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Review

Emerging protein targets for metal-based pharmaceutical agents: An update

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ABSTRACT

The peculiar chemical properties of metal-based drugs impart innovative pharmacological profiles to this class of therapeutic and diagnostic agents, most likely in relation to novel molecular mechanisms still poorly understood. However, inorganic drugs have been scarcely considered for medicinal applications with respect to classical organic compounds due to the prejudice of the relevant toxic effects indicated in certain cases. Thus, the development of improved metallodrugs requires clearer understanding of their physiological processing and molecular basis of actions. Among the various issues in the area of medicinal inorganic chemistry, target elucidation and validation is essential for identifying new therapeutic and imaging applications for metal compounds, and to develop metal complexes as molecular biological tools to detect protein activities in biological systems. Recently, various proteins/enzymes were shown to be possible targets for therapeutic or diagnostic metal complexes, including metallo-enzymes and membrane water-glycerol channels (aquaporins) with essential roles in both physiological and pathophysiological states. Herein, we present an overview of the most representative studies in the field with particular focus on the emerging protein targets – namely zinc-finger proteins, aquaglyceroporins, nitric oxide synthase, thymidine kinases and carbonic anhydrases – which have been also characterized for their interactions with metal-based compounds at a molecular level *via* different biophysical, analytical and computational methods. A chapter is also included concerning the targeting of parasite enzymes by metal compounds for the treatment of infectious diseases.

1. Therapeutic and diagnostic metal compounds

Empirical evidence for the effectiveness of metal-based therapeutics has existed for centuries, and the use of metals and metal-containing compounds in medicine dates back millennia.

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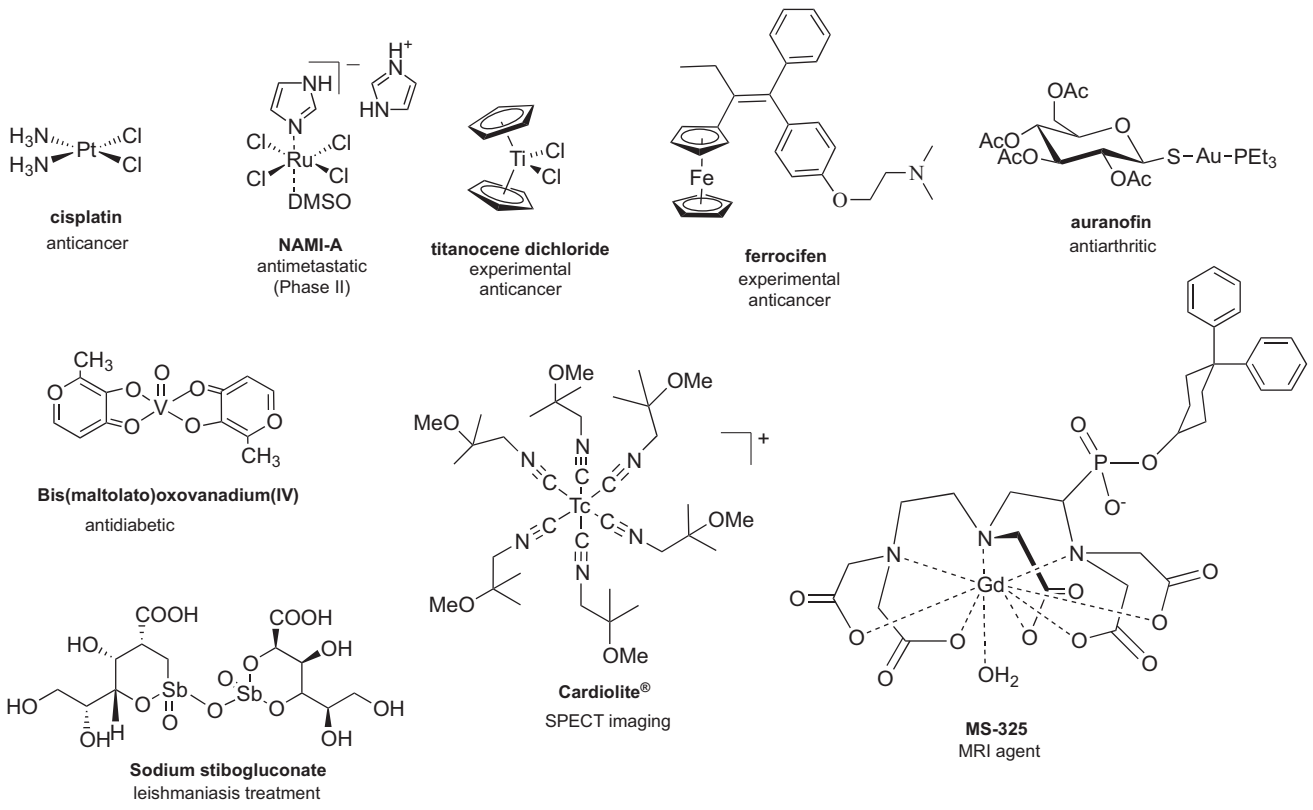


Fig. 1. Metal-based pharmaceuticals with different therapeutic and imaging applications.

Nowadays, the list of therapeutically prescribed metal-containing compounds includes platinum (anticancer), silver (antimicrobial), gold (antiarthritic), bismuth (antiulcer), antimony (antiprotozoal), vanadium (antidiabetic) and iron (anticancer and antimalarial) (Fig. 1) [1–3]. Moreover, metal compounds as diagnostic tools have also been widely explored and are successfully applied in the clinical set for imaging of diseases [4–6]. For example, lanthanides occupy a relevant place as diagnostic agents, but also have many other medically important applications, as hypophosphatemic agents for kidney dialysis patients, as luminescent probes in cell studies, and for bone pain palliation [7]. In terms of purely therapeutic agents platinum coordination compounds, and one of them, cisplatin, recognized as anticancer drug in the late 1960s, have been intensely studied for several decades [8,9]. Since then, strategies opening up new avenues are increasingly being sought using complexes of metals other than platinum such as ruthenium, gallium, iron, titanium and gold [10–17]. Thus, while non-classic platinum complexes are increasingly being developed because they do not mimic cisplatin in their modes of action, and are therefore explored to improve the pharmacological properties of the resulting compounds, metals other than platinum inherently have more or less proper preconditions for this purpose. Differences in coordination geometry, binding preferences according to the HSAB (hard and soft acids and bases) principle, important redox activity, kinetics of ligand exchange reactions, or even the simple capacity of replacement of essential metals form the chemical basis for a diversity of pharmacologically relevant interactions with biomolecules [18,19].

