of 67% as a brown solid. When this compound was tested, an encouraging IC50 value of 43 μM was determined [18]. By starting with the conversion of 4-(4-bromo-phenyl) morpholine into the morpholino-substituted fulvene, the corresponding titanocene dichloride derivative (**11**) was obtained in 61% yield as a red solid. This unbridged morpholino-substituted titanocene has an IC50 value of

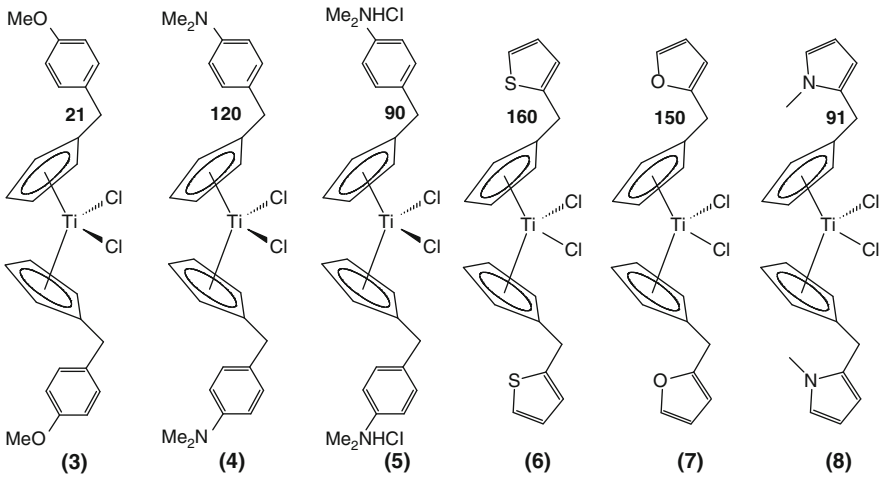


Fig. 4 Structures and IC50 values [μM] of benzyl-substituted **(3–5)** and heteroaryl-substituted **(6–8)** titanocenes on the viability of LLC-PK cells

25 μM and represents therefore, together with the *p*-methoxybenzyl-substituted titanocene **3**, one of the most cytotoxic unbridged titanocene dichlorides within this series [19].

Recently, fluorinated derivatives of Titanocene Y were synthesised; for these syntheses no direct fluorination is necessary, since commercially available benzaldehyde derivatives can be used. In the case of modifying the methoxy group to a trifluoromethoxy group in **(12),** an IC50 value of 7.3 μM was determined; introduction of fluoride into the 3 position of the phenyl group of Titanocene Y resulted in a water-insoluble titanocene derivative, while fluorination of the 2 position gave compound **(13)** with good solubility exhibiting an IC50 value of 6.0 μM [20]. All these Titanocene Y derivatives are shown in Fig. 5.

Another way of possible improvement of Titanocene Y is the exchange of chloride by another anion. With the help of anhydrous silver oxalate in THF, bis (*p*-methoxybenzyl)cyclopentadienyl titanium oxalate **(14)** (Oxali-Titanocene Y) is available in good yield; its structure is shown in Fig. 6. Oxali-Titanocene Y was found to crystallise in the orthorhombic space group Pbca (#61) with eight molecules in the unit cell [21]. The molecules show a distorted tetrahedral coordination around the central titanium atom with a Ti–O bond length of 199 pm, while the Ti–Cp(centroid) length is found to be 204 pm. The O–Ti–O angle of the distorted tetrahedron is to be only 79.0°, while the Cp(centroid)–Ti–Cp(centroid) angle is widened to 134.8° due to bulkiness of the cyclopentadienyl group.

Oxali-Titanocene Y is well soluble in DMSO/saline and shows an impressive cytotoxicity against LLC-PK with an IC50 value of 1.6 μM [21], which is a

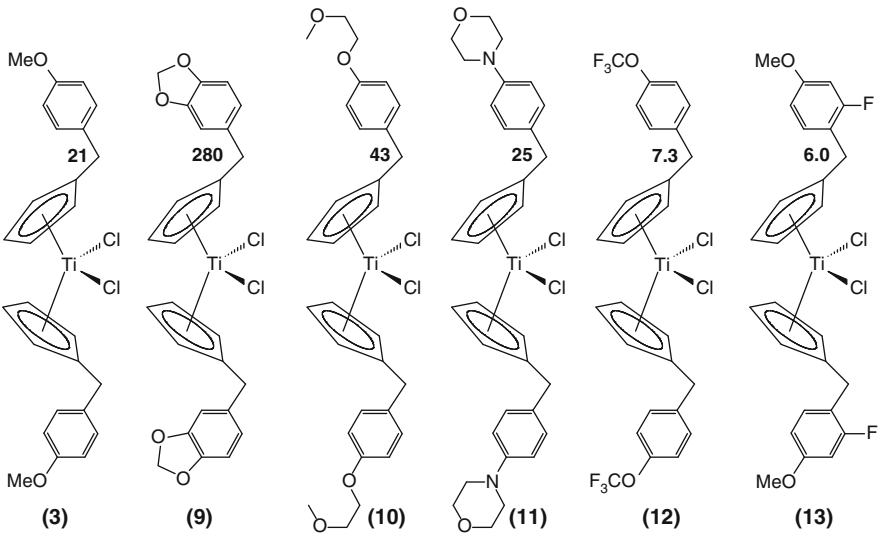


Fig. 5 Structures and IC50 values [μM] of benzyl-substituted titanocenes (**9–13**) on the viability of LLC-PK cells

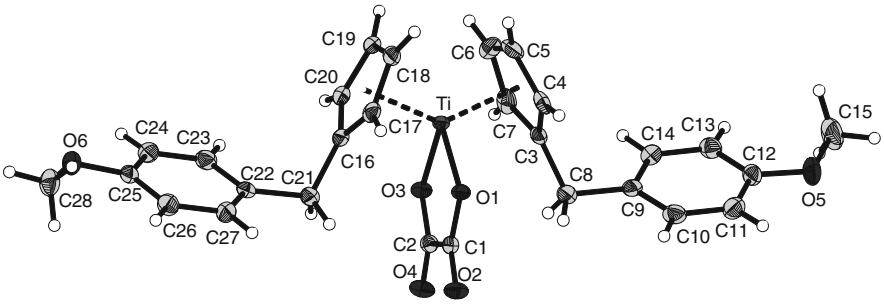


Fig. 6 Molecular structure of **14** (Oxali-Titanocene Y); (thermal ellipsoids are drawn on the 50% probability level) (after Fig. 1 from [21] with permission of Wiley-VCH Verlag GmbH & Co. © 2008 Wiley-VCH)

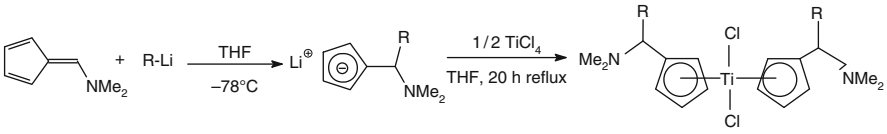
performance of a titanocene derivative better than cisplatin, which is seen for the first time on this specific cell line.

Hydridolithiation as a new synthetic approach to novel benzyl-substituted titanocene dichlorides allows the synthesis of substituted titanocenes, which are up to a 100-fold more cytotoxic than the unsubstituted Cp_2TiCl_2 itself. In general, these novel unbridged titanocene dichlorides were synthesised as solids in good overall yields of up to 77% and free of any stereo centres. They show IC50 values in the range of 280–1.6 μM with titanocenes **13** and **14** being the most cytotoxic ones. Furthermore, this represents a significant improvement compared with the analogous *ansa* compounds.

3 Chiral Mixtures of Titanocenes via Carbolithiation

Carbolithiation proved to be a very effective approach to highly cytotoxic titanocenes. The reaction of aryl or heteroaryl lithium species with 6-*N*,*N*-dimethylamino fulvene and subsequent transmetallation reaction led to dimethylamino-functionalised titanocenes with IC50 values of 6.8, 5.5 and 5.4 μM. Furthermore, this reaction proved to be tolerant to many substituents and to be a versatile tool for the synthesis of a wide variety of substituted titanocene dichlorides as seen in Scheme 3.

A first series was reported on the reaction of simple aryl lithium species with 6-*N*,*N*-dimethylamino fulvene and subsequent transmetallation. Thereby, it was possible to synthesise *para*-methoxy phenyl (**15**), *N*,*N*-dimethyl-phenyl (**16**) and benzo[1,3]dioxole phenyl-substituted (**17**) dimethylamino-functionalised titanocene dichlorides, which show IC50 values in the range of 54 μM [22]. This represents a significant improvement in cytotoxicity compared with the benzyl-substituted titanocenes by a factor of 3 for titanocene **16** (IC50 for the benzyl-substituted analogue: 120 μM) and even 10 for titanocene **17** (IC50 for the benzyl-substituted analogue: 280 μM). The sole decrease in cytotoxic activity was



Scheme 3 Synthesis of dimethylamino-functionalised titanocenes from the carbolithiation reaction of 6-*N*,*N*-dimethylamino fulvene and aryl lithium species

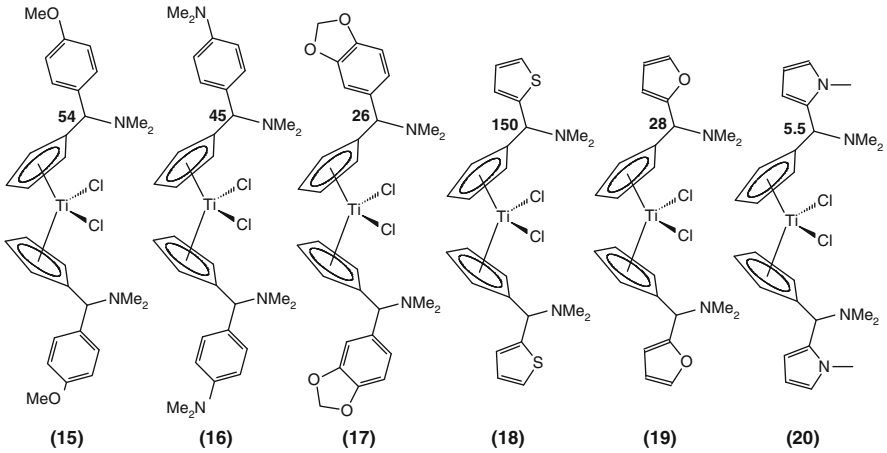


Fig. 7 Structures and IC50 values [μM] of dimethylamino-functionalised titanocenes (**15–20**) on the viability of LLC-PK cells

observed for the *para*-methoxy phenyl-substituted dimethylamino functionalised titanocene **15**, wherein the corresponding benzyl-substituted titanocene **3** affords an IC50 of 21 μM. The structures and IC50 values are presented in Fig. 7.

In line with previous experiments, a series of heteroaryl-substituted dimethylamino-functionalised titanocene dichlorides have been synthesised and evaluated [23], as shown in Fig. 7. Unfortunately, no uniform pattern compared with benzyl-substituted titanocenes was detected. A decrease in cytotoxicity was observed for the thiophenyl-substituted titanocene (**18**) from 150 μM (for the unbridged analogous compound **6**) to 240 μM. On the other side, a significant increase in cytotoxicity was observed for the furyl-substituted titanocene (**19**) (160–28 μM) and especially for the *N*-methyl pyrrole derivative (**20**), which exhibits an IC50 value of 5.5 μM. In fact, titanocene **20** is one of the most cytotoxic substituted titanocene dichlorides that have been reported up to now. This represents an impressive 400-fold increase in cytotoxic activity compared with the unsubstituted Cp_2TiCl_2, which reached Phase II clinical trials.

In a second series, the same heteroaryl lithium species were reacted with morpholino-substituted fulvene, meaning to provide a restriction in the possible coordination of the dimethylamino groups with the titanium centre together as well as increase the water-solubility of the molecule [24]. This led to three new

titanocene dichlorides **21–23** with again very mixed results in terms of the cytotoxic activity compared with the corresponding dimethylamino-functionalised titanocene. The thiophenyl-substituted titanocene **21** showed an increase in cytotoxic activity, whereas titanocenes **22** and **23** showed a decrease. Further substitutions, using imidazole and indole derivatives [25], provided no further clarification. Again, no general trend was seen with respect to the dimethylamino-functionalised analogues: although the imidazole-substituted titanocenes **24** and **25** were less cytotoxic, the indole-substituted titanocene **26** was more cytotoxic as seen in Fig. 8.

The use of different *ortho*-lithiated indole derivatives (*N*-methylindole, 5-methoxy-*N*-methylindole and *N*,*N*-dimethylaminomethylindole) led to novel titanocene dichlorides **27–29** with IC50 values of 71 μM, 37 μM and the impressive value of 8.4 μM for **28** (see Fig. 9). Again, this represents a nearly 250-fold increase in cytotoxic activity compared with Cp_2TiCl_2 [26].

These promising results led to the search for additional substitutions similar to purines, which possess a myriad of biological activities. As 7-Azaindoles are the most widely studied one nitrogen analogue of the indole ring system, with the substitution of indole C-7 by an sp^2 hybridised nitrogen providing a construct containing a hydrogen bond donor and acceptor in a rigid 3-atom arrangement, three such titanocene dichlorides encompassing this motif **(30–32)** were synthesised [27]. These novel titanocenes gave IC50 values of 87, 12 and 8.8 μM and are seen in Fig. 9.

Further lithiated small heterocyclic groups [2-thiazolyl, 5-*N*-methylpyrazolyl and 2-*N*(*N*,*N*-dimethylamino)methylimidazolyl] were the key reagents for the synthesis of novel *N*,*N*-dimethylamino-functionalised titanocene dichlorides **33–35**, which exhibit IC50 values of 61, 53 and 5.4 μM (see Fig. 10), when tested against LLC-PK [28]. This shows progress in respect to the best indole derivative and titanocene **35** with an IC50 value of 5.4 μM is next to the *N*-methyl pyrrole

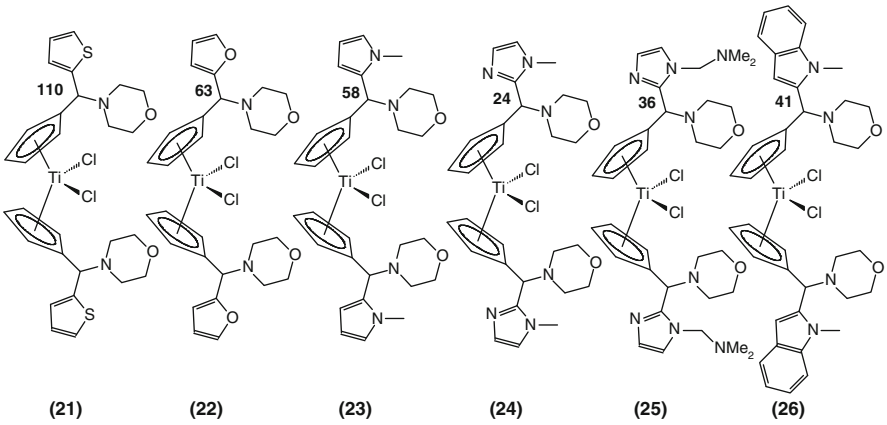


Fig. 8 Structures and IC50 values [μM] of morpholino-functionalised titanocenes (**21–26**) on the viability of LLC-PK cells

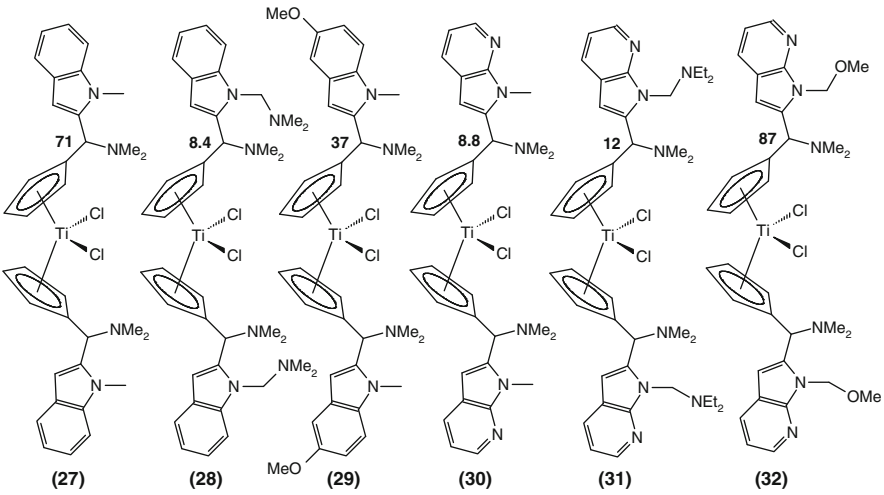


Fig. 9 Structures and IC50 values [μM] of indolyl-substituted titanocenes **(27–32)** on the viability of LLC-PK cells

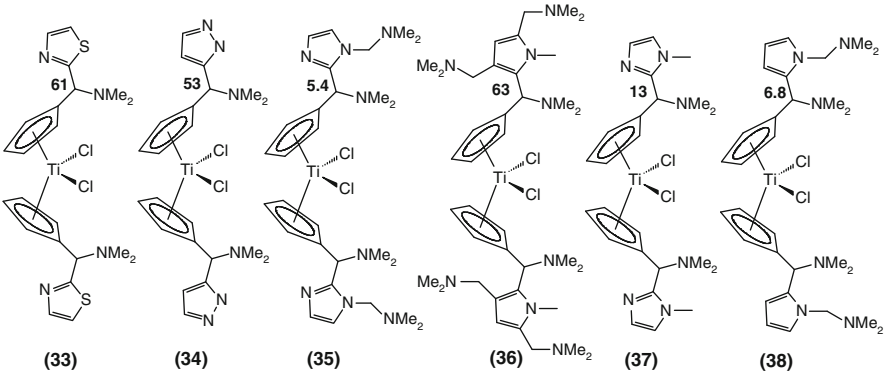
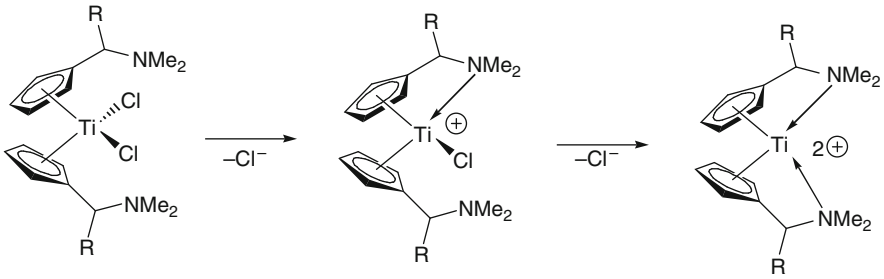


Fig. 10 Structures and IC50 values [μM] of dimethylamino-functionalised and heteroaryl-substituted titanocenes **(33–38)** on the viability of LLC-PK cells

derivative **20,** the second most cytotoxic dimethylamino-functionalised titanocene derivative published up to now.

The use of lithiated *N*-heterocyclic compounds, such as 2,4-[bis(*N*′,*N*′-dimethylaminomethyl)]-*N*-methyl pyrrole, 1-methylimidazole and *N*,*N*-dimethylaminomethyl pyrrole, gave access to novel *N*,*N*-dimethyl amino functionalised titanocenes **36–38** with promising IC50 values of 63 and 13 μM, and the very impressive value of 6.8 μM in **38**, which is illustrated in Fig. 10 as well [29].

N,*N*-dimethylamino-functionalised titanocenes presented within this review show in general an impressive high cytotoxicity in the range from 240 to 5.4 μM, depending on the substitution pattern. Compared with the unsubstituted Cp_2TiCl_2,



Scheme 4 Possible intramolecular stabilisation of mono-cation or di-cation

which reached Phase I/II clinical trials, this represents an up to 400-fold increase in cytotoxic activity and there are several compounds, which reach the target area of the lower micromolar range. This increase is believed to be the result of stabilising effects in titanocene mono- or dications through intramolecular coordination from the *N*,*N*-dimethylamino group, as visualised in Scheme 4, thereby increasing the possibility of DNA–titanocene interactions.

Unfortunately, the carbolithiation reaction of lithium species with 6-*N*,*N*-dimethylamino fulvene and subsequent transmetallation reaction leads to titanocenes with stereoisomers, as an equimolar mixture of the *R* and *S* form of the substituted lithium cyclopentadienide is formed. Subsequently, this equimolar mixture of *R* and *S* configured substituted lithium cyclopentadienide is used for the transmetallation reaction and therefore, a mixture of the 25% of *R*,*R*, 25% of the *S*,*S* and 50% of the *R*,*S* form of the chiral titanocene dichloride is obtained.

Unfortunately, the presence of stereoisomers does not allow this class of compounds to proceed directly into clinical trials. There is hope that future synthesis using chiral bases like sparteine may allow enantioselective preparation of titanocene dichloride derivatives and not only ferrocenes [30]. Additionally, further reaction pathways leading to achiral titanocenes have to be explored.

4 Achiral Titanocenes via Carbolithiation

In order to avoid the presence of stereoisomers, a further pathway including a carbolithiation reaction delivering achiral titanocene dichlorides was explored. Different aryl or heteroaryl lithium species were reacted with 6-bis-*N*,*N*-dimethylamino fulvene and therefore, the intermediate lithium cyclopentadienide and the resulting 6-bis-*N*,*N*-dimethylamino-functionalised titanocene dichlorides become achiral.

Recently, this was experimentally proven with *ortho*-lithiated furan, thiophene and *N*-methylpyrrole leading to the corresponding titanocenes with IC50 values of 270, 240 and 36 μM [31] as presented in Fig. 11. Compared with the analogous

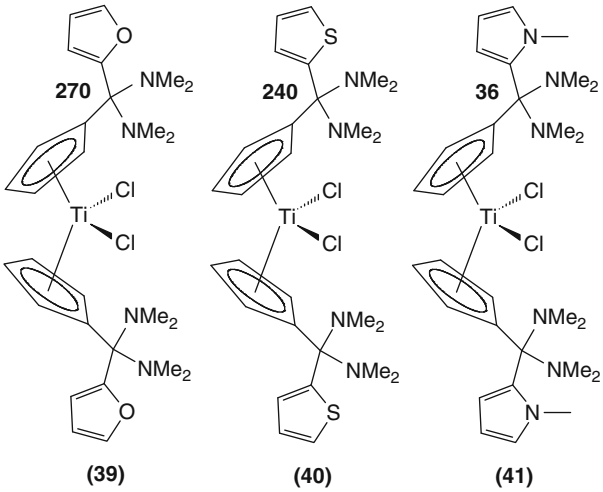
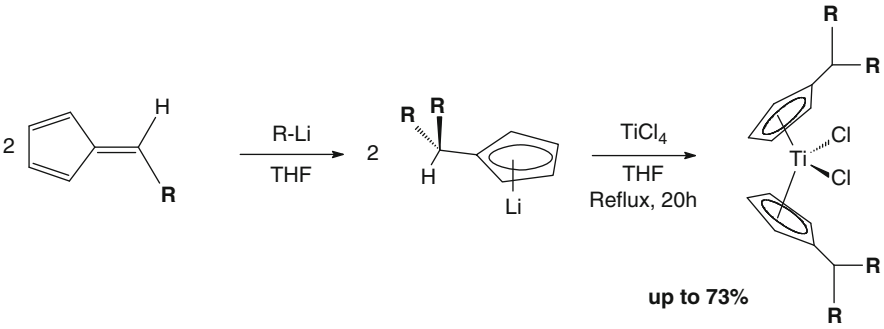


Fig. 11 Structures and IC50 values [μM] of 6-bis-*N*,*N*-dimethylamino-functionalised titanocene dichlorides (**39–41**) on the viability of LLC-PK cells



Scheme 5 Synthesis of achiral aryl and heteroaryl-substituted titanocenes from the carbolithiation reaction of 6-substituted fulvenes

6-*N*,*N*-dimethylamino-functionalised titanocene dichlorides, the new thiophenyl-substituted titanocene **40** shows similar activity, but the exceptionally high activity of the furyl- and *N*-methylpyrrole-substituted 6-*N*,*N*-dimethylamino-functionalised titanocenes **19** and **20** could not be repeated.

Within this review we describe a second reaction pathway, which includes a carbolithiation reaction and leads to achiral-substituted titanocene dichlorides also. Therefore, aryl lithium species are added to the identical substituted 6-aryl fulvenes. This leads to the formation of highly substituted, but achiral diarylmethyl-functionalised lithium cyclopentadienides as seen in Scheme 5, which can still be used in the transmetallation reaction with titanium tetrachloride.

This method was explored for the synthesis of diheteroaryl-substituted titanocenes (**42–44**). Cytotoxicity studies reveal IC50 values of 240, 140 and 32 μM, respectively [32]. The structures and IC50 values are presented in Fig. 12.

The titanocenes depicted below in Fig. 13 (**45–47**) showed IC50 values of 78, 45 and 38 μM (see Fig. 13), when tested against the LLC-PK cell line [33]. This series of diarylmethyl-functionalised titanocene dichlorides was again chosen in analogy to previously synthesised substituted titanocene dichlorides, such as benzyl- and 6-*N*,*N*-dimethylamino-functionalised titanocenes.

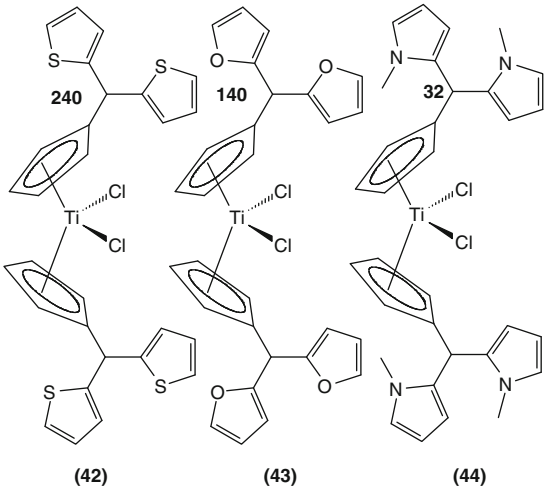


Fig. 12 Structures and IC50 values [μM] of achiral heteroaryl-substituted titanocenes (**42–44**) on the viability of LLC-PK cells

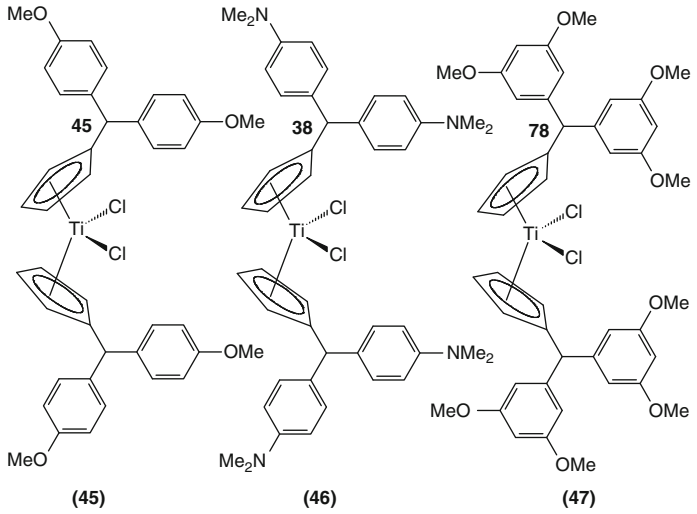


Fig. 13 Structures and IC50 values [μM] of achiral benzyl-substituted titanocenes (**45–47**) on the viability of LLC-PK cells

In summary, this novel approach to substituted titanocene dichlorides delivered compounds with a cytotoxic activity in the range of 240–32 μM, which represents an up to 60-fold increase in cytotoxicity compared with the unsubstituted Cp_2TiCl_2. The major advantage of diarylmethyl-functionalised titanocenes is the absence of stereo centres, and with further improvements, there are potential candidates for pre-clinical studies with potential for clinical trials.

5 Biological Evaluation

The cytotoxicity of substituted titanocene dichlorides is highly dependent on the substitution pattern and the structural elements. Two of the most cytotoxic titanocene dichloride derivatives synthesised using the fulvene methodology, with IC50 values of 5.5 (**20**) and 5.4 μM (**35**), included a dimethylamino-functionality. Very close to this target came two fluorinated Titanocene Y derivatives exhibiting IC50 values of 7.3 (**12**) and 6.0 μM (**13**), while Oxali-Titanocene Y (**14**) was the overall most cytotoxic derivative with an IC50 value of 1.4 μM, but does not contain chloride anymore. The structures and IC50 values are presented in Fig. 14.

A *p*-anilyl-substituted *ansa*-titanocene **48** and titanocene **3** were tested on the growth of a wide variety of tumour cells *in vitro* on a panel 36 human tumour cell lines containing 14 different tumour types investigated in a cellular proliferations assay [34]. Titanocene **3** showed a significantly higher cytotoxic activity than the *ansa*-compound and reached, on average over the whole cell panel, the activity of cisplatin within a factor of 4. Nevertheless, there were three main targets for titanocene **3** identified, which are pleura mesothelioma, uterine and renal-cell

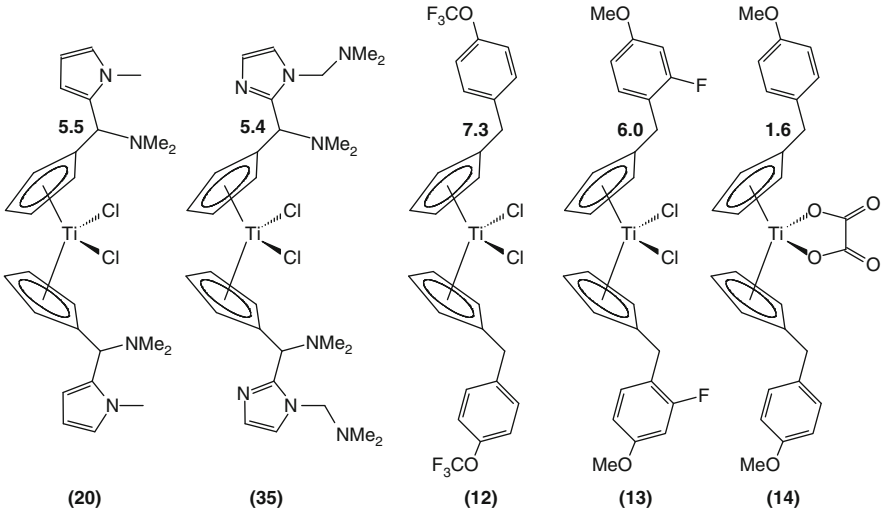


Fig. 14 The five most cytotoxic titanocene derivatives synthesised from fulvenes

cancer. Here, titanocene **3** showed a significant higher cytotoxicity than cisplatin. Furthermore, within the group of ovarian, pancreas, prostate and breast cancer, single cancer cell lines were identified which show comparable sensitivity for titanocene **3** and cisplatin.

A series of biomedical studies including ex vivo experiments and a mouse model have been undertaken using the *ansa*-titanocene **48** [35]. The ex vivo anti-tumour effect of *ansa*-titanocene **48** was studied against a total of eight freshly explanted tumours, using an *in vitro* soft agar cloning system. A concentration-dependent anti-tumour activity was observed for all samples, except melanoma. The highest activity was observed for renal-cell carcinomas and remarkable cytotoxicity was noted for the breast cancer cell MCF-7.

Furthermore, *ansa*-titanocene **48** was tested in a mouse model against Ehrlich's ascites tumour (EAT) [36]. This study showed that the treatment with *ansa*-titanocene **48** increases the lifespan of EAT-bearing mice from 25% to around 50% in a dose-dependent manner, and myelopoiesis (the formation of bone marrow or of blood cells derived from bone marrow) was not suppressed. Additionally, these experiments showed that *ansa*-titanocene **48** restores the natural killer cell function, which is reduced due to a dysfunction in EAT, and stimulates the natural killer cell-mediated cytotoxicity.

The benzyl-substituted titanocene **3**, which showed a significant higher cytotoxicity when tested *in vitro* against LLC-PK, was evaluated in a series of biomedical studies as well. Watson et al. tested titanocene **3** in comparison to two aryl-substituted *ansa*-titanocenes on prostate cancer cells, as advanced prostate cancer is not curable up to now [37]. Therefore, it was shown that all three titanocenes induced more apoptosis (programmed cell death, in contrast to necrosis) compared with cisplatin in a dose-dependent manner. Titanocene **3** had the most significant effect on the cell cycle and apoptosis.

Furthermore, the benzyl-substituted titanocene **3** was tested ex vivo against a range of freshly explanted human tumours, using an *in vitro* soft agar cloning system [38]. Titanocene **3** showed a remarkable high sensitivity against renal-cell cancer, ovarian, non-small lung and colon cancer. The good response with non-small lung and colon cancer was especially surprising. In contrast ovarian and renal-cell cancers were already predicted as good targets in the *in vitro* panel experiment performed by Kelter et al. [34].

Encouraged by these results the unbridged benzyl-substituted titanocene **3** was tested in two *in vivo* and additional ex vivo and *in vitro* experiments.

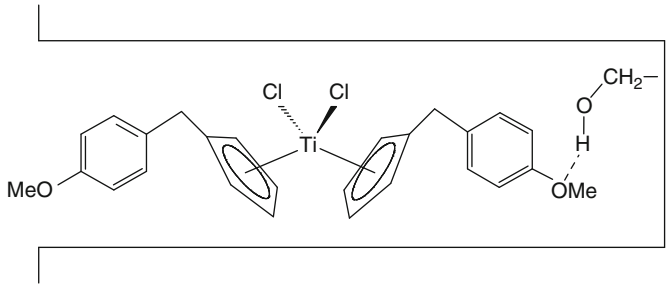
The first mouse model used was xenografted Caki-1 tumours in mice [39]. The Caki-1 mouse model represents human renal clear cell cancer, one of the prime targets identified for titanocene **3**, which was given *in vivo* in doses of 10, 20, 30, 40, 50 mg/kg on 5 consecutive days to Caki-1-bearing mice. The results showed a significant dose-dependent tumour growth reduction leading to a T/C value of 0.41 on day 38 of the experiment. The maximum tolerable dose (MTD) was determined to be 40 mg/kg per day and at this concentration it showed a superior activity compared with cisplatin, given at 2 mg/kg per day. Additionally, the *in vitro* test showed an IC50 of 36 μM for titanocene **3**, when tested against Caki-1 [39].

Recently, the caspase-dependent apoptosis triggered by titanocene **3** in epidermoid carcinoma cells A431 was shown *in vitro*. An *in vivo* study using the same cell line xenografted to mice revealed a tumour volume reduction by titanocene **3** identical to cisplatin leading to a T/C value of 0.40 on day 21 of the experiment [40]. This was followed by a xenograft experiment using the hormone-independent prostate cancer cell line PC3; this difficult to treat cancer showed a significant tumour volume reduction upon a 5-day treatment with 3 leading to an acceptable T/C value of 0.52 on day 21 of the experiment [41].

Important progress was made with respect to understanding the selective uptake of Titanocene Y (**3**) into cancer cells. In MCF-7 cell viability tests, **3** was found to be estrogenic in the absence and cytotoxic in the presence of serum albumin. In addition, it was shown that the cytotoxic effect was concentration-dependent with respect to serum albumin [42] and in a mass spectrometric investigation the binding of serum albumin and Titanocene Y could be established experimentally [43]. Further evidence for the binding mode came from molecular docking experiments [42] based on a crystal structure of an albumin–fatty acid complex; removal of an appropriately sized fatty acid molecule of palmitate allowed for the creation of a perfectly shaped binding site for **3**, which received additional stabilisation through a hydrogen bound involving the *p*-methoxy group of the anticancer drug molecule and a CH_2OH residue of a serin group from the protein as shown in Scheme 6.

Albumin binding is a particular advantage for an anticancer drug, because it is believed to exploit an inherent pathway for albumin receptor-mediated transport of drugs across the endothelial cell walls of blood vessels in tumours. This pathway is thought to be a means by which the malignant cell inherently supplies itself with nutrients and energy for rapid growth. And by doing so, the concentration of the transported anticancer drug is higher in cancer cells than in cells making up "normal" tissue, which finally can lead to the wanted selective apoptosis of cancer cells.

DNA is believed to be the most important cellular target of titanocene dichloride anticancer drugs; when **3** is reacted with a diorganophosphate a chloride-free 1:2 (Ti:P) adduct can be isolated, which shows coordination between titanium and the phosphate groups [44]. Recently, a molecular modelling study [45] was able to



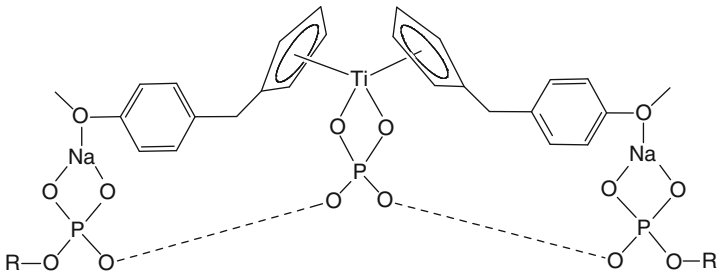
Scheme 6 Schematic coordination mode of titanocene **3** via a serin hydrogen bond in an albumin cavity

show, how titanocene **3** possibly interacts with its DNA target finally inducing apoptotic cell death. In a computational study using the force field method MM + **3** is reacted with its biological target, which is believed to be double-stranded DNA. It was found that after loss of two chloride ligands the substituted titanocene dication is able to coordinate strongly to a phosphate group by replacing a counter ion. In addition, the two *p*-methoxybenzyl groups have exactly the right length and flexibility to coordinate to two sodium counter ions bonded to two neighbouring phosphate groups, which allows **3** to become a chelating ligand strongly bonded to the surface of double-stranded DNA as seen in Scheme 7.

By doing so, the diameter and hydrophilicity of the coordinated DNA has changed significantly, which is believed to induce apoptosis of the treated cancer cell. And the mode of coordination is very different to platinum-based drugs, which are targeting the nitrogen bases adenine and preferably guanine in DNA. Therefore, it is hoped for that no cross-resistance is found for platinum- and titanocene-based drugs.

Furthermore, titanocene **3** has been studied in a very promising mouse model, using xenografted MCF-7 tumours in mice. In prior experiments titanocene **3** had been successfully tested ex vivo against freshly explanted human breast tumour cells [46]. The sensitivity of these breast tumour cells against **3** was highly remarkable over the full concentration range. Titanocene **3** showed cell death induction at the lowest concentration of 2.1 μM, well comparable to cisplatin. Therefore, for this *in vivo* experiment the human breast cancer MCF-7 cell line was xenografted into non-obese diabetic and severe combined immune-deficient (NOD/SCID) mice. Titanocene **3** was given for 21 days at 30 mg/kg per day, which represents 75% of the MTD and therefore for a longer period, which resulted in the reduction of the tumour volume to around 1/3 and not only the reduction of the tumour growth. At the end of the experiment a very promising T/C value of 0.32 was reached and this was the first time that using a substituted titanocene dichloride a shrinking of a tumour was observed.

Very recently, significant progress with respect to the mechanism of titanocene-based anticancer drugs was made. By employing human umbilical vein endothelial



Scheme 7 The principal coordination mode of the dication of titanocene **3** towards three neighbouring phosphate groups in DNA (after Fig. 2 from [45] with permission of Bentham Science Publishers Ltd. © 2008 Bentham)

(HUVE) cells, it was possible to measure the anti-angiogenic activity of several titanocene dichloride derivatives *in vitro*. These HUVE cells are grown to form spheroids, which after matrix embedding and polymerisation are stimulated to develop into vascular sprouts through addition of the vascular endothelial growth factor (VEGF). By inhibiting this sprouting through incubation with titanocene dichloride derivatives, it was possible to show that titanocene dichloride itself is a medium and Titanocene Y is a strongly anti-angiogenic compound associated with IC50 values of 19 and 4.9 μM, respectively [47], while another titanocene (**20**) was completely inactive. This experiment explains for the first time, why titanocene dichloride, which exhibits only medium to low cytotoxicity *in vitro*, is relatively efficient in xenograft mouse models *in vivo*; it is not the cytotoxicity of titanocene dichloride, but its effect on neo-vascularisation, which leads finally to reduced tumour volumes when compared with untreated controls. In addition, the success of **3** in the MCF-7 mouse model [46] is partly originating from the cytotoxic and partly from the anti-angiogenic effect; particularly, at long treatment times of 21 days the neo-vascularisation is expected to suffer, which leads to reduced tumour volumes.

The evaluation of our most cytotoxic derivative Oxali-Titanocene Y (**14**) (IC50 value of 1.6 μM against LLC-PK) led to an unexpected outcome *in vivo*. Before the xenograft experiment was performed, **14** was also tested against the proposed cell line CAKI-1 in an MTT-based cell test, which delivered an IC50 value greater than 100 μM, which means that the derivative had lost its antiproliferative effect against these specific cells. On the other hand, **14** still showed a significant anti-angiogenic activity in the HUVEC test, where an IC50 value of 14 μM was measured. Therefore, **14** was tested in the CAKI-1 xenograft mouse experiment [48] by giving it at 30 mg/kg per day on 5 consecutive days per week for 3 weeks and it induced a significant tumour volume growth reduction leading to a very good T/C value of 0.38 at the end of the experiment on day 40; this and all the other T/C values of the xenograft mouse experiments are shown in Fig. 15.

This is an experimental proof *in vivo*, that certain titanocene dichloride derivatives are cytotoxic, anti-angiogenic or both, which makes them Janus-headed

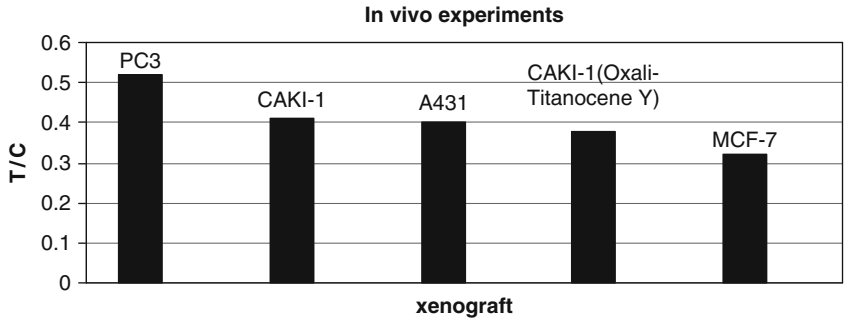


Fig. 15 T/C values for *in vivo* xenograft mouse models using the human cell lines PC3, CAKI-1, A431 and MCF-7 in connection with titanocenes **3** and **14**

bioorganometallic anticancer drug candidates for up-coming clinical testing most possibly against advanced renal-cell cancer for which no chemotherapy is available up to now.

6 Conclusions and Outlook

Reductive dimerisation of aryl fulvenes with titanium dichloride offers a general approach for the synthesis of *ansa*-titanocene dichlorides, which unfortunately show just a limited cytotoxicity. Carbolithiation of aryl or dimethylamino fulvenes followed by transmetallation delivered very cytotoxic titanocenes by reaching IC50 values of around 5 μM. Hydridolithiation of aryl fulvenes followed by transmetallation delivered titanocenes with IC50 values as low as 6 μM, but they do not contain stereo centres and therefore appear to be the better candidates for further evaluation. Moreover, anion-exchange derivatives of the titanocenes provide access to the most cytotoxic titanocene so far, with an IC50 value of less than 2 μM. As expected, the water solubility and cytotoxicity of titanocenes highly depends on the substitution pattern introduced by the fulvene. The mechanism of DNA coordination seems different with respect to cisplatin and encourages treatment of cisplatin-resistant cancer types. No myelosuppression, the activation of the immune system and anti-angiogenesis are strongly encouraging factors for the further development of fulvene-derived titanocenes. The substituted titanocenes show medium-high to high cytotoxicities in cell tests and are very promising in ex vivo renal-cell and cervix cancer experiments. In vivo data from EAT-bearing mice look promising and there are convincing results from A431, CAKI-1 and MCF-7 xenografts. Experiments in the nearby future will take the most promising candidates from the carbolithiation and hydridolithiation groups and derivatise them through anion-exchange to optimise their biological potential. It is believed that the chloride derivatives hydrolyses too quickly and are therefore not the optimal candidates for more advanced biological studies. Then this new class of titanocenes is close to clinical studies aiming for advanced and metastatic renal-cell cancer.

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Organometallics as Structural Scaffolds for Enzyme Inhibitor Design

Seann P. Mulcahy and Eric Meggers

Abstract Substitutionally inert metal-containing compounds provide new opportunities as structurally diverse and unique scaffolds for the design of protein binders. This review cites progress in this area by highlighting the use of metal complexes, including truly organometallic compounds, as inhibitors for enzymes of biological interest, such as esterases, proteases, and protein kinases. A common theme in all discussed examples is the use of the metal center as an anchor for the 3D display of organic ligands. While the metal center does not engage in any direct contacts to protein residues, it is the structural orientation of the ligands into previously unaccessible architectures that make metal complexes emerging candidates for bioactive agents with unique properties.

Keywords Enzyme inhibitors · Metal complexes · Organometallic

Contents

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1 Introduction

The identification of natural or synthetic compounds which can alter the function of biological targets, such as proteins and nucleic acids, is at the heart of medicinal chemistry. New technologies for drug discovery such as combinatorial chemistry, high-throughput screening, computer-assisted drug design, and virtual compound screening in silico have emerged during the last 20 years. Interestingly, despite these clearly extremely powerful techniques, the development of high affinity and specific compounds for a given target is still a great and often unsolved challenge. For example, not a single existing kinase inhibitor is completely specific for a particular kinase [1]. It is also surprising that while technical advances have emerged, the number of new drug launches by the top 20 pharmaceutical companies is not increasing despite more spending in research and development [2]. One limiting factor for this disappointing output can be found in a lack of structural diversity of scaffolds. For example, a recent publication revealed that the structural diversity of organic molecules, the main focus of pharmaceutical industry, is quite limited: half of the 24 million organic compounds registered in the Chemical Abstract Service (CAS) Registry can be classified with only 143 scaffolds [3]. Furthermore, the diversity of topological shapes of known organic drugs is surprisingly low: a report on the analysis of the Comprehensive Medicinal Chemistry (CMC) database demonstrated that out of more than 5,000 compounds analyzed, half of the known drugs fall into only 32 shape categories [4]. Most of the 32 frameworks contain at least two six-membered rings linked or fused together. Thus, current efforts for the design and discovery of bioactive compounds are limited to similarly structured scaffolds.

The pharmaceutical industry has relied predominantly on organic scaffolds for the design and discovery of drug candidates. However, metal-containing compounds should provide new opportunities for building 3D structures with unique and defined shapes, thus increasing the structural diversity and potentially leading to the discovery of molecules with unprecedented biological properties [5]. This article will discuss efforts into this direction. Whereas metal complexes are well established as structural scaffolds for the design of nucleic acid probes [6], their use as structural scaffolds for the design of protein binders, e.g., enzyme inhibitors, has been more or less ignored by the community of medicinal chemists and chemical biologist [5]. This article will try to demonstrate the enormous opportunities that arise from substitutionally inert metal-containing scaffolds for the design of protein binders, with a detailed discussion of a few truly organometallic compounds.

2 Metal Complexes as Structural Scaffolds

In this chapter, we will not cover work in which metal complexes are tethered to protein binders but only examples in which the metal center has a truly structural role in organizing the ligand sphere in the binding pocket of the target

protein [7]. Moreover, we limit ourselves to examples in which the metal center is believed to not being directly in contact with any residue of the protein pocket or active site.

More than 50 years ago, Dwyer systematically investigated in pioneering work the biological activities of metal complexes and, among others, discovered the bacteriostatic, bacteriocidal, and antitumor activities of chemically inert cationic polypyridyl complexes of ruthenium(II) [8–12]. Because of the high chemical and biological stabilities of such cationic ruthenium complexes, Dwyer concluded that their biological effects must be due to the cation as a whole and not to its individual components. Such ruthenium complexes also caused paralysis and death by respiratory failure when injected intraperitoneally into mice, and it was suggested that this was due to their direct inhibition of acetylcholinesterase (AChE). In fact, Dwyer et al. determined the inhibition constant (K_i) of racemic $[Ru(phen)_3]^{2+}$ for AChE to be 3.8×10^{-7} M with pronounced differences in the degree of inhibition between the Δ- and Λ-enantiomers. A structure–activity relationship revealed a strong dependence of the inhibitory potency on charge and hydrophobicity. These cationic ruthenium complexes thus most likely bind through a combination of electrostatic and hydrophobic interactions at the peripheral anionic site of AChE, which is located at the rim of the active-center gorge [13]. Mulcahy and Meggers recently followed up on this work and identified with the help of solid phase combinatorial coordination chemistry the complex **SPM1** to be a nanomolar inhibitor ($IC_{50} = 200$ nM) for AChE from *E. electricus* (Fig. 1a) [14]. Compared with the parent $[Ru(phen)_3]Cl_2$ ($IC_{50} = 10$ μM), this is an improvement by a factor of around 50-fold, just by modifying the periphery of this ruthenium polypyridine scaffold.

Tanizawa et al. designed Schiff base chelate complexes of Cu(II) and Fe(III) as inhibitors of the serine proteases trypsin and thrombin [15, 16]. The Schiff base ligands were obtained by condensing 4-formyl-3-hydroxybenzamidines or 3-formyl-4-hydroxybenzamidines with amino acids. Most interesting for the context of this review is a recently published cocrystal structure of bis(*p*-amidinosalicylidene-L-alanilato)iron(III) (Fig. 1b, **1**, $K_i = 1.6$ μM) with bovine β-trypsin. The structure revealed that the octahedrally coordinated iron is not involved in any direct interactions with amino acid residues in the active site but has a solely structural role. The phenylamidinium moiety of one of the tridentate ligands binds in the selectivity (S1) pocket, forming a salt bridge with the carboxylate of Asp189, in analogy to many organic inhibitors that target serine proteases with an arylamidinium pharmacophore moiety.

Chen et al. recently reported the stable ruthenium-oxo-oxalato cluster $Na_7[Ru_4(\mu_3\text{-}O)4(C_2O_4)_6]$ (**2**) (Fig. 1c) as a particularly potent inhibitor of HIV-1 reverse transcriptase (RT) with an IC_{50} value of 1.9 nM [17]. Apparently, the polyanionic cluster mimics polyanionic nucleic acid in its binding to HIV-1 RT through electrostatic interactions. The cluster shows promising anti-HIV-1 activity without being cytotoxic. In contrast to many polyoxometallates, this cluster has been demonstrated to be stable under physiological conditions.

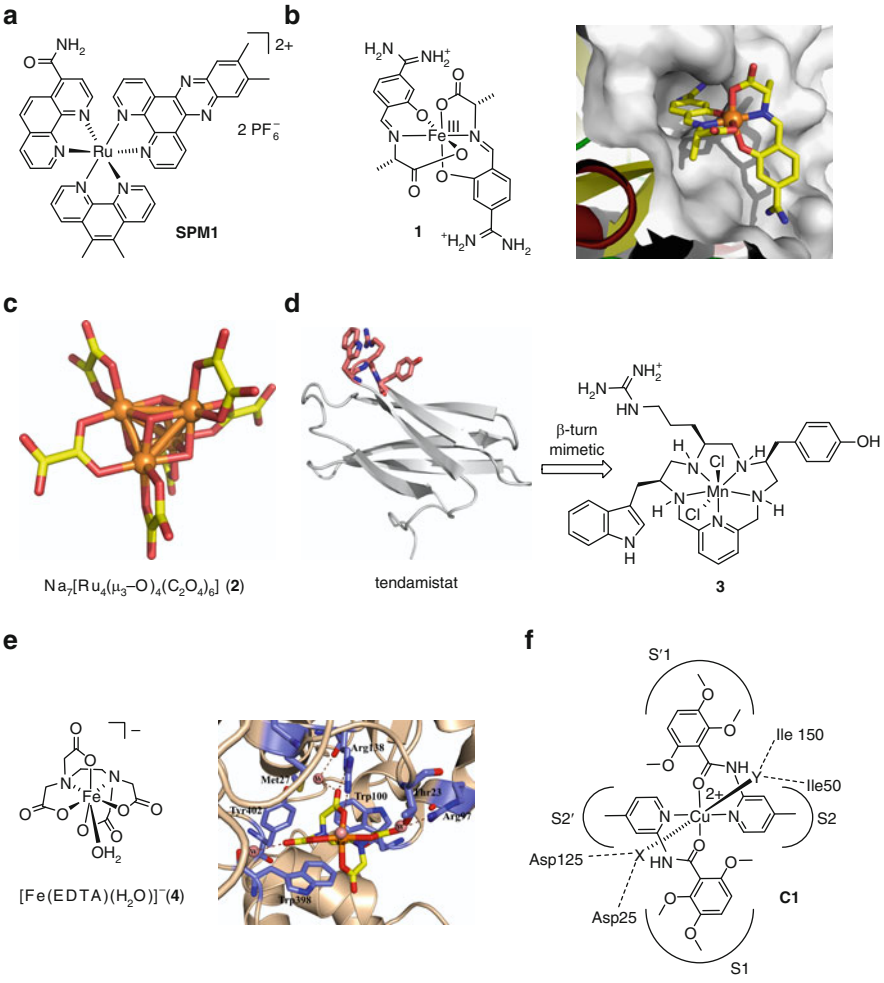

Fig. 1 Examples of metal complexes as structural scaffolds for enzyme inhibition: (**a**) Racemic ruthenium(II) polypyridine complex **SPM1** as an acetycholinesterase inhibitor (IC_{50} = 200 nM); (**b**) Bis(*p*-amidinosalicylidene-*L*-alanilato)iron(III) complex (**1**) as an inhibitor of the protease trypsin with a corresponding cocrystal structure from bovine β-trypsin (PDB code 1G3C); (**c**) Cluster $Na_7[Ru_4(\mu_3\text{-}O)_4(C_2O_4)_6]$ (**2**) as a potent inhibitor of HIV-1 reverse transcriptase (IC_{50} = 1.9 nM); (**d**) Manganese(II) azacrown (**3**) as a nonpeptidic tendamistat mimic for the inhibition of amylase; (**e**) Iron(III) EDTA complex (**4**) as a binder of the periplasmic nickel transporter protein NikA, along with a corresponding cocrystal structure (PDB code 1ZLQ); (**f**) Binding model for copper(II) complex **C1** as an inhibitor of HIV-1 protease

Marshall and coworkers proposed the utilization of metal complexes of chiral azacrowns (MACs) as a strategy to influence the conformation of these macrocycles and to fix the chiral side chains in orientations comparable with those of peptidic β-turns [18–20]. For example, the MAC complex **3** was proposed to serve as

a nonpeptidic inhibitor of amylase by mimicking the active reversed β-turn sequence Trp18-Arg19-Tyr20 of the proteinaceous amylase inhibitor tendamistat (Fig. 1d) [21, 22].

Fontecilla-Camps and coworkers found serendipitously that the heptacoordinated pentagonal bipyramidal iron complex $[Fe(EDTA)(H_2O)]^-$ (**4**) binds tightly to the bacterial periplasmic nickel transport protein NikA (Fig. 1e) [23]. A cocrystal structure revealed that the complex binds to NikA through a combination of hydrogen bonds, electrostatic, and hydrophobic contacts. For example, carboxylate groups of EDTA form hydrogen bonds with Arg97 and Arg137 and undergo additional water-mediated contacts. This is complemented by a number of hydrophobic contacts between methylene groups of EDTA and Met27, Tyr22, Trp100, and Trp398. What is particularly fascinating is that two tryptophans serve as wedges that complement the shape of the iron EDTA complex. It is noteworthy that the iron ion does not form any direct coordinative bond with amino acid residues of NikA residues and thus has mainly the purpose to organize the structure of the EDTA ligand. However, a cation-π interaction between the iron ion and Trp398, with an indole-to-metal distance of 5.5 Å, was postulated. Interestingly, since NikA has a substantially higher affinity for $[Fe(EDTA)(H_2O)]^-$ than Ni^{2+} ions, the authors suggest that a similar chelated ligand might be involved in the periplasmic Ni(II) transport by NikA.

Reboud-Ravaux and coworkers designed copper(II) complexes as substrate competitive inhibitors for HIV-1 protease (PR) [24, 25]. PR hydrolyzes viral polyproteins into functional protein products that are essential for viral assembly and subsequent activity, and PR has therefore been a prime drug target against Acquired Immunodeficiency Syndrome (AIDS). The active form of the enzyme is a C2-symmetrical dimer, in which the active site forms at the dimer interface. Reboud-Ravaux and coworkers, for example, reported the C_2-symmetrical complex **C1** as displaying a low micromolar K_i value for PR (Fig. 1f). Based on molecular modeling, it was suggested that the pyridyl group occupies the S2/S2′ pockets whereas the trimethoxybenzyl group occupies the larger S1/S1′ subsite. The copper ion would then be positioned to coordinate the catalytic water molecule in the active site and a second water ligand could interact with the flap residue Ile50. However, the high kinetic lability of these copper complexes renders them unsuitable for drug scaffolds.

These examples demonstrate the striking ability of metal ions to serve as structural centers that organize the otherwise organic ligands in the 3D space of a protein binding pocket. Their further development into new drugs hinges on their ability to elicit a desired response in vivo. Therefore, the kinetic inertness of the resulting metal-containing compounds is a crucial requirement (not fulfilled for compounds **1**, **2**, and **C1**). In addition, neutral charge is also a preferred property to assure the transport across cellular membranes (not fulfilled for compounds **SPM1**, cluster **2**, NikA binder **4**, and **C1**). Nevertheless, the complexes outlined in this chapter serve as an inspiration for the future design of molecular probes or drug candidates for a variety of diseases.

3 Organometallic Protein Kinase Inhibitors

3.1 Protein Kinases as Drug Targets

Protein kinases are currently one of the most intensively pursued classes of enzyme drug targets [26]. A variety of diseases such as cancer, diabetes, and inflammation are linked to the perturbation of protein kinase-mediated cell signaling pathways. The human genome encodes around 518 protein kinases which all share a highly conserved catalytic domain that catalyzes the transfer of a phosphate from ATP to a serine, threonine, or tyrosine residue of a substrate protein [27]. Although the ATP binding site is the major target for the design of kinase inhibitors, the conserved nature of this pocket renders the development of specific inhibitors a tremendous challenge [28]. For example, an in vitro study of the specificity of 28 commercially available organic protein kinase inhibitors, some with supposedly high specificity, revealed that all but two drugs had more than one protein target [29]. Clearly, new strategies are needed to solve the important problem of developing specific inhibitors for enzymes that are members of large and homologous enzyme families. Metal-containing compounds with their ability to build globular and rigid structures might be especially suitable scaffolds.

3.2 Staurosporine as an Inspiration for Organometallic Inhibitors

The Meggers group recently introduced a strategy for the design of highly potent and selective kinase inhibitors by modeling the design of organometallic kinase probes on the natural product staurosporine, a member of the class of carbohydrate-appended indolocarbazole alkaloids which have potent kinase inhibitory properties but suffer from poor selectivity [30]. Staurosporine binds competitively to the ATP-binding site of protein kinases through two key hydrogen bonding interactions with the hinge region and thus mimics the binding of the adenine base of ATP (Fig. 2). By using a metallo-pyridocarbazole scaffold depicted in Fig. 2, metal complexes were designed that fit into the ATP-binding site of protein kinases by mimicking the binding of ATP, staurosporine, and other typical organic inhibitors. The pyridocarbazole moiety retains the important structural features of the indolocarbazole ring that undergo the key hydrogen bonding with the hinge region of the ATP binding site, whereas additional ligands in the coordination sphere of the metal center are able to form interactions with other areas of the ATP binding site.

Through traditional medicinal chemistry approaches, including combinatorial chemistry and structure-based design, the Meggers group has developed a series of nanomolar and even picomolar protein kinase inhibitors. With the metallo-pyridocarbazole serving as the pharmacophoric model, half-sandwich scaffolds (e.g., **EA1**, **HB1052**) or truly octahedral complexes (e.g., **FL411**, **HB806**) (Fig. 3) were developed [31–42]. For example, cyclopentadienyl-CO half-sandwich

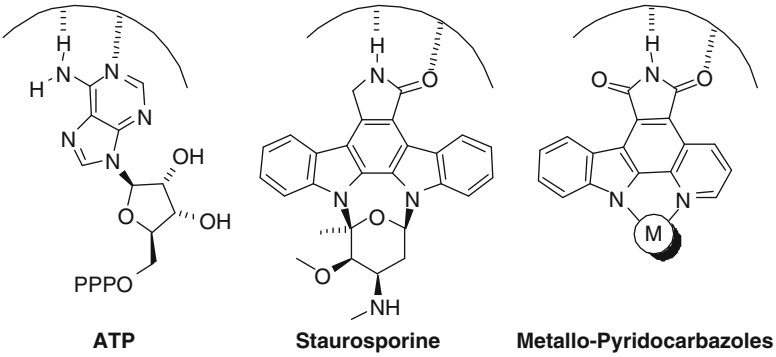

Fig. 2 Schematic representation of the hydrogen bonding contacts with the hinge region of the highly conserved ATP-binding site of protein kinases: the natural substrate ATP (*left*); the organic inhibitor staurosporine (*center*); a metallo-pyridocarbazole scaffold (*right*)

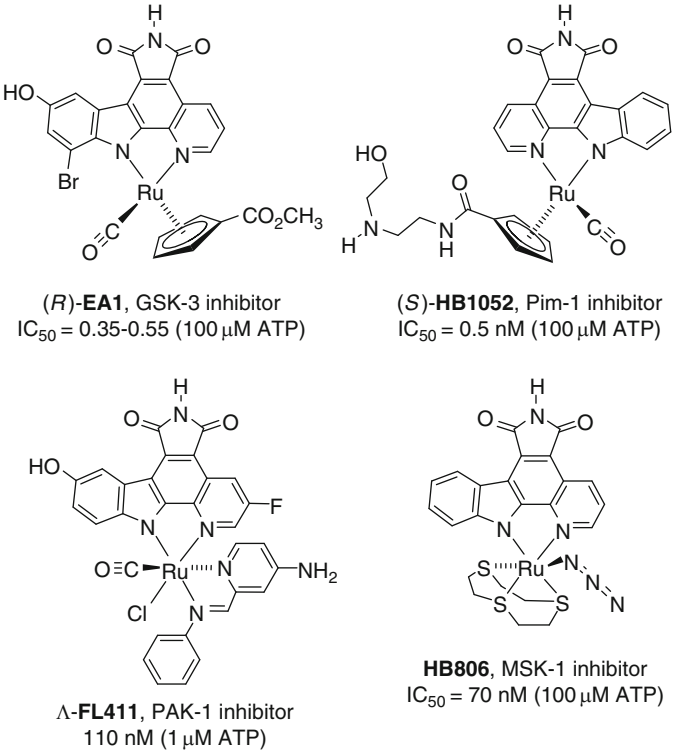

Fig. 3 A collection of potent organoruthenium protein kinase inhibitors based on the metallo-pyridocarbazole scaffold

complexes were identified as privileged lead structures for obtaining highly potent and selective inhibitors for the protein kinases GSK-3 and Pim-1 [31, 32, 34–36, 39, 41]. Such half-sandwich complexes display metal-centered chirality which, not surprisingly, confers potency and selectivity for these kinases dependent upon the absolute configuration at the metal in addition to any substitutions on the periphery of the half-sandwich scaffold. Whereas (S)-**HB1052** is very selective for Pim-1 with an IC_{50} of 0.5 nM at 100 μM ATP, it has only an IC_{50} value of 15 nM for GSK-3α (α-isoform) [34]. (*R*)-**EA1**, bearing an OH- and Br-group at the indole and a methylester at the cyclopentadienyl moiety, displays an IC_{50} of 0.35 nM for GSK-3α compared with only 35 nM for Pim-1. In fact, in a panel of 57 kinases, (*R*)-**EA1** is highly selective for just GSK-3 [32]. Such half-sandwich compounds are air-stable, moisture-stable, and can even withstand millimolar concentrations of thiols. The stability is due to the kinetic inertness of coordinative bonds to ruthenium. This, together with its cheap availability (ca. $20/g $RuCl_3$), its low toxicity, and its predictable and established synthetic chemistry, makes ruthenium one of the privileged metals for this approach. It is also noteworthy that such half-sandwich compounds have been demonstrated to inhibit their target kinases within mammalian cells, frog embryos, and zebrafish embryos [32, 41].

3.3 *Crystal Structures of Organometallic Compounds Bound to the ATP Binding Site of Protein Kinases*

Currently, seven structures of ruthenium half-sandwich complexes with protein kinases are available in the protein databank, four with Pim-1 (2BZH, 2BZI, 2OI4, 2BZJ), one with Pim-2 (2IWI), one with GSK-3β (2JLD), and one with PAK-1 (3FY0) [31, 36, 37, 39]. In addition, a cocrystal structure of an osmium half-sandwich complex with Pim-1 was published recently (3BWF) [38]. The Marmorstein group obtained two structures of half-sandwich complexes bound to the ATP binding site of the lipid kinase PI3Kγ (3CSF, 3CST) [42]. Finally, a first cocrystal structure of an octahedral organoruthenium complex with PAK-1 was published recently (3FXZ) [37]. In all these crystal structures, the metal fulfills a purely structural role and is not in direct contact with any residue in the active site.

As an example, Fig. 4 displays the crystal structure of the ultrahigh affinity ruthenium half-sandwich compound (R_{Ru},R_c)-**NP549** (Fig. 4a) bound to the ATP-binding site of GSK-3β (Fig. 4b), which is located in a deep cleft located between two lobes of the kinase domain [31]. **NP549** forms several hydrogen bonds within the ATP-binding site of GSK-3β (Fig. 4c). The maleimide moiety and the indole OH-group together establish three important hydrogen bonds to the backbone of the hinge region: one between the imide NH group and the backbone carbonyl oxygen of Asp133, a second between one of the imide carbonyl groups and the backbone NH of Val135, and a third between the backbone carbonyl oxygen of Val135 and the indole OH. The second carbonyl group of the maleimide moiety forms a

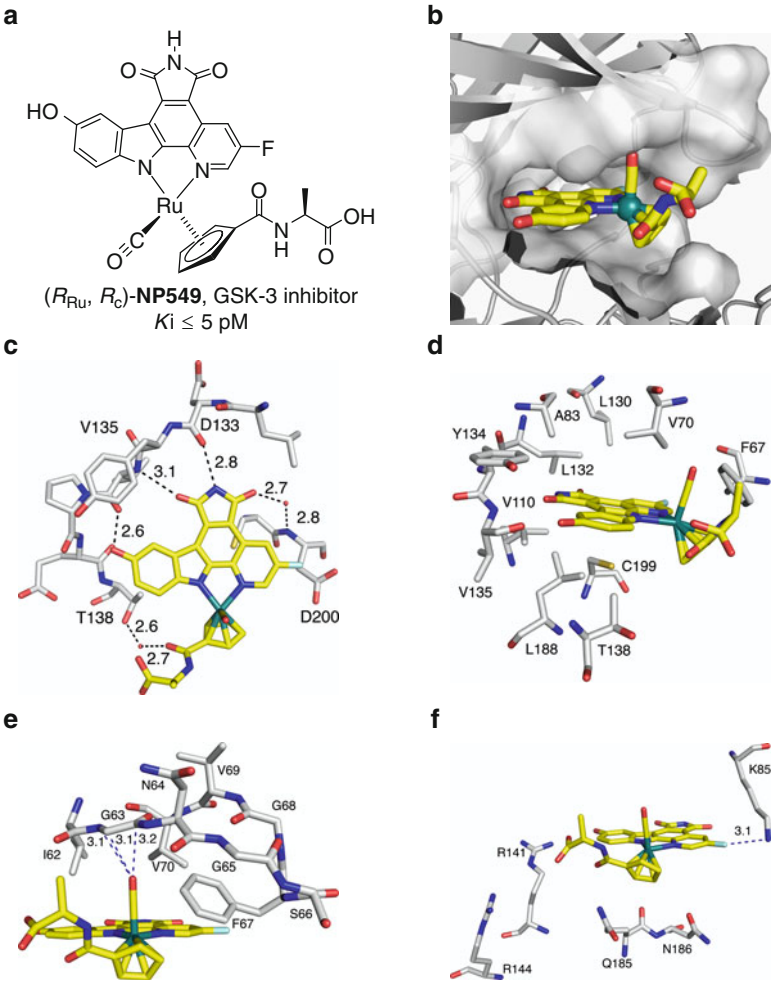

Fig. 4 Molecular interactions of an organometallic GSK-3 inhibitor with the ATP-binding site: (**a**) molecular structure of (R_{Ru},R_C)-**NP549**; (**b**) space-filling model of the active site surrounding the inhibitor; (**c**) hydrogen bonding contacts to the inhibitor; (**d**) hydrophobic interactions lining the walls of the active site; (**e**) close interactions of the CO ligand with the glycine rich loop; (**f**) weak F···H–N hydrogen bond between the pyridocarbazole and Lys85

water-mediated contact to Asp200. An additional hydrogen bond is established with the amide carbonyl group at the cyclopentadienyl moiety which is in a water-mediated contact to Thr138. Furthermore, the fluoride atom is at a close distance to the amino group of Lys85 (3.1 Å) which suggests a weak F···H–N hydrogen bond, whereas the carboxylate group is in electrostatic contact with the cationic Arg141 and Arg144 (Fig. 4f). **NP549** is also involved in extensive van der Waals contacts with GSK-3β. A hydrophobic pocket for the pyridocarbazole moiety is built by side chains from more than ten amino acids (Fig. 4d), whereas the CO

ligand stacks against the glycine-rich loop and is buried in a small pocket lined by Ile62, Gly63, and Phe67, a motif which appears to be crucial for affinity and selectivity for GSK-3β. Overall, NP549 perfectly complements the shape of the ATP-binding site and the ruthenium center is not in direct contact with any amino acid residue of the active site. With a K_i value of 5 pM or less, (R_{Ru},R_c)-**NP549** is one of the most potent protein kinase inhibitors reported to date and by almost four orders of magnitude more potent than the related natural product staurosporine (IC_{50} = 180 nM at 100 μM ATP), demonstrating that this organoruthenium structure is a privileged scaffold for the design of GSK-3 inhibitors [31].

3.4 Anticancer Properties of Organometallic Kinase Inhibitors

GSK-3 has been demonstrated to be involved in a variety of cellular processes, including sugar metabolism and Wnt signaling, and its misregulation has been shown to contribute to neurodegenerative diseases and diabetes [43, 44]. Although GSK-3 has received very little attention as a therapeutic target for cancer, several recent studies reported that the inhibition of GSK-3β induced apoptosis in colorectal cancer cells through a p53-dependent pathway [45, 46]. Further data suggest a possible mechanism for this apoptotic activity involving stabilization and upregulation of the tumor-suppressor protein p53 by GSK-3β inhibition [47, 48].

Considering this background, it is not surprising that **DW12**, being highly potent for GSK-3, Pim-1, and probably a few yet unidentified kinases, displays high cytotoxicity and apoptotic activity in cancer cells [40]. For example, incubation of the highly chemoresistant 1205Lu melanoma cell line with 1 μM **DW12** for 72 h kills more than 80% of the cancer cells. Detailed investigations revealed an apoptotic effect by strong activation of the p53 tumor suppressor protein. Intriguingly, replacing ruthenium for osmium in this organometallic kinase inhibitor scaffold afforded the isostructural complex **DW12Os** with an almost indistinguishable biological activity (Fig. 5) [38]. This is a unique example in which the replacement of a metal in an anticancer scaffold by its heavier homologue does not significantly alter the biological activity. This phenomenon can be explained by the almost identical 3D structures of the two complexes and their identical mode of action as protein kinase inhibitors. Thus, the role of the metal in **DW12** and **DW12Os** is solely structural and the biological effects of these compounds are due to the rigid and inert shapes of these complexes.

4 Conclusion

Metal-containing compounds provide new opportunities for designing unique and defined 3D globular structures and thus complement molecular diversity created by purely organic scaffolds. In this respect, a key requirement is the kinetic stability of

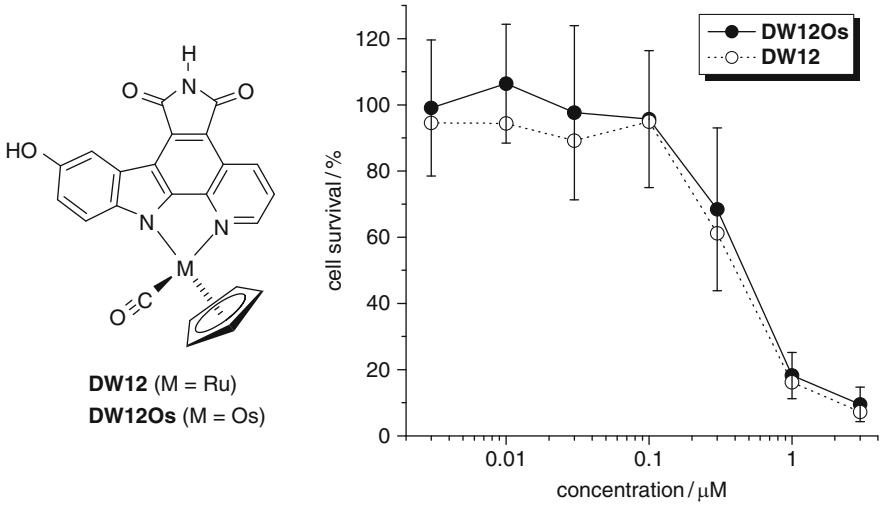

Fig. 5 Anticancer activities of the isostructural kinase inhibitors **DW12** (M = Ru) and **DW12Os** (M = Os) against the 1205Lu melanoma cell line. LC_{50} curves were determined using the MTT cell viability assay after treatment with the inhibitors for 72 h. An average of five independent experiments is shown

the coordination sphere in the biological environment. Aspects of metabolic stabilities of substitutionally inert metal complex in biological systems and corresponding potential metal-related side effects will be a critical issue to be investigated for the next generation of metal-containing compounds used in medicinal chemistry. Although not pursued by the pharmaceutical industry yet, it is only a question of time until inert metal complexes will be used routinely as structural scaffolds for the development of drugs.

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Bioorganometallic Chemistry and Malaria

Christophe Biot and Daniel Dive

Abstract This chapter summarizes recent developments in the design, synthesis, and structure–activity relationship studies of organometallic antimalarials. It begins with a general introduction to malaria and the biology of the parasite *Plasmodium falciparum*, with a focus on the heme detoxification system. Then, a number of metal complexes from the literature are reported for their antiplasmodial activity. The second half of the chapter deals with the serendipitous discovery of ferroquine, its mechanism(s) of action, and the failure to induce a resistance. Last, but not least, we suggest that the bioorganometallic approach offers the potential for the design of novel therapeutic agents.

Keywords Antimalarial · Bioorganometallic · Drug resistance ferroquine

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1 Introduction

1.1 Malaria: The Burden and the Problems

Malaria is the most common parasitical disease in the world [1]. Half of the World's population is exposed to this disease caused by a protozoan, *Plasmodium*, and transmitted by the female *Anopheles* mosquito. The clinical phase in human infection corresponds to the invasion of red blood cells (RBC) by the merozoïte form of the parasite; this follows a first phase of development of the sporozoïte in the liver after its injection by *Anopheles*. In the RBC, the parasite develops into a trophozoïte that internalizes and digests its host cell content, it then differentiates into schizont containing multiple merozoïtes which will invade new RBC. Two forms of malaria have a particularly severe prognostic: (1) cerebral malaria (mainly in young children), due to sequestration of mature parasites in the brain, the consequence of adherence of parasitized RBC to endothelium of cerebral capillary vessels, and (2) malaria occurring in pregnant women, due to mass sequestration of infected erythrocytes in placenta.

Among the five species currently recognized to infect humans, *Plasmodium falciparum* is the most deadly (the others being *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*). Among 2.37 billion people are exposed across 87 countries, malaria is the cause of 300–500 million clinical cases per year [2], but even though *falciparum* malaria is rife in nearly all tropical and subtropical areas of the World, Africa is currently the worst affected, accumulating (WHO 2004 statistics) 70% of total malaria clinical episodes which are the cause of 7% of the total number of deaths, 20% of direct child mortality (one child dies of malaria every 30 s) [3], 10% of indirect child mortality [4], and a severe prognosis for mothers and children when infection occurs during the first pregnancy [5, 6]. Of these fatal outcomes, malaria is the cause of many sequelae in the surviving patients, mainly in young children [5], and disability in adults during infectious episodes. The malaria burden,

expressed as DALYs (disability-adjusted life year), was ranked fourth out of all diseases for low-income countries in 2004, behind lower respiratory infections, diarrheal diseases, and AIDS [7]. Other heavy consequences of malaria include anemia that can increase child mortality and can markedly reduce power productivity in adults and a deficit in intellectual developments, which has consequences on the future evolution of development and productivity in concerned areas [8]. In lower income countries, malaria can represent 1–3% of Gross Domestic Product (GDP) per capita. As a result, malaria is a poverty impeding factor in developing countries that are affected by this disease [8]. To face such a challenge, the international financing was increased. The Global Fund to Fight AIDS, Tuberculosis and Malaria (GFTAM) and other supports committed US$ 991.5 million for malaria in 2007. However, among the 136 countries supported by this strategy since 2002, 16 of them, representing 710 million people living in stable transmission conditions, received an estimated combined annual support lower than US$ 0.5 per capita [9], which is not enough to fight efficiently malaria.

Vaccination, which would theoretically represent the best way to settle the problem of malaria, remains very far despite the large amount of money devoted to this research area in the last 40 years and the numbers of papers published, even if protection against malaria appears possible, based on the sum of observations collected. International organizations such as Programs for Appropriate Technologies in Health (PATH), the Malaria Vaccine Initiative (MVI), the European Malaria Vaccine Initiative (EMVI), and United States Aid (USAID) are involved in feasibility studies concerning all aspects of malaria vaccination; however, currently there are acute debates concerning acquisition of antimalarial immunity and optimization and design of a malaria vaccine [10].

An efficient fight against malaria mainly includes the control of the mosquito vector by indoor residual spraying, protection against bites by insecticide treated nets, chemoprophylaxis and chemotherapy of stated infection which gave promising results, mainly in low transmission rate areas, but failed to eradicate malaria in regions of intense transmission, even if progress was noticeable in several countries [5, 11–13]. However, the cost of these interventions is very high, representing up to 30% of domestic expenses in low-income countries and must be supported in a large part by international interventions (Multilateral Initiative on Malaria, Roll Back Malaria Project, GFTAM, Medicines for Malaria Venture), which have played an important role in the progress observed so far. Resistance, which spread both in *Anopheles* and *Plasmodium*, disproved the efficacy of the global fight program because low cost insecticides and drugs, the most interesting for concerned populations, are less and less efficient. For some insecticides as DDT, economical interests of concurrent products and environmental problems are adding to mosquito resistance problems. As a consequence, eradication programs were abandoned in the 1970s. With the extension of the resistance to chloroquine and some other low cost antimalarials, a resurgence of malaria occurred in many parts of the world, sometimes with devastating local epidemic episodes such as in Sri Lanka and Madagascar. High priority research includes development of new drugs for the treatment of malaria, the development of safe

molecules for mass drug administration in chemoprevention, the development of new insecticides for indoor residual spraying and long-lasting insecticide-treated bed nets, the development of vaccines with high transmission-blocking potential, improved access to treatment for poor populations, and cost efficiency of drug combinations [14].

Malaria chemotherapy has been based for a long time on a reduced number of drugs (mainly quinine, chloroquine, antimetabolites, and primaquine). The occurrence of resistance of *P. falciparum* to several of these antimalarials provoked the search for new active molecules. Various strategies were adopted to find new antimalarials that resulted in the industrial development of mefloquine, halofantrine, artemisinin derivatives, and several antibiotics. The spread of resistance to CQ and sulphadoxine-pyrimethamine and the decrease in susceptibility of the parasite to some of the new drugs increased interest in a new idea concerning the future of malaria chemotherapy, one interesting suggestion being the use of some already known drugs in Artemisinin Combination Therapy (ACT) to delay the occurrence of resistance.

Deciphering the *P. falciparum* genome gave access to thousands of potential new targets, as 60% of genes identified in the parasite have no equivalent orthologues in any other genome sequenced. But the validation of new targets is a long way away since it requires complex genetic reverse manipulations which can only be performed on a large scale in a few laboratories worldwide. After the validation of a target, the design of specific inhibitors is another time and money consuming step, since it requires a very fine knowledge of the target structure to design specific inhibitors. Moreover, adequate and practical methods to test a large numbers of molecules remain limited [15].

Out of the strategies targeting specific parasite probes, there are many methods used to search for new active molecules, including ethnopharmacology, medicinal chemistry, combinatorial chemistry and chemical libraries screening by high throughput screening, and drug design. These approaches have been the source of numerous potential antimalarials, but few molecules have been successful enough to enter in the queue for clinical trials.

1.2 The Digestive Vacuole of Parasite and Hemoglobin Digestion

When invading the RBC, *Plasmodium* has to solve multiple problems to successfully achieve its intraerythrocytic cycle: (1) keeping communication and exchanging tools with the extracellular compartment (necessary nutrients not being provided by its host cell); (2) to get the necessary space within the RBC to welcome it, siphoning off from the amino acids necessary for its growth; (3) to detoxify heme liberated during hemoglobin (Hb) digestion by forming hemozoin (also called malarial pigment); (4) to reorganize the surface of the RBC in order to escape to the host immune defenses (Fig. 1).

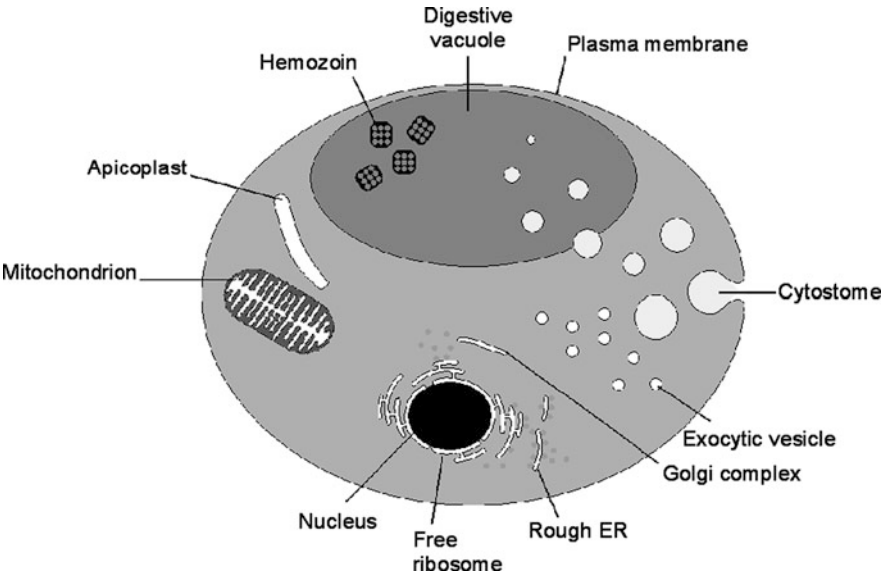

Fig. 1 Schematic representation of a malaria parasite

Digestion of Hb and heme detoxication in hemozoin appears to be very critical steps in the intraerythrocytic cycle. By providing necessary amino acids to the parasite [16], the digestion of Hb is crucial for equilibrium of osmotic pressure in the parasitized RBC in order to prevent a premature lysis before the completion of the cell cycle [17]. Such processes also provide space for the parasite to increase in size. If about 70–80% of the host cell Hb is digested by the parasite [18], only about 16% of produced aminoacids are used for protein synthesis [16, 19].

Hb digestion requires specialized structures which are organized by the parasite as soon as it enters the ring stage, but are modulated in importance throughout the cell cycle. Several mechanisms have been proposed for the capture of Hb by the parasite [20–24].

Recently, a primary process, called "big gulp" [21], occurring at the ring stage, has been proposed at the origin of the parasite digestive vacuole. The "big gulp" is different from classical macropinocytosis or phagocytosis, which is actin-dependent, as it is not affected by treatment by Cytochalasin D. Analysis of the evolution of "big gulp" structure suggests that it is at the origin of the food vacuole (containing hemozoin). Indeed, the "big gulp" goes before the food vacuole during the cell cycle, and the two structures cannot be observed simultaneously in the same parasite.

Cytostomal structures can be observed in the parasite shortly after invasion of the host cell [20–24]. The cytostome is an invagination of both parasite and parasitophorous membranes [23]. Currently, there is an active discussion about the role of actin in the formation of this structure [21, 23]. This is due to recent evidence of rab5, a marker associated with endosome formation and transport in

eukaryotes [21]. Cytostomes are formed throughout the erythrocytic cell cycle and often many are found in one parasite. Subsequent individualization of small vesicules containing Hb was observed by numerous authors [20–22, 24]. These vesicules appear at the ring stage [20] but their importance in Hb uptake becomes more important at the trophozoïte stage [21]. They are transported to the digestive vacuole and finally fuse with it. This process is actin and rab5-dependent. Digestion occurs in these structures because hemozoin can appear in them even if their fusion with the digestive vacuole is inhibited, indicating that they can acquire the necessary enzymes and factors to degrade Hb and to biocrystallize hematin [21, 24].

A cytostomal tube can be produced by the parasite, starting from a cytostome by elongation of small vesicles described above. Its formation requires actin intervention [21, 23]. Cytostomal tubes were thought to have an important role in Hb uptake by the parasite. Individual digestive vesicles were proposed to either bud off from the terminal portion of the tube or by the individualization of the different portions of the tube itself. Finally, the pigment vesicles were proposed to fuse rapidly to produce a "residual vacuole," where malarial pigment was supposed to be sequestrated [24]. Recent observations suggest a direct fuse of cytostomal tube with the food vacuole resulting from the evolution of the "big gulp" and not by individualization of vesicles [23], but these results are controversial [21]. Cytostomal tubes appear about 30 h after erythrocyte invasion and their global volume seems to be small, limiting their role in Hb uptake by the parasite.

Phagotrophy is a fourth means by which the parasite captures Hb. Many phagosomes appear at the trophozoïte and schizont stages. Unlike typical phagosome found in other eukaryotes, their formation is not actin-dependent and very similar in mechanism to the "big gulp" [21].

The four means of uptake occurred at different times during the erythrocytic cycle. Big gulp might take part in more than 90% of hemoglobin uptake at the ring stage, while small vesicules deriving from cystostomes and phagotrophy might have the prominent role in uptake during the trophozoïte and schizont stages [21]. Doubts remain about this hypothesis and concern the role of cytostomal tubes in uptake which might be more important than proposed [23].

Digestion of Hb engulfed in endocytic structures needs particular conditions which are of importance concerning the action of many antimalarials. Four aspartic proteases (falcipains), three cysteic proteases (plasmepsins), a metalloprotease (falcilysin), a dipeptidylpeptidase I, and probably aminopeptidases are involved in the digestion of Hb [25]. Hb is primarily attacked by plasmepsins and falcipains and cleaved into smaller fragments. These fragments are hydrolyzed by falcilysin in small peptides, and finally aminoacids were liberated by dipeptidylpeptidase and aminopeptidase. Inhibitors of the falcipain and plasmepsins impair or kill the parasite in vitro and in vivo, but their specificity is not strict. Knockouts experiments on several of these enzymes, individually or in combination, demonstrated that in normal culture conditions *P. falciparum* is able to acquire the important parts of the aminoacids necessary from its environmental medium (isoleucine, which is absent in Hb is entirely taken up from parasitized RBC outside compartment) as a redundant way with Hb degradation. Such experiments also showed that some

proteolytic enzymes have an overlapping role in Hb digestion and, for these reasons, plasmepsins do not represent valuable drug targets [26]. It must be noted that if Hb degradation is strictly necessary for parasite survival [26], it is not restricted to the use of produced amino acids for nutrition, but also to make room for itself [16, 19] and to maintain the osmotic pressure required to avoid lysis of the infected RBC [16, 17]. All enzymes involved in Hb digestion are more active at a pH less than 5. Consequently, the DV is an acidic compartment in the parasite and represents a primary interest in the mechanism of action of many schizontocidal antimalarials.

During hemoglobin degradation, free heme, ferriprotoporphyrin-IX or Fe(II) PPIX (Fig. 2) is released in the digestive vacuole. The toxicity of heme to the parasite has been demonstrated [27–30]; it is supposed to cause the disruption of metabolic functions by means of peroxidation of membranes and inhibitions of enzymes via the generation of oxidative free radicals [31].