Concerning metal compounds as diagnostic agents, most of the research efforts expended in the past few years in the field of radiopharmaceutical sciences/nuclear medicine are aimed at the synthesis, characterization and biological evaluation of target-specific metal-based radioactive probes for nuclear imaging (Single Photon Emission Computed Tomography – SPECT and Positron Emission Tomography – PET) or internal radiotherapy. These complexes incorporate γ-emitting radiometals for use in SPECT (*e.g.* ^{99m}Tc and ^{111}In) or β^+-emitting radiometals for PET (*e.g.* ^{68}Ga and ^{64}Cu) [20–24]. Among the different metal complexes used for SPECT-imaging, it is worth mentioning sestamibi, marketed under the trademark Cardiolite®, which is a lipophilic cation of Tc(I) stabilized by six isonitrile ligands: $[^{99m}Tc(CNR)_6]^+$(R = $CH_2C(CH_3)_2OCH_3$) (Fig. 1). This cationic radiotracer was originally developed as a SPECT myocardial imaging agent, but currently is also used for both early cancer detection and non-invasive monitoring of the tumour Multidrug Resistance (MDR) transport function. This complex is considered the unique organometallic pharmaceutical used routinely in medicine and, together with cisplatin, is among the most successful synthetic complexes for medical application, from a scientific, commercial, and healthcare point of view [25,26].

Besides the use of γ- or β^+-emitting metal-based radiopharmaceuticals for diagnostic purposes, nuclear medicine takes also advantage of complexes containing β^--emitting radiometals for internal radiotherapy. That is the case of ^{153}Sm-EDTMP (Quadramet®, EDTMP = ethylenediamine tetra(methylene-phosphonic acid)) and ^{186}Re-HEDP(HEDP = hydroxyethylidenediphosphonate) for bone-pain palliation or ^{177}Lu-[DOTA^0,Tyr^3] octreotate, a ^{177}Lu-labelled somatostatine analogue, successfully used for therapy of neuroendocrine tumours [27–29]. Metal compounds are also used in molecular Magnetic Resonance Imaging (MRI) as contrast agents. In general, the latter are paramagnetic complexes (typically Gd^{3+}-based) (Fig. 1) or super paramagnetic particles (typically iron oxides) that change the relaxation properties of water molecules that they encounter [30–32].

Recently, the major aim to study metal compounds for therapy and diagnosis stems from the wish to learn about their mechanisms of biological action in the expectation to improve selectivity, administration protocols and making new drugs. This work has

been reviewed regularly, including by some of us [25,33–41]. In this paper we will focus on the proteins/enzymes that have been more recently considered likely biological targets for metal compounds and studied at a molecular level, and we will explore the evidences of metal complexes–protein binding relevant to the drug/diagnostic agent's mechanisms of action.

2. Proteins as possible targets

The mechanisms of biological action of metal compounds for therapy and diagnosis have been widely investigated, although, in several cases still not fully elucidated. As an example, in the case of anticancer metallodrugs, DNA is not always the primary target as it appears for cisplatin [12,42–44]. In fact, many of metal-containing chemotherapic agents actually show selectivity towards proteins with respect to nucleic acids, indicating that different modes of action occur depending on the specific type of metal complex. In recent years, the general consensus on the crucial role of the interactions of metallodrugs with proteins in determining the compounds' pharmacological action, uptake and biodistribution, as well as their overall toxicity profile, resulted in an exponential increase in the number of studies. Initially, these studies mostly concerned the two major serum proteins, albumin and transferrin, involved in the transport of therapeutic metallodrugs, as well as metallothioneins, small, cysteine-rich intracellular proteins, primarily involved in storage and detoxification of soft metal ions [45,46]. Nowadays, metal-based compounds are known to bind to several classes of proteins with different roles, including transporters, antioxidants, electron transfer proteins, DNA-repair proteins, as well as proteins/peptides simply used as model systems to characterize the reactivity of metallodrugs *in vitro*, but that are also present *in vivo* [44,47–50].

Among the protein systems that have been most widely investigated as targets for metal therapeutic compounds it is worth mentioning the seleno-enzyme thioredoxin reductase (TrxR [51]), involved in the maintenance of the intracellular redox balance and overexpressed in certain cancer types, and reported to be inhibited mainly by gold compounds. In addition, several studies investigated protein kinases efficiently inhibited by ruthenium or iridium complexes [52,53], various proteases inhibited by Pt(II), Ru(II), Re(IV), Cu(II) and Co(III) complexes [54–58], and histone deacethylase (HDAC) inhibition by Pt compounds [59]. Of note in the field, the group of Meggers has pioneered an approach in which metals can also be used as building blocks for well-defined, three-dimensional constructs, and used this principle in the development of organometallic complexes that mimic organic enzyme inhibitors [44]. Notably, other studies reported on the proteasome inhibition by anticancer gold(III) complexes [60,61], as well as by Ga(III), Zn(II) and Cu(II) compounds with asymmetric ligands [62]. Finally, reversible protein tyrosine phosphatase (PTP) inhibition by anti-diabetic vanadium complexes has been widely investigated [63].

Concerning macromolecular protein targets in nuclear molecular imaging, whose expression pattern and density are linked to a certain disease, cell surface receptors (*e.g.* G-protein coupled receptors), transporters (*e.g.* glucose transport protein Glut1) and various enzymes [22,23,36] were among the most explored. Moreover, the folate receptor (FR), a cell surface protein, has been considered a promising target for diagnosis or therapy of cancer. The FR facilitates the uptake of folic acid (FA) a vitamin (B9) that is necessary for cell growth and proliferation. The FR is overexpressed in a variety of cancer types, with highest frequency observed in ovarian and endometrial carcinomas. Interestingly, besides being overexpressed in tumour, it is down-regulated in healthy adult cells. Therefore, the FR is an ideal structure for nuclear imaging using FR-targeted radiopharmaceuticals, and it has been extensively explored for diagnostic applications, namely using various radiometal complexes as recently reviewed by Muller and Schibli [64,65]. Finally, the use of radioactive metal-based probes for targeting membrane receptors with receptor-specific peptides or for *in vivo* monitoring of tumour multidrug resistance (MDR) associated to membrane transporters has been also developed, but it will not be discussed herein as it has been already comprehensively reviewed [25,36,39].

Below, we will present in detail selected proteins/enzymes that have been recently studied and characterized for their interactions with therapeutic and diagnostic metal complexes, and that, most importantly, are likely targets for these metal compounds, including zinc-finger proteins, membrane protein channels, nitric oxide synthase, the zinc enzymes carbonic anhydrases, and thymidine kinases. The last part of the review will be focused on studies of metal compounds targeting parasitic enzymes for application in the treatment of infectious diseases. Particular attention will be paid to reviewing the studies on the characterization of the metal–protein target interactions at a molecular level, using different biophysical and analytical methods.