Detoxification of the heme is achieved by hemozoin formation via a biomineralization process [32] and not via a polymerization process as previously envisaged. First, the iron II in Fe(II)PPIX is oxidized to iron III in Fe(III)PPIX or hematin (Fig. 3)

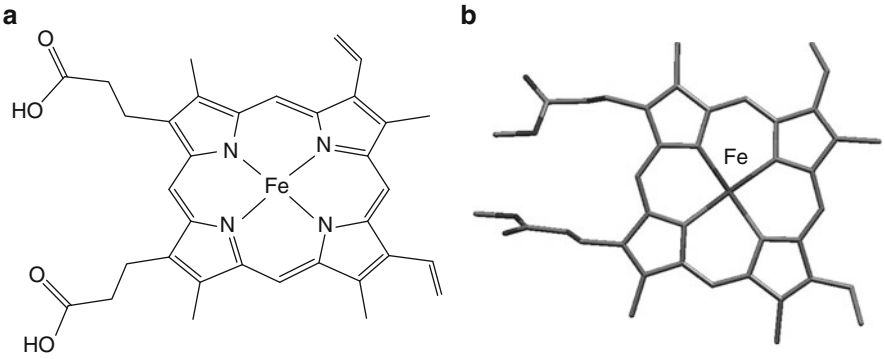

Fig. 2 (**a**) Chemical and (**b**) molecular structure of heme

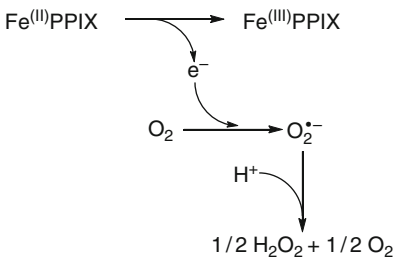

Fig. 3 Oxidation of heme in hematin

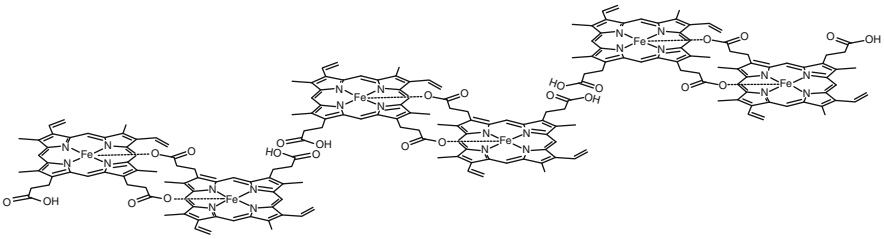

Fig. 4 Chemical structures of hemozoin

These molecules of Fe(III)PPIX are then linked into dimers through reciprocal iron-carboxylate bonds to one of the propionic side chains of each porphyrin, and these dimers form chains linked by hydrogen bonds (Fig. 4, [33, 34]).

A crystal is formed and its characteristics have been recently defined and discussed concerning the mechanism of action of antimalarials interacting with hemozoin formation [35, 36]. With the mechanism of biomineralization, the low soluble hemozoin is thus removed from the biological environment of the parasite.

However, if some doubts are remaining on how β-hematin is formed in the malaria parasite, recent results have shed light on the mechanisms involved. Among the three proposed hypotheses to explain catalysis or nucleation of the hematin biocrystallization, the role of histidin rich protein 2 (HRP2) [37] is now called into question based on: (1) the localization of the protein and the capability of HRP2 knockout parasites to form pigment [38] and (2) the fact that organisms lacking HRP2 are still able to produce a pigment similar to hemozoin [39]. There is now clear evidence that the formation of hemozoin occurs in a hydrophobic environment within or at the surface of lipids in *Plasmodium* and in other organisms producing hemozoin [40–42]. Experimental works have confirmed this hypothesis [43–47], and that the activity of some antimalarials is in clear relation with an interaction with parasite lipids [48]. The intervention of specific enzymes in biocrystallization of hemozoin has been largely discussed since the observation of heme polymerase activity in parasite extracts [49, 50]. The difficulty of purifying a protein showing such activity, the demonstration that biocrystallization could occur in absence of protein [51], and the discovery of the role of lipids have diverted interest for this hypothesis. Recently, the gene of a heme detoxification protein (HDP) was characterized. Both the recombinant protein and the authentic protein purified by immunoprecipitation were shown to interact with heme with high affinity (more than four times that of HRP2) and to catalyze very efficiently the biocrystallization in β-hematin. The catalytic activity of the protein is optimal at pH 5.2 or lower. After its synthesis, the protein seems to be exported into the infected RBC cytoplasm and is internalized again at the same time as Hb by the parasite. Knockout experiments showed that in *P. falciparum*, the hdp locus could not be mutated and authors concluded from the results obtained that the gene was certainly critical for parasite survival [52]. This protein appears well conserved within the *Plasmodium* genus [53]. The fraction of total hemozoin related to HDP activity is still under discussion.

1.3 Drug Resistance

Resistance to CQ was first reported in Columbia and Thailand in the early 1960s and has now spread worldwide. It was only in 1987 that a capital observation concerning CQ resistance was done. The Krogstad's group [54] showed that: (1) CQ-resistant parasites accumulated significantly less CQ than sensitive one; (2) this decrease in accumulation was due to a more rapid release of CQ in resistant parasites (40–50 times); (3) this accelerated efflux was slowered or inhibited by calcium channel blockers such as verapamil (Fig. 5), dilthiazem, or TMB-8 and by vinblastin and daunomycin whereas a calcium ionophore such as A23187 was not active; (4) none of these drugs affected the CQ accumulation or efflux in sensitive parasites; (5) reversion phenomenon was dependent of the acid pH of the digestive vacuole. Authors pointed out the similarity between their observations and the reversion of resistance in multidrug-resistant cancer cells which is affected in the same manner as verapamil.

In fact, at this time, two main research methods were developed independently to search the origin of CQ resistance in *P. falciparum*.

The route of multidrug resistance led to the discovery and characterization of *pfmdr1* gene and then to other transporters [55]. If *pfmdr1* was proposed to be at origin of CQ resistance, first by gene amplification [56, 57] then by polymorphim of the gene [58], field observations did not confirm this hypothesis. Nevertheless, it was shown that *pfmdr1* amplification and polymorphism were involved, possibly in association with other genetic factors [59], in the modulation of susceptibility to several antimalarials (mefloquine, halofantrine, quinine, and artemisinin) in fields studies, and in transfected parasites [60–64]. Recent studies using transfection of *Xenopus leavis* oocyte provided direct evidence of the transport of antimalarials by pgh1, the *pfmdr1* gene product [65], conducting to a new interpretation relation between pgh1 and CQ transport [66].

The other route resulted from a genetic cross between CQ-susceptible and a CQ-resistant clones of *P. falciparum* and of the genetic analysis of progeny [67]. Results showed that: (1) resistance was linked to accelerated efflux of CQ outside the parasite and susceptible to verapamil; (2) resistance level was independent from phenotype and copy number of *pfmdr1*; (3) the locus for resistance was situated on chromosome 7 and not on chromosome 5 where *pfmdr1* was found. Starting from these results, Wellems et al. completely dissected the 400 kb area suspected to contain chr 7 and finally selected a 47.5 kb area containing 13 genes, which were analyzed. The first gene (*cg2*) postulated to be at the origin of CQ resistance [68]

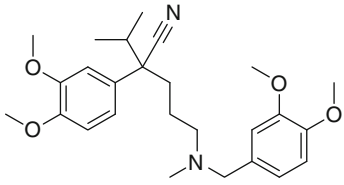

Fig. 5 Chemical structure of verapamil

was not confirmed in many epidemiological studies and by transfection experiments [69]. The discovery of a 14th gene (*pfcrt*) coding for a protein localized in the digestive vacuole membrane and carrying specific mutations in CQ-resistant parasites provided one key capital of CQ resistance [70]. The role of PfCRT protein mutations in CQ resistance was rapidly confirmed on field isolates [71, 72].

Mutations of PfCRT have been described in all CQ-resistant *P. falciparum* isolates. All CQ-resistant isolates share a common mutation at position K76T, but this mutation is never present in the gene of resistant parasites alone, in which at least 19 other points of mutations have been found [73]. Two major haplotypes were found, and several studies showed that the level of CQ resistance was associated with haplotypes [73, 74].

The mechanism by which PfCRT confers resistance to chloroquine is still under discussion [75]. The first aspect concerns the nature of the protein: whether PfCRT, postulated to possess ten transmembrane helices, is considered as a member of the drug-metabolite transporter family of proteins [55, 76–78]. Some authors discuss about the channel nature of the protein [79, 80]. It has been suggested that the change of the charged lysine to uncharged threonine affects the electrostatic interaction with the diprotonated CQ [81]. In the mutated forms, the absence of electrostatic interactions allows the drug to cross the channel (Fig. 6). This results in the efflux of the drug out of the DV [81]. Note here that the reduced accumulation can be partially reversed by verapamil, a lipophilic compound (Fig. 5).

Other experiments have shown that the mutation K76T is not the only factor to modulate the action of PfCRT. The Cooper group [82] demonstrated that mutations in transmembrane domains 1, 4, and 9 altered susceptibility to CQ, quinine, and quinidine. Photoaffinity labeling of PfCRT with a novel perfluorophenylazido CQ [83] provided new information concerning interaction of the drug with the protein. Mutations conferring CQ resistance increased the CQ-associated H^+ leak in the

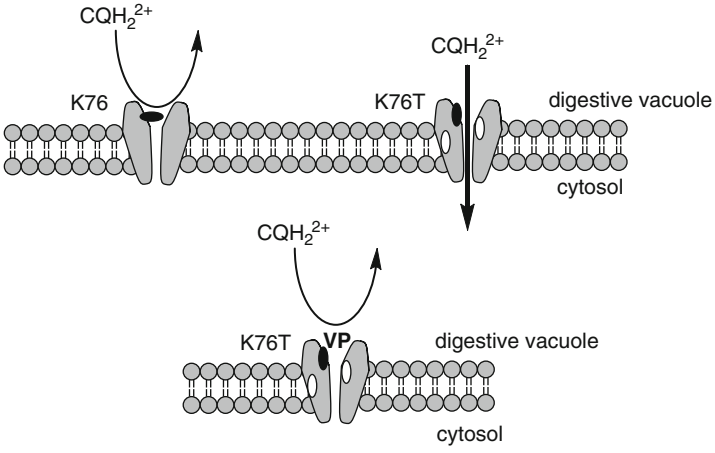

Fig. 6 Schematic representation native and mutant (K76T) PfCRT. Postulated mechanism of the efflux of charged CQ reversed by verapamil

parasite digestive vacuole [84]. Recent observations have shown that PfCRT may exert its protective role even in erythrocytic stages when hemoglobin digestion and production of pigment is not very active, as in rings and late schizonts [85]. On the other hand, we should keep in mind that CQ resistance has a biological cost for the parasite. In some geographical areas, the abandonment of CQ use results in resurgence of susceptible phenotypes [86, 87]. As PfCRT is considered by some authors as a metabolite/drug transporter, it can be expected that mutations in such a protein can modify the physiology of parasite. Comparison of transcriptomes of clones carrying wild type PfCRT, with clones carrying a single mutation in the gene showed that the mRNA level from 45 genes was modified compared with the wild type parent. Most of the up-regulated genes are involved in invasion, cell growth and development, signal transduction, and transport activities. Some genes encode proteins involved in transport and/or regulation of cytoplasmic or compartmental pH [88].

Current knowledge is in favor of intervention of more than one gene in modulation of CQ resistance, even if PfCRT appears to play the main role. It seems to be difficult to simultaneously target several genes to overcome CQ resistance, especially when some of these factors remain to be precisely identified. However, the reversal of Q resistance, by verapamil and related drugs, is a common characteristic of all CQ-resistant strains of *P. falciparum* and open ways for future therapeutics [89, 90].

2 Metal Complexes as Antimalarials: An Overview

Due to the success of *cis*-platin in anticancer therapy, medicinal chemists have rapidly begun to evaluate the utility of metal complexes as antimalarials [91–94]. We will not report all of the bioinorganic compounds that have been synthesized and tested. In the following, we will just focus on the most representative agents. For example, we will not review the metal chelators.

In order to exploit the antimalarial activity of CQ, coordination of CQ by different metals has been explored. The triphenylphosphinegold(I)–CQ complex (Fig. 7) was found to have a ninefold higher activity in vitro than CQ against FcB1 and FcB2 Colombian CQ-resistant strains of *P. falciparum*. The complexation of Au to CQ also increased the in vivo susceptibility of *P. berghei* to CQ [95].

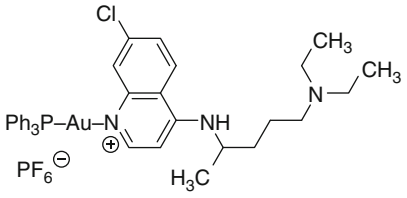

Fig. 7 Chemical structure of the gold CQ complex

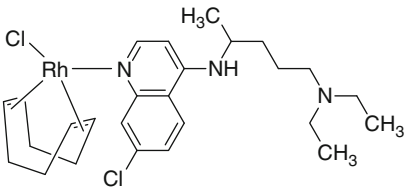

Fig. 8 Chemical structure of the rhodium CQ complex

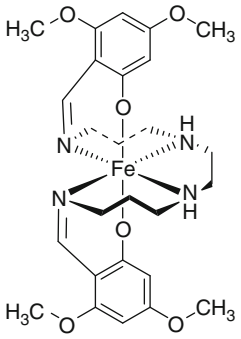

Fig. 9 Chemical structure of the schiff-base phenolate iron complex

To the contrary, coordination of CQ by the organometallic moiety Rh(COD)Cl (Fig. 8) did not significantly alter the in vitro antimalarial activity of CQ. In in vivo tests, equivalent concentrations of free CQ reduced the parasitemia by 55%, while for the complex CQRh(COD)Cl the reduction reached 73% without any sign of acute toxicity being observed up to 30 days after treatment [96].

Schiff-base phenolate metal complexes (Fig. 9) have been shown to inhibit both HB3 CQ-susceptible and FCR3 CQ-resistant parasites and correlate in a free metal-independent manner with their ability to inhibit hemozoin formation in vitro [97].

More recently, the synthesis of an amine–phenol complex of gallium(III) was described (Fig. 10). The antimalarial activities were in the micromolar range against the HB3 CQ-susceptible and Dd2 CQ-resistant strains. The authors proposed that the cytotoxic targeting properties of these kinds of metal complexes lie in the spatial orientation of substituents on the peripheral part of the molecule [98].

2.1 Ferrocenic Molecules with Antimalarial Properties

The synthesis of ferrocenyl sugars as exemplified by Fig. 11 and their antimalarial activities have been reported. Whereas the organic parent compound, an ellagitannin derivative, trideca-O-methyl-α-pedunculagin, displayed no antimalarial activity, the diferrocenyl glucoside showed inhibitory activity similar to that of quinine against the FCR3 CQ-susceptible strain [99].

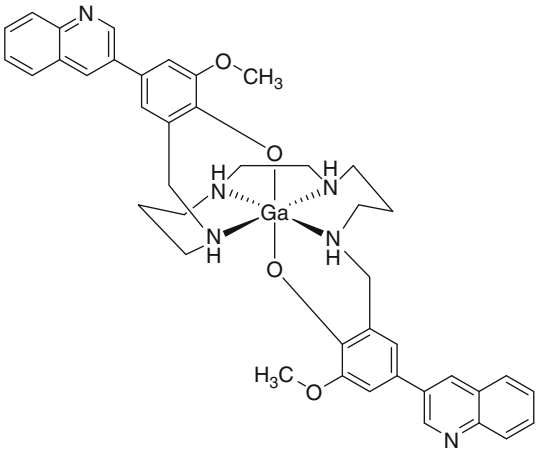

Fig. 10 Chemical structure of the amine–phenol gallium complex

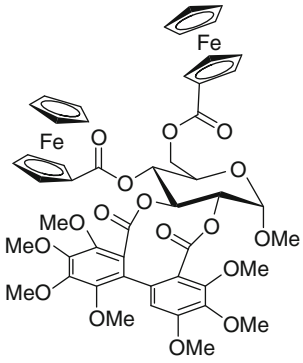

Fig. 11 Chemical structure of the ferrocenyl sugar

A series of ferrocenyl chalcones as exemplified by the two compounds in Fig. 12 were evaluated for in vitro antimalarial activity against the K1 CQ-resistant strain. Disappointing low activities in the micromolar range were reported [100].

The results showed that the location of ferrocene influenced the ease of oxidation of iron II and the polarity of the carbonyl linkage. These parameters were found to influence antiplasmodial activity. Better antimalarial activities were obtained when the ferrocene moiety is adjacent to the carbonyl group [101].

In order to exploit the cationic redox behavior of methylene blue as antimalarial agent, new ferrocene benzimidazolium conjugates (Fig. 13) have been designed, synthesized, and tested against the *P. falciparum* malarial parasite strain NF54. Compound with a thiophene moiety as R group showed $IC_{50} = 40$ nM similar to that of CQ ($IC_{50} = 20$ nM) and artemeter ($IC_{50} = 25$ nM). Unfortunately, no data about their in vivo activity is available [102].

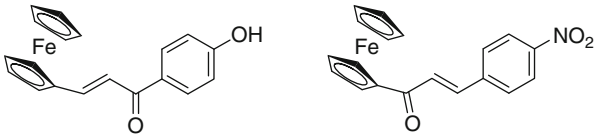

Fig. 12 Chemical structures of selected ferrocenyl chalcones

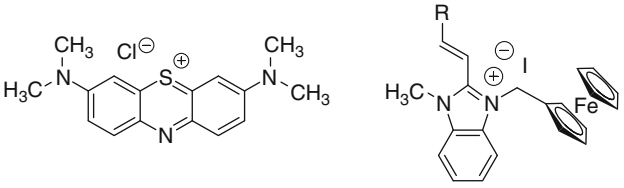

Fig. 13 Chemical structures of methylene blue and ferrocenic benzimidazolium salts. R is an (hetero)aromatic ring

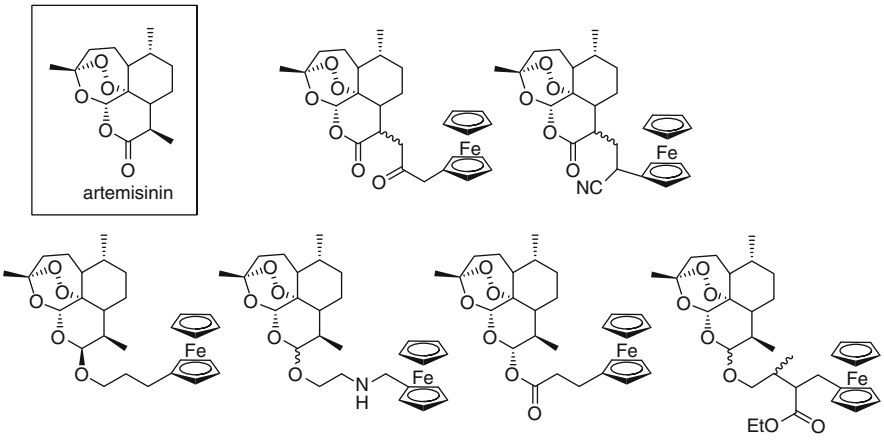

Fig. 14 Chemical structures of artemisinin and the ferrocenyl conjugates

2.2 Ferrocene Conjugates with Antimalarial Drugs Other Than Chloroquine

2.2.1 Artemisinin

Introduction of a ferrocene core in the chemical structure of artemesinin (Fig. 14) did not enhance antimalarial activity compared with artemesinin (or dihydroartemisinin) alone.

Whatever the chosen linker (ether, ester, amine, . . .) between both fragments, no improvement of the antimalarial activity was noted. It was therefore concluded that

incorporation of the ferrocene moiety into an artemesinin skeleton did not improve its activity [103, 104].

2.2.2 Atovaquone

A similar strategy based on the coupling of active fragments led to the design of two new series of atovaquone conjugates (Fig. 15). The compounds were tested on both *Toxoplasma gondii* and *P. falciparum* [105]. The very lipophilic derivatives with an aliphatic chain of 6–8 carbon atoms were active on atovaquone-resistant ATO *T. gondii* clone. Only antimalarial activities in the micromolar range were reported against the 3D7 CQ-susceptible strain and Dd2 CQ-resistant strain. Whereas 1,4-naphthoquinone derivatives, such as atovaquone, were reported to interfere with the oxidation of dihydroorotate, no data for these derivatives is available.

2.2.3 Mefloquine and Quinine

Exploiting the structure-based strategy, the quinuclidinyl and the piperidinyl side chains of quinine (QN) and mefloquine (MF) were, respectively, substituted with a ferrocene moiety while maintaining a basic amino group (Fig. 16). In vitro, lower activities than the parent compounds were reported [106]. In acidic aqueous solution, these ferrocenyl analogs seemed to be unstable, leading to the formation of the presumably inactive carbeniums.

2.2.4 Ferrocenyl Pyrrolo[1,2-a]quinoxaline Derivatives

Inspired by the bioorganometallic approach, two series of ferrocenyl pyrrolo[1,2-*a*] quinoxalines were designed and prepared (Fig. 17). The derivatives were tested in vitro against three different strains of *P. falciparum*: F32, FcB1, and PFB [107]. The best results (IC_{50} between 30 and 70 nM in comparison to CQ $IC_{50} = 225$ nM) were observed in the first series with a bis(3-aminopropyl)piperazine as a linker. These compounds were tested for their ability to inhibit β-hematin formation. For all but one case, the derivatives did not interfere with hemozoin formation

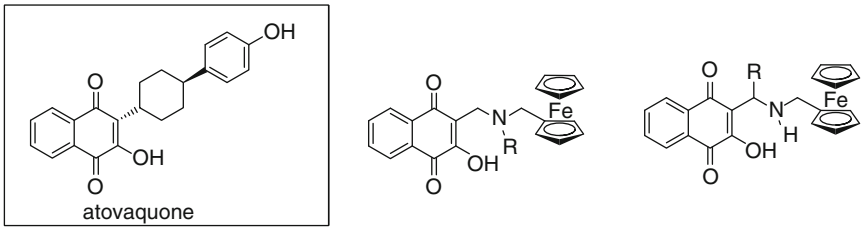

Fig. 15 Chemical structures of atovaquone and the ferrocenyl conjugates

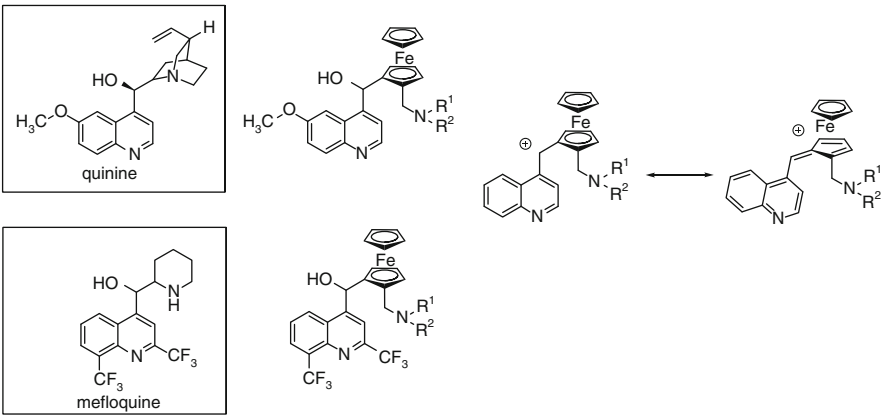

Fig. 16 Chemical structures of quinine and mefloquine, and their ferrocenyl conjugates. The hypothetical carbenium is presented on the *right*

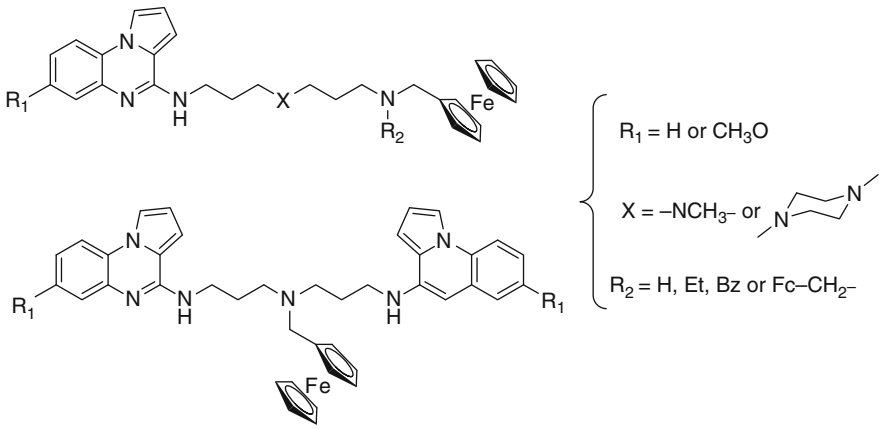

Fig. 17 Chemical structures of the ferrocenyl pyrrolo[1,2-a]quinoxaline derivatives

suggesting a different mechanism of action compared with the classical 4-aminoquinoline family. Their toxicity being too high, the development was stopped.

2.2.5 Ciprofloxacin

Quinolones and fluoroquinolones are widely used against bacteria and mycobacteria [108]. Recently, it has been shown that, similarly to *T. gondii*, at pharmalogical concentrations, ciprofloxacin (Fig. 18) acts on *P. falciparum* [109]. Among all the (fluoro)quinolones active on bacteria and tested against *P. falciparum* strains (3D7 or NF54-R), none of them showed an activity better than 1 μg/mL (the most

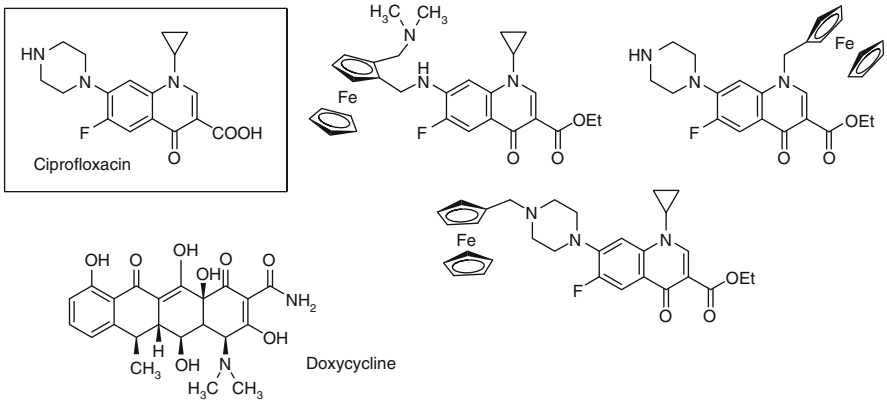

Fig. 18 Chemical structures of ciprofloxacin and its ferrocenyl conjugates. Doxycycline is the reference compound

active proved to be grepafloxacin, gatifloxacin, and moxifloxacin) [110]. Moreover, several quinolones proved to be active in vitro against CQ-susceptible (D6) and CQ-resistant (Dd2) *P. falciparum* strains [111, 112].

Curiously, although hundreds of analogs of ciprofloxacin have been synthesized and structure–activity relationship has been analyzed, the bioorganometallic strategy has not been previously envisaged. Therefore, new ciprofloxacin derivatives bearing a ferrocenyl substituent at position N(1) or at C(7) of the quinolone ring were designed (Fig. 18).

A lead compound bearing a ferrocenyl moiety at position N(1) was identified. This derivative is more active than Ciprofloxacin and Doxycycline. The activity is remarkably constant regardless of the level of resistance to CQ of the strains. Contrary to other antibiotics, no "delayed-death" effect was noted. Isobologram analysis showed that this compound exerts an antagonist effect with the main quinoline-containing antimalarials. In vitro results have to be confirmed in vivo to check the bioavailability of the molecule and its potential interest as a new antimalarial [113].

2.3 Ferrocene Conjugates with Chloroquine

First, CQ was associated with the ferrocenecarboxylic acid by a weak salt bridge interaction (Fig. 19). Indeed, the ferrocene moiety may independently potentiate the activity of CQ by enhancing oxidative stress. Regrettably, this hypothesis was proven wrong. In vitro against the SGE2 CQ-susceptible and the FCM17 CQ-resistant strains, this salt was even less active than CQ diphosphate alone, suggesting an antagonist effect between both parts [114].

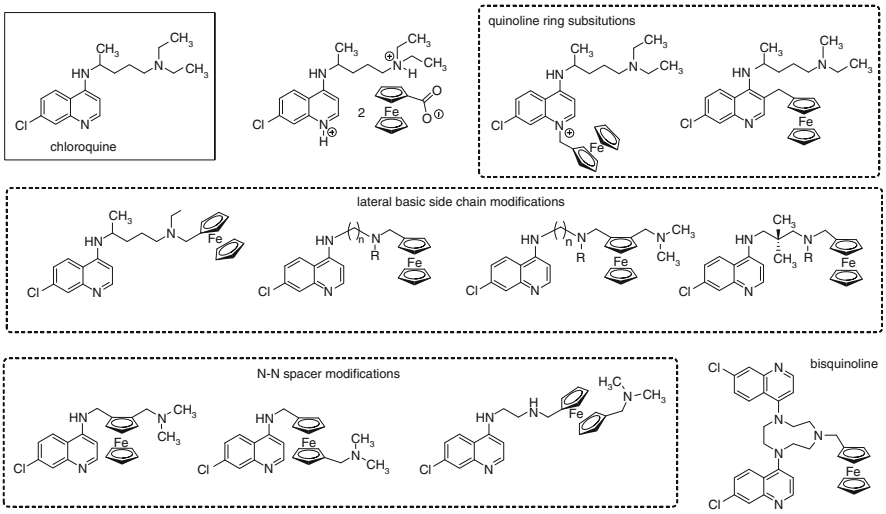

Fig. 19 The template, chloroquine **1**, and the ferrocene conjugates **2–12**. R is an alkyl or a ferrocenylmethyl group, *n* varying from 2 to 6

2.3.1 Quinoline Ring Substitutions

With the same idea in mind, i.e., limiting the number of reaction steps to reduce the costs of production, direct condensation of the ferrocenylmethyl (Fem) moiety on the endocyclic nitrogen of the CQ afforded the quaternary ammonium salt (Fig. 19). Nevertheless, such modification of CQ abolished the activity of the parent molecule on both Dd2 CQ-resistant and HB3 susceptible *P. falciparum* strains [115]. We can hypothesize that the charged species should not be able: (1) to cross the membrane; (2) to stack over the ferriprotoporphyrin ring due to unfavorable electrostatic interaction and/or steric hindrance.

Against the same laboratory strains, a low antimalarial activity was also observed with compound where the quinoline cycle is substituted at the C-3 position by Fem (Fig. 19). Here again, the bulky ferrocenyl group should sterically hinder the stacking interaction between the quinoline ring and heme and/or Fem should modify the charge density distribution on the quinoline cycle [115].

2.3.2 Lateral Side Chain Modifications

Particular attention was devoted to studying the impact of the introduction of the ferrocenyl moiety into the lateral side chain of CQ, implicated in DV targeting. Moreover, the length of the side chain and the distance between the two exocyclic nitrogen atoms may both affect resistance against 4-aminoquinolines by *P. falciparum* [116, 117]. 4-aminoquinolines with shorter (two or three carbon atoms)

or longer side chains (10 or 12 carbon atoms) than CQ are more active against CQ-resistant *P. falciparum*. It has been suggested that these molecules had an N···N spacing which is less suited for binding with the putative CQ transporter and are therefore less efficiently extruded from the food vacuole.

A series of CQ analogs (Fig. 19) characterized by the presence of the ferrocenyl group attached to the terminal basic nitrogen atom of the CQ lateral chain were synthesized and tested [118–120]. Whereas most analogs were found to be more active than CQ, they did not present an optimal activity compared with others. For compounds with a benzylcarbamoyl substituent (R), the chain length (n) made no significant difference to the efficacy in D10 CQ-susceptible strain, but a decrease in efficacy with an increase in the length of the methylene spacer was noted in the K1 CQ-resistant strain [120]. Compounds with the Fem group at the end of the side chain showed an activity which decreased rapidly with the level of CQ-resistance among the *P. falciparum* clones tested. Clearly, a cross resistance could be postulated to emerge very quickly. There was no significant correlation between either the liphophilic character or the in vitro inhibition of hemozoin formation and IC_{50} values among these CQ analogs [118, 119]. Bis-ferrocenyl conjugates led to erratic activities and impossibility to measure precise IC_{50} values, mainly due both to stability and solubility problems in culture medium [118, 119]. A decrease of the efficacy was observed between the linear and branched propylamino chain derivatives (Fig. 16). Introduction of methyl groups in the side chain was not favorable to the antimalarial activity [118, 119].

2.3.3 N-N Spacer Modifications

Incorporation of the ferrocene core in the lateral side chain of CQ between the two amine atoms has led to the design of Ferroquine (FQ, SR97193). This compound was shown to be extremely active against both CQ-sensitive and CQ-resistant *P. falciparum*. FQ was also the most active derivative in vivo and was considered as a lead compound early on (More details in Sect. 3) [121]. As 1,2-unsymmetrically substituted ferrocenes are chiral molecules, an effort was made to design an achiral version of these derivatives. The easiest solution was to move the second substituent to the other cyclopentadienyl cycle. These achiral 1,1′-substituted ferrocene analogs (Fig. 19) were much more active than CQ but exhibited four times lower activity against the K1 CQ-resistant strain than against the D10 CQ sensitive strain [122]. Unfortunately, no in vivo data were available for the comparison of the substitution patterns: 1,2 vs. 1,1′.

2.3.4 Bisquinolines

In the search for quinoline compounds that evade the resistance problem, bisquinolines were found very promising being active against CQ-resistant strains [123]. The ferrocenyl bisquinoline (Fig. 19) remained more efficient on the Dd2 CQ-

resistant strain than CQ although this compound was less active on the HB3 CQ-sensitive strain, than FQ [124].

3 Ferroquine: A New Candidate Antimalarial Drug

The presence of the ferrocene moiety within the lateral chain of CQ is the main condition required to retain a strong antimalarial activity on CQ-resistant *P. falciparum*. FQ was rapidly identified as a lead compound with sufficiently potent in vitro and in vivo activities to meet candidate nomination requirements.

3.1 The Chemistry of Ferroquine

The synthesis of FQ (Fig. 20) is simple and quite economical, which renders FQ attractive for the development of an antimalarial drug intended for use in areas, concerned by malaria, that are mostly overlaying with low-income countries. FQ was obtained starting from the commercially available *N*,*N*-dimethyl-1-ferrocenylmethanamine. The ferrocenic aldehyde results from a C–C bond formation, a two-step sequence involving metallation with *tertio*-butyllithium and a reaction with DMF. This step has been previously studied and the 1,2 orientation of the two substituents of the cyclopentadienyl has been unambiguously established [125]. The aldehyde is converted to the corresponding oxime, which is then reduced to the primary amine. The SN_{Ar} reaction between the amine and 4,7-dichloroquinoline leads to the desired FQ [121].

FQ possesses planar chirality due to its 1,2-unsymmetrically substituted ferrocene moiety (Fig. 21). In the laboratory, a synthetic method was developed to provide separate pure enantiomers of FQ [126]. The approach included a biocatalytic kinetic resolution using *Candida rugosa* lipase (*C.r.*l.). The key step (Fig. 22)

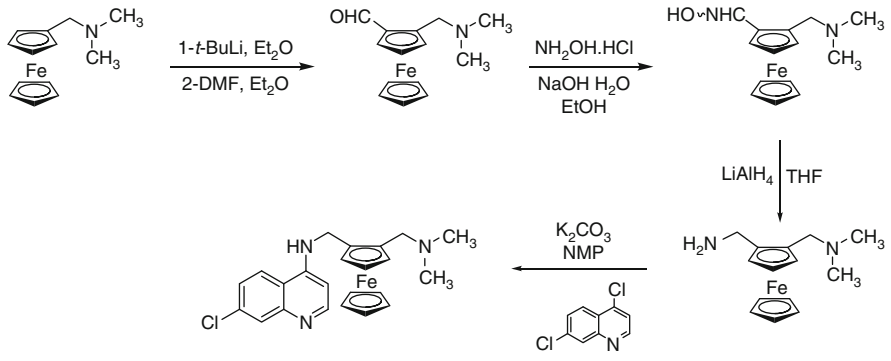

Fig. 20 Chemical synthesis of ferroquine

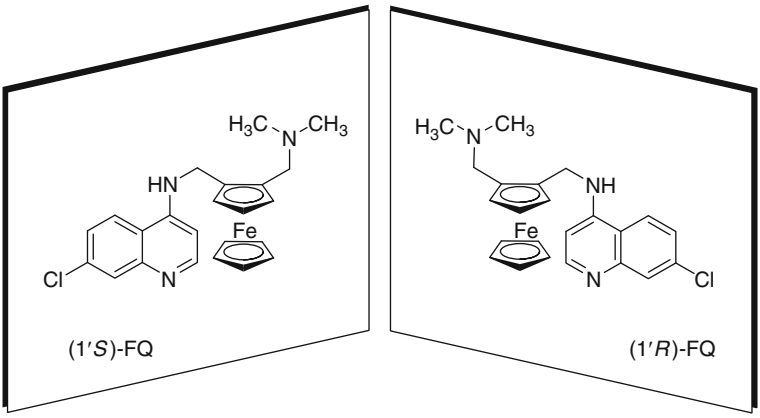

Fig. 21 Ferroquine enantiomers

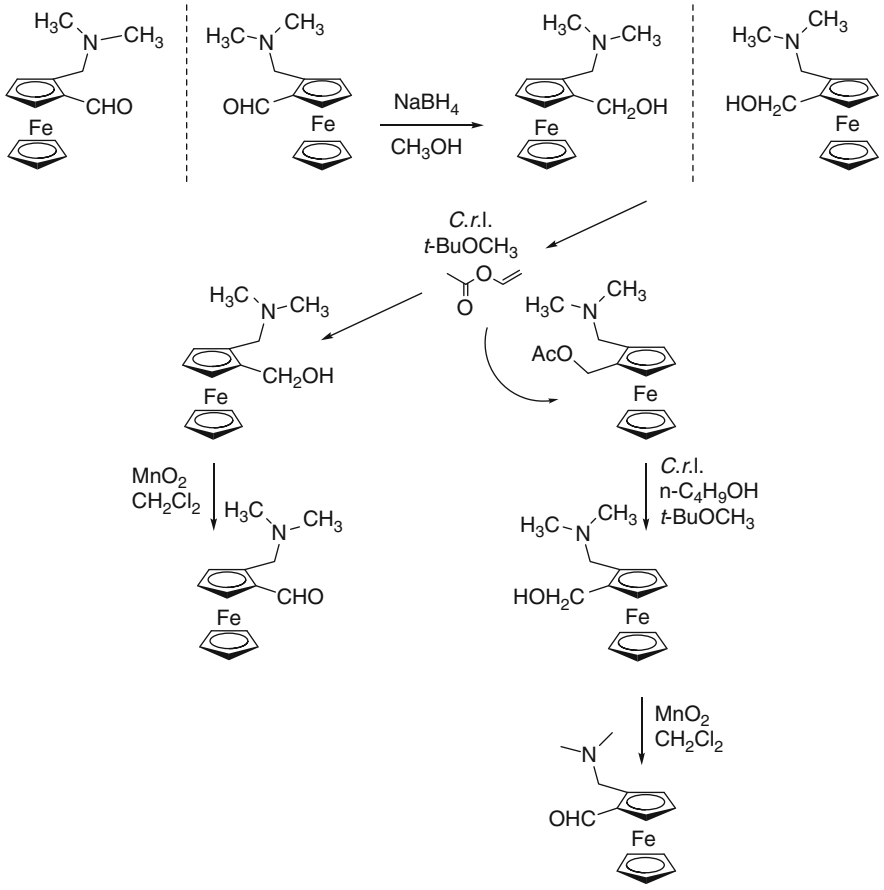

Fig. 22 Key steps of the synthesis of FQ enantiomers by enzymatic resolution

is enantioselective acetylation of primary hydroxy group leading to the formation of enantiomerically enriched acetate. The remaining untransformed alcohol was isolated in an extremely high enantiomeric excess (ee > 98%). The deacylation of the enriched acetate was undertaken by means of a transesterification process catalyzed also by *C.r.*l. This reaction provides the other alcohol with high enantiomeric purity (ee > 98%). Finally, both amino alcohols were oxidized to the corresponding carboxaldehydes and then engaged in the total synthesis of (1′*R*)-FQ and (1′*S*)-FQ.

Note here that for (semi)industrial scale production, both enantiomers were obtained starting from the racemic mixture by preparative chiral chromatography.

Activity of pure enantiomers was compared with the racemate in vitro and in vivo. In vitro, the FQ enantiomers and the racemate were found to be equally active against the CQ-susceptible and CQ-resistant *P. falciparum* strains HB3 and Dd2. In vivo, both enantiomers were slightly less active than the racemic mixture against CQ sensitive and CQ resistant *P. vinckei vinckei*, suggesting an additive or a synergetic effect between both enantiomers. Moreover, (1′*R*)-FQ displayed a slightly improved curative effect than (1′*S*)-FQ suggesting minor differences in pharmokinetics properties. Actually, the in vitro cytotoxicities of (1′*R*)-FQ and (1′*S*)-FQ or the racemate appeared similar in the L5178Y cell proliferative assay. As no critical adverse affect was observed during Phase I and IIa of clinical trials with racemate, the enantiomeric mixture was adopted in further Phase IIb trials in association with artesunate [127].

The apparent partition coefficients (log *D*) of CQ and FQ were measured at vacuolar (5.2) and cytosolic (7.4) pHs (Table 1, [128]). At cytosolic pH, FQ was over 100-fold more lipophilic than CQ, whereas the difference in lipophilicity is only slight at acidic DV pH (about threefold).

In terms of basicity, pK_as of the two drugs indicate that FQ is less basic than CQ (Table 1, [129]). Crystal structure of FQ (Fig. 23) shows the presence of a strong internal hydrogen bond between the anilino (N11) group and the tertiary amino N(24) [128]. This, together with the electron donating properties of the ferrocene moiety, should explain the decreased pK_a values.

In the absence of special transport mechanisms (which is not proven, see further in the text), the vacuolar accumulation ratios (VAR) could be predicted from a derivation of the Hendelson–Hasselbach equation using the calculated log *D* values. Indeed, for a diacidic base, the log *D* is a function of the acidity constants (pK_a) and the partition coefficient (log *P*):

$$\log D_{\text{calculated}} = \log P - \log[1 + 10\text{^}(\text{p}K_{a1} - \text{pH}) + 10\text{^}(\text{p}K_{a1} + \text{p}K_{a2} - \text{pH})].$$

Table 1 Experimental pK_a, log *P*, log *K*, IC_{50}, and log *D* at two different pH reflecting the cytosolic and vacuolar environment

Cpd	pK_{a1}	pK_{a2}	log *P*	log $D_{5.2}$	log $D_{7.4}$	log *K*
CQ	10.03	7.94	4.63	−1.20	0.85	5.52
FQ	8.19	6.99	5.1	−0.77	2.95	4.95

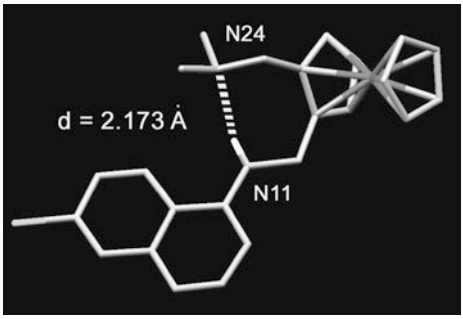

Fig. 23 Crystal structure of neutral ferroquine (CCDC 262108). The *dashed line* indicates the intramolecular hydrogen bond

So, when using this formula, FQ (VAR = 6,402) would not be expected to accumulate in the DV as much as CQ (VAR = 19,521). Nevertheless, in view of the high degree of variability of the methods (the pK_a were determined in a dioxane–water mixture and the log *P* in octanol–water mixture), some errors could be included in the results.

Instead of the calculated log *D* values, the experimental log *D* values can also be exploited, and the VAR were calculated as follows:

$$\mathrm{VAR} = 10\text{^}(\log D_{7.4} - \log DpH).$$

Using this formula, FQ is expected to accumulate 50-times more than CQ in the parasitic DV. However, both mathematical models could be subject to suspicion of bias as special transport mechanism(s) could not be excluded and are even strongly suspected. Indeed, the binding to free heme should contribute to uptake [130, 131].

The debate of the "real" concentration of FQ in the DV compared with CQ is still open. Experimental data will be urgently needed to validate the correct mathematical models.

3.2 *Ferroquine Derivatives*

In order to study if structural modifications of FQ might improve its antimalarial activity, several derivatives were prepared and tested.

Tertiary amines (Fig. 24, $R^1 = R^2 = C_2H_5$ or $R^1 = C_2H_5$ and $R^2 = CH_3$) showed strong antimalarial activity, especially against the CQ-resistant Dd2 and W2 strains [118]. These compounds were two- to tenfold more active than CQ and as active as FQ. Secondary amines (R^1 = H and $R^2 = CH_3$ or C_2H_5 or $C(CH_3)_3$) also possess antimalarial activity comparable to that of FQ. All these compounds exhibited better inhibitory activity against the Dd2 strain than CQ itself [132].

These structural modifications allow us to conclude that the in vitro antimalarial activity was not disturbed by slight modifications in the lateral basic side chain. To the contrary, when a second ferrocenyl group was introduced on the terminal nitrogen atom, the efficacy of compounds was markedly attenuated [118].

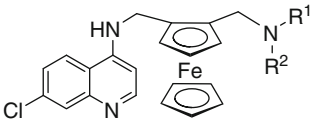

Fig. 24 Chemical structures of the analogs of ferroquine modified on the basic amino group

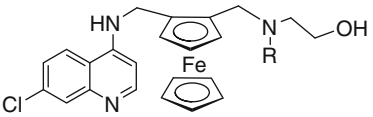

Fig. 25 Chemical structures of hydroxyferroqines. R is a hydrogen or an alkyl group

Three FQ derivatives (Fig. 25) with R = H, CH_3, or C_2H_5, closely mimicking the antimalarial drug hydroxychloroquine (HCQ), have been prepared [119]. These compounds differed from FQ in their side chains on the basic nitrogen atom N (25). Introduction of a hydroxyl group provides the expected reduction of cytotoxic effects compared with FQ. Moreover, these metallocenic compounds inhibited in vitro growth of *P. falciparum* far better than CQ. The best results were obtained when R was an ethyl substituent. The high potent antimalarial activity of this compound was confirmed on 25 Cambodian field isolates. This derivative showed almost the same level of activity as that of FQ. As expected, a high correlation ($r^2 = 0.7129$) with the IC_{50} of FQ was noted within the isolates tested. This suggests that the two compounds have a similar mode of action and/or uptake by the parasite.

Oxidative metabolization of tertiary amines like CQ or FQ occurs via the cytochrome P-450 system (see Sect. 3.4 for more details). The main metabolites are generated by side-chain de-alkylation leading first to the monodesalkyl and then to the didesalkyl derivatives. A similar oxidative pathway should mediate the de-alkylation of hydroxyferroquines (R = CH_3 or C_2H_5) as shown for FQ. The loss of the ethanol fragment generates the secondary amine. The other C–N cleavage generates the active mono-*N*-desmethyl-(dMFQ) and mono-*N*-desethyl-FQ (dEFQ), respectively. By comparison with HCQ and FQ, it is tempting to suggest that during a clinical use of hydroxyferroquines (R = CH_3 or C_2H_5), formation of these active metabolites may occur and participate in the global activity of the parent products.

Moreover, this new class of bioorganometallic compounds exerts antiviral effects with some selectivity toward SARS-CoV infection. These new drugs may offer an interesting alternative for Asia where SARS originated and malaria has remained endemic.

A chimeric ligand approach (Fig. 26) was used to combine the properties of FQ and those of thiosemicarbazones (TSC) [133].

Indeed, derivatives of TSC have shown potent antimalarial activities [134, 135]. Due to their intrinsic metal (e.g., iron) chelating properties, the mechanism of action of TSCs is believed to result from the generation of reactive oxygen radicals [136]. To the contrary, the presence of the ferrocene moiety within the lateral chain is the main condition required to retain a strong antimalarial activity on CQ-resistant *P. falciparum*.

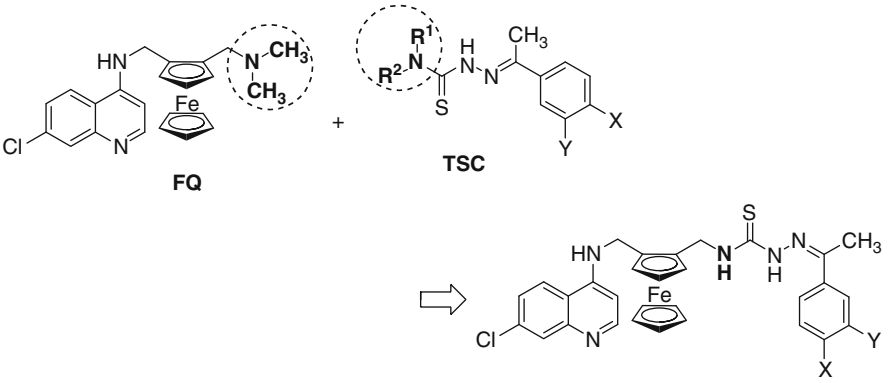

Fig. 26 Chimeras of FQ and TSC. The *dashed circles* indicate the merged groups

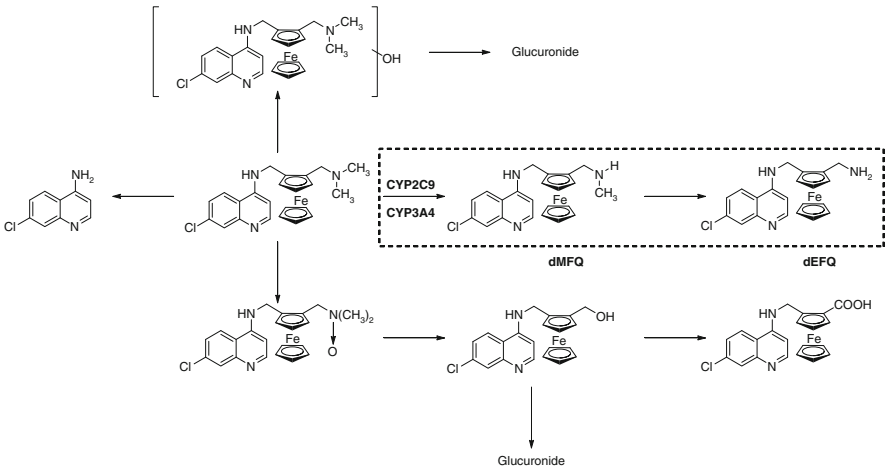

Fig. 27 Proposed metabolic pathway of ferroquine in human hepatic models. Main metabolites are in the *dashed line box*

As can be seen in Fig. 27, a covalent binding between both active fragments was envisaged by merging the amino groups. In order to compare the contribution of each fragment, analogs without the ferrocenic moiety and analogs without the 4-aminoquinoline moiety were also synthesized. Chimeras of TSC and FQ were the most active derivatives against four different strains of *P. falciparum*. Nevertheless, the corresponding purely organic derivatives showed comparable potency. Contrary to previous results, introduction of the ferrocene moiety did not increase antimalarial activity. Here again, we noticed no significant difference in the activity of ferrocenyl compounds between CQ-susceptible and CQ-resistant parasites.

3.3 Specific Pharmacology

The first tests performed in vitro on *P. falciparum* clones or acclimated isolates [114, 121, 129] showed that the molecule had an activity equivalent to that of CQ on CQ susceptible parasites and was as efficient on CQ resistant parasites with similar IC_{50} for both parasites phenotypes. These results were confirmed on other *P. falciparum* clones which were tested in other laboratories [122, 137].

This preliminary data incited us to explore in vivo activity of FQ on murine malaria models. Subcutaneous administration of FQ to mice infected by *P. berghei* N, according to the "four-days blood schizonticidal test" [138], showed that drug IC_{50} and IC_{90} values were, respectively, 1.22 and 1.95 mg/kg/day as against 1.39 and 2.7 mg/kg/day for CQ [129]. More than IC_{50} tests, curative tests showed the efficacy of CQ, but also the limits of some strains for in vivo tests (unpublished results). We conducted the curative test as the "four-days blood schizonticidal test," but in place of parasitemia determination at day 4, mice are monitored for survival throughout a period of 2 months after the end of treatment, with regular controls to verify if mortality is related to parasite infection [126, 129]. Experiments showed that the curative dose for CQ on the *P. berghei* N strain was 50 mg/kg/day (to be compared with the IC_{90} reported above). Under the same conditions a daily dose of 8.39 mg/kg/day of FQ cured 14/15 mice. Similar results were obtained with *P. yoelii* NS strain, with no curative effect of CQ at 60 mg/kg/day vs. 14/30 mice cured with 10 mg/kg/day of FQ, 3/3 cured at 15 mg/kg/day, and 5/5 cured at 20 mg/kg/day (unpublished results). In all these experiments, the last surviving parasites were observed in immature RBC (reticulocytes). The affinity of *P. berghei* and some *P. yoelii* strains for immature RBC is well known [139]. To get round this problem, we selected a strain of *P. vinckei vinckei* which did not invade immature red cells [129]. In these conditions, FQ cured mice equally from CQ-susceptible or CQ resistant *P. vinckei* strains at a dose of 8.4 mg/kg/day whilst CQ cured the CQ sensitive line at 31 mg/kg/day and was not curative for CQ resistant line at a subtoxic dose of 58.9 mg/kg/day. One promising result was that FQ was equally curative given subcutaneously or orally, which was an indication of its good bioavailability [126, 129].

In parallel, extensive studies on *P. falciparum* field isolates in Gabon [140–142], Senegal [143], Cambodia [118, 119, 144], and the Thailand Burmese border [145] corroborated the efficacy of FQ on the parasite whatever its resistance level to chloroquine or to other commonly used antimalarials: mefloquine, quinine, halofantrine, and artemisinin derivatives [146, 147]. The cross reactivity observed in some studies with CQ was limited and it was demonstrated that it was caused by differences in initial parasitemia among isolates at the start of the assays [141]. Independance of susceptibility of *P. falciparum* with phenotypic variation of *pfcrt* gene, responsible for CQ resistance, could be suspected from these results, but this was demonstrated at the molecular level on Cambodia isolates [148] and extended further on other genes currently involved in resistance to aminoquinoline antimalarials [89, 90].

Assays were done to select a *Plasmodium* clone resistant to FQ. All attempts on *P. falciparum* failed. Parasites appearing after 35 days pressure were unable to develop, even in absence of the drug in the medium [148]. Experiments were conducted in vivo on a murine strain of *P. yoelii NS* and resulted in a multi-resistant phenotype (CQ, mefloquine and FQ) with a very low pathogenicity. The strain was able to develop only in immature erythrocytes and maximum parasitemia was about 2%. Mice were very often able to cure themselves from parasites, either under pressure of FQ or in absence of drug. Moreover, FQ resistance was not fixed genetically, in opposition with CQ resistance [146]. Just removing drug pressure resulted in a rapid loss of resistance to FQ, which was already observed on an artemisinin-resistant phenotype of the same species [149]. When the strain under FQ pressure was cryopreserved, it had lost its resistance by the time the mouse was inoculated again. In these two conditions the strain remained resistant to CQ. Analysis of pycrt and pymdr1 did not show mutation compared with the wild type line in critical regions known for phenotypic resistance [146]. This result posed questions not only concerning the potential mechanism of resistance to FQ in *Plasmodium*, but also on the origin of CQ resistance in *P. yoelii*. Recent results obtained on *P. berghei* and concerning the translocation of the gene of a multidrug resistance-associated protein (*mrp* gene) from chromosome 13/14 to chromosome 8 in the chloroquine resistant strain RC might be an interesting lead for comprehension of mechanisms involved in FQ resistance [150].

In conclusion, all specific pharmacology studies showed that FQ was very active on all strains of *P. falciparum* currently resistant to already used antimalarials. The susceptibility to the drug is not dependant to resistance phenotypes already determined. Experiments to induce resistance in vitro or in vivo show clearly that the fit cost of FQ resistance is extremely high for the parasite and disproves its pathogenicity. Nevertheless, one may keep in mind that resistance or decreased susceptibility occurred for all antimalarials currently used. Such resistance could emerge if FQ were used extensively in the future. One way to decrease or to overcome the risk is to use FQ in an ACT [127], with artesunate, as it is planned during its phase II of clinical assays. Out of all cautions taken to delay the occurrence of resistance, experimental studies remain to be performed to select a clone resistant to FQ. The molecular analysis of such resistance might provide useful tools for monitoring the risk of decreased susceptibility of parasite in area when FQ is used.

3.4 Metabolism, ADME, and Toxicology

In humans, CQ is metabolized in the liver, mostly by oxidation via the cytochrome P-450 enzyme system, leading to *N*-desethylchloroquine and then didesethylchloroquine [151]. Successive dealkylation of the side chain ultimately produces the 7-chloro-4-aminoquinoline. In comparison to CQ, the metabolism of FQ was

examined in different in vitro animal and human hepatic models. FQ is also metabolized via a major dealkylation pathway into the mono-*N*-desmethyl FQ (dMFQ) and then into di-*N*,*N*-desmethyl FQ (dEFQ). Other minor metabolic pathways were also identified (Fig. 27). Cytochrome P-450 isoforms 2C9, 2C19, and 3A4 and, possibly in some patients, isoform 2D6 are mainly involved in FQ oxidation [148].

Interestingly, dMFQ remains as active as FQ on the CQ-susceptible strain 3D7 and less active than FQ but much more active than chloroquine on CQ-resistant strain W2. The other metabolites were less effective than FQ (eight- to tenfold according to their IC_{50} values), and their IC90 (>>100 nM) values show that they would not be efficient in parasite elimination [148]. Previously, dMFQ was found as active as FQ on CQ-susceptible strain (HB3) and on a moderate CQ-resistant strain (Dd2). dEFQ was found only twofold less active than ferroquine on Dd2 [132].

As these two metabolites are present in significant concentration in blood after administration of FQ, they should be involved in the global antimalarial activity of FQ.

FQ exhibited a good transepithelial transport in a Caco/TC7 monolayer intestinal epithelium model, independent of pH, and not influenced by pgp inhibitors. This indicates a good oral absorption, confirming the experimental results already obtained during in vivo curative assays on mice using an oral way administration [126, 129]. Moreover, the drug shows a low transfer rate through the blood brain barrier in an in vitro model of transendothelial transport across bovine capillary endothelial cells. Both by intravenous and oral administration, FQ showed an extensive plasma clearance, a large volume of distribution, and a large bioavailability (60%). Plasma concentrations are high in a range of 3–8 h after oral administration and decrease slowly during the first 24 h. A large distribution was observed in liver and in the brain. These properties are close to those already observed for CQ. Concerning the erythrocytic/plasma distribution, the three models tested (rat, macaque monkey, and human) converged toward a high level of accumulation of FQ in RBC (68–85%), close or even better than CQ itself.

In a 14-day test on rats, using an equivalent dose of CQ which caused 100% mortality, FQ caused only about 7% mortality. The liver, and to a lesser extent the kidney, appeared to be the two main target organs. Phase I and IIa of clinical experiments confirmed the good tolerability of FQ and its lower toxicity compared with CQ.

Concerning embryotoxicity and mutagenicity, FQ was found negative on the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) test [152]. FQ and its enantiomers, solubilized in aqueous solutions, were negative in the Ames test [153]. FQ responded negatively in the micronucleus in vitro and in vivo assays conducted under GLP Standards. Interestingly, in the same kind of experiments, CQ was found to be weakly mutagenic and genotoxic [154].

In conclusion, FQ showed a good pharmacological profile after oral absorption, and experimental and clinical phase I assays showed a very good tolerance to FQ given single dose, even at high dosage. These results, associated with the activity of FQ and its first metabolite against CQ-resistant *P. falciparum*, are a good omen for

the future development of FQ as a new antimalarial, confirmed by current passing of the drug in Clinical Phase IIb.

3.5 A Brief Industrial Development Story

1994: First chemical synthesis of FQ in the Laboratory of Catalysis of Lille (now Catalysis and Solid State Chemistry, UMR CNRS 8181), ENSCL, University of Sciences and Technologies, Lille, France.
1995: French and International Patents of FQ and derivatives.
1994–1995: First tests of FQ on Gabon acclimated isolates of *P. falciparum* by Pascal Millet.
1996: First tests on laboratory clones of *P. falciparum* and on rodent malaria in vivo by Daniel Dive.
1997: First contacts with a pharmaceutical industry (Pierre Fabre Medicament, PFM), which asks key questions about the molecule for development.
1997–2001: A crystallized form of FQ is obtained.
Synthesis of FQ enantiomers by C. Biot. Specific pharmacology on *P. falciparum* and on rodent malaria in vivo by Daniel Dive.
2001: PFM stops its activities regarding malaria.
New contacts with another pharmaceutical group (Sanofi Aventis).
FQ is licensed by SA. Preliminary development.
2001–2004: Tests of FQ on field isolates in Gabon, Senegal, Cambodia.
06/2003: Development of FQ by SA.
2004: Beginning of Phase I clinical trials (first in humans).
2005: First administration to human in Gabon.
2006: FQ is an international nonproprietary name.
2006: Patent on association between FQ and an artemisinine derivative for treating malaria (SA).
2007: Beginning of Phase II clinical trials.
2008: Comparative safety and activity study with FQ/Artesunate vs. Amodiaquine/Artesunate in African adult patients.

4 Mechanism(s) of Action of Ferroquine

The antimalarial activity of FQ was initially compared with that of the purely organic CQ in an effort to understand how the presence of the ferrocene contributes to the antiplasmodial property. Over the years, the mechanism of CQ has been (and is still) the subject of many discussions and arguments. Nevertheless, there is strong evidence that the action of CQ is linked to its localization in the DV of the parasite and to the inhibition of the formation of hemozoin ([37], see [155–157]).

4.1 Inhibition of Hemozoin Formation

FQ formed a complex with hematin in solution with a stoichiometry of 1:1 (log $K = 4.95 \pm 0.05$) [128]. Moreover, in the presence of FQ, hematin is no more converted into β-hematin and a dose-dependent inhibition of β-hematin formation was obtained. The IC_{50} of FQ was 0.8, whereas the IC_{50} of CQ was 1.9. This clearly shows that FQ is a strong inhibitor of β-hematin formation and even more potent than CQ [128]. The molecular electrostatic potential (MEP) surfaces have been computed at the DFT-B3LYP level of theory for neutral and diprotonated FQ and CQ. In both cases, FQ and CQ show considerable similarity in the quinoline area. Since this part of the molecule is thought to directly interact with hematin by stacking interaction, a similar mode of interaction between these active drugs (FQ or CQ) and hematin was suggested [128].

4.2 Specific Drug Targeting

However, both the basicity and lipophilicity of FQ are significantly different to that of CQ. The lipophilicity of FQ and CQ is similar when protonated at the putative food vacuole pH of 5.2 (log $D = -0.77$ and -1.2, respectively), but differ markedly at pH 7.4 (log $D = 2.95$ and 0.85, respectively). In addition, the pK_a values of FQ are lower ($pK_{a1} = 8.19$ and $pK_{a2} = 6.99$) than those of CQ (10.03 and 7.94, respectively). As a free base, FQ is more than 100-fold more lipophilic than CQ; this difference may explain the remarkable activity of FQ compared with CQ. Indeed, FQ may target the lipid site of hemozoin formation more efficiently, and thus more powerfully inhibit the process of hemozoin crystallization. To validate or disprove this theory, incubating parasites with [^{14}C] FQ [158] and localizing it by electron microscope autoradiography and subcellular fractionation is envisaged [37] .To avoid the use of radioactive material, an alternative method will be to use an electron-dense tracer as ruthenoquine (the ruthenium analog of FQ) to study its uptake by ultrastructural studies of treated parasites.

4.3 A Critical Intramolecular Hydrogen Bond

The solid-state structure of neutral FQ is stabilized by a strong intramolecular hydrogen bond ($d = 2.173$ Å) between the anilino N(11) and the tertiary amino N(24) (see Chap. 3.1, [128]). When in solution with a low dielectric constant (chloroform, $k = 5.5$) such as the lipid environment, NMR data show that the spatial structure of FQ is much the same as in the crystal [159]. This peculiar shape leads to an increase of its hydrophobicity and results in the rejection of the bulky ferrocenyl moiety toward the outside [157].

Combined spectroscopic and structural investigations revealed that the extended structure of FQ in polar solvent changes to a more compact conformation via an additional intramolecular hydrogen bond in apolar solvent (Biot et al. 2008). So, the role of this noncovalent interaction on the antimalarial activity was questioned.

To this end, an analog (FQ-Me) bearing a methyl group instead of a hydrogen atom on the anilino N(11) was synthesized (Fig. 28). This compound shared similar physicochemical properties with FQ and was also able to inhibit the β-hematin formation (Table 2). Nevertheless, this structural modification led to significant reduction in activity against CQ-susceptible and CQ-resistant strains (Table 2). The presence of a hydrogen bonding interaction in the lateral side chain of FQ should contribute to the antiplasmodial activity [157]. These results are in accordance with previous observations where FQ analogs (a) and hydroxyferroquines (b) including an intramolecular H-bond in their side chain were shown much more active than molecules lacking this noncovalent interaction.

The flip/flop H-bond between the open conformation of the diprotonated FQ and the folded conformation of the neutral FQ should contribute to the transport from water to the hydrophobic membranes (Fig. 29).

The hydrophobic ferrocene moiety should establish favorable van der Waals interactions with lipid structures involved at the interface with aqueous content of the DV, positioning FQ in the same catalytic site as hematin [157]. This preferential location of FQ should cause inhibition of formation of hemozoin more efficiently than CQ does.

Moreover, tight binding interactions between FQ and lipids suggest that the concentration of free FQ in water is decreased and FQ is no longer a substrate for

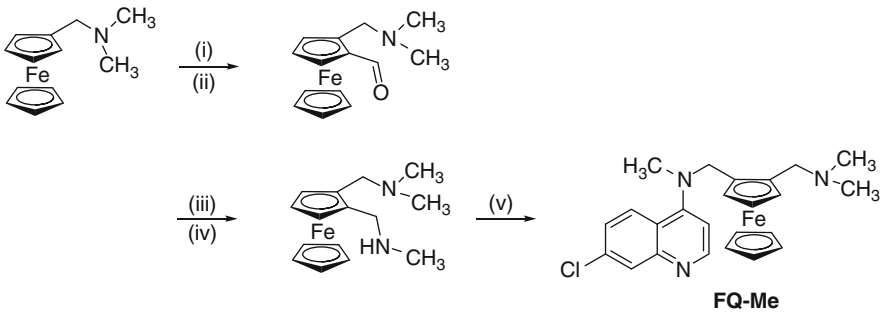

Fig. 28 Chemical synthesis of the methyl FQ derivative (FQ-Me). (**a**) *t*-BuLi, Et_2O; (**b**) DMF; (**c**) CH_3NH_2, THF; (**d**) $NaBH_4$, MeOH; (**e**) 4,7-dichloroquinoline, K_2CO_3, NEt_3, NMP, 135°C

Table 2 Biological, biochemical, and physicochemical properties of FQ and its methylated derivative

	3D7	IC_{50} (nM)		Lipophilicity		Basicity	
		W2	FCM29	log $D_{7.4}$	log $D_{5.2}$	pK_{a1}	pK_{a2}
FQ	3.5	7.1	10.0	2.95	−1.20	7.00	8.45
FQ-Me	99.8	200.9	230.1	3.04	−0.56	7.05	8.80

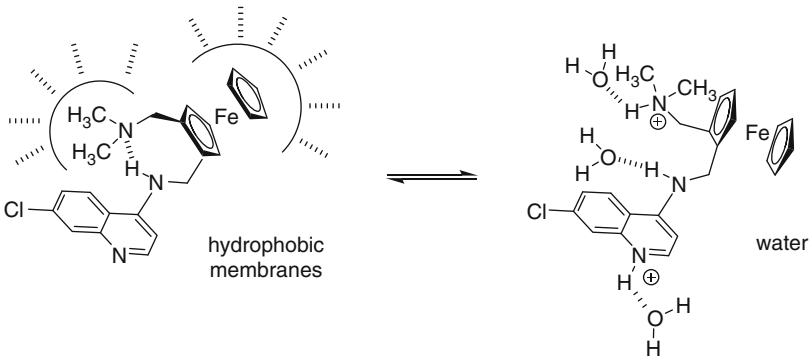

Fig. 29 Proposed flip/flop H-bond that may help transport of FQ through the hydrophobic membranes

the efflux transporter(s) associated with CQ resistance. More biophysical and biological approaches will be necessary to refine this hypothesis and are currently being developed by our groups.

4.4 Production of Reactive Oxygen Species

In the DV of the parasite, free heme is rapidly converted to hematin, i.e., iron II is oxidized in iron III. During this process, electrons liberated promote the formation of reactive oxygen species (ROS) such as superoxide anion radicals and hydrogen peroxide [160]. ROS can cause cellular damage. Hydrogen peroxide may also be used for the peroxidative degradation of heme. In this context, the influence of H_2O_2 on the redox behavior of FQ and implications for antimalarial activity was questioned.

Under the specific conditions (acidic and oxidizing) mimicking the parasite DV, FQ shows a reversible one-electron redox reaction [161]. This leads to the formation of ferriquinium and generation of hydroxyl radicals (Fig. 30), with kinetics which are relevant for an antimalarial activity on *P. falciparum*.

CQ has been shown to induce ROS production in astroglial cells via a signaling pathway involving NFκ-B [162, 163]. Nevertheless, in the RBC devoid of a nucleus, such a pathway cannot occur. Additional spin trapping experiments demonstrate that CQ is not able to produce OH• radicals by itself in the presence of H_2O_2 at the acidic pH of the parasite DV. On the contrary, using the same experimental conditions, hydroxyl radicals are produced by FQ at micromolar concentration [161]. Whereas the production of OH• is weak, it should be sufficient to induce significant damage.

FQ might thus strike the parasite not only via direct inhibition of hemozoin formation but also by production of lethal hydroxyl radicals. These radicals are known to be particularly aggressive toward unsaturated fatty acids present in

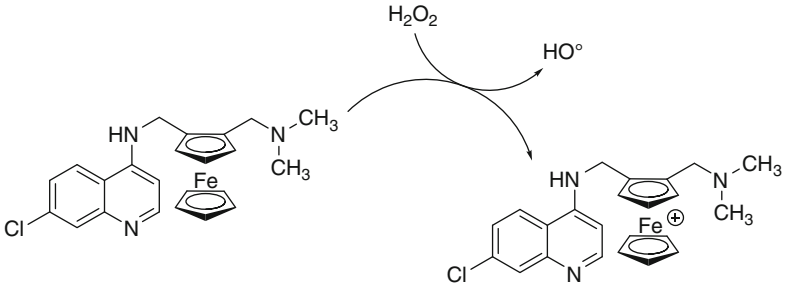

Fig. 30 Production of hydroxyl radicals by ferroquine

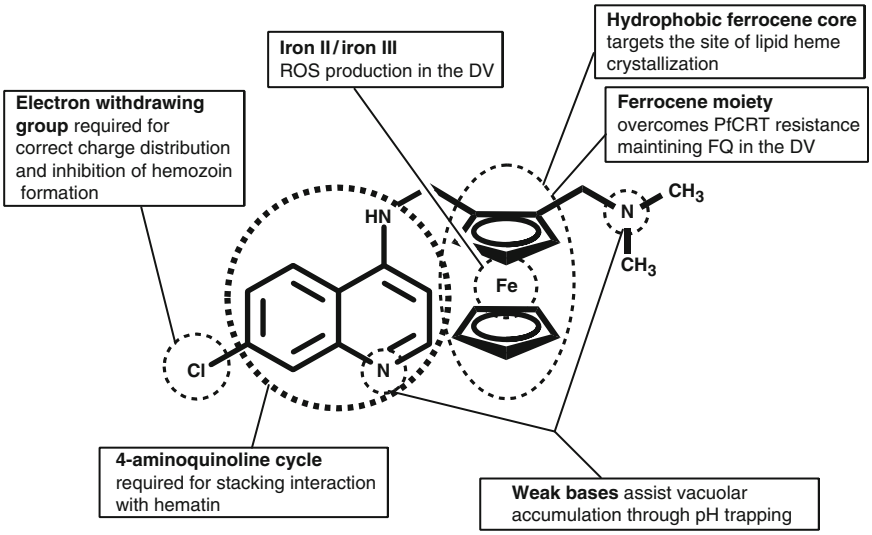

Fig. 31 Proposed structure–activity relationships for FQ

membrane phospholipids and to promote an extensive chain reaction of their peroxidation products [164]. The alteration or the destruction of these structures should then influence heme detoxification processes and could result in a complementary harmful effect on the parasite. Further experiments will be necessary to provide answers to these hypotheses.

In conclusion, the activity of FQ is due to more than one route (Fig. 31). Due to its physicochemical properties, FQ could specifically target the lipid site of hemozoin formation. Its mechanism of action should be in part similar to that of CQ, based on the inhibition of hemozoin formation. Upon the specific (acidic and oxidizing) DV conditions, production of radical oxygen species (ROS) by FQ should be sufficient to promote significant damage on membranes of the parasite DV. The strong activity of FQ on CQ-resistant clones and isolates of *P. falciparum* suggests a fundamental difference in interaction with resistance mechanisms of the parasite.

5 Conclusion

The contribution of organometallic chemistry in the development of new antimalarials is promising in its potential uses, but so far the outcome remains limited. The attempts to insert an organometallic moiety in already known antimalarials were based on the idea to improve their activity or to overcome the resistance of the parasite to the parent drug. Results remained, for most of them, within expectations, except for CQ analogs and the serendipitous discovery of FQ. The extensive structure–activity studies, realized on the ferrocenic CQ conjugates, provided important information about the role of the ferrocene core in antimalarial activity. The position of the ferrocenyl moiety in the lateral side chain of CQ was probed with precision. The results formed the basis for new ideas and quinine, melfoquine, and artemisinin were derivatised. The influence of the ferrocenyl moietyl on the lipophilicity is really important when considering that an antimalarial drug has to cross at least three membranes before reaching the parasite cytoplasm and five to six membranes to reach the inner part of the parasite mitochondria or the plastid. The fine analysis of FQ properties led to the underlining of the possible specific role of the ferrocene core in the antimarial activity via the production of hydroxyl radicals. The presence of a specific intramolecular hydrogen bond in the molecule seems to be an important factor for its activity. It was postulated that this weak interaction confers to FQ a potency to interact with membrane lipids and to be specifically present at the site of action when it can be the most efficient. The insertion of the ferrocene moiety in the weak base CQ undoubtedly increased its ability to target the parasite digestive vacuole and possibly enables the drug to escape the resistance mechanisms.

FQ (in phase IIb of clinical trials) represents the first organometallic antimalarial currently in the "pipe-line" of promising drugs. Moreover, the pursuit of studies regarding the mechanism of action of FQ and the possible mechanisms of resistance appears promising not only for chemotherapy of malaria, but also as the basis for new concept in drug design.

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Biomedical Applications of Organometal–Peptide Conjugates

Nils Metzler-Nolte

Abstract Peptides are well suited as targeting vectors for the directed delivery of metal-based drugs or probes for biomedical investigations. This chapter describes synthetic strategies for the preparation of conjugates of medically interesting peptides with covalently bound metal complexes. Peptides that were used include neuropeptides (enkephalin, neuropeptide Y, neurotensin), uptake peptides (TAT and poly-Arg), and intracellular localization sequences. To these peptides, a whole variety of transition metal complexes has been attached in recent years by solid-phase peptide synthesis (SPPS) techniques. The metal complex can be attached to the peptide on the resin as part of the SPPS scheme. Alternatively, the metal complex may be attached to the peptide as a postsynthetic modification. Advantages as well as disadvantages for either strategy are discussed. Biomedical applications include radiopharmaceutical applications, anticancer and antibacterial activity, metal–peptide conjugates as targeted CO-releasing molecules, and metal–peptide conjugates in biosensor applications.

Keywords Bioorganometallic chemistry · Medicinal organometallic chemistry · Metal carbonyls · Metallocenes · N-heterocyclic carbenes · Peptides · Peptide Bioconjugates · Peptide synthesis · Solid phase synthesis · Tris(pyrazolyl)borate ligands

Contents

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Abbreviations

AMP	Antimicrobial peptide
Boc	*tert*.-Butoxylcarbonyl
CORM	CO-releasing molecule
Cu-AAC	Cu-catalyzed azide-alkyne coupling reaction
Enk	Enkephalin
Fc	Ferrocenyl
Fca	1′-Aminoferrocene-1-carboxylic acid
Fer	Ferrocenyl-alanine
Fmoc	Fluorenyl-methoxycarbyl
HATU	*O*-(Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium
HBTU	*O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium
HMBA	4-Hydroxymethylbenzoic acid
MIC	Minimum inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHC	N-heterocyclic carbene
NLS	Nuclear localization signal
NPY	Neuropeptide Y
PADA	Picolylamine-*N*,*N*-diacetic acid
Pgl	Propargyl-glycine
PNA	Peptide Nucleic Acid
SAR	Structure–activity relationship
SPPS	Solid-phase peptide synthesis
TBTU	*O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate
Tp	Tris(pyrazolyl)borate
tpm	Tris(pyrazolyl)methane

The common three letter codes for amino acids are used throughout. Unless specifically noted, stereochemically pure L amino acids are assumed. Peptides are consistently written from N- to C-terminus in standard peptide nomenclature. In a peptide, "Gly" corresponds the fragment "$HNCH_2CO$." For example, H-Gly-NH_2 is glycine carboxamide ($H_2NCH_2CONH_2$) while Ac-Gly-Ala-OH is *N*-acetylated glycyl alanine.

1 Introduction

An important aim in drug research is specificity, meaning in this context that any drug should ideally target only the organism or organ that it is designed for. Nonspecificity will result in side effects. For example, the main molecular target of the anticancer drug cisplatin is DNA. Because DNA is present in cancer cells, but equally in all other cells, cisplatin has little specificity for cancer, resulting in severe side effects of the drug, in this case nephrotoxicity and neurological disorders. This problem of specificity has been tackled by many researchers, and numerous approaches have been proposed. One very promising strategy is to conjugate (potential) drugs with (bio)molecules, often called vectors, which provide specificity for certain cell or tissue types or at least organs. These vectors can be essential nutrients for cells (e.g., sugars or amino acid) or cofactors (e.g., vitamin B_{12} as described elsewhere in this book). Cell specificity has also been achieved by conjugating drugs to antibodies. While providing good target binding, these are large macromolecules and have the problems usually associated with such macromolecules, i.e., limited diffusion rates and fast clearance. Also, chemical modification of antibodies is not straight-forward and difficult to achieve in a directed, unambiguous fashion. Lastly, given the high molecular weight of the antibody, the drug-to-vector ratio is poor in terms of molecular weight.

Finally, peptide conjugates are an option that has a lot of potential and is the topic of this chapter. Peptides are oligomers of the naturally occurring L amino acids, usually between 5 and 50 (some researchers draw the line already at 30) amino acids in length. Peptides serve as messengers and signaling molecules in the body, and literally hundreds of peptides with a physiological function are known. Physiologically, they have only limited stability because they are degraded by peptidases. For practical applications, their half life can be improved by incorporation of unnatural and/or D amino acids or chemical modifications of the peptide bond, e.g., by reducing the amide bond or using N-methylated amino acids. The chemistry of peptides is extremely well established, many peptides are commercially available, and so are a variety of reagents for their preparation. Therefore, it is quite surprising to observe the relative scarcity of metal derivatives of peptides. It is the topic of this chapter to introduce the reader to organometal–peptide conjugates. First, I will discuss the underlying chemistry and then present some applications of the so-obtained bioconjugates in the second part of this chapter.

While no comprehensive review of this topic has been published before, several overview articles on related topics were published in last few years. Dirscherl and König have summarized the solid-phase synthesis of metal–peptide conjugates in general [1]. Grotjahn have presented an interesting labeling experiment of the peptide hormone secretin with an (Arene)Ru complex [2–4]. Oligomers of metal complexes were prepared by solid-phase synthesis techniques but without any amino acids or even biological applications [5], by Heinze and coworkers who has also summarized this field [6]. Erker and coworkers studied the reaction of metal fragments such as “Jordan’s cation” with small model peptides, which leads to

N,O-coordinated bent metallocenes [7]. Structural aspects of 1,1′-substituted metallocene peptide conjugates, which have relevance to turn structures in peptides, were also reviewed [8]. Finally, peptide conjugates of (organic) drugs have received considerable attention as they offer the possibility for targeted tumor therapy. An earlier book chapter covers metal conjugates of peptides and peptide nucleic acids (PNA) in detail [9]. Two book chapters on "Bioorganometallic Chemistry" [10, 11] as well as a more specialized (and extensive) one on the bioorganometallic chemistry of ferrocene cover peptide conjugates as well [12]. Our own work on metallocene–peptide conjugates has recently been summarized [13]. van Koten has reviewed their work on Pt group metal complexes with so-called "pincer" ligands, of which some peptide conjugates were reported for biosensor applications [14]. Apart from and beyond the metal complexes covered herein, several peptide conjugates of compounds from the well-known platinum anticancer drug family were prepared and their biological activity explored [15–19].

2 Chemical Synthesis of Metal–Peptide Conjugates

To understand the problems associated with the synthesis of organometal–peptide conjugates, one has to analyze the underlying chemistry in more detail. First, peptides host a variety of chemical functionality, e.g., amino and alcohol groups, thiols, carboxylates, heterocycles, and amide bonds. This chemical diversity makes regiospecific functionalization a challenge. Second, many organometallic complexes are sensitive to oxidation by air or hydrolysis in water. Therefore, the metal complexes have to be chosen carefully, not only in terms of stability against air and water, but also with relation to the chemistry that is required for peptide synthesis (see below). While it was historically assumed by many researchers that *almost all* organometallic compounds are unsuitable for biological applications, the examples we show herein (and in other chapters) certainly disprove this general notion. It is, however, true that the choice of metal complexes for peptide synthesis is rather complex. It is hoped that the examples below are sufficiently encouraging by demonstrating that all issues associated with chemical stability can be solved satisfactorily.

In principle, there are two possible sequences of linking the metal covalently to the peptide (see Fig. 1). First, the metal complex is added during the solid-phase peptide synthesis (SPPS) while the peptide is still on the resin (Fig. 1, top). Alternatively, the metal complex can be added in solution *after* the peptide synthesis has been completed on the resin and the peptide was cleaved and purified (Fig. 1, bottom). The first scheme has all the benefits of solid-phase synthesis, that is, high yield of the conjugation reaction and purity of the conjugate. It does require, however, that an excess of the metal complex is readily available and that the metal complex is stable enough to survive all subsequent manipulations, including in particular cleavage from the resin. For very sensitive metal complexes, this may not be feasible and therefore, the second option comes into play. Also, this second scheme is obviously preferable for radioactive metal complexes.

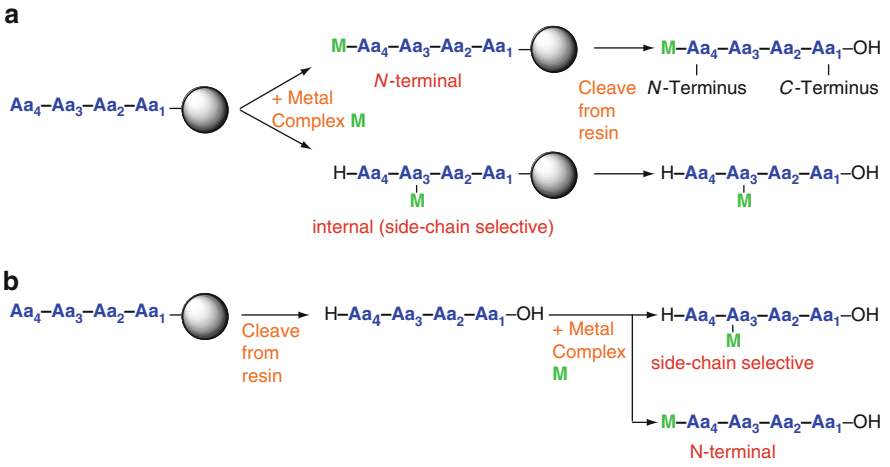


Fig. 1 Two principally different alternatives for the chemical synthesis of metal–peptide bioconjugates: (**a**) On the resin (*top*) and post-SPPS (**b**) (*bottom*). Functionalization can be N-terminal or on one of the amino acid side chains

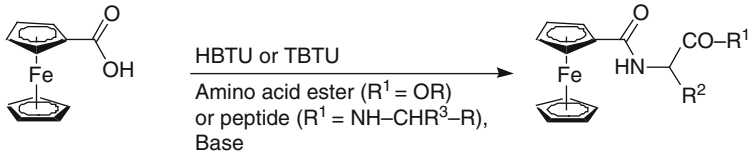


Fig. 2 General scheme for the coupling of ferrocene carboxylic acid to amino acids and peptides

2.1 *Synthesis in Solution*

The chemistry of conjugates of amino acids with metal complexes has been summarized by Beck and coworkers [20]. Also, it is beyond the scope of this review to include all amino acid conjugates with metal complexes. We will only touch a few selected examples herein.

The reaction of activated carboxylic acids with the amino group of amino acids and peptides is probably the most straight-forward conjugation reaction. It has been used, in the context of this chapter, for the functionalization of amino acids and small peptides by several groups. In most cases, ferrocene carboxylic acid or derivatives thereof were used as the metal fragment. Most work in this field was not intended for biomedical applications, but this chemistry has been amply reviewed [6, 8, 9, 12, 21–29]. In particular, the structural implications of the resulting ferrocene–peptide conjugates were categorized and analyzed in detail [8, 24]. A general reaction scheme is given in Fig. 2. The use of coupling reagents has been critically reviewed [30]. In our hands, common coupling agents like HBTU or

TBTU give good results. Coupling reactions using isobutyl-chloroformate also work well in solution, with the added advantage that only volatile side products form which facilitates work-up and purification.

We have also prepared a propargylic acid derivative of the pentapeptide enkephalin (see below), which was reacted, after cleavage and purification, with $Co_2(CO)_8$ in solution to yield the $Co_2(CO)_6$(alkyne) derivative [31]. Similarly, other $Co_2(CO)_6$ alkynyl peptides were synthesized from the preprepared peptide and $Co_2(CO)_8$ in solution (see Sect. 2.2.2 and the section below on biological activity) [32]. In a related fashion, the $Co_2(CO)_6$ moiety can also be introduced C-terminally (see Sect. 2.2.3) or internally (see Sect. 2.2.2).

2.2 Solid-Phase Peptide Synthesis

The vast majority of peptides is synthesized by SPPS techniques. SPPS is a sequential synthesis technique, in which the peptide is assembled amino acid after amino acid on solid support. It was invented in 1963 by Bruce Merrifield, who received the Nobel prize in chemistry in 1984 for this milestone in synthesis methodology. In order to achieve highest possible chemical selectivity, the incoming amino acid is N-terminally protected by a temporary protecting group (in most cases discussed in this chapter, the Fmoc group), and all functional side chain residues are also protected by permanent protecting groups. The Fmoc group is removed after successful coupling and the next amino acid can then be added to the growing peptide chain. At the end of this synthesis scheme, the peptide is cleaved from the resin and at the same time, all permanent side chain protecting groups are removed. This last step is performed, in most cases, with more or less concentrated acids. A general scheme for solid-phase synthesis is given in Fig. 3. Several different places and times for inserting the metal complex are conceivable as indicated in the figure. Naturally in all schemes, the metal complex has to survive all subsequent synthesis steps, and in particular the final cleavage from the resin.

2.2.1 N-Terminal Derivatization

In the most obvious way, a metal complex can be added to the N terminus of the final peptide on the resin (bottom path in Fig. 3). This requires very similar chemistry as discussed above for solution metallation. The metal complex is treated more or less as "another amino acid." HBTU and TBTU are the preferred coupling reagents in this case, also HATU might have advantages in difficult cases. We find that for less reactive metal complexes, e.g., cobaltocenium carboxylic acid, longer activation and coupling times of up to 24 h may be needed.

In fact, the first organometal–peptide bioconjugates were reported by a French group in the 1980s, all following this scheme [33–36]. However, there were problems with the isolation and purification of many of these compounds, as

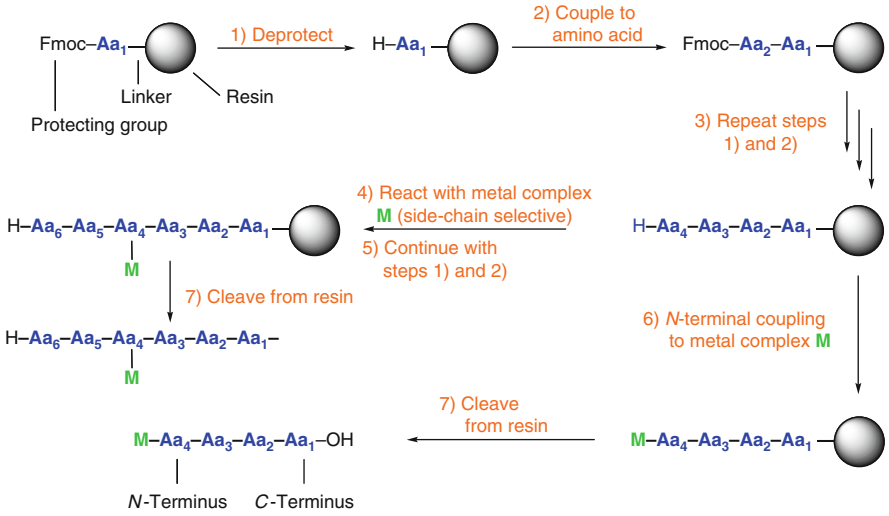


Fig. 3 General scheme for solid-phase peptide synthesis (SPPS)

noted and honestly reported by the authors at the time [33]. In hindsight, the problem was probably the harsh conditions of cleavage that were standard at the time, requiring concentrated HF. It is very likely that the metallocenes (mostly ferrocene derivatives and cymantrene) decomposed under those harsh conditions. After these initial failures, the field lay dormant for more than 10 years.