2.1. Zinc-finger proteins

Zinc is an essential metal in biology, being essential for growth and development. In fact 10% of the human genome encodes zinc proteins, representing *ca.* 3000 proteins of which 427 are zinc-finger (ZF) proteins. Zinc can bind in different ways in protein structures and so be classified into two main categories: (i) *catalytic zinc* in enzymes, where the binding site has a readily exchangeable water ligand coordinated and there can be up to three ions available for catalysis, and (ii) *structural zinc* with only protein residues in the coordination sphere, with general coordination of the type S_4, S_3N or S_2N_2 [66,67]. In this latter case, zinc is not a direct participant in the conveyed interactions of the protein with other residues or molecules, but maintaining a tridimensional secondary/tertiary structure is essential for protein function. There are also intermediate cases such as the Ada protein, where the zinc is coordinated tetrahedrally only by aminoacid residues (four cysteines) although one of the residues is a catalytic cysteine site [68]. This was the first observed case of a residue bound to Zn^{2+} and acting as catalytic element, therefore not fitting this protein in either of the main categories.

Classically, zinc-finger (ZF) proteins belong to the *structural zinc* family where the zinc ion structurally organizes small peptidic domains (or bigger domains in case of multiple zinc ions) and different coordination, interactions and arrangements can contribute to the structural and functional variety of these proteins [68]. ZF proteins were shown to be intimately involved in a wide range of functions in DNA repairing, recognition, transcription, replication, apoptosis and metabolism. A remarkable example was the first described ZF motif in the transcription factor TFIIIA from the clawed toad *Xenopuslaevis* [69], exhibiting a diverse array of structure and functions, the latter involving important cellular processes such as transcription, DNA repair, cellular signalling, metabolism and apoptosis. All of these processes are essential for cell growth and development, thus, having direct implications in health and disease, and so zinc-fingers are recognized more frequently as possible medicinal targets. In fact, these domains show a thermodynamic preference for Zn^{2+} to the detriment of other endogenous metal ions and, in case of metal substitution or coordination residue mutation, the protein function can be impaired or lost. Coordination compounds can affect ZF domain conformation either *via* zinc substitution or *via* oxidative damage, and therefore, may be important in the development of new therapeutic drugs [70].

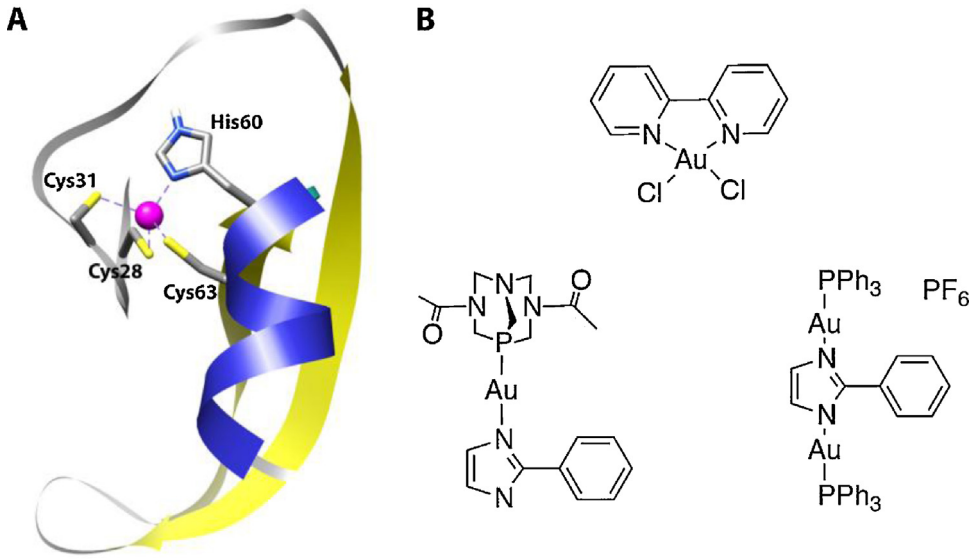


Fig. 2. (A) Ribbon representation of the NH_2-terminal zinc finger of PARP-1, with the zinc binding motif shown as stick model. The Zn atom is depicted as a magenta sphere. This figure was generated using the pdb2DMJand Chimera software (http://www.cgl.ucsf.edu/chimera). The 106 amino acid sequence is as follows: GSSGSSGMAESSDKLYRVEYAKSGRAS**C**KK**C**SESIPKDSLRMAIMVQSPMFDGKVPHWY**H**FS**C**FWKVGHSIRHPDVEVDGFSELRWDDQQKVKKTAEAGGSGPSSG (Zn-binding residues Cys-28, Cys-31, His-60, and Cys-63 in bold letters), (B) Gold(III) and gold(I) complexes as PARP-1 inhibitors.

Another important family of zinc-finger proteins includes the enzymes poly(adenosine diphosphate (ADP)-ribose) polymerase (PARPs), essential proteins involved in cancer resistance to chemotherapies. Moreover, PARPs play a key role in DNA repair by detecting DNA strand breaks and catalyzing poly(ADP-ribosylation) [71], and consequently, PARPs have been referred to as "the guardian angels" of DNA. Notably, PARP-1, the most studied member of the PARP family, is characterized by the presence of two long zinc-fingers (ZF-PARPs, also termed as nick-sensors), that are positioned upstream of the catalytic domain [72], and mediate specific nicked DNA recognition [73]. PARP-1 also binds to platinum-modified DNA [74,75], and a systematic *in vitro* study was recently conducted in which the effect of PARP-1 inhibition on the ability of nuclear proteins to bind platinum-modified DNA was evaluated by photo- cross-linking experiments [76]. According to these results the activity of PARP, following exposure to platinated DNA, resulted in the dissociation of DNA-bound proteins. Moreover, PARP inhibitors were able to sensitize some, but not all, of the cell lines towards cisplatin. Other studies describe the binding of PARP-1 to platinum 1,2-d(GpG) and 1,3-d(GpTpG) intrastrand cross-links on duplex DNA [77] and a more recent report demonstrated that PARP-1 differentiates between normal and platinum-damaged DNA, having higher binding affinity for the cisplatin 1,2-d(GpG) cross-links than for the unplatinated DNA or other types of cisplatin–DNA cross-links [74]. In this latter study it was also shown that PARP-1 may shield the DNA lesion from repair and triggers a cytotoxic response. Overall, in spite of these numerous studies, the activity of PARP upon cisplatin treatment remains controversial and not fully understood.