During this time, solid-phase (peptide) synthesis techniques developed rapidly, and many more options for resins, linkers, and side chain protecting groups are now commercially available. As a demonstration, our group published the successful SPPS of the pentapeptide enkephalin with a (histidinato)Mo(allyl)CO_2 complex, which is very sensitive even to dilute acids. After protonation of the ally ligand, the complex decomposes rapidly. This synthesis was achieved by using a suitable resin and the HMBA linker that can be cleaved by concentrated ammonia, thereby avoiding acidic conditions in the presence of the metal complex altogether [37]. We and the Schatzschneider group have since published several more metal complexes that were N-terminally coupled to enkephalin (see Fig. 4). Enkephalin is a five-amino acid neuropeptide. It exists in two forms which differ only by the C-terminal amino acid (Leu or Met). The more abundant [Leu^5]-enkephalin has the primary sequence Tyr-Gly-Gly-Phe-Leu and is a natural ligand for the opiate receptor. In our work, we have exclusively worked with [Leu^5]-enkephalin (Enk) and derivatives thereof. While we have explored the biological activity of metal-enkephalin derivatives in some cases (see below), we have not chosen Enk for its biological function, but rather for its ease of preparation with only five amino acids and one side chain protecting group (Tyr).

We have prepared Enk with ferrocene and cobaltocenium carboxylic acid attached to its amino end [38]. In a series of papers, we have explored the use of the tris(pyrazolyl)borate (Tp) ligand family [39–41]. Boron-functionalized,

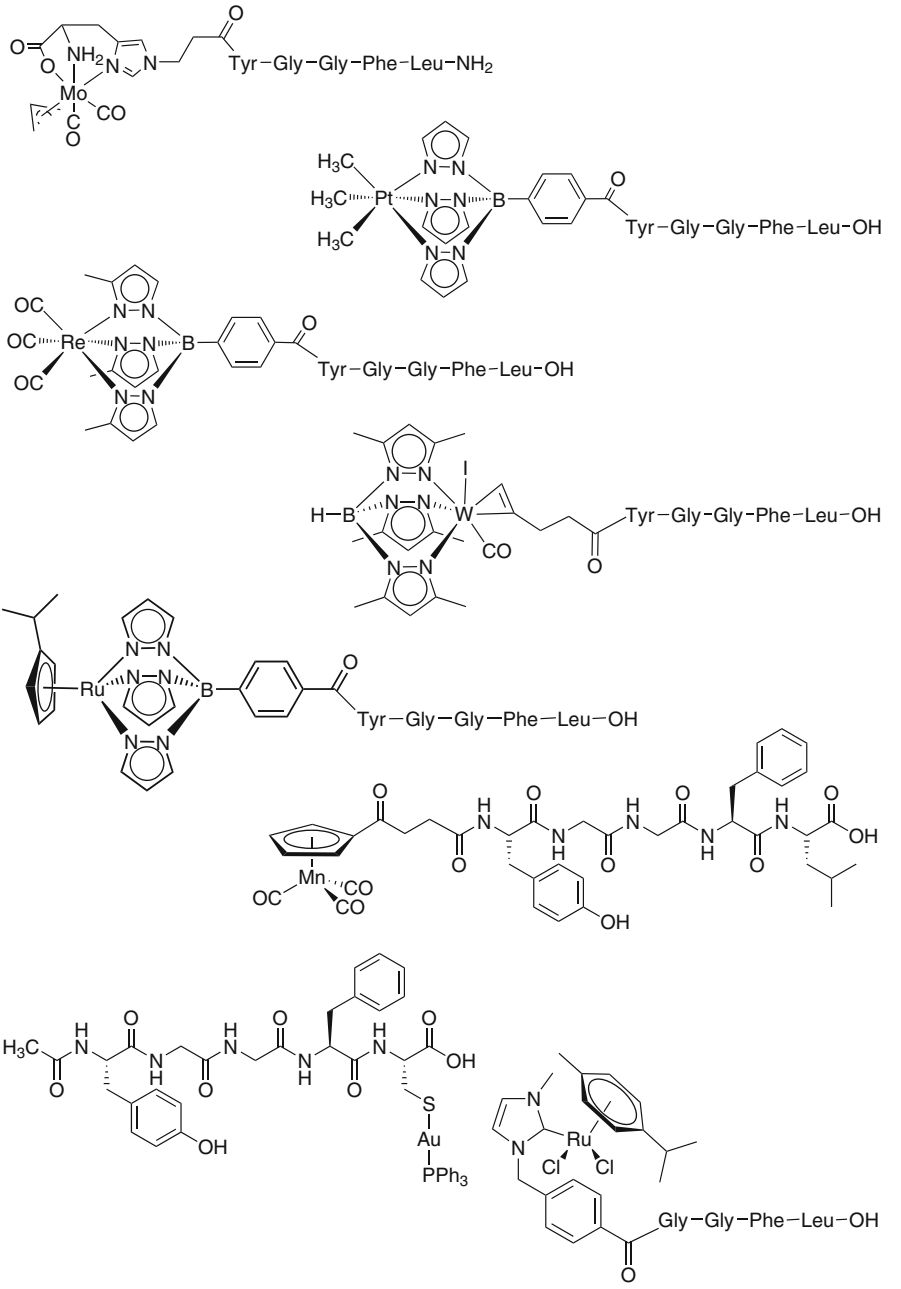


Fig. 4 Organometallic derivatives of the neuropeptide Enkephalin

so-called "third generation," Tp ligands can be prepared which have a benzoic acid functionality. These ligands can be converted into a variety of metal complexes, which can then be used for the labeling of peptides. Two examples are shown in Fig. 4. The X-ray single crystal structure of a (Tp)$PtMe_3$ derivative with phenylalanine was also reported [39]. This compound, as well as a related (Tp′)$Re(CO)_3$ complex, was successfully linked to enkephalin (Tp′: tris(3-methylpyrazolyl)-borate) [40]. In both cases, however, the standard methods for SPPS were carefully optimized by prior test reactions on single amino acids. Binding of a mixed Cp/Tp Ru complex to enkephalin was only possible for a carefully selected mixed-sandwich ruthenium derivative (Fig. 4) [41]. We have also used another $Re(CO)_3$ complex with the 3,3-bis(2-imidazolyl) propionic acid ligand in bioconjugates with PNA oligomers [42]. As another interesting class of commonly used ligands in organometallic chemistry, we have synthesized *N*-heterocyclic carbenes (NHC) with carboxylic acid functionality that could be used in SPPS. One example of a stable Ru(arene)(NHC) complex on a truncated Enk derivative is also shown in Fig. 4 and the [43]. Finally, we have added a (Tp′)W(alkyne)CO complex to the amino group of Enk (shown in Fig. 4) and the peptide neurotensin. In this case, pentynoic acid served as a bifunctional linker for peptide conjugation as well as for metal complexation to the triple bond [44].

Schatzschneider and coworkers prepared the manganese-containing carboxylic acid Cym–CO–CH_2–CH_2–COOH (Cym = cymantrene, $CpMn(CO)_3$), which could be coupled to several peptides such as enkephalin (Fig. 4) [45], neurotensin (NT) and pseudoneurotensin (pNT), the simian virus nuclear localization sequence (NLS), and TAT peptides [46]. This work also introduces microwave-assisted peptide coupling reactions for the metal complex. An interesting case arises for branched peptides. Thus, a derivative of the native hormone human calcitonin with a branching lysine residue in the middle has been reacted with a keto acid derivative of cymantrene ($CpMn(CO)_3$) [47]. While the metal resides at the N terminus of the main chain peptide, branching at this lysine residue creates a second N-terminal site at the k7 side chain, which is available for further derivatization. This concept was used in the present study for labeling with carboxy-fluorescein [47]. In a thorough follow-up study, several more cymantrene derivatives of this peptide were investigated to optimize the linker between the organometallic group and the peptide [48]. The cytotoxicity as well as cellular uptake and localization of these conjugates was explored, see below in Sect. 3.

A derivative of the naturally occurring [Leu^5]-Enk is [Cys^5]-Enk, which has a cysteine instead of leucine as the C-terminal amino acid. This peptide was prepared on Wang resin, the cysteine was selectively deprotected, and a Au(PPh_3) fragment complexed to the thiol group. Following a modified cleavage procedure, the Au-Enk peptide was isolated in >90% purity as the first synthetically Au(I) peptide prepared by SPPS [49]. Schatzschneider and coworkers have used a cationic $Mn(CO)_3$ complex with an alkyne-functionalized tris(pyrazolyl)methane (tpm) ligand for N-terminal peptide modifications via Sonogashira coupling and "click" chemistry (see Fig. 5) [50].

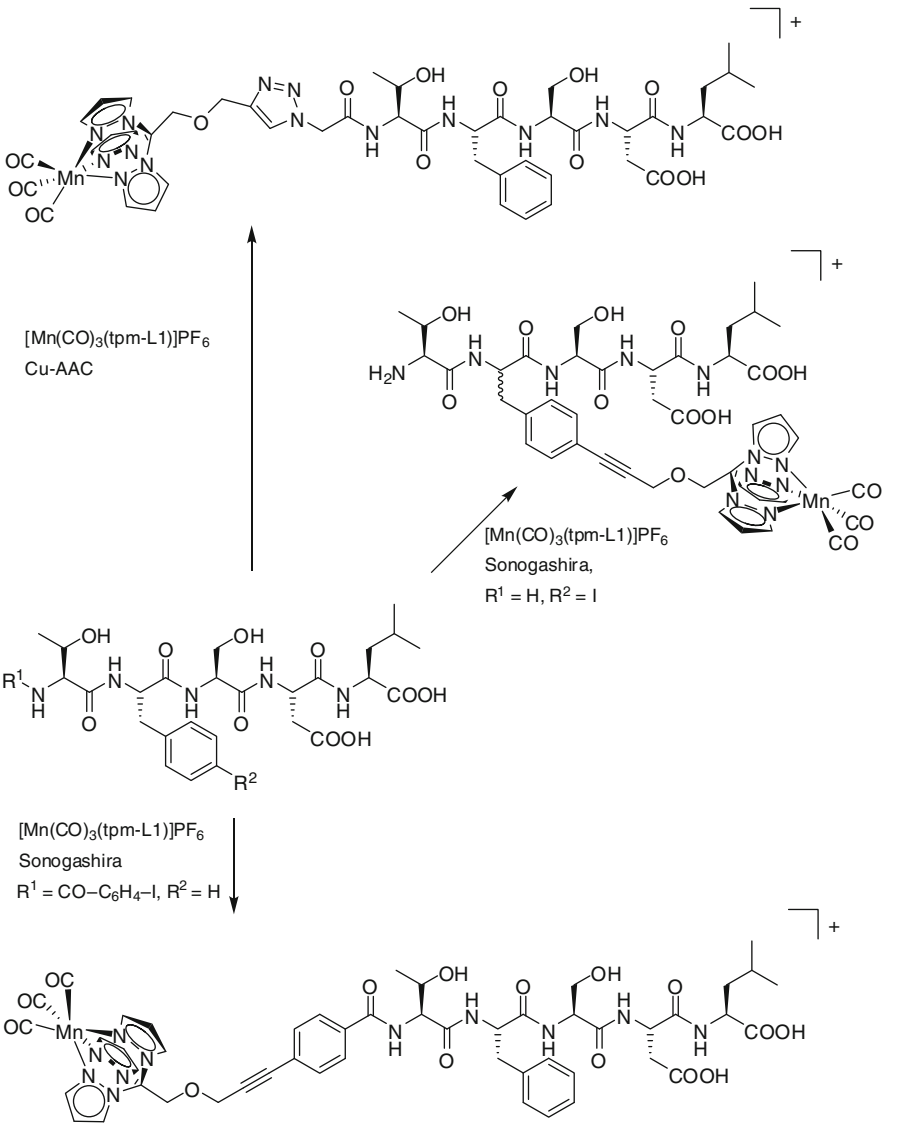


Fig. 5 Examples which demonstrates the versatility of an alkyne-functionalized [(tpm)Mn $(CO)_3]^+$ complex for both derivatization strategies, as well as the regio-flexibility with this chemistry

2.2.2 Side Chain Derivatization

A different strategy has to be employed for placing the metal complex at a side chain of the peptide. One possibility would be to choose an orthogonally protected side chain, i.e., a Lys residue from which the protecting group can be removed

under conditions which leave the rest of the peptide intact. The free amino group could then be reacted with an activated acid complex much as described in the section above.

Another strategy is to introduce yet a different chemical functionality, which can be used for derivatization in a regioselective manner. To this end, we have used the Pd-catalyzed C–C bond forming reaction between alkynes and aromatic iodides (Sonogashira coupling) [51, 52], as well as the Cu-catalyzed [2 + 3] dipolar cycloaddition reaction between alkynes and azides (Cu-AAC) [53–55]. While the uncatalyzed reaction has long been studied by Huisgen and coworkers, the Cu-catalyzed variant has recently gained popularity as the prototypical "click reaction" [55].

We have reported the Pd-catalyzed Sonogashira coupling of ferrocene alkyne derivatives as metal probes to iodophenylalanine-containing peptides. 4-Iodophenylalanine was incorporated into dipeptides and the neuropeptide [Leu^5]-enkephalin by SPPS, thereby creating a functional group suitable for the Sonogashira coupling. The reaction with two different ferrocene alkynes resulted in the corresponding ferrocene-labeled derivatives, which were obtained in good yield and purity (Fig. 6) [56]. Sonogashira coupling was also used to link a $[(tpm)Mn(CO)_3]^+$ complex

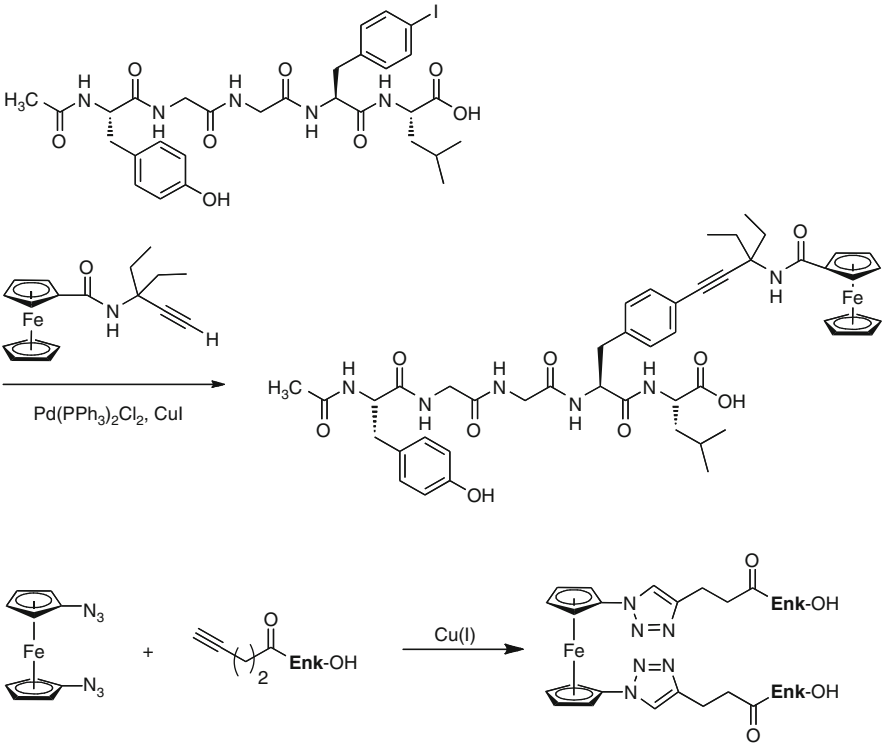


Fig. 6 Two examples for the use of the Sonogashira reaction (*top*) and the Cu-AAC ("click chemistry," *bottom*) for the modification of peptides with organometallics from our group

(tpm = tris(pyrazolyl)-methane) to several model peptides [50]. Similarly, the same tpm-alkyne derivative was coupled N-terminally to the same azide-functionalized peptides by Cu-AAC (see Fig. 5) [50].

The click chemistry approach has been used by our group to prepare a range of amino acid-triazole ferrocene and 1,1′-bis(amino acid-triazole)–ferrocene conjugates [57]. By using the N-terminal pentynoic acid enkephalin, the respective ferrocene mono- and 1,1′-bis-peptide derivatives were obtained (see Fig. 6 for an example). The mono-substituted Fc-triazole-Enk was also obtained by performing the Cu-AAC reaction on the resin [57]. Chemically related but with a different background, we have used the Cu-AAC to generate protein-resistant SAMs on Au surfaces [58, 59]. While other groups have used this versatile reaction, inter alia, to modify DNA oligomers [60–62] or prepare compounds of medicinal interest [63], we have used similar chemistry to obtain metal-modified oligomers of PNA for biosensor applications [64–66].

Once an alkyne moiety is introduced into a peptide, e.g., as propargyl glycine (Pgl) in place of a simple glycine in Enk, it can of course be used directly for complexation to a metal complex. Along those lines, we have reacted $Co_2(CO)_8$ with the peptide Ac-Tyr-Gly-Pgl-Phe-Leu-NH_2, to yield the $Co_2(CO)_6$ complexed to the Pgl alkyne side chain [32]. In a real SPPS scheme with a pre-formed metal-alkyne complex, the amino acid building block [Tp*WI(CO)(η^2-Fmoc-Pgl-OH)] was incorporated to yield the side-chain substituted Enk derivative H-Tyr-Gly-[Tp*W(I)(CO)(η^2-Pgl)]-Phe-Leu-OH (Tp*: tris(3,5-dimethylpyrazolyl)-borate) [44].

In a slightly different approach, the introduction of a metal complex is also possible as a metallo amino acid. For example, ferrocenyl-alanine (Fer) was prepared as early as 1957 [67]. Also, stereoselective preparations [68–71] and its biological activity were reported [72], and the compound has been inserted into peptide sequences just as a normal amino acid would (see Fig. 7 for an example) [35, 71]. In this case, the metallocene constitutes the side chain. Alternatively, the amino and carboxylic acid function can be on two different Cp rings, yielding, in the simplest possible case, 1′-aminoferrocene-1-carboxylic acid (Fca, see Fig. 7 for an example). This compound has also been reported in the literature, although it was difficult to obtain in pure form [73, 74]. It has since been incorporated into oligomers

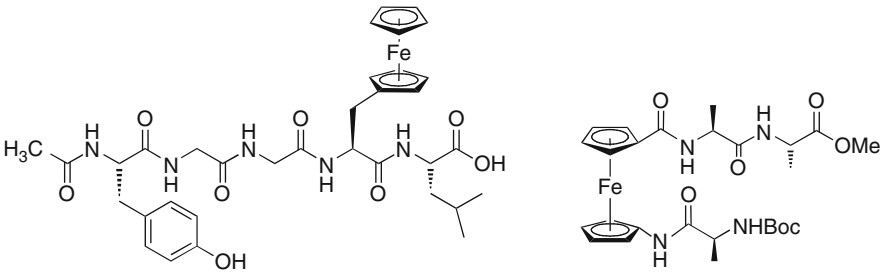


Fig. 7 Peptides containing ferrocenyl-alanine as one amino acid (Ac-Tyr-Gly-Gly-Fer-Leu-OH, *top*) and 1′-aminoferrocene-1-carboxylic acid (Boc-Ala-Fca-Ala-Ala-OMe, *bottom*)

and peptides as one amino acid [75–81]. Interestingly, Janda and coworkers have synthesized and used suitably substituted Fca derivatives to generate antibodies for enantioselective catalysis (Diels-Alderases) [82]. These workers also published a single crystal structure of a ferrocene-containing antibody [83]. Novel ferrocene-derived amino acids were also reported and incorporated into peptides by the groups of Schmalz [84] and Erker [85, 86]. Heinze and Hempel reported on the incorporation of (organo)metal-based amino acids into fluorophore-derivatized peptides by SPPS [87], while Kirin et al. included the bis(pyridylamine) ligand into the side chain of peptides, which could then be turned into metal complexes [88, 89].

2.2.3 C-Terminal Modification

A C-terminal modification is certainly most difficult to achieve during standard SPPS, since the peptide is usually linked to the resin via its carboxylic acid group. Functionalization at this group, therefore, requires a so-called safety-catch linker. While such linkers are available, and some are also commercial, they have rarely been used in metal-peptide chemistry. We have used aminoferrocene in standard peptide coupling reactions with Boc-protected amino acids to form Boc-Aaa-NH-Fc, where Aaa were several different amino acids [90]. In our hands, direct C-terminal functionalization with metal compounds such as aminoferrocene has been difficult. A successful example was the introduction of a dicobalt-hexacarbonyl unit onto the C terminus of Enkephalin (see Fig. 8). Strictly speaking, though, the Enk was C-terminally functionalized with an alkyne unit upon cleavage, and the Co carbonyl moiety was introduced only post-SPPS in solution (see Sect. 2.1 above) [32].

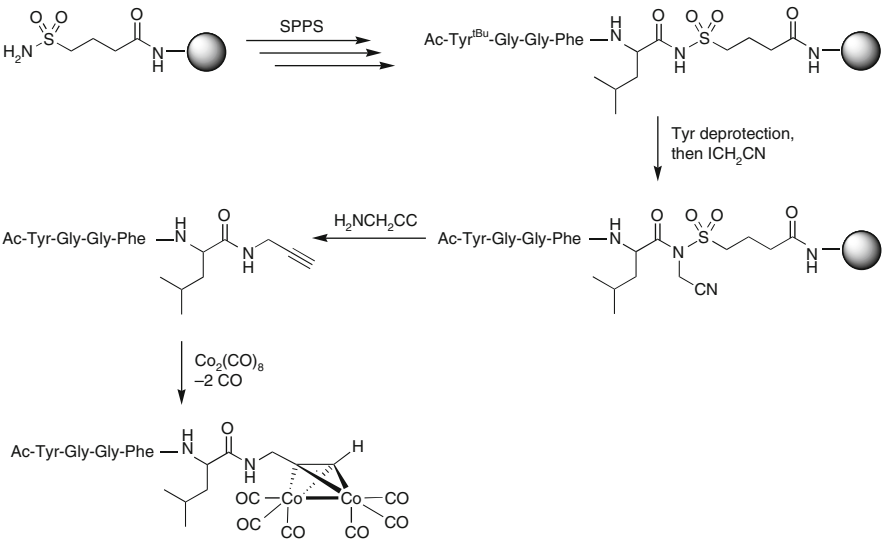


Fig. 8 Synthesis of an enkephalin derivative with a C-terminal Co carbonyl metal complex

3 Biomedical Applications

Applications of metal–peptide conjugates in general were published in several areas, also extending the range of subjects covered herein. We are restricting the discussion below (1) to metal–peptide conjugates with *organometallic* compounds and (2) to biomedical applications only.

3.1 Radiopharmaceutical Applications

This area is covered almost exclusively with one class of compounds, namely the $^{99m}Tc(CO)_3$ moiety for radiolabelling and the $Re(CO)_3$ congener of the metastable ^{99m}Tc isotope. Generally, labeling of the peptide is performed with the $^{99m}Tc(CO)_3$ core and a suitable bifunctional chelator as described in [91]. The current status and opportunities for radiolabelling of peptides with Tc(I) or Re(I) species as described above have been reviewed frequently [92–96]. We have reviewed previous work on $Tc(CO)_3$-labeled peptides [9] and will hence limit our discussion here to very few selected examples that may serve to highlight novel chemistry in this very important field.

An early paper describes the use of picolylamine-*N*,*N*-diacetic acid (PADA) as a chelator for the $Tc(CO)_3$ core as a radiolabel for biologically stabilized neuropeptide Y (NPY) analogs [97]. This paper serves as an illustration of this general approach. As a more natural ligand, suitably substituted histidine derivatives were introduced as versatile ligands for the radiolabelling with Tc(I) [98]. More recently, Schibli and coworkers reported a so-called "click-to-chelate" approach [99], which combines the Cu-AAC (see Sect. 2.2.2) with suitable amino acids. A chelating moiety is generated, which involves the newly formed 1,2,3-triazole ring, upon the click reaction. If the reaction is performed in the presence of the $[Tc(H_2O)_3(CO)_3]^+$ precursor, then a radiometal-chelate ring is formed in one step with high selectivity. This reaction is extremely versatile and has since been exploited for a number of different biomolecules, including, inter alia, peptides [99–101]. A general reaction scheme is shown below in Fig. 9. Dual-labeling with fluorescein and a Tc(I) radiolabel has also been reported [102].

Beck-Sickinger and coworkers have explored medicinal applications of NPY. The NPY receptor is overexpressed on numerous tumor cells, and hence this

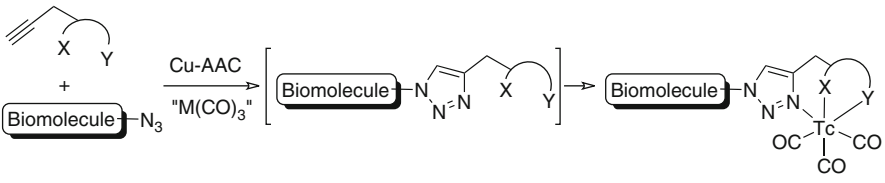


Fig. 9 General reaction scheme for the "click-to-chelate" approach by Schibli and coworkers

peptide has potential not only for diagnostic applications in radio imaging, but also for radio therapy. The principal suitability of NPY was demonstrated some years ago by this group [97]. More recently, the binding of $Re(CO)_3$-labeled peptides with the NPY-receptor and its cellular internalization was demonstrated. Radio imaging studies were then carried out on mice as well as on healthy humans and individuals suffering from breast cancer. Unfortunately, however, for these studies unspecific labeling with higher-valent ^{99m}Tc compounds was used [103]. Even so, a good tumor-to-background ratio was achieved. It will be interesting to see the results if specific labeling with a high-affinity ligand for the $Tc(CO)_3$ fragment would be used. The work from this group and their collaborators has been reviewed [93, 95].

3.2 *Anticancer Activity*

Today, testing for antiproliferative activity is usually done in colorimetric cell culture assays, such as the MTT assay (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide). As described in the introduction, the combination of cell-targeting peptides with cytotoxic metal complexes has the potential of providing more specific drug candidates with less side effects. However, this concept has so far only rarely been realized.

Nonetheless, cytotoxicity testing was performed on metal–amino acid bioconjugates. In a series of papers, Kenny and coworkers describe the antiproliferative activities of conjugates of ferrocene with amino acids and dipeptides. In some cases, good IC_{50} values down to low μM numbers were observed, and some structure–activity relationships (SAR) could be derived [104–108]. Their most active compound so far, N-(6-ferrocenyl-2-naphthoyl)-glycine-L-alanine ethyl ester, has an IC_{50} value of 1.3 μM against the H1299 lung cancer cell line [105], slightly lower than even cisplatin on the same cell line. Cell cycle analysis shows an arrest in the G2 phase for these ferrocene peptides [104]. Very recently, ferrocene tri- and tetrapeptides were also prepared and tested [109], but had lower activity (>50 μM) than the smaller ferrocene dipeptides. Also, all ferrocenoyl derivatives of the oligo-peptides prepared in our group so far (e.g., of enkephalin and peptides discussed in Sect. 3.4 below) had IC_{50} values >1 mM and are thus considered nontoxic [38, 110, 111]. Miklan et al. found that ferrocenyl acrylic acid exhibited remarkable activity against a human leukemia cell line, which was not improved by conjugating this compound to oligo-Arg peptides. In contrast, the corresponding conjugates of ferrocene carboxylic acid did show significantly better activity against the same cell line [112].

The cytotoxicity of other metal complexes was also investigated. Neundorf, Schatzschneider, and coworkers found that the cymantrene derivative of the branched cell-penetrating peptide hCT(18-32)-k7 mentioned in Sect. 2.2.1 shows high cytotoxicity (IC_{50} = 36 μM) against the MCF-7 cell line, while the cymantrene complex as well as the peptide alone is nontoxic [47]. Likewise, the attached

metal complex alters the intracellular distribution such that the metal–peptide conjugate accumulates in the cell nucleus. This redistribution, compared to the peptide alone which remains in the cytoplasm, may be related to the conjugate's activity [47]. Further detailed structure–activity studies on related derivatives were carried out by the same groups [48, 113]. Originally, these complexes were conceived as CO-releasing molecules (CORMs), a currently very active area of bioorganometallic chemistry (see [114]).

Interesting antiproliferative activity was also observed upon light activition of peptide conjugates of metal carbonyl complexes [115]. While the original idea was to open a coordination site for interaction with biomolecular targets by irradiation, it may be that CO release or singlet oxygen generation is more likely modes of action eventually.

The cytotoxicity of the $Co_2(CO)_6$ alkyne derivative of enkephalin described in Sect. 2.1 was investigated by our group, and IC_{50} values between 15 and 50 μM were obtained, depending on the cell line [31]. This is about one order of magnitude worse than the activity an $Co_2(CO)_6$ alkyne derivative of acetyl salicylic acid, which is the lead structure of this class of compounds [116–118].

3.3 Antibacterial Activity

In the literature, (small) peptides with antibacterial activity are known for over 20 years now [119]. They have been tested also in clinical settings; however, no real breakthrough has been made with simple, linear peptides. Interference with the bacterial membrane has been suggested as the main mode of action for these antimicrobial peptides (AMPs) [120, 121]. We have discovered that modification of simple, small peptides with metallocenes (ferrocene or cobaltocenium) not only increases the peptides' activity in certain cases [122, 123]. More importantly, metallocene substitution can alter the specificity for Gram-positive or Gram-negative bacteria, respectively. For example, the ferrocenoyl pentapeptide FcCO-Trp-Arg-Trp-Arg-Trp-NH_2 has a MIC value of 7.7 μg ml^{-1} against the Gram-positive *S. aureus* but about fourfold decreased activity against the Gram-negative *P. aeruginosa* [123]. This behavior is in contrast to the 20 amino acid naturally occurring peptide antibiotic pilosulin 2, which is four times more active against *P. aeruginosa* and has an MIC of only 16 μg ml^{-1} against *S. aureus*. Shortening or changing the amino acid sequence decreases activity for the metallocene derivatives. Also, replacement of ferrocene with the positively charged cobaltocenium carboxylate eliminates any differential activity for Gram-positive or Gram-negative strains [123]. It appears that the mode of action of these and related metallocene–peptide conjugates resembles the detergent Triton X-100 [124]. A recent proteomics study by mass spectrometry on differential protein expression of *Corynebacterium glutamicum* sheds more light on the mode of action by this peptide [125].

3.4 Cell Uptake and Intracellular Localization

We have initiated a research program to study the use of small peptides as vectors to direct covalently attached (organo)metal complexes to specific loci inside mammalian cells. In this setting, the attached peptide serves as a vector to direct the attached metal complex to any specific place inside a cell, where it can exert its activity with high specificity. This question of target specificity is in fact already discussed in the introduction, and it was originally the starting point for our investigations.

The synthesis of metal derivatives of such cellular signaling peptides involves more functionalized amino acids and is therefore more involved than most of the examples presented above. In addition, a fluorescent marker needs to be introduced into the metal–peptide conjugate to enable visualization by (confocal) fluorescence microscopy. This required yet another functionality to be available, e.g., the primary amino group of a lysine side chain. We found that metallocene derivatives of the simian virus 40 derived nuclear localization signal (NLS) were successfully translocated into the nuclei of HepG2 cells (Fig. 10 left, a) [111]. Moreover, and unexpectedly, they were readily taken up by the cells, when in fact the acetylated (and nonmetallated) NLS derivatives gained no entry into these cells. The results were later confirmed with scrambled NLS sequences, which also gained no entry into this cell line [110]. None of these metallocene heptapeptides were toxic at concentrations up to 100 mM, and the overall integrity of the cells was confirmed by propidium iodide staining. Interestingly also, metallocene derivatives of the TAT(48–60) peptide, which is derived from the HI virus, remained in the cytoplasm of the same cell line (Fig. 10, middle, b) [126]. Moreover, only the ferrocene-TAT derivative, but not the isostructural cobaltocenium derivative, was at all incorporated into these cells (Fig. 10, right, c). Representative microscopy pictures are shown in Fig. 10, with the corresponding metallocene–peptide conjugates shown in Fig. 11. Colocalization studies using confocal fluroescene microscopy revealed a lysosomal uptake mechanism [13].

Cellular uptake and subcellular distribution of organometal–peptide conjugates was also investigated by Schatzschneider and coworkers, as described above [47].

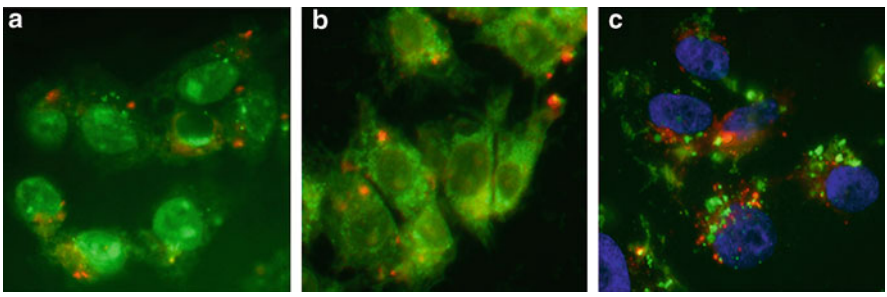
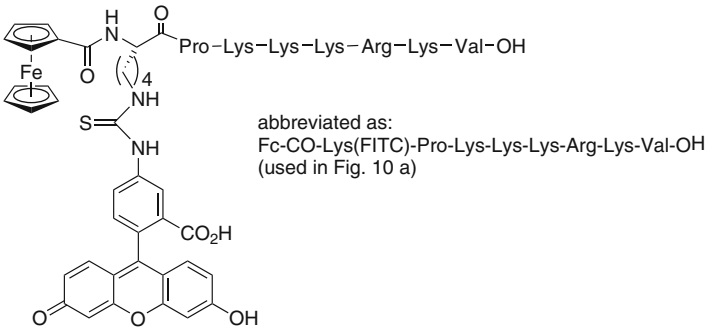


Fig. 10 Fluorescence microscopy images of metallocene peptides in HepG2 cells. See text for details



Fc-CO-Lys(FITC)-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-OH (Fig. 10 b)

Cc^{+}-CO-Lys(FITC)-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-OH (Fig. 10 c)

Fig. 11 Metallocene–peptide conjugates used for the study in Fig. 10

3.5 Biosensors and Molecular Recognition

Organometallic complexes in general have frequently served in biosensors. Most importantly, ferrocene and its derivatives have been used as electrochemically active groups for electrochemical biosensors [9, 12, 25]. Kraatz and coworkers have studied inhibitors for the cysteine protease papain in some detail. The ferrocenoyl tetrapeptide Fc-Gly-Gly-Tyr-Arg-OH was identified as a potent competitive inhibitor of papain with mM affinity [127]. Toward the design of protein-sensitive electrochemical probes, the same group has recently extended this approach toward electrochemical screening of papain inhibitor peptide libraries [128, 129]. Given the pathophysiological importance of cysteine proteases, such an assay may provide alternative screening opportunities for lead identification and optimization. As mentioned above, the van Koten group has pioneered work on cyclometallated "pincer" ligands of Pt and Pd. Such complexes could be covalently linked to proteins, as well as to peptides by solid-phase synthesis methods. The resulting complexes could serve as colorimetric sensors for small molecules and toxins such as I_2 and SO_2 [14, 130–132]. Moreover, these highly colored complexes could be useful color biomarkers in solid-phase (peptide) synthesis.

4 Summary

In this chapter, we describe the synthesis of peptides with covalently bound organometallic fragments, as well as the activity of such conjugates. Systematically, the metal complex can be bound to the C terminus or the N terminus of the peptide, the latter being far more explored simply for practical reasons during

solid-phase synthesis. Alternatively, functional amino acids can be introduced and a suitable metal fragment may thus be connected to the side chain of those amino acids. To this end, the Pd-catalyzed Sonogashira coupling and the Cu-catalyzed azide-alkyne coupling (Cu-AAC, also called "click reaction") were found most useful. The reduction of *p*-nitro-phenylalanine to *p*-amino-phenylalanine might provide another alternative as the reduction chemistry is orthogonal to common peptide chemistry. Although the reaction has already been used to bind, e.g., bis (picolyl)amine ligands to peptides and PNA oligomers [133], its potential for organometallic functionalization remains yet to be uncovered.

Following either of the above schemes, metal functionalization can be achieved on the resin or after cleavage of the peptide from the resin in solution. Both schemes have particular advantages and disadvantages and were indeed explored already. In any case, the necessary chemistry will be governed by the chemical stability of the metal complex. In our work, the stability of any new metal complex is usually tested on small model peptides on the NMR or HPLC/MS scale before a long peptide synthesis with this new metal complex is initiated. In most cases, an optimized synthesis with the metal complex appropriately introduced will usually yield the peptide conjugate in >90% purity, as established by analytical HPLC. Remaining impurities can be removed by preparative HPLC. In many cases, also mixtures of peptides can be purified by (semi)preparative HPLC, but the conditions need to be optimized with particular respect to the chemical properties of the metal complex. For reliable and reproducible biological results, the identity and purity (ideally >95%) of all peptide bioconjugates should be established and rigorously monitored. In this way, chemists can provide tailor-made and high-quality tools for the study of biological problems.

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Organometallic Radiopharmaceuticals

Roger Alberto

Abstract Although molecular imaging agents have to be synthesized ultimately from aqueous solutions, organometallic complexes are becoming more and more important as flexible yet kinetically stable building blocks for radiopharmaceutical drug discovery. The diversity of ligands, targets, and targeting molecules related to these complexes is an essential base for finding novel, noninvasive imaging agents to diagnose and eventually treat widespread diseases such as cancer. This review article covers the most important findings toward these objectives accomplished during the past 3–4 years. The two major available organometallic building blocks will be discussed in the beginning together with constraints for market introduction as imposed by science and industry. Since targeting radiopharmaceuticals are a major focus of current research in molecular imaging, attempts toward so-called technetium essential radiopharmaceuticals will be briefly touched in the beginning followed by the main discussion about the labeling of targeting molecules such as folic acid, nucleosides, vitamins, carbohydrates, and fatty acids. At the end, some new strategies for drug discovery will be introduced together with results from organometallic chemistry in water. The majority of the new results have been achieved with the $[^{99m}Tc(OH_2)_3(CO)_3]^+$ complex which will, though not exclusively, be a focus of this review.

Keywords Technetium · Carbonyl complexes · Technetium cyclopentadienyl · Vitamin B12 · Molecular imaging

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Contents

1 Introduction

The application of organometallic compounds in life sciences represents the advent of bioorganometallic chemistry as an independent research direction within biological and medicinal inorganic chemistry. Recent bioorganometallic chemistry turned into a flourishing field of research (and hopefully soon into routine application) mainly due to the promise related to organometallic tamoxifen analogs as introduced by Jaouen et al. [1]. As witnessed by the past International Symposia in Bioorganometallic Chemistry (ISBOMC), bioorganometallic chemistry has a multitude of facets. Application of organometallic compounds in, e.g., cancer therapy is certainly one of the major forces driving the field [2, 3], but thematically related application in bioanalytical chemistry is impacting as well [4, 5]. Mimicking naturally occurring (organometallic) enzymes such as hydrogenases with organometallic complexes further powers our field. Dihydrogen as a major energy source in the future requires aqueous catalysts. Rauchfuss et al. reported substantial progress in this direction, employing organometallic mimics of the natural models [6, 7]. Complementing organometallic small pharmaceuticals with larger, targeting biomolecules such as peptides or PNAs has recently been reported by Metzler-Nolte et al. introducing a further aspect previously almost exclusively occupied by classical coordination compounds, if metals were playing a role at all [8].

Traditionally, complexes containing one or more metal–carbon M–C bonds are considered to be water- and air-sensitive and therefore not suitable for application in any field relying on an aqueous environment. This simplified view of the world has been changed with organometallic models of hydrogenases or applications in medicinal fields as outlined above. Although most of organometallic complexes are indeed sensitive in water and/or air, ligands such as cyclopentadienyl, arenes,

carbon monoxide CO, allyles, or isonitriles have shown to stabilize partly uncommon oxidation states and metals even under strongly challenging conditions. Sadler and Dyson et al. showed for instance the versatility of the $[(\eta^6\text{–}C_6H_6)Ru]^{2+}$ motif for applications in cancer therapy [9, 10]. The arene ligand has been successfully complemented with a variety of other polyaromatic ligands to elucidate structure–activity relationships with respect to their therapeutic indexes, underscoring the impact of organometallic complexes in medicine [11].

The application of organometallic compounds in radiopharmacy is different from other fields of bioorganometallic chemistry [12]. Whereas novel, metal-based agents for cancer therapy are selected from essentially all available organometallic complexes (provided they are stable under physiological conditions), complexes to be applied in molecular imaging have to be synthesized on site, at least for diagnostic but, likely, also for therapeutic purposes. This constraint is imposed by the short half-life times of the radionuclides, in particular for ^{99m}Tc with its $t_{1/2}$ of 6 h. The yield must exceed 98%, be reproducible and the procedure tolerates variations from the standard synthetic protocols since hospital laboratories have often different instrumentations and customs. These features limit the number of complexes and building blocks substantially.

Organometallic chemistry in radiopharmacy follows two strategies which current status is outlined below in more detail for actual examples. The first strategy involves the synthesis of a building block in a Kit-like setup. The building block is then submitted to the labeling of the targeting molecule of choice. This strategy includes the opportunity of using such Kits for research but is not really appreciated in clinical routine. The second method is the "all in one Kit." All the components of the imaging agent are present, enabling the production of the targeted compound in one single step. Actually available, commercial Kits in routine application obey this principle. The development of novel radiopharmaceuticals (organometallic or "classical") aims at proceeding from method 1 to 2. These basic considerations are outlined in Fig. 1.

There are two extremes for the two strategies mentioned above. For the first strategy stands the bis-arene-Tc(I) compounds of the form $[(C_6H_6)_2{}^{99m}Tc]^+$, highly appreciated by the author but which never exceeded the basic research Kit 1 stage, probably for synthetic reasons [13]. The second strategy is represented by the production of the well known Cardiolite® Kit for myocardial imaging, the most successful (for commerce and health care) radiopharmaceutical and a classical organometallic complex [14].

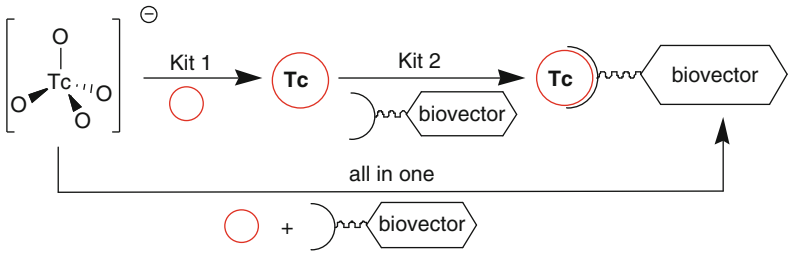

Fig. 1 The two strategies in producing a radiopharmaceutical. Two-step approach with precursor complex above, all in one procedure for routine clinical application below

The building block strategy 1 is currently much more distinct than the "all in one" approach but this is rather related to the fact that new radiopharmaceuticals are not in the stage of clinical development than due to the impossibility of proceeding from option 1 to 2 for the currently available complexes.

2 Building Blocks for Organometallic Radiopharmaceuticals

In the research for novel radiopharmaceuticals, the building block concept comprises an invariable complex fragment which is produced in one step and which features a number of coordination sites easily to be substituted by a ligand group pendent to a targeting bioactive molecule (second generation radiopharmaceuticals). Optionally (first generation radiopharmaceuticals), a ligand with high affinity may coordinate to the building block. Coordination of this ligand will make up the final radiopharmaceutical whereby neither the ligand for itself nor the building block is pharmaceutically active (technetium essential approach). There are currently three building blocks under study. The first one is the $[^{99m}Tc(CO)_3]^+$ fragment in the form of $[^{99m}Tc^{I}(OH_2)_3(CO)_3]^+$ **1** [15] and the second $[^{99m}Tc^{I}(NO)(CO)_2(OH_2)_3]^{2+}$ **2**, both based on technetium in the oxidation state +I [16]. The third is represented by the nonorganometallic $^{99m}Tc^{III}$ building block $[^{99m}Tc^{III}(NS_3)]^0$ **3** (with NS_3 being a tetradentate umbrella type ligand), which has a high affinity for binding C-ligands, mainly isonitriles and which obey the mixed ligand [4 + 1] concept [17, 18]. These building blocks are depicted in Fig. 2.

To mirror the current status of these building blocks in the research for novel organometallic radiopharmaceuticals, we will focus in this chapter on small molecules. The three building blocks, especially $[^{99m}Tc^{I}(OH_2)_3(CO)_3]^+$ and $[^{99m}Tc^{I}(NO)(CO)_2(OH_2)_3]^{2+}$, have widely been studied for peptide and antibody labeling. These topics are discussed in [19].

From a physico-chemical point of view, the three building blocks are different and offer the opportunity of selecting for a particular target the appropriate one. The preparation of the building blocks is, however, rather different. Whereas **1** is available in the so-called IsoLink® Kit from Covidien (Tyco-Mallinckrodt), the preparations of **2** and **3** are still not at that advanced stage. Building block **2** is prepared from **1** with $[NOHSO_4]$ under acidic conditions and is unstable at pH > 7.5,

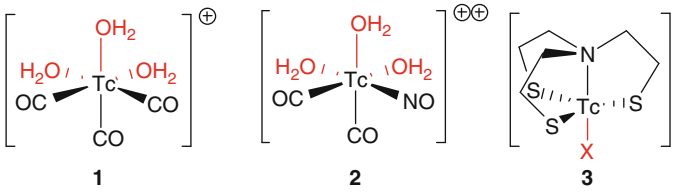

Fig. 2 The three fundamental building blocks and precursor complexes in organometallic radiopharmaceuticals in *red* the sites which can be substituted by targeting molecules (second generation) or ligands for technetium essential radiopharmaceuticals (first generation)

likely due to hydrolytic NO cleavage and subsequent reoxidation [16, 20, 21]. The coordination chemistry of the two complexes **1** and **2** has been compared in detail. Whereas **2** binds very tightly to some basic ligand frameworks like iminodiacetic acid (H_2IDA) or nitrilo-triacetic acid (H_3NTA), the aforementioned instability and the possibility of forming different stereoisomers are the difficulties, especially when working on the no carrier added (n.c.a) level with ^{99m}Tc [22]. Complexes based on **3** were prepared from a Tc^{III}(EDTA) complex (first step) and subsequent reaction with a mixture of the umbrella type NS_3 together with the isonitrile ligand in a methanol/water mixture [23–25]. Whereas labeling and coordination chemistry with **2** would merit more attention due to the hydrophilicity of the formed complexes, **3** has been widely explored for different targets as outlined in more detail below [26]. Clearly, coordination or organometallic chemistry with **1** is developed best and recent studies will cover the larger part of this review.

3 Technetium Essential Organometallic Radiopharmaceuticals

A major target in radiopharmacy is still the development of novel myocardial imaging agents, not only due to the success of Cardiolite® but also due to the need for an agent with improved pharmacokinetics [27, 28]. The tris(pyrazolyl)methane (tpz) ligand has shown its versatility together with the building block **1** and with respect to a variety of targeting molecules [29]. Santos et al. applied this and related pyrazolyl-based ligands for peptides and central nervous system (CNS) ligands. The tpz ligand offers the opportunity of derivatization on the azole ring system for substantially modulating the in vivo characteristics and pharmacokinetics, thereby enabling systematic structure–activity relationships for drug finding and development. Relevant progress was recently made by introducing two or three –CH_2O–CH_3 groups in the azole rings. The corresponding complexes, e.g., [^{99m}Tc$(CO)_3${HC[3,4,5-$(CH_3OCH_2)_3$pz]$_3$}]$^+$ **4**, showed in animals fast and stable heart uptake together with very rapid liver clearance. The results provide an impetus for pursuing this very interesting new candidate for myocardial imaging [30, 31].

Attempts toward myocardial imaging agents but different for the one above were the combination of the MIBI (2-methoxyisobutylisonitrile) ligand with **1** to receive complex **5**. Whereas the exact composition of the complex(es) was not unambiguously clear, excellent heart uptake was found encouraging to continue research with this classical ligand [32]. A combination of a PNP ligand bearing a pendent crown ether bound to the central nitrogen ligand with the [^{99m}Tc$(CO)_3$] core (**6**) gave nice labeling features and good heart uptake [33–35]. The structures of the few novel compounds for myocardial imaging are depicted in Fig. 3.

The research toward novel myocardial imaging agents based on organometallic complexes showed promising results. To achieve an improved agent applied in clinical routine does, however, not only require excellent properties for the good of the patient, but also has to be accepted by commercial companies willing to go through the clinical trial phases. Otherwise, improved compounds may be available but are never established routinely for patients simply for commercial reasons [36, 37].

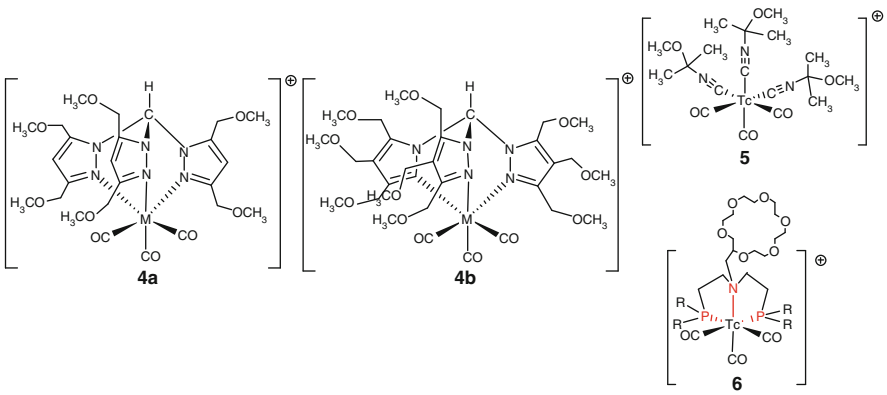

Fig. 3 Novel complexes for myocardial imaging based on the $[^{99m}Tc(CO)_3]^+$ core

An important class of technetium essential radiopharmaceuticals for the assessment of organ functions are still the renal imaging agents. The commercially available agent Tc-MAG3 is one of the most widely applied radiopharmaceuticals and, as for the myocardial imaging agents, compounds with improved pharmacokinetics are sought for. Renal radiopharmaceuticals need to be small and highly hydrophilic. Despite the inherent lipophilicity of complexes with the $[^{99m}Tc(CO)_3]^+$ core, it was recently possible to combine polyamino-polycarboxylate type ligands like NTA^{3-} with **1** to yield the di-anionic complex $[^{99m}Tc(NTA)(CO)_3]^{2-}$ **7**. Complex **7** showed improved pharmacokinetic properties in rats over the standard compound ^{131}I-hippuric acid assessing the appropriateness of organometallic ^{99m}Tc compounds over radioiodinated ones [38]. Application wise, it should be kept in mind that the price of a radiopharmaceutical is a major issue for market introduction and compound **7** would certainly represent one of the least expensive renal imaging agents. Furthermore, compound **7** can be prepared in a one-step synthesis in quantitative yield as required for strategy 2 outlined in the introduction. It should be emphasized at this point that the labeling chemistry with either fragment **1** or **2** is very classical coordination chemistry with Werner-type ligands. From a different point of view, both precursors can be considered as a different kind of aqua-ions. In contrast to "normal" aqua-ions, these moieties have only three binding sites left for coordination to a wide variety of ligands [39]. Accordingly, the search for renal clearing agents has also been investigated with other basic ligands such as lanthionine (lan). Due to the five potential coordinating sites in lan^{2-}, only coordination via the nitrogen and the sulfur has been found. The complex $[^{99m}Tc(lan)(CO)_3]^-$ **8** formed quantitatively and was the first organometallic radiopharmaceutical subjected to human studies [34, 35, 40]. The compound showed excellent renal clearance comparable to ^{131}I-hippuric acid. A thorough synthetic study for assessing the coordination behavior with similar ligands has been performed to clarify the structures of various complexes with ligands of the polyamino-polycarboxylate type (**9**) [41]. These basic ligands have also been used for studying new hypoxia imaging agents [42]. The complexes mentioned in this section are shown in Fig. 4.

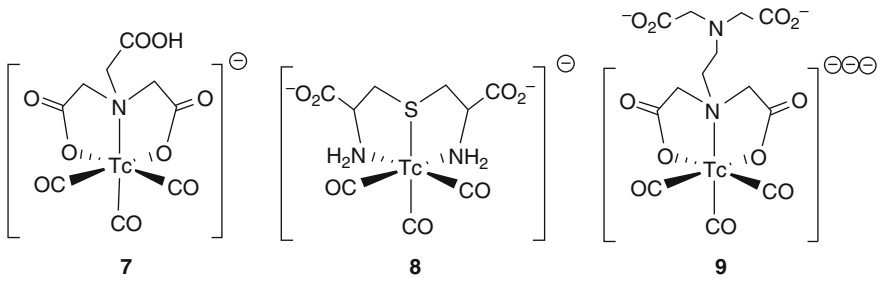

Fig. 4 Novel $[^{99m}Tc(CO)_3]^+$ based complexes for renal imaging

Whereas the search for novel technetium-essential radiopharmaceuticals with improved pharmacokinetics and costs is still of high interest, research was clearly not focused anymore in this field. We discussed above studies which were based on organometallic complexes and it becomes clear that they add value to this field and introduced alternative opportunities as compared with the purely coordinative compounds. It also stands to reason that there is a need for such complexes from a medicinal point of view; however, the most intense research is clearly devoted to targeting radiopharmaceuticals due to assumed higher specificity. Current status of chemistry with organometallic complexes toward targeted diagnosis and therapy is described in the following sections.

4 Targeting Organometallic Radiopharmaceuticals

The combination of a metal complex, organometallic or Werner type, with a small biological receptor ligand is highly challenging. The metal-based label has often a molecular weight which maybe factors higher than that of the vector itself. Different physico-chemical behavior of the pharmaceutical governs the biological behavior and pharmacokinetics of the radiopharmaceutical and receptor affinity are affected. Efficient radiopharmaceutical finding has to rely on screening methods, which are only possible based on a building block principle. The basic complexes **1**–**3**, but also the nitrido core $[Tc \equiv N]^{2+}$, introduced this opportunity [43–46].

4.1 Fatty Acids

The labeling of fatty acids aims at a noninvasive diagnosis of myocardial diseases. The labeled fatty acids should follow the fatty acid metabolism which is distinct in the heart. Radioiodinated iodo-phenyl fatty acid [47] is the golden standard which features have to be exceeded by a corresponding ^{99m}Tc complex. Since all attempts

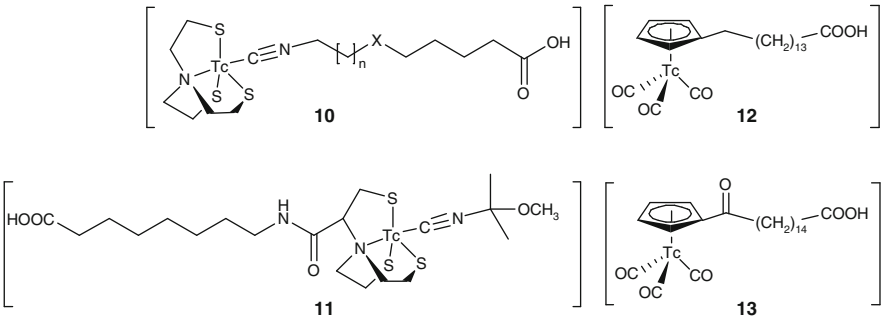

Fig. 5 Novel fatty acid-based complexes for myocardial imaging

to label fatty acids with classical coordination compounds essentially failed, organometallic complexes offer new opportunities. The labeling of fatty acids has mainly been the field of the [4 + 1] approach (vide infra) in which a Tc^{III} complex protected with an umbrella-type ligand was bound to the fatty acid via an isonitrile function. Two examples, complexes **10** and **11**, are shown in Fig. 5. Systematic studies, enabled by the building block principle, varied chain lengths, position of the chelate, and additional functionalities. Thereby, the accumulation in or washout from liver as well as heart uptake could be influenced. However, no compound could be found for which the two properties were properly combined [24, 25, 48]. These findings represent very well the obstacles to be overcome when conjugating a comparably small complex to a "simple" biological molecule such as a fatty acid.

Major efforts were also put in labeling of fatty acids via piano stool cyclopentadienyl (Cp) complexes. The chemistry with the HCp ligand will be described in more detail below. To use of cyclopentadienyl as a ligand in labeling and in water describes nicely how new approaches have to be found for performing aqueous organometallic chemistry. Different approaches have been described and the first purely aqueous approach paved the way for the synthesis of piano stool complexes directly in water [49, 50]. Organic methods using ferrocene (Fc) as a source for cyclopentadienyl were introduced earlier but the synthetic conditions are inappropriate for routine application [51]. Still, along this way, fatty acid derivatives have been labeled and biologically studied. The hypothesis behind the use of Cp is its close structural similarity to phenyl rings. The compounds are shown in Fig. 5. Uehara et al. prepared the analog **12** to the golden standard iodo-hippuric acid [52]. Although the biodistribution of **12** does not lead to a novel fatty acid-based compound for myocardial imaging, it could be shown by metabolite studies that **12** was recognized by the fatty acid metabolism and metabolized to **13**. From a biological point of view, this is an important finding since it proves the possibility of derivatizing a small biological molecule with an organometallic moiety under retention of enzymatic recognition [53, 54].

Further studies with carboxyl rich thioether ligands have been provided. The ligands stabilize the $[^{99m}Tc(CO)_3]^+$ core. One uncoordinated carboxylate group makes them hydrophilic but can also be used to covalently link this ligand to targeting molecules [34].

4.2 Targeting the Folate Receptor with Organometallic Complexes

The Folic acid receptor is a very attractive target for imaging a number of tumors. It is overexpressed on cancer cell lines and the same time down-regulated on healthy cells. The receptor has a high picomolar affinity for folic acid and should therefore tolerate derivatizations as imposed by a metal complex. The folate receptor is also expressed in normal tissues such as kidneys, lungs, or placenta, which are involved in retention and concentration of the vitamin, which makes it difficult to achieve high target/nontarget ratios. With respect to organometallic radiopharmaceuticals, folates and antifolates have been studied mostly with the building block **1**. Folic acid was, for instance, derivatised via an ethylene-diamino spacer with DTPA. Although a classical Werner type ligand, DTPA has a high affinity for the $[^{99m}Tc(CO)_3]^+$ core yielding hydrophilic complex as required for the labeling of hydrophilic biomolecules [55]. It could be shown with the bioconjugate of folic acid **14** that the vector underwent receptor-mediated uptake; hence, the folate was still recognized and transported into the intracellular space. However, high levels of radiopharmaceuticals were also found in nontargeted tissue due to the presence of folate receptors in these organs. This finding mirrors the problems related to selective targeting very well. Although appropriate, organometallic radiopharmaceuticals can conveniently be synthesized, target to nontarget ratios are frequently found to be low.

An earlier study applied a different linker and the frequently used tridentate chelator picolylamine-monoacetic acid (PAMA, Fig. 6). The ligand PAMA was either conjugated via a spacer to the glutamic acid group in folic acid or directly to the carboxylate in the pteroic acid subunit of folic acid to receive compounds **15** and **16**, respectively. As before, it was found that these conjugates could quantitatively be labeled with the building block **1** and that the new radiopharmaceuticals were competitive inhibitor toward the folic acid receptor with comparable affinities as folic acid itself [56]. Preclinical evaluation of these radiotracers gave promising results, which stimulate further development in the targeting of the folate receptor. Binding constants for instance are in the 2 nM range for the folic acid derivatives but about 15 nM for the pteroic acid compound (folic acid 7 nM). Clearance of the radiotracers from the blood pool and from nontargeted tissues was efficient and tumor to blood ratio reached approximately 200–350 24 h p.i. Small animal SPECT/CT allowed visualization of folic acid receptor-positive tumors [57]. For further enhancement of tumor uptake, folic acid expression was stimulated by the concomitant application of labeled folic acid and antifolates that lead to a much better target to nontarget ratio as a further step toward imaging the folic acid receptor [58]. More recently, a further conjugate **17** has been introduced to folic acid, which used a tridentate quinoline-based ligand. The complexes of this ligand do not only allow a stable labeling but enable also confocal fluorescence microscopy for assessing the localization of the complex within the cells. In addition, it was found that these derivatives of folic acid displayed significant cytotoxicity which could lead to a combined action of diagnosis and therapy with the same organometallic complex [59]. The complexes discussed in this section are shown in Fig. 6.

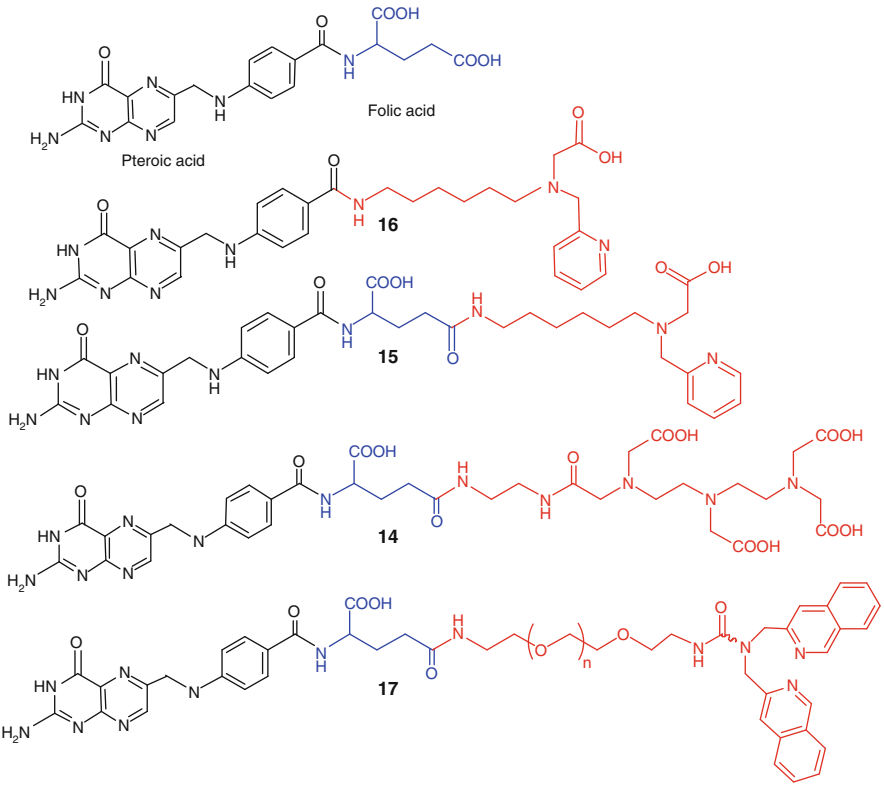

Fig. 6 Radiopharmaceuticals of folic acid. The pteroic acid is *black*, pteroic acid + glutamic acid (*blue*) gives folic acid

The folic acid receptor represents a challenging but a promising target for organometallic radiopharmaceuticals. Data as available so far clearly demonstrate that folic acid can be derivatised with a variety of ligands under retention of affinity for the FR. Pharmacokinetics can be improved by the administration of additional pharmaceuticals influencing the expression of the FR which leads to improved imaging properties. Folic acid may therefore not only be of interest for imaging but also for therapy using further organometallic complexes of the, e.g., $[Ru(arene)]^{2+}$ type with established cytotoxic action.

4.3 Competing with PET: Carbohydrates Labeled with ^{99m}Tc Organometallic Complexes

One of the most successful radiopharmaceuticals in oncology (and in other fields of noninvasive diagnostic medicine) is certainly ^{18}F-labeled glucose, 2-^{18}F-2-deoxy-glucose, so-called FDG [60]. FDG is taken up by the glucose transporters GLUT,

bound to hexokinase and phosphorylated at the 6-position. The next step in the enzymatic degradation is inhibited by the fluoride at the 2-position. Thus, the 6-phosphate-glucose is not converted any further but remains captured in the cell due to its negative charge. Those cells with highest glucose and hexokinase activity demand will accumulate most ^{18}FDG, the ultimate reason for its versatility in imaging. A recent review has highlighted the biology of glucose in general and of derivatised carbohydrates in particular [61]. To complement radiopharmaceuticals in nuclear medicine with a ^{99m}Tc-labeled glucose (or other carbohydrates) is most attractive for scientific but also for economic reasons. Since GLUT is highly selective, it is generally considered as impossible of conjugating a bulky metal complex under retention of transport activity. Still, it has been shown in literature that a variety of glucose derivatives were transported by GLUT indeed [62, 63]. To find a compound which is accepted by the hexokinase enzyme is not such a problem as will be seen later; however, active uptake into the cell is the major barrier to be overcome. The labeling of small molecules such as carbohydrates points again to the question about a complex as small as possible with properties comparable to the targeting moiety. Therefore, this is a typical question for organometallic complexes since small coligands will result in a small complex.

Efforts to produce a labeled glucose have substantially been intensified over the past couple of years. With respect to organometallic compounds, the 1-OH position has been derivatised with the imino-diacetate ligand and labeled with the $[^{99m}Tc(CO)_3]^+$ core. Both α- and β-anomer **18** were received in pure form, the α-anomer for 2-deoxyglucose and the β-anomer for glucose [64]. It should be noted that labeling was quantitative and the hydroxy groups in the carbohydrates did not compete for the ligand. Later on, Schibli et al. introduced glucose derivatives which were derivatised at the 3-position with iminodiacetate (**19**), histidine (**20**), or the aforementioned PAMA tridentate ligand (**21**) bound via a C2 spacer [65, 66]. The C3 position has been chosen since it seems to tolerate some derivatizations without complete loss of recognition by the major transporter GLUT1 [67]. These studies were complemented by additional derivatizations of glucose also at the 6-position. In all cases, well defined and stable complexes have been received. Phosphorylation studies with hexokinase showed a moderate activity of the two compounds derivatised at the 2-position but none of the complexes was transported by GLUT1 reflecting the high selectivity of this transporter [66]. Some of these basic complexes are depicted in Fig. 7.

New approaches have recently been introduced by Orvig et al. developing a strategy based on a different set of ligands and partly including functionalities on the carbohydrate for coordination. In a pure chemical study based on glucosamine, they conjugated a hydroxybenzyl moiety at position 2. Radiolabeling or conjugation to the "cold" $[Re(CO)_3]^+$ moiety implied tridentate coordination to the aromatic –OH group, the reduced amine at C2 and coordination to 3-OH (**22**) representing the first (and so far only) example for which glucose functional groups were employed in coordination [68]. The glucosamine is a convenient starting material for the preparation of glucose conjugates. Based on the well known ability of the 2,2′-dipicolylamine ligand to strongly bind **1**, this ligand was introduced via amide

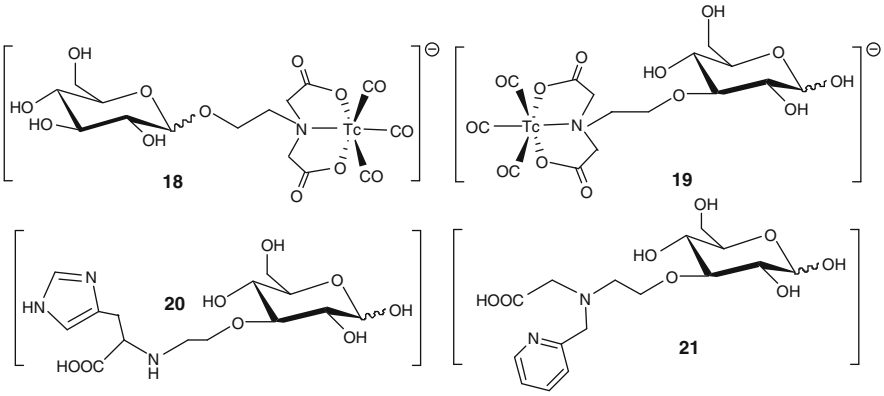

Fig. 7 Different glucose derivatives which have been labeled with the $[^{99m}Tc(CO)_3]^+$ core

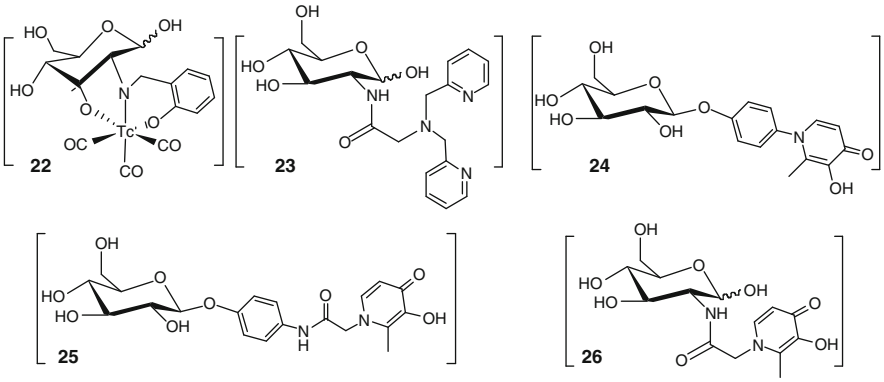

Fig. 8 Glucose derivatised at various position. No cell uptake of the labeled compounds was found

formation to glucosamine to yield the carbohydrate conjugate **23** [69]. Radiolabeling gave >99% yield and excellent serum stability was found. In an extension to this study, four carbohydrates derivatives based on glucose, xylose, and mannose and the glucosamine derivative mentioned before were synthesized and characterized, partly even by X-ray structure analysis [70, 71]. Although the compounds have all required characteristics for becoming radiopharmaceuticals according to the needs from routine preparation, none showed uptake and/or phosphorylation. This was concluded from the fact that tumor washout paralleled the one of blood, hence, no retention in the tumor as expected from a negatively charged compound to be expected after conversion by hexokinase. Similar biological results were reported with carbohydrate appended 3-hydroxy-4-pyridinone complexes of $[^{99m}Tc(CO)_3]^+$ and the rhenium analogs (**24**–**26**) [72]. No substrate or inhibitory activity was observed. The structure of relevant carbohydrate conjugates is depicted in Fig. 8.

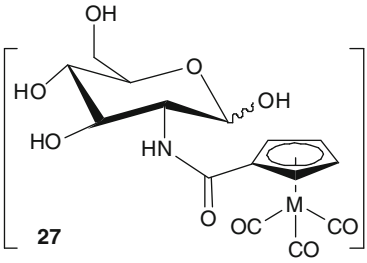

Fig. 9 A piano stool derivative of glucosamine

Some other approaches toward labeled glucose have been reported. Valliant et al. derivatised glucose at C1 via an ether spacer to carboranes, a novel and most interesting ligand system (see below). They prepared and fully characterized the corresponding ^{99m}Tc and Re complexes but did not give evidence of biological activity so far [73, 74]. A ligand with electronic similarity to the carboranes is cyclopentadienyl (Cp). Cp represents one of the smallest tridentate ligands with a mol. wt. of 65. From a topological point of view, it would match best the small size of glucose. Accordingly, two glucosamine based Cp ligands have been prepared. The rhenium complex was prepared via activation of the carboxylic acid group in $[(C_5H_4–COOH)Re(CO)_3]$ and coupling to glucosamine (**27**). Labeling with ^{99m}Tc was performed via the single ligand transfer procedure (SLT) [75] in DMSO, starting from the ferrocene derivative of glucosamine (Fig. 9) [76]. In vitro evaluation of these compounds showed some retention of biological activity and competitive inhibition of hexokinase with a Ki value about three times larger than FDG. However, phosphorylation was not found but the results at least suggest that the small size of the $[CpRe(CO)_3]$ complex might be beneficial for finding new glucose analogs with retained biological activity.

A number of basic studies toward the application of carbohydrates as ligands for the $[M(CO)_3]^+$ entity have been described. Klüfers et al. demonstrated the ability of the hydroxy groups in a variety of carbohydrates to bind in different, pH and stoichiometry dependent ways to rhenium. A variety of complexes were structurally characterized [77]. Finally, two 1-thioglucose entities have been coupled to a central 4,4′-bipyridine ligand. These unique conjugates could be labeled at high dilution, exhibited perfect in vitro stability but were not yet subjected to biological studies [78].

Despite many efforts toward ^{99m}Tc-labeled glucose with organometallic or coordination compounds, none has been successful so far. The major obstacle of being transported by GLUT1 might be the limiting step. However, the principle possibility of transporting organically derivatised carbohydrates has been shown and let at least expect that ^{99m}Tc derivatives of glucose may work as well. The impact such a compound would have justifies further research in this field, however, probably along less obvious routes than simply the pendent approach.

4.4 Targeting Enzymes: ^{99m}Tc-Labeled Thymidine for TKs

The labeling of nucleosides or nucleotides is a persisting topic in nuclear medicine. Most approaches and compounds have been prepared with the PET radionuclides ^{11}C or ^{18}F but scarcely with ^{99m}Tc or other metallic radionuclides. Relevant targets for labeled nucleosides is the human cytosolic thymidine kinase hTK1 and the herpes simplex virus type 1 thymidine kinase (HSV1-TK), both strongly over-expressed in cancer cells. Based on X-ray structure analyses of the viral enzyme, it was predicted that the least bioactivity affecting position for derivatization would be the 5′-OH group in 2-deoxy-thymidine. Accordingly, Schibli et al. introduced the IDA chelator to this position via an amide linkage and alkyl spacers of variable lengths (**28**–**29**) [79–81]. Deoxythymidine (dT) is phosphorylated in vivo by the two enzymes hTK1 and HSV1-TK to dTMP. A versatile radiopharmaceutical should bind to the kinase and competitively inhibit this enzymatic reaction. For the assessment of such an inhibitory action, the rhenium models were subjected to in vitro studies. The organometallic derivatives revealed competitive inhibition toward hTK1 but not toward HSV1-TK. Inhibition increased with increasing spacer length. These features prohibit the use of the corresponding ^{99m}Tc complexes for gene therapy monitoring but might have potential as proliferation markers in tumor diagnosis. Zubieta et al. introduced the dipicolyl-amine ligand to the 5′-OH position and directly to an amine at the 2′-position to yield **30** and **31** [82]. Both **30** and **31** could be labeled with **1** but the selectivity of these compounds toward the targeted enzyme hTK-1 has yet to be evaluated.

More recently, thymidine has been derivatised at N3 with tridentate ligands yielding after labeling neutral (**32**), anionic, and cationic (**33**) complexes, respectively. In addition, the spacer lengths have been altered to receive a first insight into structure–activity relationships. It could be shown that the neutral thymidine derivative **32** with a C10 spacer and a cysteine-based ligand was phosphorylated by hTK1 to a comparable extent as dT itself. Generally, neutral and anionic complexes were more accepted than cationic ones. These encouraging results should power further fine-tuning of this class of important radiopharmaceuticals [83]. A selection of complexes discussed in this section is shown in Fig. 10.

4.5 Very Small and Essential Biomolecules: α-Amino Acids

Labeled amino acids are a persisting lead structure in the search for novel radiopharmaceuticals. Due to their small size, amino acids are again typically labeled with PET radionuclides or with iodine isotopes. Accordingly, iodinated and fluorinated tyrosine and phenylalanine are a main focus in this research. Amino acids are actively taken up by transmembrane protein transporters, for instance, the L-type amino acid transporter 1, LAT1. Since LAT1 is strongly over expressed on many cancer cell lines, amino acids are attractive metabolic tracers for imaging enhanced proliferation. In contrast to the GLUT1, the L-type amino acid transporter LAT1

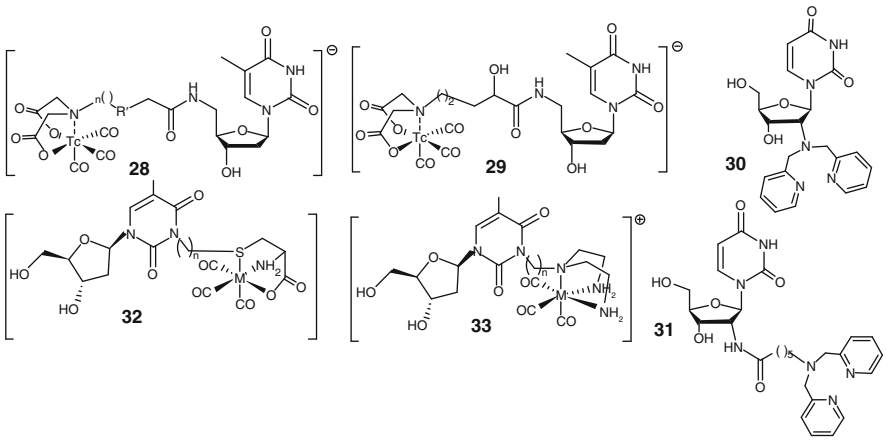

Fig. 10 Thymidine derivatives labeled with the $[^{99m}Tc(CO)_3]^+$ core and showing binding to TKs

carries a variety of neutral and lipophilic amino acids. It is therefore not specific to one single amino acid and careful design might even enable the transport of pendent metal complexes despite its relatively large size as compared with the amino acid entity. It has been stated that LAT1 exhibits a relatively broad and symmetrical selectivity but strongly asymmetric substrate affinity [84–86]. Based on the design of an organometallic complex as small as possible, we introduced the tripod chelator 2,3-diamino-propionic acid (**34**). Despite of having very favorable coordinating properties toward the $[^{99m}Tc(CO)_3]^+$ core, this tripod is scarcely used in the labeling of biomolecules. We introduced the amino acid moiety via alkyl spacers of various lengths to the bridgehead carbon (**35**). The corresponding rhenium complex **36** showed active transport into cells with a Ki value of 308 μM (phenylalanine 72 μM) and a C4 spacer. The affinity showed a distinct spacer lengths dependence [87]. Interestingly, attaching the amino acid via a C4 spacer to N3 (**37**) resulted in a complete loss of affinity, underscoring not only the size of the pendent metal complex but also its topology. Preliminary in vivo studies showed rapid excretion from the animal likely due to their inherent hydrophilic character. However, it could be shown that amino acids can be labeled with organometallic ^{99m}Tc under retention of biological affinity. This is surprising considering the small size of the lead in comparison to the albeit small but comparatively large metal complex. Future research must go toward the introduction of cyclopentadienyl to mimic phenylalanine and to further reduce the complex size. Basic complexes are depicted in Fig. 11.

4.6 Vitamin B_{12}: An Organometallic Coenzyme for Organometallic Complexes

Vitamin B_{12} (**38**) is the largest vitamin known to date. It is responsible for two essential intracellular enzymatic processes. B_{12} deficiency results in severe diseases

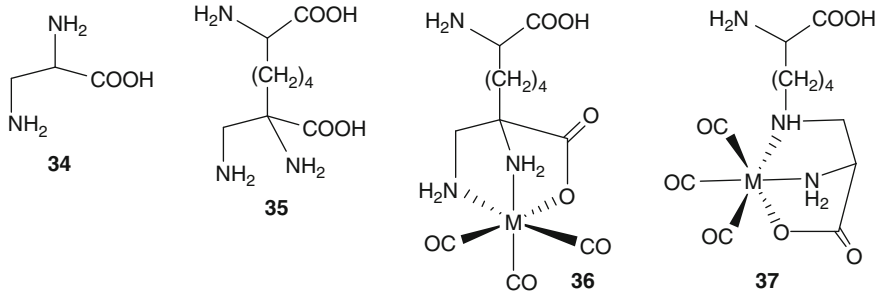

Fig. 11 Labeled artificial amino acids which were recognized by the L-type amino acid transporter LAT1 (**36**) whereas the isomer **37** lost any affinity

such as megaloblastic anemia and neurological malfunctions and ultimately leads to death [88]. It is known that rapidly proliferating cells have an increased demand for B_{12} rendering this vitamin a carrier for radionuclides or cytotoxic agents. Studies for the radiolabeling of B_{12} with ^{99m}Tc and ^{111}In have been performed since a number of years. Labeling strategies were based on coordination compounds and the chelators introduced at 2′-OH function in the ribose portion and the b- or the d-acid in the corrin framework [89, 90]. Although good tumor accumulation was found, confirming the versatility of B_{12} as a targeting vector, a drawback of these studies was the very high accumulation in liver and kidneys, the organs in which B_{12} bound to its transporter transcobalamin II (TCII) is stored. The B_{12} conjugates under study did not allow a fine tuning of the structure since the ligand used for ^{99m}Tc was DTPA. The versatility of organometallic closed-shell complexes came now into play. As demonstrated in the chapters above, a wide variety of chelators can be used for complex **1**, consequently enabling a systematic study of the influence of the complex to the pharmacokinetic of the radiopharmaceutical. We initially focused on the derivatization at the 2′-OH position using various chelators such as histidine (**39**) or PAMA. We found excellent labeling and unaffected binding to haptocorrin (HC) and TCII. Animal distributions, however, also showed preferential accumulation in kidneys, thus, in terms of biodistribution, no advantage over other radiolabeled B_{12} compounds [91, 92]. We introduced the chelators via spacers to the different acids at the corrin ring [93–95] and found binding selectivity to HC and TCII depending on the spacer lengths and the type of chelator. Derivatives at the b-acid with PAMA turned out to be of particular interest since they bound to HC but not to TCII. Surprisingly, these compounds also showed high tumor accumulation but very little retention in the kidney as expected for a useful radiopharmaceutical [96]. Since receptor-mediated endocytosis and kidney accumulation are both a consequence of TCII binding, this surprising result is still controversially discussed and only patient studies will prove or disprove the biological behavior. Some selected B_{12} derivatives are depicted in Fig. 12.

Inspecting the structure of vitamin B_{12}, one recognizes the cyanide bound to Co^{III} in native B_{12}. Whereas metal bridging cyanides are one of the longest known structural features in inorganic chemistry (Prussian Blue), this cyanide has never

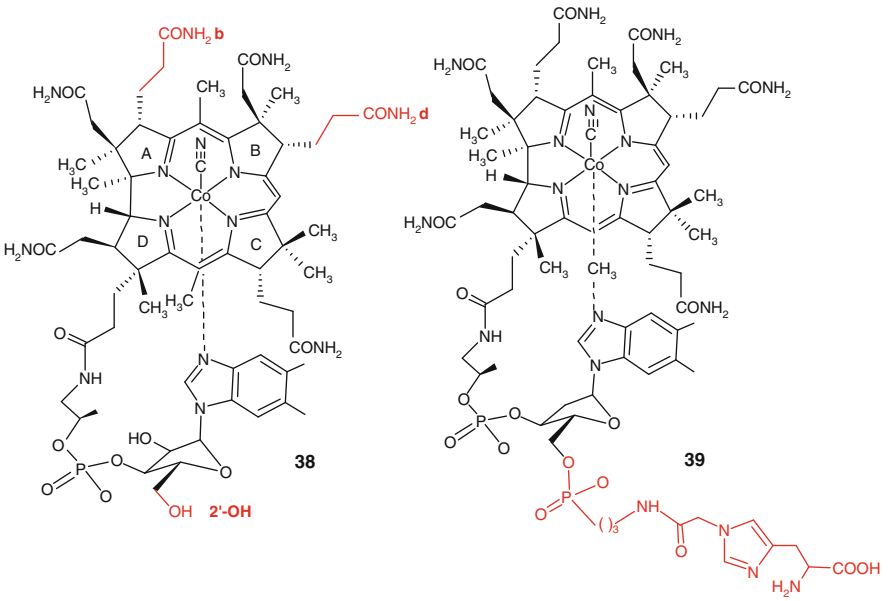

Fig. 12 Native Vitamin B_{12} (**38**) with the most common derivatization sites in *red*. One example (**39**) is shown in which the 2′-OH group was derivatised with histidine, a very strong chelator for the $[^{99m}Tc(CO)_3]^+$ core

been considered for such a purpose. Again, organometallic complexes came into play and we could show that complex **1** and some derivatives stably coordinated to the N of cyanide, affording a heterodinuclear B_{12} derivative with a central Co–CN–Tc or Re motive [97]. After binding to cyanide, complex **1** has in principle two coordination sites left, still being occupied with water or halide ligands. These two positions can be occupied with bidentate ligands thereby enabling a fine-tuning of binding properties and pharmacokinetics. The Re or Tc centers can even mediate the binding of different kind of bioactive molecules or fluorescence markers. We exemplified this principle not with Tc but with cytotoxic platinum complexes [98]. All complexes conjugated via the cyanide showed still binding to HC and TCII. They should therefore been taken up into the cells via the TCII receptor. It was found that the rhenium as well as the platinum complexes obeyed this expectation. It was a decisive finding that the derivatives were still recognized by the intracellular adenosylation assay being converted to the coenzyme B_{12} under cleavage of the metal complex. This approach is therefore a method of using B_{12} as a Trojan Horse for radioactive and cytotoxic organometallic complexes [98].

Although B_{12} seems to be a "complicated" biomolecule, it is a very versatile carrier for robust organometallic compounds for various purposes. Our finding implies to use, e.g., ruthenium-based agents along the same line, concealing it behind the B_{12} framework and release in the intracellular space.

4.7 Targeting the Cell Nucleus

A recent review characterized the cell nucleus as a "no-go" area [99]. This statement is certainly correct with respect to the activity toward DNA as a target but not to its potential importance. Considering the high linear energy transfer (LET) of Auger electron or alpha-emitters, they could efficiently deposit a very high dose at a critical machinery in the cell. The incentive is to find a combination between bioactive molecules, selective for cells, and conferring entry into the nucleus. TAT peptides possess the capability of carrying various substances into the nucleus, but are not cell specific [100]. A novel concept for finding the nucleus is required. Such a concept, we call it a trifunctional radiopharmaceutical for cell nucleus targeting, is schematized in Fig. 13.

The first function is for selectively targeting a receptor overexpressed on cancer cells, particularly a peptide [19]. The second function is the nucleus penetrating molecule and the third the metal complex itself, eventually mediating the coupling of the two other functions. Upon entry into the cell, the first function should be cleaved allowing the remaining conjugate to enter the nucleus. Applying ^{99m}Tc complexes, the effect of its Auger electrons on cell viability was studied. Organometallic complexes play a crucial role in this design since they are robust and allow the combination of the two functions by simple coordination to the central organometallic moiety. The metal-mediated binding of two targeting principles must rely on molecules which can be bound through ligands of low denticity but still providing sufficient kinetic stability to not decompose under physiological conditions. We have established the radiotoxicity of ^{99m}Tc Auger electrons by applying two of the above mentioned functions, the complex and a nuclear localizing signal peptide [101]. The ligand for this purpose was an aliphatic triamine providing a positively charged complex with high electrostatic affinity for the negatively

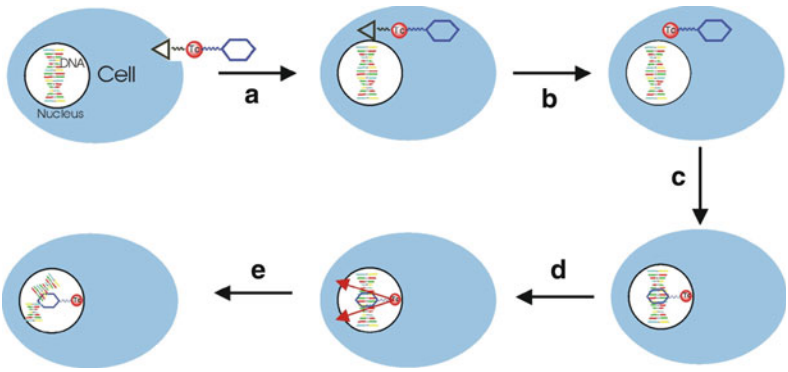

Fig. 13 The principle mode of action of trifunctional radiopharmaceuticals (**a**) cell receptor-specific binding and receptor-mediated endocytosis (**b**) cleavage of targeting function (e.g., petide) (**c**) uptake into the nucleus and intercalation into DNA, bringing the radionuclide close to the double strand. (**d**) Auger or α-emission and (**e**) double or single strand break leading to cell death. Coupling of the two biological function via the organometallic complex

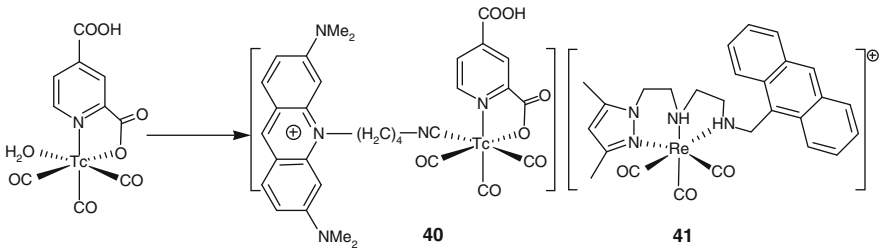

Fig. 14 The synthesis of a nuclear targeting complex according to the [2 + 1] principle. The first function for nucleus targeting is acridine orange, the dipicolinic acid may serve for binding the second function. The organometallic complex mediates the coupling of the two biological vectors

charged DNA backbone. Radiotoxicity by DNA double-strand breaks could be assessed [101]. In order to visualize the uptake in the nucleus, we introduced a nucleus targeting intercalating molecule into the complex with the acridine orange lead structure (**40**, Fig. 14).