Within this framework, one of us recently described the ZF enzyme poly(adenosine diphosphate (ADP)-ribose) polymerase 1 (PARP-1) inhibition properties of different metal compounds including cisplatin, and of a series of gold-based compounds with phosphine or bipyridyl ligands (Fig. 2) [78,79]. Interestingly, gold(III) complexes were among the most efficient in inhibiting purified PARP-1 followed by gold(I) compounds (IC_{50} in the nanoM range). Moreover, the gold complexes were able to efficiently inhibit PARP-1 in cancer cell extracts treated with the compounds, but only in certain human cancer cell lines.

Additional information on the reactivity of the metal complexes with PARP-1 N-terminal zinc-finger domain was obtained by high-resolution electrospray ionization Fourier-transform ion cyclotron mass spectrometry (ESI-FT-ICR MS) [78]. An excellent correlation between PARP-1 inhibition in protein extracts and the ability of the complexes to bind to the zinc-finger motif (in competition with zinc) was established. The results support a model whereby displacement of zinc from the PARP-1 zinc finger by other metal ions leads to decreased PARP-1 activity, and to formation of the so-called "gold-finger" (Fig. 3).

Concerning ZF transcription factors, human DNA polymerase-α is inhibited by cisplatin *via* coordination with the cysteine residues on the protein's C4-ZF motif [80], causing tertiary structure distortion and displacement of the Zn ion. Ralph et al. have also shown by an ESI-MS approach that platinum compounds can interfere with binding of the transcription factor PU.1-DBD to a dsDNA molecule containing its consensus-binding site [81]. Moreover, cisplatin has also been recently reported to affect the conformation of the apo-form of the breast cancer susceptibility protein 1 (BRCA1) RING finger domain forming intra- and intermolecular Pt-BRCA1 adducts, where a preferential platinum-binding site was found at His-117 [82]. The same authors investigated the functional consequences of the *in vitro* platination of the BRCA1 RING domain by cisplatin and analogues, which resulted in the inhibition of the ubiquitin ligase activity of BRCA1 [83].

Of note, platinum(II) complexes have been reported to interact with the C-terminal finger of the HIV nucleocapsid NCp7 zinc finger leading to zinc ejection [84]. These latter studies show the opportunity of exploiting metal-based drugs as new classes of anti-HIV agents based on inhibition of HIV NCp7 function and targeting protein Cys residues [85]. Recently, the same authors showed that a platinated single-stranded oligonucleotide can alter the structure of a model ZF peptide and characterized this interaction at a molecular level by NMR spectroscopy [86]. The ZF conformation change results from the formation of an adduct between the platinated oligonucleotide and the peptide, stabilized by strong H-bonding interaction. Most importantly, these results have shown that the extent and rate of zinc displacement by inorganic compounds can be modulated by the nature (metal, ligands) of the reacting compound, and that DNA-tethered

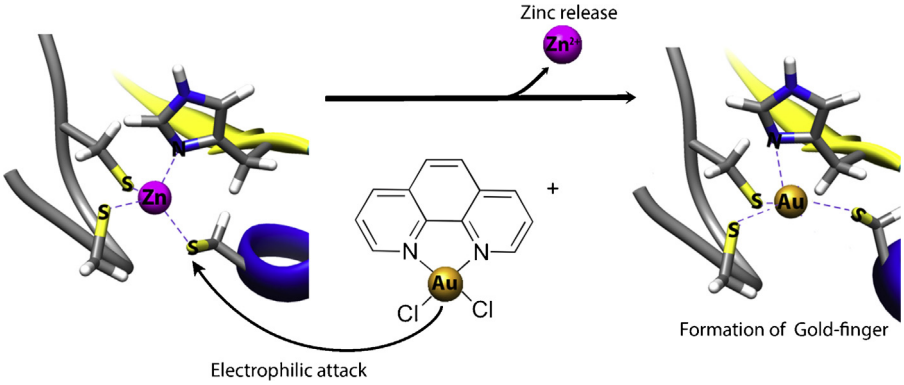


Fig. 3. Model of the possible interaction between gold(III) complexes and PARP-1 N-terminal ZF domain.

coordination complexes may be designed to target specific ZF motifs.

2.2. Aquaporins

Aquaporins belong to a highly conserved group of membrane proteins called the major intrinsic proteins (MIPs) that form a large family comprising more than 800 integral membrane proteins [87] that are involved in the transport of water and small solutes such as glycerol, nitrate and urea [88,89]. Nowadays, aquaporins have been recognized in nearly all-living organisms (reviewed by Kruse et al. [90] and Gomes et al. [91]). Unicellular organisms such as bacteria or yeasts usually possess one or two aquaporin genes encoding water channels [92,93], but a few more aquaporin genes are found in the genome of multi-cellular organisms such as plants, which may contain 38 putative aquaporin genes [94].

In mammals, 13 isoforms (named AQP0–AQP12) have been identified so far and can be divided into two major groups on the basis of their permeability characteristics: those primarily permeable to water (called orthodox aquaporins, AQP0–AQP2, AQP4–AQP6 and AQP8) and those permeable to small solutes including glycerol (called aquaglyceroporins, AQP3, AQP7, AQP9, and AQP10), emphasizing the essential nature of response to osmolarity and the need for conductance of glycerol. Glycerol, a three-carbon backbone tri-alcohol, is a key component of the majority of phospholipids and an important metabolite. Once inside the cell, glycerol is phosphorylated by glycerol kinase, maintaining the inward gradient that drives inward glycerol flux. Additionally, a third subgroup of aquaporins named super-aquaporin or unorthodox aquaporins (AQP11 and AQP12) were identified and showed a lower homology to other AQPs; their cellular localization and function are still obscure [95].

Three-dimensional structural analyses of AQPs have revealed a tetrameric assembly of four identical monomers each behaving as a water channel [96–100]. Fig. 4A and B shows different views of this tetrameric structure here exemplified for the bovine *b*AQP1. The monomers interact with two of their neighbours and form the tetramer with a central pore that excludes water molecules [101]. It has been suggested that this pore is involved in gas conduction [102,103] and could function as a gated cation channel [104,105].

The most remarkable feature of the aquaporin channels is their high selectivity and efficiency on water or glycerol permeation. Aquaporins allow water/glycerol to move freely and bidirectionally across the cell membrane, but exclude all ions including hydroxide, hydronium ions and protons [101], the latter being essential to preserve the electrochemical potential across the membrane. Although classical aquaporins are still considered mostly specific for water, AQP1 permeation by small polar solutes was recently proposed where an inverse correlation between permeability and solute hydrophobicity was found [107]. In the pore region, the water specificity is achieved by the presence of particular residues conferring size constrictions and/or charge characteristics that enable water molecules to pass through, while preventing permeation to protons or any solutes above 2.8 Å. The cytoplasmic and periplasmatic entry of the pore in the aquaporin monomer offers several water–wall residue interactions, mainly with carbonyl groups. After passing these first interactions, wall regions with different hydrophobic/hydrophilic characteristics determines selectivity, conduction rate and open/closed state of the pore.