Acridine orange was derivatised with a monodentate isonitrile ligand and coupled according to the [2 + 1] principle [102] to the $[Re(CO)_3]^+$ or the $[^{99m}Tc(CO)_3]^+$ core. By confocal fluorescence microscopy, the resulting complex could nicely be localized in the cell nucleus. Preliminary radiotoxicity studies with the ^{99m}Tc analog showed strong effect on cell viability implying a radiotoxic effect of the emitted Auger electrons. Control experiments with ^{99m}Tc outside the cells did not affect cell viability [103]. When combining the nucleus targeting functions with a peptide for achieving receptor selectivity, uptake into the cytoplasm was found. Since the targeting bombesin peptide was not cleaved from the trifunctional molecule, the acridine orange could not carry the complex into the nucleus. The single step of this concept has been proven; however, more efforts are needed to turn nucleus targeting from a "no-go" area into a promising concept.

A recent study aimed in a similar direction. Santos et al. conjugated the well known intercalator anthracene to a pyrazolyl-based tridentate amine ligand (**41**), providing a positive charge to the complex and ensuring water solubility [104]. By different spectroscopic methods, they could show high DNA affinity of this complex and confirm an intercalative mode of binding. In vitro cell studies furthermore indicated uptake and retention in the cell nucleus. As in the case of Agorastos et al., however [103], these bifunctional conjugates have to be complemented with a third receptor specific function to target cancer cells rather than penetrating unspecifically any cells.

The small number of studies aiming at targeting the nucleus of specific cells underscores the assignment of this field as (so far) "no-go." However, the high LET of, e.g., Auger electrons of ^{99m}Tc, $^{188/186}Re$, or other radionuclides implies the need for substantially less activity to achieve the same biological damage (dose) than with common β-based therapeutic approaches [105–107]. To target the nucleus is highly challenging since at least two biological barriers have to be overcome. It represents a field for which organometallic complexes are tailor-made. The kinetic

stability of many metallic building blocks enables the combination of various targeting molecules by coordination to the metal center and, thus, an efficient way of drug finding and drug development with a new perspective and scope.

4.8 *Drug Finding and Development: The Single Amino Acid Chelate Approach*

As emphasized in the introduction and through all the preceding sections, it would be versatile of having a single ligand system which could be applied to, e.g., combinatorial syntheses of peptides or at any desired place in the structures of biological macromolecules. Such a strategy would enable a highly efficient drug discovery if based on one single chelator matching the conditions for, e.g., peptide syntheses. The introduction of bifunctional ligands stabilizing the $[Tc = O]^{3+}$ core was problematic with respect to altering the conjugation position. Therefore, only punctual variations were possible. Valliant and Zubieta et al. presented a chelating system which is based on one single amino acid and which terminal amino group could conveniently be derivatised with two pyridines (**42**) or, alternatively for fluorescence studies, with two quinoline ligands (**44**). A recent review describes the background and the state of the art of this system comprehensively [108]. They called their building block a single amino acid chelate (SAAC), the principle of which is depicted in Fig. 15.

The major advantage of SAACs is that they are based on natural (e.g., lysine) or artificial amino acids that enable a straight forward and systematic variation of the spacer between ligand and biomolecule binding functionality. Since the ligand does not contain coordinating groups which need to be protected, these bifunctional amino acids can directly be subjected to automated peptide syntheses (**43**), making the syntheses of a multitude of peptides possible without tedious protection chemistry. To illustrate the utility of this system, the lysine-based ligand bismethylpyridylamine was introduced at various positions in the chemotactic fMLF peptide (**45**, Fig. 16) [109]. Due to the high stability of the organometallic complex, this strategy cannot

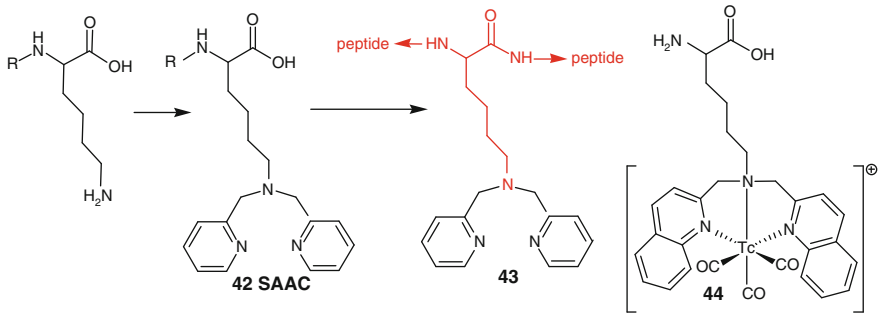

Fig. 15 The newly introduced principle of single amino acid chelators (**42**, SAAC) which can directly be subjected to automated solid phase peptide syntheses

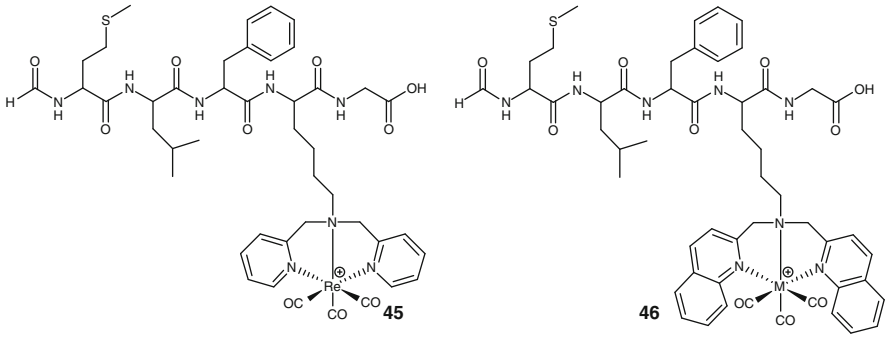

Fig. 16 Examples for the incorporation of SAAC in peptides, $[fMLFTAMG]^+$ (**45**) and for fluorescence microscopy $[fMLF\{Re(CO)_3–SAACQ\}G]^+$ (**46**)

only be applied to the free ligand (for later labeling with ^{99m}Tc) but also to the preformed $[Re(CO)_3]^+$ complex, an approach hardly possible with other building blocks due to their instability toward strongly acidic conditions. It is noteworthy that one of the so prepared complexes bound better to granulocytes than the parent peptide. Clearly, the strategy allows systematic and timely syntheses of peptide libraries with the SAAC integrated at any desired position in the strand, thus, a fast approach to drug discovery and drug development.

A further option using the SAAC approach is the introduction of fluorescence by replacing the pyridine with quinoline groups to yield so-called SAACQ. The corresponding rhenium complexes are fluorescent with appropriate properties such as long fluorescence half-life time and large Stokes shift [110, 111]. Whereas radioanalytical methods do not allow to localize radioconjugates with cellular resolution without destroying the cells, fluorescence microscopy has the resolution. Thus, by switching from classical SAAC to the SAACQ methodology, the localization of a biomolecule in the cell can be monitored directly, thereby enabling an indirect tracing of the analogous radioconjugate as well. This principle was nicely confirmed with the fMLF peptide (**46**) and a permeation chemotactic peptide for labeling stem cells. Both peptides were derivatised with SAACQ and allowed, e.g., showing effective labeling of neural stem cells [112]. Further examples with nucleosides or carbohydrates have been described in previous sections of this chapter and other examples are available in literature [113, 114].

The SAAC strategy is an excellent example for the superiority of applying organometallic complexes over coordination compounds. Keeping the building block intact, in these cases the $[^{99m}Tc(SAAC)(CO)_3]^+$ entity, it can be driven to any position in a targeting molecule with one single synthetic methodology and giving access to a broad number of new molecules. This is not immediately possible with coordination compounds of rhenium or technetium since their coordination sphere is normally saturated with strong ligands. It should also be emphasized that the basic SAAC can be replaced by other tridentate systems and subsequently subjecting them drug discovery along the same route. Doubtless, the potential behind SAACs is enormous and its exploitation will be intensified in near future.

5 New η^5-Coordinating Ligands: Cyclopentadienyl and Carborane Complexes

Cyclopentadienyl $[C_5H_5]^-$ is one of the most prominent ligands in organometallic chemistry but has scarcely found entry into radiopharmaceuticals due to the absolute need of preparing the corresponding complexes directly from water [12, 39, 115–117]. Piano stool complexes of the cymantrene $[(\eta^5\text{–Cp})M(CO)_3]$ type would be interesting since the Cp-ring mimics phenyl rings in purely organic pharmaceuticals as prototypically shown by Jaouen et al. with ferrocifen, the ferrocene analog of tamoxifen [118–120]. The incentive of synthesizing ^{99m}Tc complexes has been tackled by Wenzel et al. who prepared the first Cp-complex $[(\eta^5\text{–Cp})^{99m}Tc(CO)_3]$ along a nonaqueous route [51, 121]. The approach has later been modified in the double ligand transfer (DLT) approach by Katzenellenbogen et al., but still applying organic solvents [122, 123]. We introduced for the first time a fully aqueous approach to Cp complexes [49, 50] and a SLT approach starting from ferrocene precursors [75]. The Cp-chemistry has been reviewed in detail in an earlier article in this series and therefore only recent approaches will be discussed [116].

The previous approaches, though feasible in water, were relatively limited in terms of biomolecules conjugated to the Cp-ring and with respect to the stability of the Cp derivatives. When we attempted to extend the type of Cp-derivatives to cyclopentadiene-carboxylic acid [C_5H_5–COOH], we found that not only the monomeric unit could coordinate to $[^{99m}Tc(OH_2)_3(CO)_3]^+$ but also its Diels-Alder dimerized form $[(C_5H_5\text{–COOH})_2]$, the so-called Thiele's acid (**48**). Since Thiele's acid does not thermally monomerize under our aqueous conditions, the formation of $[(C_5H_4\text{–COOH})^{99m}Tc(CO)_3]$ **47** must have been induced by metal-mediated cleavage (Fig. 17) [124].

Furthermore, we could show that this reaction mechanism to produce Cp-complexes of ^{99m}Tc is not limited to carboxylate derivatives of Cp but that also further functionalities can be conjugated to receive, e.g., amides (**49** and **50**) [125]. This reactivity implies that, comparable to the SAAC approach, Thiele's acid derivatives can be incorporated in, e.g., a peptide strand and directly be labeled with $[^{99m}Tc(OH_2)_3(CO)_3]^+$. The preparation of cyclopentadienyl complexes with a variety of functionalities attached to the cyclopentadienyl ring stands for a more routine introduction of this important tridentate ligand in radiopharmaceutical organometallic complexes.

An electronic surrogate of the cyclopentadienyl ligand are carbon containing polyhedral boron clusters of the type $[R_2C_2B_9H_9]^{2-}$ (**51**). The pioneering work around this ligand has been provided by Valliant et al. who prepared a variety of complexes $[Re(R_2C_2B_9H_9)(CO)_3]^-$ (**52**) and their ^{99m}Tc analog along a purely aqueous approach [126, 127]. Substantial progress has been made for the syntheses of such complexes with various substituents on the carboranes cage. In an improved synthesis, Sogbein et al. found that the presence of fluoride substantially facilitated the reaction for a variety of basic and substituted metallocarboranes with Re and ^{99m}Tc [128]. In an excellent study, it was found that the presence of fluoride in the

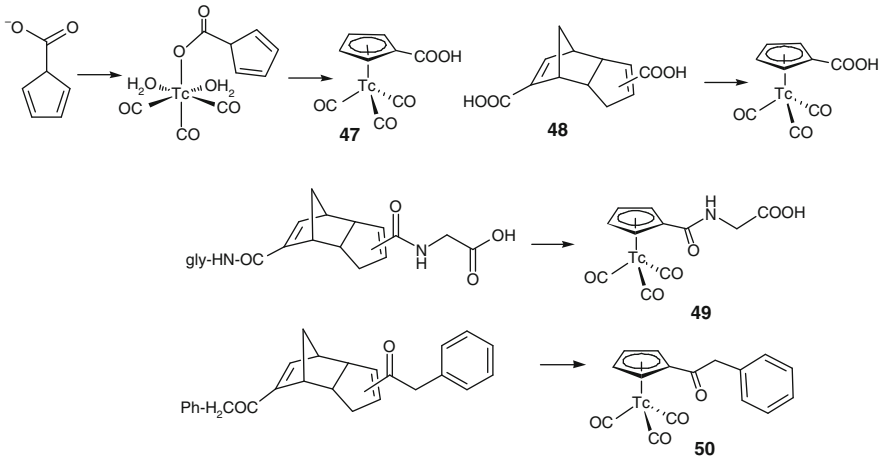

Fig. 17 Metal-mediated cleavage of Thiele's acid (**48**) and further examples **49** and **50** with derivatives of **48**

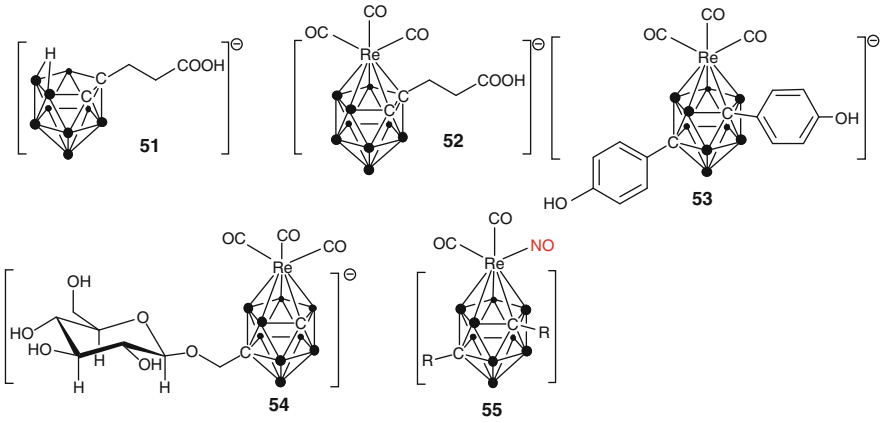

Fig. 18 Major classes of carborane-based rhenium and technetium complexes

standard IsoLink kit of the preparation of $[^{99m}Tc(OH_2)_3(CO)_3]^+$ enabled the one pot formation of a series of ^{99m}Tc carborane in essentially quantitative yield. It can be concluded that these carboranes belong to those complexes which can be prepared in one single step from $[^{99m}TcO_4]^-$ as required for routine clinical application. Although the mechanism and the role of fluoride remain to be determined, the new kit formulation allows the labeling of a variety of carboranes, thus, introducing this important class of η^5-type ligands into routine organometallic radiopharmaceutical research as well (Fig. 18) [129]. The potential of metallocarboranes with ^{99m}Tc and Re was recently assessed with a new class of probes for imaging the estrogen receptor (**53**) [130]. Additionally, carboranes have been coupled to glucose (**54**) and even be nitrosylated to receive the neutral radioconjugate **55** [74].

Carboranes, at a first glance at least exotic for biological applications (apart from boron neutron capture therapy), have become a fundamental class of chelators for organometallic radiopharmaceuticals. Their preparation, derivatization, and conjugation have reached a level of routine feasibility and they merit broader attention concerning classes of targeting molecules. The combination of molecular imaging opportunities with heavily boron-loaded compounds will allow to quantify the accumulation of boron clusters as a base for improved boron neutron capture therapy.

6 Conclusion and Perspectives

The application of organometallic complexes in radiopharmacy and molecular imaging has made substantial progress over the past 3–4 years. This progress is not only due to more labeled biomolecules but rather due to new strategies and ligands, opening access to novel targets and labeling opportunities. Although it is not likely that a new ^{99m}Tc radiopharmaceutical (organometallic or Werner type) will appear in near future, the research efforts in bioorganometallic chemistry open more and more gates, which will ultimately lead to novel radiopharmaceuticals able to efficiently compete with the currently favored PET imaging molecules. Apart from application, the progress outlined in the preceding chapters should also underline the importance of fundamental chemistry. Many of the examples briefly introduced before comprise relevant knowledge not immediately important for application purposes but contribution to general knowledge of technetium and rhenium chemistry. It should be emphasized at this point that fundamental research will lead to improved strategies for molecular imaging since it provides novel labeling opportunities and access to new targets.

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Carbon Monoxide: An Essential Signalling Molecule

Brian E. Mann

Abstract Carbon monoxide (CO), like nitric oxide (NO), is an essential signalling molecule in humans. It is active in the cardiovascular system as a vasodilator. In addition, CO possesses anti-inflammatory, anti-apoptotic and anti-proliferative properties and protects tissues from hypoxia and reperfusion injury. Some of its applications in animal models include suppression of organ graft rejection and safeguarding the heart during reperfusion after cardiopulmonary bypass surgery. CO also suppresses arteriosclerotic lesions following angioplasty, reverses established pulmonary hypertension and mitigates the development of post-operative ileus in the murine small intestine and the development of cerebral malaria in mice as well as graft-induced intimal hyperplasia in pigs. There have been several clinical trials using air–CO mixtures for the treatment of lung-, heart-, kidney- and abdominal-related diseases. This review examines the research involving the development of classes of compounds (with particular emphasis on metal carbonyls) that release CO, which could be used in clinically relevant conditions. The review is drawn not only from published papers in the chemical literature but also from the extensive biological literature and patents on CO-releasing molecules (CO-RMs).

Keywords Cardiovascular disease · CO-releasing molecules · Heme oxygenase · Inflammation · Medicinal chemistry · Metal carbonyls · Organ transplantation · Reperfusion injury

Contents

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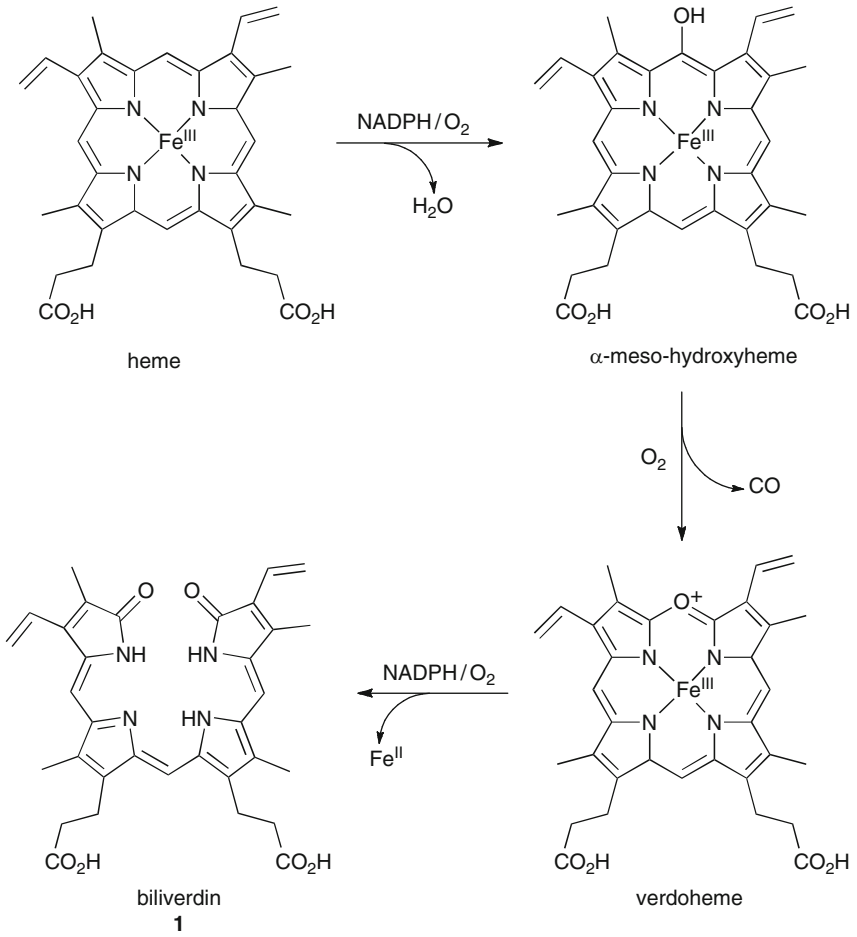
Abbreviations

AEGL3	Acute exposure guideline level 3 – life-threatening health effects or death
AMP	Adenosine monophosphate
ANG	Angiotensin
ASMC	Airway smooth muscle cells
BAY	41-2272 5-cyclopropyl-2[1-(2-fluorobenzyl)-1H-pyrazolo [3,4-b] pyridine-3-yl] pyrimidin-4-ylamine
cGC	Guanylyl cyclase
cGMP	5′-cyclic guanylyl phosphate
CORM-1	$[Mn_2(CO)_{10}]$
CORM-2	$[RuCl_2(CO)_3]_2$
CORM-3	[RuCl(glycinate)$(CO)_3$]
CORM-A1	$Na[H_3BCO_2H]$
CO-RMs	CO-releasing molecules
COX-2	Cyclooxygenase-2

DC	Dendritic cell
DMSO	Dimethylsulfoxide
EC	Endothelial cells
ERK1/2	Extracellular signal-regulated kinase-1 and -2
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
iNOS	Inducible NO synthase
LPS	Lipopolysaccharide
Mb	Myoglobin
MbCO	Carboxymyoglobin
MMP	Matrix metalloproteinase
mRNA	messenger Ribonucleic acid
mTALH	Medullary thick ascending limb
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NANC	Non-adrenergic/non-cholinergic
NF-κB	Eukaryotic transcription factor
Nox4	Gene that encodes the enzyme NADPH oxidase 4
PC-12	A cancer cell line
PMN	Human neutrophils
PPAR-gamma	Peroxisome proliferator-activated receptor-gamma
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
sFlt-1	Soluble fms-like tyrosine kinase-1
STZ	Streptozotocin
THF	Tetrahydrofuran
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
VEGFR-2	Vascular endothelial growth factor-2
YC-1	1-benzyl-3-(5′-hydroxymethyl-2-furyl)indazole

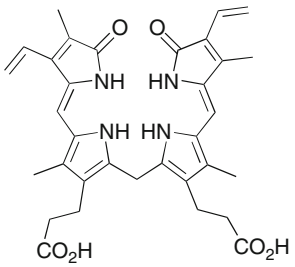
1 Introduction

During the 1940s, it was found that all humans produce carbon monoxide (CO) and breathe out a few cm^3 of this gaseous molecule every day. Most of the CO produced in the body (86%) originates from the catabolism of heme by heme oxygenase to give initially biliverdin, **1**, Fe^{II} and CO (see Scheme 1). Subsequently, the biliverdin is reduced to bilirubin, **2**, by biliverdin reductase. This is one of the most familiar enzyme processes that occurs in the body. An injury forming a bruise liberates heme with O_2 coordinated giving the familiar initial red-purple colour.



Scheme 1 The catabolism of heme by heme oxygenase to give Fe^{II}, CO and biliverdin

The catabolism uses three molecules of O_2 per heme and consequently the bruise goes blue with the loss of O_2 from the heme. As the heme is converted to bilirubin, the bruise goes yellow. The yellow bilirubin is also observed in urine and jaundice. A mixture of sources, including peroxidation of lipids, bacteria, photo-oxidation and xenobiotics accounts for the remaining 14% [1]. Healthy humans produce around 20 μM h^{-1} (ca. 10 cm^3 d^{-1}) of CO [2–4], but elevated levels are found in the breath of humans suffering from a number of diseases including asthma [5, 6], bronchiectasis [7], cystic fibrosis [8, 9], diabetes [10] and rhinitis [11]. All three final products of the catabolism of heme by heme oxygenase (Fe^{II}, CO and bilirubin) are biologically active. However, in this review I will focus primarily on the biological activities of CO with emphasis on the development of CO-releasing molecules (CO-RMs) as potential pharmaceuticals.



bilirubin
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An excellent review of "Pharmacological and Clinical Aspects of heme oxygenase" has recently appeared which gives excellent coverage of applications of the heme catabolism products, CO, biliverdin/bilirubin and Fe^{2+}/ferritin in medicine and their mechanism of action [12]. This is essential background to the present review which concentrates on compounds, principally metal carbonyls, which release CO when administered to mammals with the information being drawn from both papers and patents. There are two forms of heme oxygenase. Heme oxygenase-1 is inducible and heme oxygenase-2 which is constitutive [12].

Recent reviews with some emphasis on medical applications of CO and CO-releasing molecules include "CO and NO in Medicine" [13], "Use of carbon monoxide as a therapeutic agent: promises and challenges" [14] and "Chemistry and biological activities of CO-releasing molecules (CO-RMs) and transition metal complexes" [15].

2 The Role of CO In Vivo

There have been extensive studies on the role of CO in vivo. It has been shown to have a wide range of functions, including:

- Anti-inflammatory
- Protection from tissue reperfusion injury
- Vasodilatation
- Anti-apoptotic
- Anti-proliferative
- Anti-hypoxia

Some examples of its application in animal models include:

- Suppression of organ graft rejection. Mouse to rat cardiac transplantation was performed. One group of rats was exposed to 400 ppm CO in air for 2 days following the operation and survived for 50 days. The other group breathed air, and survived only 5–7 days [16].
- CO improves cardiac energetics and safeguards the heart during reperfusion after cardiopulmonary bypass surgery [17].

- Arteriosclerotic lesions in rats can result from chronic graft rejection and from balloon injury following angioplasty but are suppressed by CO [18].
- Established pulmonary hypertension in rats is reversed by CO [19].
- The development of post-operative ileus in the murine small intestine of mice is suppressed by CO [20].
- CO prevents the development of experimental cerebral malaria in mice [21].
- CO prevents graft-induced intimal hyperplasia in pigs [22].

As a result of these and many other investigations of the use of CO in animal models, there is considerable interest in extending the application to humans. CO gas is being used in clinical trials, where the carbon monoxide is breathed as a mixture with air with concentrations of usually up to 250 ppm. So far this has resulted in several clinical trials. A preliminary trial using up to 500 ppm CO produced no reduction in anti-inflammatory effects in a systemic inflammation model in humans, as 250 ppm for 1 h did in rodents [23]. A phase II trial entitled "Anti-inflammatory effects of inhaled carbon monoxide in patients with chronic obstructive pulmonary disease" has been completed [24]. This was a preliminary trial using 100–125 ppm CO and produced encouraging results. Another five trials are in progress; a Phase I trial entitled "Carbon Monoxide to Prevent Lung Inflammation" [25], a Phase II trial entitled "Safety and Tolerability Study of Inhaled Carbon Monoxide in Kidney Transplant Patients" [26] and after a Phase I trial "Effects of Heme Arginate in Healthy Male Subjects" [27], a Phase II/III trial is examining "Does Heme Oxygenase-1 Induction Ameliorate Cardiac Injury After Myocardial Infarction?". This trial also uses heme arginate as a substrate for heme oxygenase [28]. The fifth, a Phase II trial, is a "Study of Inhaling Carbon Monoxide to Treat Patients with Intestinal Paralysis After Colon Surgery" [29].

3 Sites of Action of CO

There is at present limited information on the sites of action of carbon monoxide. Hemes and cytochromes are the most likely sites. However, direct evidence is generally missing with the exception of guanylyl cyclase.

Stability constants alone do not give the answer and have to be used in conjunction with kinetics. CO binds very strongly to hemoglobin with an equilibrium constant, 583 μM^{-1} for type A sheep hemoglobin [30]. The binding constant to targets and probable targets are considerably smaller. Raw 264.7 macrophage cells are frequently used to study the effects of CO in reducing NO production, detected usually as $[NO_2]^-$, presumably by deactivating NO synthase. Recently, it has been proposed that the activity of CO in these cells is due to its coordination to cytochrome *c* oxidase [31, 32]. However, yeast cytochrome *c* peroxidase has a small $K \sim 0.003\ \mu M^{-1}$ for CO binding [30]. Cytochrome *d* is a more promising target with cytochrome *d* from *Escherichia coli* having $K \sim 13\ \mu M^{-1}$ [33].

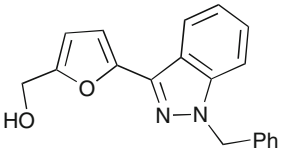
Despite the small stability constant, CO has been shown to be involved in deactivating inducible NO synthase, $K \sim 0.003\ \mu M^{-1}$ [34] and activates guanylyl cyclase, $K \sim 0.004\ \mu M^{-1}$ [35]. However, many biological studies have shown that

CO administered as a mixture with air and transported around the body in the blood stream is biologically active.

A rationale for the effectiveness of CO gas despite the high binding constant to hemoglobin may lie with a report in 1977 [36], which indicated that CO coordinated to heme in hemoglobin is not toxic while that dissolved in the plasma is. When air containing CO, typically at 250 ppm, meets deoxygenated blood in the lungs, the O_2 will bind much faster to the heme on account of its much higher concentration and somewhat faster rate constant [30]. As a result, the CO dissolves in the plasma and replaces the O_2 bound to the hemoglobin during the next few minutes [37]. This gives time for the CO to be delivered to CO binding sites throughout the body. However, the solubility of CO in human serum is only 0.63 mM at 1 atm CO [38]. Hence at the 250 ppm CO typical administered, the CO concentration in serum will only be around 0.16 μM if it is assumed that the solubility in the plasma can be treated as obeying Henry's law.

3.1 Guanylyl Cyclase

Guanylyl cyclase, cGC, is activated by NO and CO to convert guanylyl triphosphate to 5′-cyclic guanylyl phosphate, cGMP, which is an important signalling molecule. NO activates cGC 200-fold over its resting state [39], while CO only activates it fourfold. This is in spite of the small CO binding constant, $K \sim 0.004\ \mu M^{-1}$ [35]. Both CO and NO coordinate to the heme to give six-coordinate iron. In the case of NO coordination, what happens next is still uncertain, but it is probable that the histidine moves away to give a five-coordinate Fe–NO complex. Addition of YC-1, 1-benzyl-3-(5′-hydroxymethyl-2-furyl)indazole, **3**, in addition to CO activates the sGC to the same extent as NO [40], raising the possibility that CO acts with a second signalling molecule. The compound, BAY 41-2272, 5-cyclopropyl-2[1-(2-fluorobenzyl)-1H-pyrazolo [3,4-b] pyridine-3-yl] pyrimidin-4-ylamine, **4**, also enhances the activation of sGC by CO [41–43].



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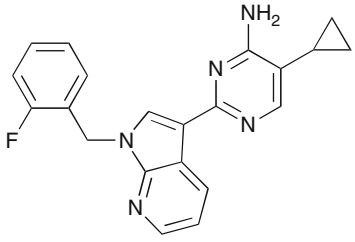

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3.2 Na^+ and K_{Ca}^+ Channels

Heme oxygenase-2 is an oxygen sensor for a calcium dependent potassium channel [44]. Knock-down of heme oxygenase-2 activity was re-instated by CO.

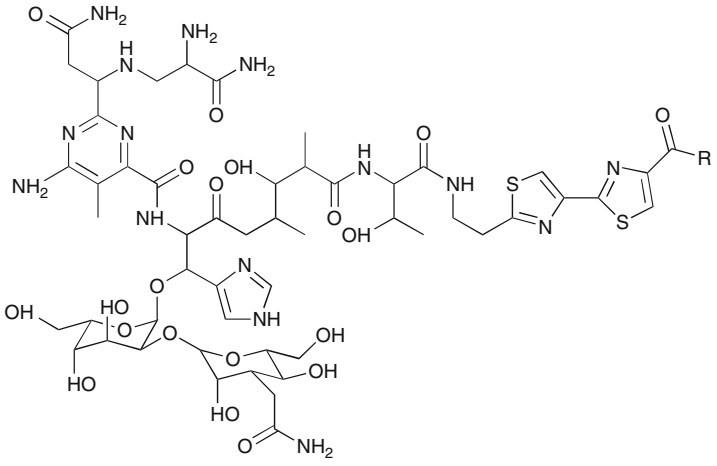
Subsequently, it was found that a motif in the S9–S10 part of the C-terminal is essential for CO activation [45]. A similar behaviour has been observed for epithelial sodium channels [46]. In the case of the human BKCa subunit, cysteine residues are important for channel sensitivity to CO [47].

3.3 Hemes and Cytochromes

Although there is no direct evidence that the coordination of CO to hemes and cytochromes has a role in CO signalling apart from in guanylyl cyclase, it is probable that this is the case. CO binds strongly to hemes and cytochromes, and one of the major roles of CO is to protect from reperfusion injury and reactive oxygen species. This requires the activation of oxygen. This can involve cytochromes or the generation of peroxynitrite from NO generated by NO synthase. There are numerous reports showing that CO suppresses NO generation in macrophages. This probably involves CO coordination to the heme in NO synthase. The generation of reactive oxygen species involves coordination of O_2 to cytochromes that is blocked by CO coordination. It has recently been reported that cystathionine beta-synthase is a carbon monoxide-sensitive regulator of bile excretion due to CO coordination to a heme [48].

3.4 Other Possible Binding Sites for CO

Heme oxygenase catabolises heme to give CO, biliverdin and Fe^{II}. The generation of Fe^{II} opens the possibility of Fe^{II} coordination to biological sites followed by CO coordination. There are many possibilities based on model compounds. For example, Fe^{II} reacts with cysteine to give [Fe(cysteinate)$_2$] which takes up CO to give [Fe(CO)$_2$(cysteinate)$_2$] [49, 50] and with bleomycin, **5**, followed by CO to give a Fe–CO complex [51, 52].



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In addition to simple coordination, the CO could be involved as a reducing agent. The water–gas shift reaction occurs in the bacterial enzyme, CO dehydrogenase/acetyl coenzyme A synthase ([53] and references therein) but it has not been observed in mammals. However, in vitro, CO reduces aquocobalamin, vitamin B_{12a}, Co^{III}, to vitamin B_{12r}, Co^{II}, [54–57] which raises the possibility that the water gas shift reaction could occur in humans.

4 CO-Releasing Molecules (CO-RMs)

4.1 Initial Discovery

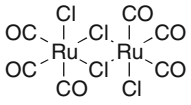
The first report of using metal carbonyls as CO-releasing molecules (CO-RMs) for medical applications, especially involving the cardiovascular system, came in a patent submission in 2001 that was published in part in 2002 [58]. A wide range of compounds were examined as CO-RMs along with biological tests including CO-binding to myoglobin, vasodilation, inhibition of NO production by macrophages and survival of mice after heart transplantation. Subsequently, CO-RMs have found many applications in biological/medical research but have not yet got to the clinic. Emphasis is placed on the few CO-RMs that have received substantial biological usage but the other CO-RMs that have been reported only once or twice will be noted.

4.2 Ruthenium CO-RMs

Although the original patent contained a range of ruthenium carbonyls, the biological work has involved just two: $[RuCl_2(CO)_3]_2$ (CORM-2) and [RuCl (glycinate)$(CO)_3$] (CORM-3). $[RuCl_2(DMSO)_4]$ is commonly used as a control substance.

4.2.1 $[Ru(CO)_3Cl_2]_2$ (CORM-2)

$[RuCl_2(CO)_3]_2$, **6**, is commercially available and is used as a solution in DMSO [58, 59]. When dissolved in DMSO, $[RuCl_2(CO)_3]_2$ rapidly gives a ca. 40:60 mixture of **7** and **8**. Subsequently, **8** isomerises to **9**.



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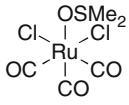

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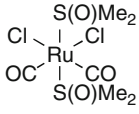

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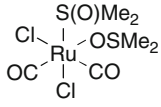

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As the original reports showed that $[RuCl_2(CO)_n(DMSO)_{4-n}]$ can be interconverted by adding and removing CO, the ratio of **7** and **8** is likely to depend on CO concentration [60].

On account of the commercial availability of $[RuCl_2(CO)_3]_2$ and its being one of the first reported CO-RMs, it has attracted considerable attention. Recently, it was reported that it required Fe^{II} to stimulate the release of CO; however, the Fe^{II} is coordinated to heme, and this is consistent with a common observation for CO-RMs that CO release is faster in the presence of myoglobin [61].

To date there have been approximately 80 papers reporting the biological use of CORM-2. These have covered a wide range of applications. CORM-2:

- Inhibits lipopolysaccharide-induced nitric oxide production in RAW264.7 cells [58, 62, 63]
- Inhibits both O_2^- and NO production and down-regulates HO-1 expression in LPS-stimulated macrophages [64]
- Activates PPAR-gamma which inhibits iNOS but not COX-2 expression in LPS-stimulated macrophages [65]
- Inhibits IL-1β-induced iNOS expression [66]
- Protects PC12 cells from peroxynitrite-induced apoptotic death [67, 68]
- Attenuates the *N*-ω-nitro-L-arginine-induced vasoconstriction [69]
- Produces relaxation in gastric fundus and jejunum via sGC and activation of K_{Ca} channels [70]
- Has been used to investigate the role of the sGC α_1/α_2 subunits in the relaxant effect of CO and CORM-2 in murine gastric fundus [71]
- Produces internal anal sphincter relaxation [72]
- Produces small relaxation responses of the airway epithelium [73]
- Inhibits NAD(P)H oxidase cytochrome b_{558} activity, increases oxidant production by the mitochondria and inhibits ASMC proliferation and phosphorylation of the ERK1/2 mitogen-activated protein kinase and expression of cyclin D1 in human airway smooth muscle [74]
- Decreases sFlt-1 release and inhibited VEGFR-2 phosphorylation [75]
- Induces vasorelaxation [76]
- Inhibits proliferation of IL-1β-stimulated vascular smooth muscle cells [77]
- Blocks hemin-induced Egr-1 expression [78]
- Inhibits the inflammatory response induced by cytokines in Caco-2 cells [79]
- Attenuates the inflammatory response in the lung of thermally injured mice [80, 81]
- Attenuates the inflammatory responses in liver of mice with severe burns [82]
- Attenuates the inflammation in the liver of septic mice [83]
- Regulates vascular tone in cirrhotic rat liver [84]
- Is a novel regulator of the inflammatory process in osteoarthritic chondrocytes [85]
- Attenuates the production of inflammatory cytokines, prevents burn-induced ROS generation, and suppresses the oxidative stress in the small intestine of burnt mice by interfering with the protein expression of iNOS [86]

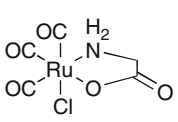
- Protects human osteoarthritic chondrocytes and cartilage from the catabolic actions of interleukin-1 beta [87]
- Modulates the inflammatory response in the liver of thermally injured mice [88]
- Inhibits acute necrotizing pancreatitis in rats [89, 90]
- Protects against H_2O_2-induced injury [91]
- Pretreatment significantly protects p53-deficient cells from the NO donor *S*-nitroso-*N*-acetylpenicillamine-induced cell death [92]
- Activates calcium-dependent potassium channels [44] and sodium channels [46]
- Causes reversible, voltage-independent Ca^{2+} channel inhibition [93]
- Inhibits human cardiac L-type Ca^{2+} channels, [94]
- Reliably and repeatedly activates Slo1 BK channels [95], activates non-selective cation current in human endothelial cells [96]
- Inhibits L-type Ca^{2+} channels [97]
- Has been used to show that cysteine residues in the C-terminal tail of human BKCa alpha subunit are important for channel sensitivity to CO [47]
- Is a rapid modulator of recombinant and native P2X(2) ligand-gated ion channels [98]
- Attenuates leukocyte infiltration in the small intestine of thermally injured mice [99] and in the renal tissue of thermally injured mice [100]
- Attenuates leukocytes sequestration in the liver of burnt mice [101]
- Profoundly inhibits proliferation of human pulmonary artery smooth muscle cells [102]
- Is a possible therapeutic drug for pulmonary hypertension [103]
- Rescues heme oxygenase-1 deficient mice from arterial thrombosis in allogenic aortic transplantation [104]
- Increases the velocity of thrombus growth and strength [105, 106] and decreases fibrinolysis [107, 108] in human plasma
- Has antiproliferative effects on human T cells [109]
- Has a protective effect in ischemia-induced acute renal failure [110]
- Protects against ischemia/reperfusion injury in isolated rat hearts [111]
- Significantly reduces anoxia/reoxygenation-induced apoptosis in cardiomyocytes [112]
- Reduces cell death of cardiomyocytes evoked hy H_2O_2 [113]
- Mediates heme oxygenase 1 induction via Nrf2 activation in hepatoma cells [114]
- Provides photoimmune protection from tumours in mice [115]
- Has an effect on the immunological activation of guinea-pig mast cells and human basophils [116, 117]
- Enables stem cells to form spheres in a serum-free medium and thus can be maintained in an undifferentiated state for a long period of time [118]
- Suppresses LPS-induced nuclear translocation of p50 [102], provides cytoprotection [119]
- Inhibits MMP-1 and MMP-2 mRNA expression in the human lung epithelial cell line A549 [120]

- Protects against lung-injury induced by limb ischemia–reperfusion [121]
- Induces active chloride secretion in Caco-2 cells [122]
- Protects against endotoxin-induced renal injury [123]
- Induces concentration-dependent alterations in the electrophysiological properties of axons in mammalian spinal cord [124]
- Regulates enteric neuron migration [125]
- Has provided evidence that CO acts as an inhibitory neurotransmitter in the longitudinal muscle of the C57BL/6 J mouse distal colon [126]
- Promotes angiogenesis in human micro-vessel endothelial cells [127]
- Attenuates LPS-induced activation of endothelial cells [128]
- Attenuates IL-18-induced endothelial cell death [129]
- Inhibits high dose glucose-induced ICAM-1 expression via AMP-activated protein kinase and PPAR-gamma activations in endothelial cells [130]
- Inhibits DC immunogenicity induced by TLR ligands [131]
- Protect against cell death of cerebellar granule neurons and the increase in ROS induced by 3-nitropropionic acid [132]
- Significantly impairs the growth of *E. coli* and *Staphylococcus aureus* [133–135]
- Plays an important role in the antimicrobial process without inhibiting the inflammatory response during microbial sepsis [136]
- Inhibits LPS-induced high-mobility group box 1 release in vitro and improves survival of mice in LPS- and cecal ligation and puncture-induced sepsis model in vivo [137]
- Produces an inflammatory response in sepsis in mice and its mechanism was investigated [138]
- Inhibits tissue factor expression in sepsis [139]
- Has been applied to the treatment of pyrohemia in its early stage [140]
- Protects Chinese hamster ovary cells after α-particle irradiation [141].

4.2.2 [Ru(CO)$_3$Cl(glycinate)] (CORM-3)

[Ru(CO)$_3$Cl(glycinate)], **10**, was in the first group of compounds to be described as a CO-releasing molecule. Specifically, it was the first water-soluble CO-RM to be synthesised and tested in biological systems. Among other actions, it protects cardiac cells and isolated mice hearts from ischemia–reperfusion injury and reduces cardiac transplant rejection in mice [58, 142]. It has a complicated solution chemistry. When dissolved in water, it immediately reacts giving [Ru(CO)$_2$(CO$_2$H)Cl(glycinate)]$^-$ with a $pK_b = 10.9$ [143]. The resulting solution is acidic, typically ca. pH 2.5. [Ru(CO)$_2$(CO$_2$H)Cl(glycinate)]$^-$ is a mixture of two of the three possible isomers, **11**–**13**. Both these isomers undergo two more pH-dependent equilibria, the first at ca. pH 6, $pK_b = 5.7$ and 6.6 and the second at ca. pH 10, $pK_a = 9.1$ and 10.4. It is unknown whether the first of these equilibria produce isomers of

$[Ru(CO)_2(CO_2)Cl(glycinate)]^{2-}$ or $[Ru(CO)_2(CO_2H)(OH)(glycinate)]^-$ and it is probable that the second equilibria produce isomers of $[Ru(CO)_2(CO_2)(OH)(glycinate)]^{2-}$. If a solution containing $[Ru(CO)_2(CO_2H)Cl(glycinate)]^-$ is acidified with dilute aqueous HCl to generate $[Ru(CO)_3Cl(glycinate)]$, $[Ru(CO)_3Cl_2(NH_2CH_2CO_2H)]$ is also produced. Further reaction with Cl^- yields $[Ru(CO)_3Cl_3]^-$. As a result of these problems, it is difficult to get $[Ru(CO)_3Cl(glycinate)]$ as a pure compound and it is normally used as a mixture with typically 10% $Na[Ru(CO)_2(CO_2H)Cl(glycinate)]$. This does not affect the use of $[Ru(CO)_2(CO_2H)Cl(glycinate)]^-$ as a CO-RM as the pH-dependent equilibria produce the same mixture of compounds in aqueous solution independent of the ratio of $[Ru(CO)_3Cl(glycinate)]$ and $[Ru(CO)_2(CO_2H)Cl(glycinate)]^-$ in the preparation. However, it is essential to check each preparation of $[Ru(CO)_3Cl(glycinate)]$ for purity. If there is too much $[Ru(CO)_2(CO_2H)Cl(glycinate)]^-$, the solid will be cream to yellow in colour and can be sticky. Alternatively, if too much acid is used in the preparation, $[Ru(CO)_3Cl_2(NH_2CH_2CO_2H)]$ is also produced which can be identified in the ^1H NMR spectrum in CD_3OD [143]. It is also essential to check each preparation for CO loss to myoglobin.