The orthodox aquaporin and the aquaglyceroporin subfamily share a common protein fold. It comprises six membrane-spanning helices plus two half-helices with their positive, N-terminal ends located at the centre of the protein and their C-terminal ends pointing towards either side of the membrane. The helices surround the 20-Å-long and 3-4-Å-wide amphipathic AQP channel. AQPs are identified by two asparagine–proline–alanine (NPA) sequence motifs located at the ends of the two quasi 2-fold related half-spanning helices. The selectivity filter (SF), a constricted region formed by four residues near the periplasmic/extracellular entrance, provides distinguishing features that identify the subfamilies. In water selective AQPs this region is smaller and more polar and contains a conserved histidine residue, while in aquaglyceroporins it is larger and more hydrophobic with two conserved aromatic residues [108]. Two conserved constriction sites are present in the channel. An aromatic/Arg (ar/R) constriction is located at the extracellular pore mouth. Its diameter determines whether or not solutes, such as glycerol and methylamine, can pass the AQP in addition to water [107,109,110]. Furthermore, the positively charged residues in this region form an energy barrier for protons. The second constriction resides in the centre of the channel, where the positive ends of the two half-helices meet. The helix dipole moments add up to a full positive charge, and the resulting electrostatic field poses another energy barrier for cations [111]. Fig. 4C shows a schematic representation of the pore region of *b*AQP1 (mesh area), indicating the location of the residues lining the pore wall relevant for selectivity and the two half helices that behave as dipoles with the positive charge oriented towards the centre of the pore.

Aquaporins are expressed throughout the body with high concentration in kidney and in red blood cells, being detected in many epithelia and endothelia involved in fluid transport, but also in non-fluid transporting tissues, like skin, fat and astroglia. Phenotype analysis of aquaporin knockout mice has revealed a variety of important physiological roles of AQPs in the urinary concentration mechanism, glandular fluid secretion, brain swelling, neural excitability, fat metabolism, skin hydration and more recently, cell migration (for a review see [112]). Malfunctions of aquaporins are associated with diseases such aspolycystic kidney disease,

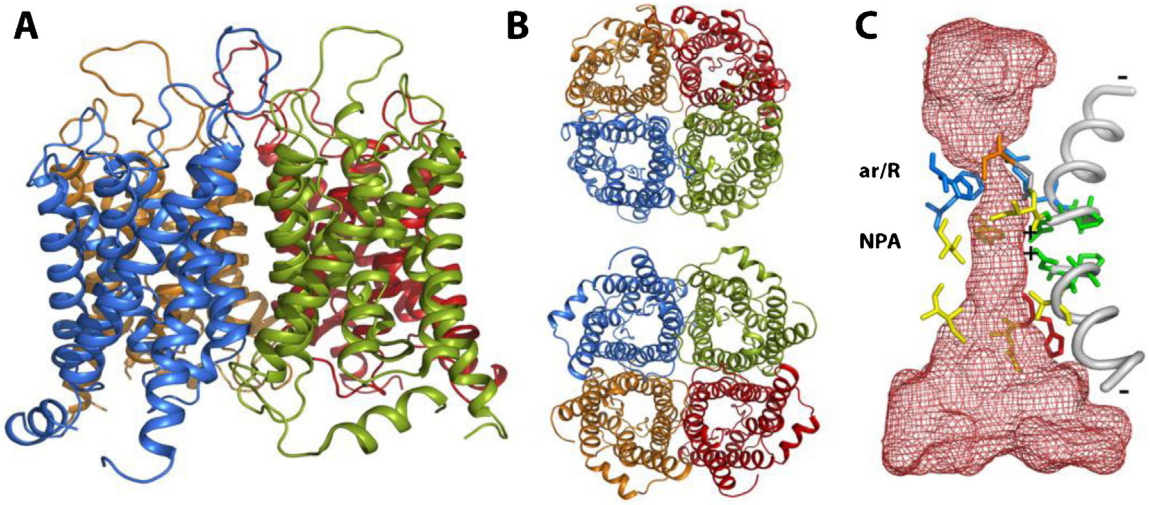


Fig. 4. Structure of bAQP1. (A) Tetrameric side view, (B) tetrameric top and bottom views, (C) monomeric structure with the red mesh indicating the pore region, the half helices dipoles and the hydrophilic and hydrophobic residues lining the pore together with the aromatic/Arg (ar/R) and NPA selective filters. The crystal structure of bAQP1 was downloaded from the PDB archive (entry code 1J4N) [reference: doi:10.2210/pdb1j4n/pdb] and processed in the PyMOL software [106].

nephrogenic diabetes insipidus, cataract, brain oedema, neurological disorders as well as in the development of obesity and cancer.

AQP1 is one of the most studied isoforms and is mainly found in erythrocytes and renal proximal tubules [113]. AQP1 water channels allow water to move freely and bidirectionally across the cell membrane, but exclude all ions including hydroxide, hydronium ions and protons [101], the latter being essential to preserve the electrochemical potential across the membrane. Notably, when compared with other mammalian orthodox aquaporins (namely with AQP1), the AQP3 isoform is moderately permeable to water, but highly permeable to glycerol and possibly to urea [88]. AQP3 expression has been reported in several mammalian tissues besides human erythrocytes [114], such as kidney collecting ducts, epidermis, urinary, respiratory and digestive tracts [115]. In particular, recent studies have correlated AQP3 glycerol permeation with skin tumorigenesis [116] and demonstrated an aberrant AQP3 expression in tumour cells of different origins, particularly aggressive tumours [117–119] suggesting that AQP3 might be a novel target of diagnostic and prognostic value. In view of the broad expression profile and the wide range of pathologies in which aquaporins are implicated, there is a considerable potential for transferring knowledge of AQP structure, function and physiology to the clinic, and certainly there is great translational potential in aquaporin-based therapeutics. AQP modulator drugs are predicted to be of broad potential utility in the treatment of several diseases such as kidney diseases, cancer, obesity, glaucoma, brain oedema and epilepsy [120]. Moreover, analysis of AQP involvement in the life-cycle of pathogenic protozoan parasites [121] suggest additional opportunities for pharmacological intervention in the treatment of human diseases.

There are at present very few reported AQP inhibitors that are suitable candidates for clinical trials. Though mercurial compounds such as $HgCl_2$ [122] inhibit several AQPs, these substances are non-selective in their action and extremely toxic. Other inorganic salts such as $AgNO_3$ and $HAuCl_4$, that are prone to interact with sulfhydryl groups of proteins, and inhibit water permeability in plasma membrane from roots. In particular $AgNO_3$ efficiently inhibits water permeability in human red blood cells (hRBC) (EC_{50} = 3.9 μM) [123]. Various other candidate blockers of AQP1 have been also reported, including tetraethyl-ammonium [124], acetazolamide [125] and DMSO [126]; however, other studies indicated little or no AQP1 inhibition by tetraethylammonium salts or acetazolamide [127] and apparently inhibition by DMSO results from an osmotic clamp effect rather than true inhibition [128]. Recently, several articles reported AQP4 inhibition by a series of arylsulfonamides, antiepileptic drugs and related molecules, with strong inhibition at low micromolar concentrations [129]; yet, these results could not be confirmed, with no inhibition activity found even at high concentrations of any of the putative AQP4 inhibitors [130]. The sulfonamide compound bumetamide was recently reported to inhibit AQP1 and AQP4 [131], and novel small molecule inhibitors of AQP9 glycerol permeability were recently disclosed [132] but due to their limited solubility, they seem not suitable for *in vivo* experiments.

Within this framework, some of us recently reported on the potent and selective inhibition of AQP3 by two water-soluble gold(III) coordination compounds [Au(phen)Cl_2] Cl (phen = 1, 10-phenatroline, Auphen) and [Au(dien)Cl] Cl_2,(dien = diethylentriamine, Audien) (Fig. 5) [133]. The effect of the compounds was tested by stopped-flow spectroscopy on human red blood cells (hRBC) that specifically express large amount of AQP1 and AQP3, and further confirmed on transfected in rat adrenal medulla pheochromocytoma (PC12) cells with overexpression of either AQP1 or AQP3. Both compounds are tetracoordinated gold(III) complexes with square planar geometry in which the Au(III) oxidation state is stabilized by the presence of nitrogen atoms on the phenantroline and diethylentriamine ligands. Auphen and Audien resulted to inhibit AQP3 in hRBC with an $IC_{50} = 0.78 \pm 0.08$ μM and $IC_{50} = 16.62 \pm 1.61$ μM, respectively, while having only a modest inhibitory effect on AQP1 water permeability [133].

The mechanism of gold inhibition was also investigated through DFT calculations on the interactions of gold(III) complexes with model amino acids side chains, indicating the thiolate form of Cys as the most favourable binding site for Au(III) complexes both in the intact or mono-hydroxo species [133]. To further investigate the

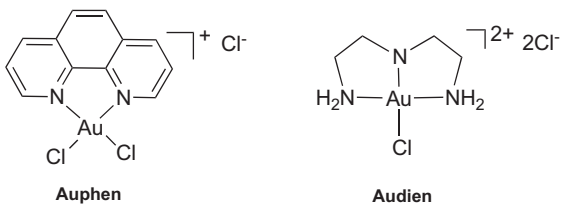


Fig. 5. Gold(III) compounds as AQP3 inhibitors.

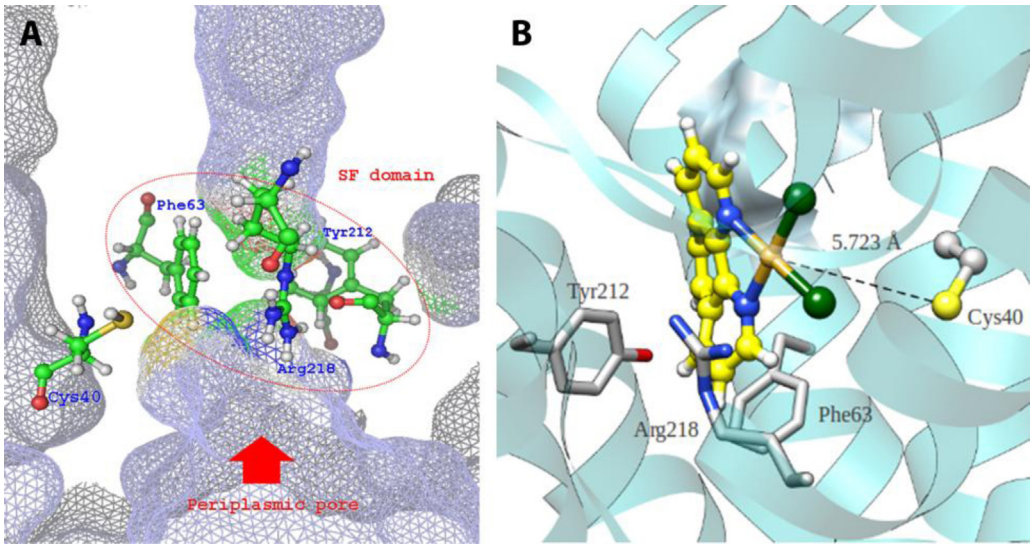


Fig. 6. (A) Molecular surface of AQP3 periplasmic pocket. Represented are the selectivity filter (SF) domain residues together with Cys-40 side chain and the contributor atoms to the molecular surface. (B) Interaction diagram of Auphen inside the AQP3 perisplasmic pocket. Calculated homology modelling structures (pdb format) of AQP3 are available at http://www.ff.ul.pt/fct/dataset_s1.pdf, and were processed here in the Maestro environment [134].

mechanisms of AQP inhibition by gold compounds at a molecular level and explain the compounds' selectivity for AQP3, molecular modeling studies were undertaken allowing the identification and characterization of protein binding pockets. The well-established presence of an extended hydrophobic area in the periplasmic region of AQP3 was confirmed, while a marked hydrophilic character was indicated in the same region of AQP1. This important difference might account for a higher binding affinity of the Auphen complex for AQP3 with respect to Audien, resulting in higher inhibition potency. Indeed, the phenantroline ligand is likely to establish favourable hydrophobic interactions at the entrance of the glyceroporin channel. Most importantly, the mapping of the periplasmic surface allowed establishing the possible gold binding sites and their exposure on both aquaporin isoforms. In AQP1 none of the cysteine, methionine or histidine residues appear to be accessible for gold binding, whereas in AQP3 the thiol group of Cys-40 is projected towards the periplasmic space approaching the channel pore and, therefore, it was proposed as a likely candidate for binding to gold(III) complexes (Fig. 6A).

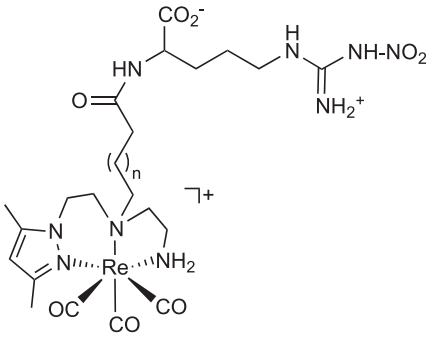
The investigation of non-covalent binding of Auphen and Audien and their monohydroxo species at AQP1 and AQP3 by docking approaches provided evidence for a slightly stronger binding affinity of the gold(III) compounds towards the AQP3 periplasmatic pore. The docking analysis showed also the possibility for the gold complexes to reach the SF domain of AQP3 in closer proximity with respect to AQP1, therefore allowing the compounds to bind at protein sites closer to the constriction pore of the aquaglyceroporin (Fig. 6B). Indeed, it is known that the AQP1 channel cross-section size is the major determinant of selectivity for larger amphipathic molecules such as glycerol [108] and these same steric restrictions might also apply to gold(III) complexes.