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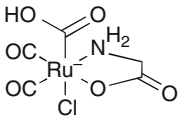

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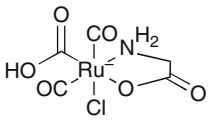

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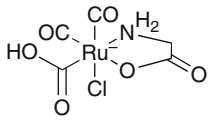

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The mechanism of CO-release is unknown. In pure water, CO release is very slow but in the presence of myoglobin it is very rapid with $t_{1/2} \leq 2$ min. When dissolved in phosphate buffer for 24 h, $[Ru(CO)_3Cl(glycinate)]$ is deactivated and no longer releases CO. This combination of properties makes $[Ru(CO)_3Cl(glycinate)]$ ideal for use as a CO-RM. Solutions can be prepared and administered without significant loss of CO, and then release CO after administration. $[Ru(CO)_3Cl(glycinate)]$ deactivated with phosphate buffer provides a reference sample which does not release CO so that the effects of CO can be investigated.

Approximately, 50 papers have appeared describing applications of $[Ru(CO)_3Cl(glycinate)]$ (CORM-3) in biological systems. It

- Is well tolerated by pigs [144] and cynomolgus monkeys at 4 mg kg^{-1} [145] and has anti-inflammatory and immunomodulatory properties which may result in clinical benefit to allo- and xenografted organs [146]
- Enables pretreated cardiac cells to become more resistant to the damage caused by hypoxia-reoxygenation and oxidative stress, enables isolated hearts reperfused in its presence (10 μM) after an ischemic event to display a significant recovery in myocardial performance and to produce a marked and significant reduction in cardiac muscle damage and infarct size [142]
- Prolongs the survival rate of transplanted hearts using a model of cardiac allograft rejection in mice [142]

- Causes vasodilatation [147, 148]
- Causes both vasoconstriction through the generation of ROS which CO can negate producing vasodilatation [149]
- Relaxes rat aorta through activation of the guanylyl cyclase/cGMP system, and the modulation of K^+ channels and reduces the expression of adhesion molecules by EC and PMN through a superoxide-dependent mechanism [117]
- Reduces infarct size in vivo when administered at the time of reperfusion [150]
- Induces late preconditioning against myocardial infarction [151]
- Exerts beneficial effects against ischemia/reperfusion-induced injury through its abilities to release CO which mediates a cardioprotective action by regulating tissue Na^+, K^+ and Ca^{2+} levels [152]
- Exerts a positive inotropic effect in doxorubicin-induced cardiomyopathy [153]
- Is a potent enhancer of angiogenesis [154]
- Inhibits expression of vascular cell adhesion molecule-1 and E-selectin independently of heme oxygenase-1 expression [155]
- Shows positive inotropic effects in the isolated perfused rat heart thus strengthening muscle contraction [156]
- Has been used to develop the use of miniaturised conductance catheters to measure left ventricular function [157]
- Inhibits human platelets aggregation by a mechanism independent of soluble guanylate cyclase [158]
- Improves myocardial function after cold storage with preservation solution supplemented with it [159]
- Blocks interleukin-18-mediated NF-κB-PTEN-dependent human cardiac endothelial cell death [129]
- Reduces the development of post-operative ileus via modulation of MAPK/heme oxygenase-1 signalling and reduction of oxidative stress [160]
- Has a beneficial effect on vascular relaxation in diabetes in rats [161]
- Has been used to investigate how carbon monoxide restores vascular function in type 1 diabetes [162], and how enhancement of NANC relaxation was significantly impaired in STZ-treated rats [163]
- Produces a significant decrease in endothelial cell sloughing in diabetic rats [164]
- Reduces nitrite generation from macrophages and hence reduces inflammation [62]
- Attenuates lipopolysaccharide- and interferon-gamma-induced inflammation in microglia [165]
- Significantly reduces TNF-α and IL-1β proinflammatory responses in healthy monkeys and prolonged exposure to it was well tolerated [145]
- Has been used to evaluate the effects in an in vitro model of cardiovascular inflammation [166]
- Modulates thrombin-induced neuroinflammation in BV-2 microglia [167]
- Activates Ca^{2+} sparks and reduced global Ca_I^{2+} in smooth muscle cells of newborn piglet brain slice arterioles [168]

- Reduces joint inflammation and erosion in murine collagen-induced arthritis [169]
- Protects against cisplatin-induced nephrotoxicity [170] and in ischemia-induced acute renal failure [110]
- Improves renal function at reperfusion following pretreatment [171]
- Protects renal tubular epithelial cells against cold-rewarm apoptosis [172]
- Inhibits tubuloglomerular feedback in kidneys [173]
- Protects against ischemia–reperfusion injury in an experimental model of controlled non-heartbeating donor kidney [174]
- Improves renal function during reperfusion during warm preservation [175]
- Could be utilised as adjuvant therapeutics in University of Winconsin solutions to limit the injury sustained by donor livers during cold storage prior to transplantation [176]
- Stimulates the Ca^{2+} activated big conductance K channels in cultured human endothelial cells [177]
- Impairs alveolar fluid clearance by inhibiting epithelial Na channels [178]
- Modulates leukocyte–endothelial interactions under flow [179]
- Modulates polymorphonuclear leukocyte migration across the vascular endothelium by reducing levels of cell surface-bound elastase [180]
- Could increase cavernous tissue cGMP [181]
- Could provide a possible therapeutic strategy to improve cell viability during neural differentiation in applications that use stem cell technology [182]
- Abrogates polymorphonuclear granulocyte-induced activation of endothelial cells and mast cells [183]
- Has effects in a coincubation model of rat mast cells with human neutrophils [184]
- Reduces intraocular pressure [185]
- Reduces cholesterol synthesis [186]
- Rescues mice from lethal sepsis by supporting mitochondrial energetic metabolism and activating mitochondrial biogenesis [187]
- Results in a strong inhibition of *E. coli* cell growth. *S. aureus* was more resistant, particularly under aerobic conditions [134, 188]
- Exerts bactericidal activity against *Pseudomonas aeruginosa* and improves survival in an animal model of bacteraemia [189].

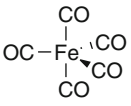
4.2.3 Other Ruthenium Compounds

A number of ruthenium compounds were examined in the 2002 CO-RM patent for CO release but on account of the success of $[Ru(CO)_3Cl(glycinate)]$ as CO-RM, they have not been investigated further [58]. The compounds include $[Ru(CO)_3Cl_2L]$ L = DMSO, THF, guanosine, guanine and triacetylguanosine, $[Ru(CO)_3Cl$ (amino acidate)] amino acid = cysteine, isoleucine, serine, alanine, glutamine, arginine, lysine, valine, threonine and proline, $[Ru(CO)_3ClL_2]^+$, L = cytidine,

guanosine, guanine, $[Ru(CO)_3\{(O_2CCH_2)_2E\}]$ E = O, NH, $[Ru(CO)_2Cl_2]_n$ and $[Ru(CO)_2Cl_2(DMSO)_2]$.

4.3 Iron CO-RMs

$[Fe(CO)_5]$, **14**, and $[Fe_2(CO)_9]$, **15**, along with other metal carbonyls have been reported in a patent as being applied as a transdermal patch or cream [190]. Subsequently, $[Fe(CO)_5]$ was shown to release CO to myoglobin on photolysis [58, 59].



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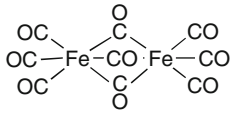

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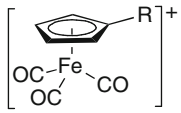
4.3.1 Heme-Based Carriers

A polyethylene glycol-conjugated hemoglobin derivative and carbon monoxide carrier, CO-MP4, is able to deliver CO to the circulation and reduces ischemia/reperfusion injury in rats, providing the first evidence for this drug as a CO therapeutic agent [191, 192].

4.3.2 $[CpFe(CO)_3]^+$ and Its Derivatives

$[CpFe(CO)_3]^+$, **17**, R = H, was found to release CO rapidly in 2002 [58]. This led to detailed study of derivatives with substitution of the cyclopentadienyl ring and replacement of a carbonyl by a halide [193, 194]. By making small changes in substituent, it was possible to tune the half-life for CO release over nearly an order of magnitude. The compounds showed low cell toxicity and between acceptable and good inhibition of nitrite formation using murine RAW264.7 macrophages. The compounds and their biological results are summarised in Table 1.



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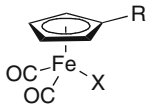

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$[CpFe(CO)_2(pyrone)]^+$, pyrone = 4-methoxy-6-methyl-2-pyrone and 3-bromo-4-methoxy-6-methyl-2-pyrone were examined as potential CO releasing molecules but failed to release [195].

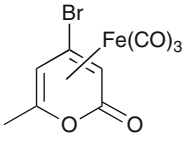
Table 1 Half-lives for CO release to myoglobin and measurements of cell viability, cytotoxicity and nitrite formation using murine RAW264.7 macrophages [193, 194]

Compound/Ion	$t_{1/2}$/min	Cell viability	Cyto-toxicity	$[NO_2]^-$
17, R = H	69			
17, R = CO_2Me	42	***	***	*
17, R = $CO_2CH_2CH_2OH$	62	***	***	*
17, R = CH_2CO_2Me	225	***	***	*
17, R = $CH_2CH_2CO_2Me$	285	***	***	*
18, R = CO_2Me, X = Cl	63	***	***	**
18, R = CO_2Me, X = Br	38	***	***	**
18, R = CO_2Me, X = I	48	***	***	**
18, R = CO_2Me, X = NO_3	170	***	***	**
18, R = H, X = Cl	350	***	NA	NA
18, R = H, X = Br	200	***	***	*
18, R = H, X = I	150	***	***	*

*Greater than 50% cell viability or less than 50% cell toxicity at 10 μM CO-RM concentration or greater than 50% nitrite production inhibition at 100 μM CO-RM concentration
**Greater than 50% cell viability or nitrite production inhibition, or less than 50% cell toxicity at 50 μM CO-RM concentration
***Greater than 50% cell viability or less than 50% cell toxicity at 100 μM CO-RM concentration or greater than 50% nitrite production inhibition at 10 μM CO-RM concentration

4.3.3 [(η^4-2-pyrone)Fe(CO)$_3$]

A number of compounds [(η^4-2-pyrone)Fe(CO)$_3$] have been tested for CO release and toxicity. Three were found to release CO, **19–21**, and were also tested for toxicity with IC_{10} = 132, 89 and 95 μM for **19**, **20** and **21**, respectively. **19** causes vasodilatation of aortic rings pre-contracted with phenylephrine [196]. Subsequently, this was extended to cell viability, cytotoxicity and inhibition of nitrite production by using murine RAW264.7 macrophages and compound **22** was included [197]. Of these compounds, **19** was the most promising.



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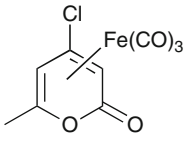

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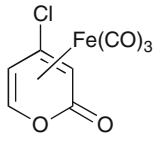

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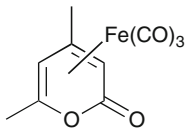

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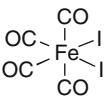
4.3.4 Other Iron CO-RMs

[$FeI_2(CO)_4$], **23**, loses CO very rapidly to myoglobin [198]. *mer*-[$FeI_2(CO)_3(PR_3)$], **24**, R = OMe, CH_2OH, *cis*, *cis*, *cis*- and *cis*, *cis*, *trans*-[$FeI_2(CO)_2\{P(OMe)_3\}_2$], **25** and **26**, lose CO more slowly with $t_{1/2}$ between 235 and 764 min [198]. [$Fe(SPh)_2(CO)_2(NH_2CH_2CH_2NH_2)$] and [$Fe(SPh)_2(CO)_2$(2,2′-bipyridyl)] have been shown to cause vasodilatation [58].



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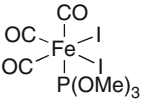

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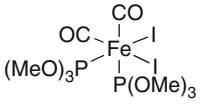

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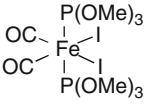

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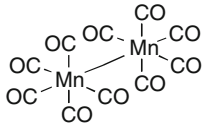
4.4 Manganese CO-RMs

4.4.1 $[Mn_2(CO)_{10}]$, CORM-1

The use of $[Mn_2(CO)_{10}]$, **27**, as a CO-RM was first described in the first publications on the subject [58, 59] where it was shown to cause vasodilatation when photolysis was used to liberate CO. As a consequence of $[Ru(CO)_3Cl_2]_2$ and $[Ru(CO)_3Cl$ (glycinate)] being easier to use, these CO-RMs are generally used in preference. Nevertheless, there have been a few biological applications of $[Mn_2(CO)_{10}]$. CO released from $[Mn_2(CO)_{10}]$ on photolysis has been reported to dilate isolated pressurised cerebral arterioles of newborn pigs [199] and piglet pial arterioles [200, 201], activate KCa channels in newborn arteriole smooth muscle cells by increasing apparent Ca^{2+} sensitivity of α-subunits [202], control of renal hemodynamics and glomerular filtration in kidney [203] and attenuate the inflammatory response in the lung of thermally injured mice by decreasing leukocyte sequestration and interfering with NF-Kβ activation, protein expression of ICAM-1, thus suppressing endothelial cells pro-adhesive phenotype [81].



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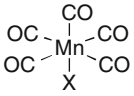
Note: The reader should be aware that some authors have described $[Ru(CO)_3Cl_2]_2$ as CORM-1 rather than CORM-2.

4.4.2 $[Mn(CO)_5X]$, X = Cl, Br, I

$[Mn(CO)_5Br]$, **28**, X = Br, strongly reduces the viability of *E. coli* and *S. aureus*. The presence of hemoglobin removed the bactericide activity [188]. $[Mn(CO)_5X]$, **28**, X = Cl, Br, I, release CO to myoglobin with $t_{1/2}$ = 970 min (X = Cl), $t_{1/2}$ = 245 min (X = Br) and $t_{1/2}$ = 384 min (X = I) [204]. A subsequent measurement on $[Mn(CO)_5Br]$ gave values ranging from 1,049 to 1,434 min [198].



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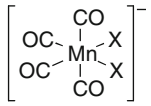
4.4.3 $[Mn(CO)_4X_2]^-$, X = Br, I, SC(O)Me [13, 204]

$[Mn(CO)_4Br_2]^-$, **29**, X = Br, releases CO very rapidly to myoglobin with $t_{1/2} < 2$ min. It shows no cytotoxicity at 100 μM. $[Mn(CO)_4I_2]^-$, **29**, X = I, releases CO to myoglobin with $t_{1/2} = 12$ min. $[Mn(CO)_4\{SC(O)Me\}_2]^-$, **29**, X = MeC(O)S, releases CO to myoglobin with $t_{1/2} = 32$ min but even at 100 μM it does not suppress $[NO_2]^-$ formation by murine RAW264.7 macrophages. However, it does produce rapid vasodilatation.



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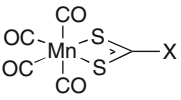
4.4.4 $[Mn(CO)_4(\eta^2\text{-}S_2CR)]$, R = NEt_2, $NMeCH_2CO_2H$, $N(CH_2CH_2OH)_2$, OEt, and $[Mn(CO)_4\{\eta^2\text{-}S_2P(OEt)_2\}]$ [204]

$[Mn(CO)_4(\eta^2\text{-}S_2CNEt_2)]$, **30**, X = NEt_2, releases CO rapidly to myoglobin, $t_{1/2} < 2$ min; it is cytotoxic at 50 μM and suppresses $[NO_2]^-$ formation by murine RAW264.7 macrophages at 10 μM. The introduction of polar groups reduces the toxicity so that $[Mn(CO)_4\{\eta^2\text{-}S_2CN(CH_2CH_2OH)_2\}]$, **30**, X = $N(CH_2CH_2OH)_2$, and $[Mn(CO)_4(\eta^2\text{-}S_2CNMeCH_2CO_2H)]$, **30**, X = $NMeCH_2CO_2H$, behave similarly but are less cytotoxic. $[Mn(CO)_4(\eta^2\text{-}S_2COEt)]$, **30**, X = OEt, releases CO more slowly, with $t_{1/2} = 72$ min. It has some cytotoxicity at 50 μM while at 10 μM it suppresses $[NO_2]^-$ formation by murine RAW264.7 macrophages. $[Mn(CO)_4\{\eta^2\text{-}S_2P(OEt)_2\}]$, **31**, has $t_{1/2} = 6$ min. It shows no cytotoxicity at 100 μM while at 50 μM it suppresses $[NO_2]^-$ formation by murine RAW264.7 macrophages.



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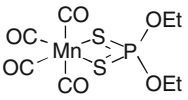

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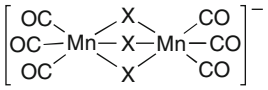
4.4.5 $[Mn_2(CO)_6X_3]^-$, X = Cl, OAc [204]

$[Mn_2(CO)_6Cl_3]^-$, **32**, X = Cl, releases CO very rapidly with $t_{1/2} < 2$ min. It shows no cytotoxicity at 100 μM and limited suppression of $[NO_2]^-$ formation by murine RAW264.7 macrophages. $[Mn_2(CO)_6(OAc)_3]^-$, **32**, X = OC(O)Me, releases CO more slowly but still rapidly with $t_{1/2}$ ~7 min. It shows no cytotoxicity at 100 μM while at 50 μM it suppresses $[NO_2]^-$ formation by murine RAW264.7 macrophages. It also produces rapid vasodilatation.



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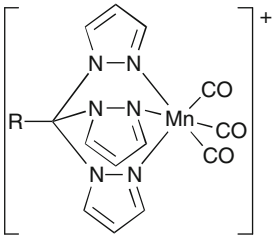
4.4.6 Other Manganese Compounds

Although CO release for $[(MeC_5H_4)Mn(CO)_3]$ was not detected, the introduction of a CO_2H group into the cyclopentadienyl ring in $[\{Me(HO_2C)C_5H_3\}Mn(CO)_3]$ results in very slow CO release to myoglobin with a half-life of ca. 3,000 min [193].

Cell viability studies of HT29 colon cancer cells treated with the CO-releasing compound $[Mn(CO)_3(tpm)]PF_6$, **33** R = H, revealed a significant photoinduced cytotoxicity comparable to that of established agent 5-fluorouracil [205]. Subsequently, $[Mn(CO)_3(tpm)]PF_6$, **33**, was modified by replacing R with peptides to permit cellular recognition [206]. **34**, R = H, Me, releases nearly 2 mol of CO to myoglobin on photolysis while **35**, E = lone pair, O, S, releases approximately 1 mol of CO. The n-octanol/water partition coefficients were also measured [207].



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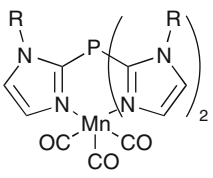

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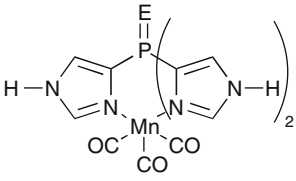

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CO release from a range of other manganese compounds has been reported but are generally slow CO releasers [204].

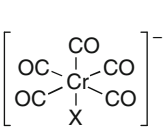
4.5 Vanadium

$[NEt_4][V(CO)_6]$ releases CO to myoglobin with a concentration dependent $t_{1/2}$, at 20 μM $t_{1/2}$ = 1,546 min, at 40 μM $t_{1/2}$ = 965 min and at 60 μM $t_{1/2}$ = 685 min [198].

4.6 Chromium

$[NEt_4][CrX(CO)_5]$, **36**, X = Cl, Br, I, $[NEt_4][\{Cr(CO)_5\}_2X]$, **37**, X = Cl, Br, $[Cr\{=C(OCH_3)CH_3\}(CO)_5]$, **38**, release CO to myoglobin [198]. $t_{1/2}$ values of around 200 min were found. $[\{Cr(CO)_5\}_2X]^-$, X = Cl, Br, is an intermediate of CO loss from $[CrX(CO)_5]^-$.

$[\{(MeO)_2C_6H_4\}Cr(CO)_3]$, **39**, releases CO to myoglobin on photolysis or on stirring in the presence of a heme coated electrode [61].



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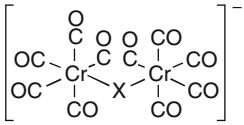

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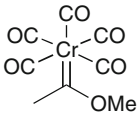

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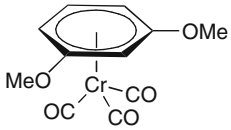
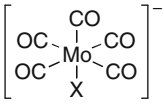


39

4.7 Molybdenum

4.7.1 $[Mo(CO)_5X]^-$, X = Cl, Br, I, and $[Mo\{=C(OMe)Me\}(CO)_5]$

$[Mo(CO)_5Br]^-$, ALF-62, **40**, X = Br, strongly reduces the viability of *E. coli* and *S. aureus*. The presence of hemoglobin removed the bactericide activity [188]. The symptoms of arthritis decrease in rats when treated with $[Mo(CO)_5Br]^-$ [208]. $[NEt_4][MoX(CO)_5]$, **40**, X = Cl, Br, I, releases CO to myoglobin [198]. $t_{1/2}$ values of 718–2,440 min were found. The CO release is quicker for the carbene, $[Mo\{=C(OMe)Me\}(CO)_5]$, **41**, with $t_{1/2}$ values between 90 and 356 min [198].



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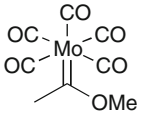

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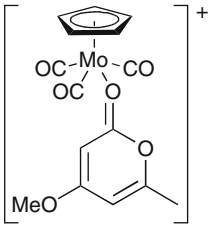
4.7.2 $[CpMo(CO)_3(pyrone)]^+$

Two compounds, $[CpMo(CO)_3(pyrone)]^+$, pyrone = 4-methoxy-6-methyl-2-pyrone, **42**, and 3-bromo-4-methoxy-6-methyl-2-pyrone, **43**, were examined as potential CO releasing molecules. Both release CO to myoglobin. $[CpMo(CO)_3$(3-bromo-4-methoxy-6-methyl-2-pyrone$)]^+$, **43**, which showed the faster CO release also showed good cell viability, cytotoxicity and inhibition of nitrite production using murine

RAW264.7 macrophages and vasodilatation [195]. Note that the claim in this paper that $[CpMo(CO)_3(3\text{-bromo-4-methoxy-6-methyl-2-pyrone})]^+$, **43**, was the most rapid CO releaser reported to date is incorrect. There are a number of ruthenium CO-RMs, including $[Ru(CO)_3Cl_2]_2$ and $[Ru(CO)_3Cl(glycinate)]$, reported in the initial patent that release to myoglobin so rapidly that the first measurement gave >10 μM MbCO from 20 μM compound giving $t_{1/2} < 2$ min [58].



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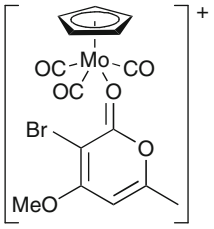
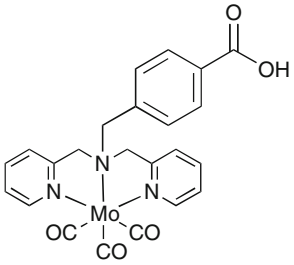


43

4.7.3 $Mo(CO)_3$ L, L = nitrilotriacetate, 4-[[bis(2-pyridinylmethyl)amino] methyl]-benzoate, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetate, histidinate

The molybdenum tricarbonyls, **44**, **45** and **46**, have been reported as being antibacterial with **45** and **46** being active against H. pylori [134] while oral administration of ALF-186, **47**, prevents the induction of a gastric ulcer by the NSAID, indomethacin [209].



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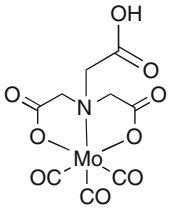

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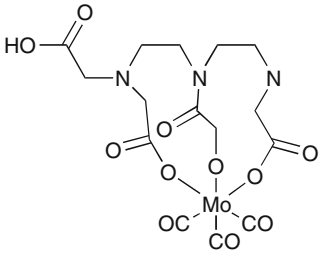

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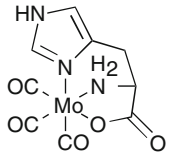

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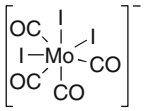
4.7.4 $[NEt_4][MoI_3(CO)_4]$

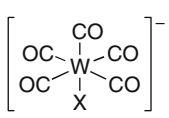
$[NEt_4][MoI_3(CO)_4]$, **48**, loses CO to myoglobin with $t_{1/2}$ between 90 and 356 min [198].



48

4.8 Tungsten

$[NEt_4][WX(CO)_5]$, **49**, X = Cl, Br, I, releases CO to myoglobin [198]. $t_{1/2}$ values of 1,758–3,488 min were found. The CO release is slower for the carbene, $[W\{=C(OMe)Me\}(CO)_5]$, **50**, X = {=C(OMe)Me} with $t_{1/2}$ values between 3,966 and 7,924 min [198]. $[NEt_4][WI_3(CO)_4]$, **51**, and $[W(\kappa^2\text{-acac})_2(CO)_3]$, **52**, also release CO with $t_{1/2}$ values of 93–205 min and around 76 min, respectively [198]. Near-UV photolysis of $[W(CO)_5\ P(C_6H_4\text{-}4\text{-}SO_3)_3]^{3-}$, **53**, in an aqueous buffer solution leads to the high quantum yield release of a single CO. In aerated media, one CO is slowly released from the $[W(CO)_4(H_2O)\{P(C_6H_4\text{-}4\text{-}SO_3)_3]^{3-}$ photoproduct owing to autoxidation of the tungsten centre [210].



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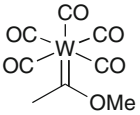

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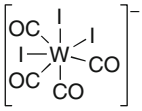

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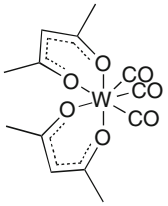

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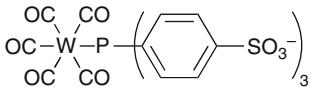

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4.9 Cobalt

It has been observed that for $[(\mu_2\text{-}R^1C_2R^2)Co_2(CO)_6]$, the alkyne substituents significantly affect the rate of CO-release, cytotoxicity and cell viability of **54** (see Table 2) [211].

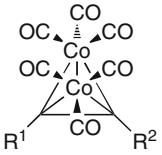

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Table 2 Half-lives for CO release, toxicity and cell viability data for $[(\mu_2\text{-}R^1C_2R^2)Co_2(CO)_6]$

R^1	R^2	$t_{1/2}$/min[a]	Toxicity[b]	Cell viability[c]
CO_2H	H	32.6	84.6% (5.6)	35.1% (12.2)
CO_2H	CO_2H	1.6	6.6% (5.6)	79.6% (10.0)
CH_2OH	H	67.8		
Bu^t	H		3.3% (3.6)	86.6% (5.2)
Ph	H	0	82.9% (4.3)	24.4% (7.9)
Ph	Ph	0		

[a]Averaged over values reported
[b]Toxicity at 100 μM, expressed as a percentage of a standard treated with detergent
[c]Cell viability at 100 μM – the ability of the treated cell to metabolise substrate, expressed as a percentage of a fully functioning cell
Standard deviation values are provided in brackets

4.10 Boron. Na[H_3BCO_2H], CORM-A1

Although boron carbonyls are only stable at very low temperature, H_3BCO reacts with hydroxide to give $[H_3BCO_2]^{2-}$, which is stable at room temperature and is normally isolated as the mono sodium salt, $Na[H_3BCO_2H]$ [212]. It was patented as a source of CO source for preparation of transition metal carbonyl complexes [213]. It is also a good source of CO for medical applications [214]. It readily releases CO at physiological pH.

$$[H_3BCO_2]^{2-} \overset{H^+}{\rightleftharpoons} [H_3BCO_2H]^- \overset{-[OH]^-}{\rightleftharpoons} [H_3BCO] \xrightarrow{H^+} 3H_2 + \left[B(OH)_4\right]^- + CO$$

At pH 7.4, $[H_3BCO_2]^{2-}$ releases CO relatively quickly at 37°C with a $t_{1/2} = 21$ min [214, 215]. It has received a number of tests for biological activity. It causes vasorelaxation [214, 215]. However, although it caused significant coronary vasodilatation in perfused rat hearts, it had no effect on myocardial contractility [156]. It reduces ANG II-mediated increases in superoxide production in mTALH cells [216, 217]. CORM-A1 enhances proliferation and motility of endothelial cells in vitro by 50% vs. air controls, and in vivo, it accelerates reendothelialization of the denuded artery by day 4 after injury vs. day 6 in air-treated animals following balloon angioplasty [218]. It irreversibly inhibits amiloride-sensitive short-circuit currents in H441 human bronchiolar epithelial cells and primary rat alveolar type II cells by up to 40% [178]. It causes an increase in renal blood flow and a decrease in vascular resistance in mice kidneys [219] and ameliorates vascular activity, energy metabolism and renal function at reperfusion [171]. There are cerebroprotective effects against seizure-induced neonatal vascular injury in newborn pigs [220]. A cytoprotective role for the heme oxygenase-1/CO pathway during neural differentiation of human mesenchymal stem cells has been identified [182]. It blocked TNF-α-induced reactive oxygen species accumulation and apoptosis consistent with the antioxidant and antiapoptotic roles of the end products of HO activity [221]. Glutamate-induced reactive oxygen species production and apoptosis

were blocked by CORM-A1 and by bilirubin consistent with the antioxidant and cytoprotective roles of the end products of HO activity [222]. It inhibits Nox-4 generated $[O_2]^-$ and TNF-α stimulation leading to apoptosis in brain cell endothelial cells [223].

4.11 Organic Compounds as Sources of CO

Several organic sources of CO have been identified. The simplest source is to provide hemin for heme oxygenase to catabolise to carbon monoxide, biliverdin/bilirubin and Fe^{II} [224]. Haloalkanes, especially CH_2Cl_2, $CHBr_3$ and CHI_3, are metabolised to CO. These compounds are capable of inhibiting the proliferation of vascular smooth muscle cells, protecting the vasculature against oxidative stress and injury, modulating the activity of various immune system cells, inhibiting the production of pro-inflammatory cytokines and enhancing production of anti-inflammatory cytokines, thereby being effective in the treatment of conditions associated with adverse proliferative or inflammatory responses [225, 226]. More recently tertiary aldehydes have been shown to give CO and when administered to rat models of arthritis significantly reduced paw edema and improved the arthritic indexes in these animals [227, 228].

5 Detection of CO Release

An excellent review of methods of measurement of CO in biological systems has been published [1]. The rate of CO release from CO-RMs is dependent on conditions, with myoglobin frequently producing faster CO release than pure water or a phosphate buffer. Many CO-RMs are oxygen and light-sensitive so that it is important that measurements are carried out in the absence of O_2 and/or light. Most measurements are carried out at 37°C. Manometric and GC measurements rely on the low solubility of CO in water. At 1 atm CO, the solubility is ca. 1 mM in pure water at 25°C [229]. This does not normally cause problems as experimental conditions produce a low partial CO pressure and the gas space is normally larger than the solution space.

5.1 Manometric

Gas evolution can be measured manumetrically. The gas is evolved in a closed vessel and the volume of gas evolved determined using a manometer [188].

5.2 Gas Chromatography

CO can readily be determined in the gas phase using gas chromatography [230]. As with manometric CO determination, it relies on the low solubility of CO in water.

5.3 CO Electrode

World Precision Instruments have produced a CO electrode, which has been used to determine the CO concentration in solution [215, 231]. The electrode can be calibrated using $Na_2[H_3BCO_2]$ [232]. As the electrode consumes CO during the measurement, the resulting data has to be fitted to a double exponential corresponding to the liberation of CO from the CO-RM and the consumption of CO by the electrode.

5.4 Myoglobin

When myoglobin reacts with CO to form carboxymyoglobin, the electronic spectrum changes significantly permitting electronic spectroscopy to be used to monitor the release of CO from a CO-RM [59]. As myoglobin also reacts readily with dioxygen, it is necessary to carry out the measurements in the presence of the reducing agent dithionite. CO-RMs frequently release CO much faster in the presence of myoglobin rather than in a simple buffer. Normally commercial myoglobin is used which is often considerably impure and its concentration is determined using the known extinction coefficient, $\varepsilon_{555} = 12{,}920\ \text{L mol}^{-1}\ \text{cm}^{-1}$ [233]. Myoglobin reacts rapidly with CO, $k = 0.38\ \mu\text{M s}^{-1}$, with a high binding constant, $16.9\ \mu\text{M}^{-1}$. As a result it removes the CO rapidly. This could affect the rate of CO release if CO loss from the CO-RM is reversible. In this, it differs from the other measurement techniques where either the CO is not removed or is slowly consumed.

6 Mechanisms of CO Release

There have been no detailed studies of the mechanism CO release by CO-releasing molecules. Photolysis is established for $[Fe(CO)_5]$, $[Mn_2(CO)_{10}]$ [58, 59], $[Mn(CO)_3(tpm)]$, **33–35**, [205–207] and $[\{(MeO)_2C_6H_4\}Cr(CO)_3]$ [61], but will also apply to many CO-RMs and it is prudent to keep CO-releasing molecules in the dark. Protonation is established as the mechanism of CO release for $[H_3BCO_2]^{2-}$ [215]. It is probable that the release of CO from $[Ru(CO)_3Cl(glycinate)]$ results

from replacement of the chloride and/or glycinate by *trans*-labilising ligands such as thiols and/or sp^2-nitrogen ligands. It is also probable that CO is released from some CO-RMs by oxidation and for this reason it is wise to keep CO-releasing molecules in an inert atmosphere as far as possible. This could in part account for the facile CO release from Mn(I) CO-RMs.

There has been an attempt to model CO release from $[Mn_2(CO)_{10}]$, $[Ru(CO)_3Cl_2]_2$ and $[Ru(CO)_3Cl(glycinate)]$ in aqueous solution in the presence of Cl^-, $[HPO_4]^{2-}$ or NO [234]. A number of species were identified. The most significant result was that the reaction of NO with $[Ru(CO)_3Cl_2]_2$ and $[Ru(CO)_3Cl(glycinate)]$ is endothermic. This supports the belief that inhibition of nitrite formation by macrophages using $[Ru(CO)_3Cl_2]_2$ or $[Ru(CO)_3Cl(glycinate)]$ is due to inhibition by CO rather than coordination of the NO intermediate to ruthenium. Unfortunately, the calculations are flawed. For $[Ru(CO)_3Cl_2]_2$, the calculations were done on the dimer while it is insoluble and reacts with a coordinating solvent, L, to give $[Ru(CO)_3LCl_2]$, where L is usually DMSO [59]. Similarly, $[Ru(CO)_3Cl(glycinate)]$ reacts with hydroxide at pH 3 to give $[Ru(CO)_2(CO_2H)Cl(glycinate)]^-$ (two isomers observed) and at around pH 6 with a second hydroxide to give $[Ru(CO)_2(CO_2H)(OH)(glycinate)]^-$ (isomers) or $[Ru(CO)_2(CO_2)Cl(glycinate)]^{2-}$ (isomers) [142]. Unfortunately, the calculations were performed on $[Ru(CO)_3Cl_2]_2$ and $[Ru(CO)_3Cl(glycinate)]$ rather than the species actually present in a typical buffered aqueous solution used for biological work.

7 Some Potential Applications of CO Gas and CO-RMs in Medicine

There is considerable interest in the use of carbon monoxide in medicine. There are three principal methods being used.

- Heme oxygenase-1 is inducible and a number of compounds have been identified which induce it so as to produce carbon monoxide, biliverdin/bilirubin and Fe^{2+}/ferritin [12]. These include aspirin, statins, curcumin (a component of turmeric) and resveratrol (a component of red wine). The induction of heme oxygenase takes many hours so that such treatments are applicable to chronic conditions. Aspirin and statins are now prescribed for the cardiovascular system but the significance of the heme oxygenase is unproven. The compounds that are catabolised to give CO provide an alternative procedure.
- Most of the applications of CO gas in medicine are for short-term treatments, often associated with surgery. The carbon monoxide is breathed as a mixture with air, usually at around 250 ppm. There is currently uncertainty about what level of CO gas concentration can be administered safely. The American Environmental Protection Agency gives 330 ppm as the maximum 60 min exposure at the AEGL3 level where greater exposure could result in life-threatening health effects or death. This comes down to 150 ppm at 4 h exposure [235]. In

the light of the trials on humans using 500 ppm CO for 1 h any effect, these recommendations appear to be over cautious [23]. The administration of CO gas is coupled with monitoring the concentration of carboxyhemoglobin and this is normally limited to 10%.

- CO-releasing molecules (CO-RMs) have the advantage that they can be administered locally in controlled quantities and to specific locations. The result is that it is possible to generate a local high CO concentration without administering a dangerous quantity to the whole body. As some CO-RMs are absorbed by cells, CO can be administered to weak CO binders without most of it being captured by hemoglobin. Unlike CO gas, there is no risk of exposing the medical staff to dangerous quantities of CO.

Although the development of CO-RMs as pharmaceuticals is several years behind that of CO, there have been many important applications in animal models. Some significant developments are:

- Isolated hearts were subjected to ischemia for 30 min followed by reperfusion, modelling a heart attack. When [$Ru(CO)_3Cl$(glycinate)] was added to the reperfusion fluid, the hearts showed a significant reduction in cardiac muscle damage and infarct size compared with hearts reperfused without [$Ru(CO)_3Cl$(glycinate)] [142]. This offers a method to reduce reperfusion damage whenever blood flow is re-established to regions where it has been restricted, for example following angioplasty or following surgery. This has led to using CO-RMs in conjunction with organ transplantation when the transplanted organs generally perform better when perfused with solutions containing CO-RMs during the period out of the body. Better outcomes have been observed after CO-RM treatment during transplantation for heart [111, 142, 159] or kidney [110, 171, 172, 174, 175, 236].
- CO-RMs are generally effective at reducing inflammation. This is potentially of value for the treatment of arthritis [85], inflammatory responses following thermal injury [80–82], inflammatory responses following sepsis [83, 136], gastric ulcers [234], bacterial infection [134, 140] and neuro-inflammation [167].
- CO-RMs affect the condition of the veins and arteries. They induce vasodilatation [147, 156, 219, 237], inhibit platelet aggregation [158] and are anti-apoptopic. These properties make CO-RMs of interest in the treatment of diabetes and Alzheimer's disease.

8 Patents

When research started on the use of metal carbonyls as CO releasing molecules for applications in medicine in 2000, it was virtually a virgin area with a considerable background of work involving CO gas. As a result, patents were used to secure a broad area for commercial development. The first patent was due to Hemocorm in

2001 and provide protection for the use of a wide range of metal carbonyls, primarily in mainly the cardiovascular system [58]. In 2002, Alfama submitted a similar patent that added to the areas with patent coverage [238].

Subsequently, further patents were added by Hemocorm and Alfama covering specific applications and groups of compounds. This includes:

- Using metal carbonyls to deliver carbon monoxide to isolated organs of humans and other mammals to limit post-ischemic damage [239]
- The combination of a metal carbonyl compound and a guanylate cyclase stimulant (e.g. YC-1) or stabiliser for the therapeutic delivery of carbon monoxide [240]
- The use of boranocarbonates for the therapeutic delivery of carbon monoxide [214]
- Compositions comprising organometallic molybdenum compounds for treating cancer [241]
- Molybdenum carbonyl complexes useful for inhibiting tumour necrosis factor (TNF) production and for treating inflammatory diseases [242]
- Molybdenum carbonyl complexes for treating rheumatoid arthritis and other inflammatory diseases [208]
- The use of CO-releasing molybdenum carbonyl complexes for the treatment of inflammatory diseases [188]
- The use of cyclopentadienyl metal carbonyls and related compounds as CO-RMs [193]
- The use of CO-RMs to treat infections [134]
- The use of some manganese carbonyls as CO-RMs [204]
- The prevention of gastric ulcers by carbon monoxide (using ALF-186, **47**) [209].

The Alfama patent coverage also includes the use of tertiary aldehydes to be metabolised to give CO [227, 228].

During 2008, Alfama and Hemocorm merged to give a single company with excellent patent coverage of the field.

In addition to the Alfama-Hemocorm patents, there are other relevant patents:

- The use of heme proteins with CO coordinated [191, 243–245]
- The use of metal carbonyls, especially $[Fe(CO)_5]$ and $[Fe_2(CO)_9]$, trans-dermally [190]
- Treating vascular disease and modulating the inflammatory and immune processes using carbon monoxide-generating compounds, especially CH_2Cl_2 [225, 226]
- Devices such as soft tissue implants and methods for inhibiting fibrosis comprising non-gaseous CO, e.g. adsorbed in a zeolite, with anti-fibrotic activity [246]
- A method of treatment and prophylaxis of events, conditions and diseases of the systemic vasculature and immune function to decrease cardiovascular risk and pathogenic infection mentions manganese carbonyl [247]
- Composition for treating autoimmune disease comprising heme oxygenase-1 inducer or CO donor capable of inhibiting proliferation of CD4+ T-cell causing autoimmune disease [248]

- Multipotent stem cells derived from human adipose tissue and cellular therapeutic agents comprising the same, using $[Ru(CO)_3Cl_2]_2$ [118]
- The application of carbon monoxide-releasing molecules ($[Ru(CO)_3Cl_2]_2$) in the treatment of pyohemia in early stage [140].

In addition to these patents are a substantial number concerning the use of CO gas and some of these patents include the possibility of using a CO-RM as the source of CO.

The strength of patents in the area makes it very difficult for researchers to develop new CO-releasing molecules as pharmaceuticals. To take a new compound from the bench to the clinic is very expensive and can only be done commercially with very strong patent coverage. As many compounds release CO making it possible to find alternatives, secure patent coverage requires a very broad range of compounds patent protected and Alfama is the only company with the patent coverage to do this.

9 The Future

After the initial discovery of $[Mn_2(CO)_{10}]$, $[Ru(CO)_3Cl_2]_2$, $[Ru(CO)_3Cl(glycinate)]$ and $Na_2[H_3BCO_2]$ as CO-releasing molecules, nearly all the published biological investigations have concentrated on this selection of molecules. In this review, it has been shown that there are many other CO releasing molecules and it is to be expected that they will be investigated in a broad range of medical applications.

As yet there have been few investigations of the uptake of CO-RMs by cells but this is where CO-RMs have a great advantage over the competing method of administration of CO as a mixture with air that is breathed in, coordinates to hemoglobin and is then transmitted to the whole body by blood flow. On account of the high binding constant of CO to hemoglobin, e.g. type A sheep hemoglobin, $K = 583\ \mu M^{-1}$ [30] and the much lower binding constant to possible sites such as inducible NO synthase, $K = 0.00255–0.0051\ \mu M^{-1}$ [34], guanylyl cyclase, $K = 0.003846\ \mu M^{-1}$ [35], or cytochrome bo, $K = 1.7\ \mu M^{-1}$ [249], carboxyhemoglobin is unlikely to efficiently transfer CO to these sites. Binding of CO to oxygenated hemoglobin is relatively slow [36], providing a mechanism to deliver CO from the lungs dissolved in the plasma. The CO should be rapidly removed by the hemoglobin once it delivers oxygen within the body. However, CO gas therapy is effective showing that the take up of CO gas from the plasma is slow, presumably as a result of the CO having to enter the blood cells.

In contrast, $[Ru(CO)_3Cl(glycinate)]$ [250] and $[Mo(CO)_5Br]^-$ [134] have been shown to be taken up by cells providing a mechanism to deliver CO directly to cells without it being trapped by hemoglobin. When $[Ru(CO)_3Cl(glycinate)]$ is administered to mice, the carboxyhemoglobin concentration does not increase but reduces infarct size due to reperfusion injury following coronary artery occlusion [150]. In contrast, $[Mo(CO)_5Br]^-$ strongly reduces the viability of *E. coli* and *S. aureus* but

the presence of hemoglobin removed the bactericidal activity which implies CO release outside the cell [188].

There has been little or no work done on investigating the rate of uptake by cells or the rate of CO release in cells and what happens to the CO-RM once it has entered a cell. This is of particular importance as much of the research is done using mice and rats to develop protocols for administration to humans. For example, if the application is organ transplant, a CO-RM that is rapidly absorbed by the cells of a small organ may only protect the more accessible parts of a much larger human organ. There is now a range of CO-RMs available and it is time for the development of CO-RMs that are optimised for specific applications. It is therefore essential to investigate the pharmacokinetics of CO-RMs and for any chemist developing CO-RMs to be part of a team developing CO-RMs for a specific application.

In a recent review of the manganese CO-releasing molecules patent [204], it was concluded that "there are multiple potential clinical candidates from the data presented and future work is expected to develop these CO-RMs into clinical products for a myriad of pathophysiological processes and disease states" [251].

The challenge is still to get CO-RMs into the clinic.

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