Interestingly, both Auphen and Audien have been previously reported to possess anticancer properties *in vitro*. In this context, we cannot exclude that inhibition of AQP3 might influence the biological effects of the compounds towards cancer cells, although other studies need to be performed to validate such hypothesis. In conclusion, the selective and potent inhibitory effect (in the low microM range) of Au(III) complexes bearing nitrogen donor ligands towards AQP3, together with their limited toxicity and high water solubility, makes them suitable candidates for future *in vivo* studies and disclose novel metal-based scaffolds for AQP drug development.

2.3. Nitric oxide synthase

Nitric oxide synthase (NOS) is the enzyme responsible for the catalytic oxidation of L-arginine (L-Arg) to L-citrulline and nitric oxide (NO), an endogenous free radical, which is a key signalling mammalian mediator in several physiological processes (*e.g.* vasodilation, neurotransmission, host-defence and platelet aggregation) [135]. NOS is a heme-containing enzyme that presents three structurally distinct isoforms. Two of them are constitutively expressed, being Ca^{+2}-dependent (nNOS [neuronal NOS, NOS1] and eNOS [endothelial NOS, NOS3]). The third isoform is Ca^{+2}-independent (iNOS, NOS2) and is inducible. The three isoforms differ in their tissue distribution and biological role [136]. The low levels of NO resulting from the activity of the constitutive isoforms (eNOS and nNOS) regulate blood pressure, platelet aggregation and neurotransmission. The iNOS is expressed and induced at a transcriptional level by inflammatory stimuli (*e.g.* interferon, IFN-γ and bacterial lipopolysaccharide), and the relatively high levels of NO produced by this isoform contribute to the pathophysiology of several diseases, such as stroke, hypertension, cancer, ischaemia, inflammation, colitis, and rheumatoid arthritis [137–139]. Therefore, the *in vivo* imaging of NO or NOS expression would allow earlier diagnosis, earlier treatment, better prognosis and individualized patient management of various diseases linked to NO/NOS deregulation [140,141]. Taking into consideration the interest of one of us in the design of innovative radiometal-based complexes as probes for *in vivo* molecular imaging of NOS, a set of $M(CO)_3$-complexes (M = ^{99m}Tc, Re) containing pendant NOS-recognizing units have been designed [142–145].

At this stage it is worth mentioning that the development of novel ^{99m}Tc-based complexes for imaging applications implies always the use of a solution of sodium pertechnetate ($Na[^{99m}TcO_4]$) in saline as radioactive precursor. The dilute nature of this solution (10^{-8}–10^{-10} M) makes the structural characterization of the resulting ^{99m}Tc complexes impossible by the current analytical methods (*e.g.* elemental analysis, NMR and IR spectroscopy). One of the simplest ways to overcome this issue is to compare the chromatographic behaviour of the ^{99m}Tc complexes with that of the corresponding compounds prepared at the "macroscopic" scale with natural rhenium ("cold metal"). Indeed, technetium and rhenium, transition metals of group 7 of the periodic table, share similar coordination chemistry, and, consequently, rhenium



***fac*-[Re(CO)$_3$(Pz-Prop-N$^{\omega}$-NO$_2$-L-Arg)]** (n = 1)
***fac*-[Re(CO)$_3$(Pz-Hex-N$^{\omega}$-NO$_2$-L-Arg)]** (n = 4)

Fig. 7. Rhenium tricarbonyl complexes with a pendant N$^{\omega}$-NO$_2$-L-Arg moiety as inhibitors of nitric oxide synthase.

complexes can be used as non-radioactive ("cold") surrogates of the respective ^{99m}Tc complexes.

Among the various Re(CO)$_3$-complexes mentioned above for NOS probing, prepared as "cold" surrogates of the analogue ^{99m}Tc(CO)$_3$-complexes, those containing a pendant N$^{\omega}$-NO$_2$-L-arginine moiety, an L-Arg derivative with known inhibitory ability towards NOS, displayed the most favourable targeting properties. In contrast, the complexes bearing a pendant L-Arg moiety, the natural substrate of NOS, lost completely the ability to recognize the enzyme. In the most promising complexes, the N$^{\omega}$-NO$_2$-L-Arg pendant moiety is linked through its R-NH$_2$ or R-CO$_2$H group and an alkyl spacer of variable length to the M(CO)$_3$ core, which is stabilized by a tridentate bifunctional chelator of the pyrazolyl-diamine type (Pz). The complexes containing conjugates with a propyl*fac*-[Re(CO)$_3$(Pz-Prop-N$^{\omega}$-NO$_2$-L-Arg)] or an hexyl spacer*fac*-[Re(CO)$_3$(Pz-Hex-N$^{\omega}$-NO$_2$-L-Arg)], in which the R-NH$_2$ group of the inhibitor is involved in the conjugation to the metal centre, presented remarkable affinity for purified iNOS, being similar to that of the free non-conjugated inhibitor (K_i = 3–8 μM) in the case of the complex bearing the 6-carbon linker (K_i = 6 μM) (Fig. 7).

Interestingly, the metal complexes presented higher inhibitory potency than the respective metal-free conjugates. Additionally, these complexes permeate also through RAW 264.7 macrophage cell membranes, interacting specifically with the target enzyme in the cytosol, as confirmed by the suppression of NO biosynthesis (30–50%) in LPS-treated macrophages. The respective ^{99m}Tc(CO)$_3$-complexes also presented the ability to cross cell membranes, as demonstrated by internalization studies in the same cell model. Preliminary biodistribution studies in LPS-pretreated mature female C57BL6 mice suggest that the complexes accumulate in tissues with iNOS upregulation.

Aiming to shed some light on the specific protein (iNOS)/ligand (rhenium complexes) interactions and to establish a preliminary structure–activity relationship, Oliveira et al. [146] performed a molecular docking study to evaluate the binding modes of the N$^{\omega}$-NO$_2$-L-arginine-containing conjugates and of the corresponding rhenium complexes. Molecular dynamics simulations were used to refine the conformations obtained by docking and to identify the most prevalent interactions between the Re complexes and the iNOS isoform, more specifically, between the "Re(CO)$_3$" metallic fragment and the active site of the enzyme. The higher inhibitory effect of the rhenium compound with the hexyl spacer (*fac*-[Re(CO)$_3$(Pz-Hex-N$^{\omega}$-NO$_2$-L-Arg)]) arises from the stronger, unique, electrostatic interactions observed between the "Re(CO)$_3$" core and the residues Arg-260 and Arg-382 (Fig. 8A). This interaction, is only possible due to the higher flexibility associated to the C6-carbon linker when compared to the C3 linker present in the other rhenium analogue. Moreover, the computational studies demonstrated that the metal centre plays a key role in the organization and orientation of the organic ligands, defining the overall shape of the inhibitors that fit better in the active pocket of iNOS (Fig. 8B).

Brought together, computational methods may be useful for predicting the affinity of putative novel rhenium and technetium complexes. Such an approach may provide strategies for the design of novel metal-based substrates/inhibitors with unique shapes and higher structural diversity with the aim of targeting NOS *in vivo* more effectively.

2.4. Carbonic anhydrases

Mammalian carbonic anhydrases (CAs) are zinc metalloenzymes that comprise 16 different isozymes, among which several cytosolic forms (CA I–III, CAVII), four membrane-bound isozymes (CA IV, CA IX, CA XII, and CA XIV), one mitochondrial form (CA V), as well as a secreted CA isozyme, CA VI. These enzymes catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are involved in crucial physiological processes connected with respiration and transport of CO$_2$/bicarbonate between metabolizing tissues and lungs, pH and CO$_2$ homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes. As will be discussed shortly, many of these isozymes are important targets for the design of inhibitors with clinical applications [148]. Among other biomedical applications (*e.g.* treatment of glaucoma by lowering of the intraocular pressure with CA inhibitors), CAs have been considered validated drug targets for cancer diagnosis and therapy since there is overexpression of specific isozymes in certain tumour types. Indeed, CA isozymes IX and XII are overexpressed in cancer cells of many hypoxic tumours, being involved in critical processes connected with cancer progression and response to therapy [148].

The Zn(II) ion of CAs is essential for catalysis. X-ray crystallographic data showed that the metal ion is situated at the bottom of a 15 Å deep active site cleft, being coordinated by three histidine residues (His-94, His-96, and His-119) and a water molecule/hydroxide ion (Fig. 9A). The inhibition and activation of CAs are well understood processes, with most types of inhibitors binding to the metal centre, whereas the activators bind at the entrance of the active site cavity where they participate in the proton shuttling between the metal-coordinated water molecule and the environment [149]. Aromatic/heterocyclic sulfonamides and their derivatives, such as sulfamates and sulfamides are the most investigated types of organic carbonic anhydrase inhibitors (CAI) [150,151] having various biomedical applications as diuretics or as drugs for the treatment or prevention of a variety of disorders such as antiglaucoma drugs, anticonvulsants, antiobesity, anticancer, analgetic and antiinfective agents. Sulfonamides and their bioisosteres bind to the metal ion from the CA active site in deprotonated form, as anions, by replacing the metal-coordinated water molecule/hydroxide ion, which is necessary for catalysis, and thus exerting their inhibitory mechanism [152]. Recently, novel interesting chemotypes, in addition to the sulfonamides, were discovered, many of which are based on natural products, such as phenols/polyphenols, phenolic acids, and coumarins [153].

In this respect, metal-based compounds of various types were also studied for their CA inhibition properties. For example, metal complexes of heterocyclic sulfonamides (all clinically used CA inhibitors) have been reported to possess very efficient CA inhibitory properties, and their mechanisms of action have been explained as being due to both sulfonamidate anions, as well as

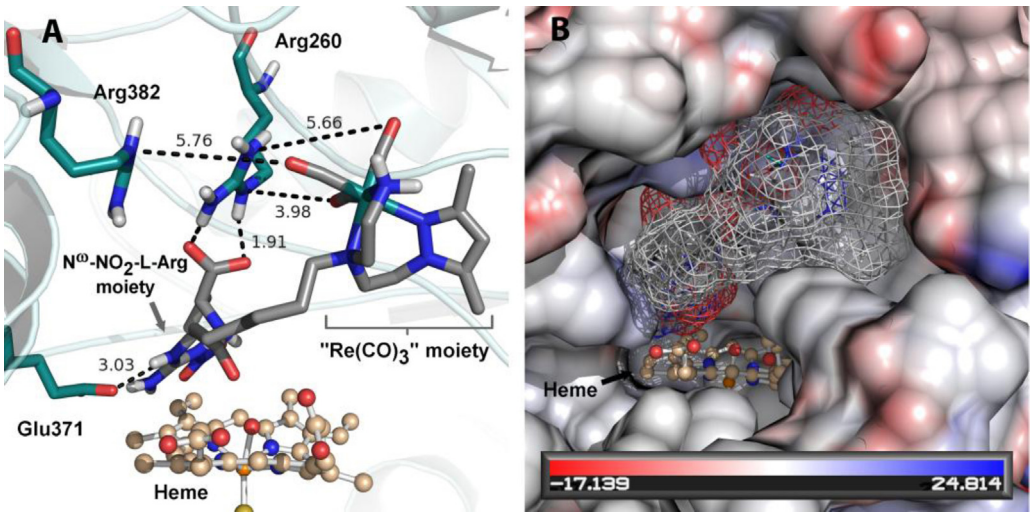


Fig. 8. (A) Proposed structure of *fac*-[$Re(CO)_3$(Pz-Hex-N^{ω}-NO_2-L-Arg)] in complex with iNOS obtained by MD simulation. The following colour scheme is used: nitrogen in blue, oxygen in red, sulfur in yellow, iron in orange and rhenium in deep teal. All distances shown in Å. (B) Molecular surface of the active site of the same system coloured according to electrostatic potential. The figure was generated using the VASCo PyMOL plug-in [106,147].

metal ions (formed after dissociation of the complex in dilute solutions) which interact thereafter with different binding sites of the enzyme. Metal ions incorporated in such complexes mainly included transition metal ions such as Zn(II), Cu(II), Co(II) and Ni(II), as well as lanthanides(III) [154].

Most recently, more structurally elaborated complexes, were designed and tested as CAI. Among them, BR30 bears a benzenesulfonamide moiety and a cupric iminodiacetate (IDA-Cu^{2+}) unit (Fig. 9B). Its rationale is to incorporate in the same molecule an aromatic sulfonamide fragment (that would coordinate to the zinc ion from the active site) and copper(II)-iminodiacetic (IDA) moieties that may bind to His-64 on the rim of the active sites of the various isoforms [155]. The crystal structures of human carbonic anhydrases I and II complexed with BR30 and other inhibitors of the same family have been determined. The ionized NH– group of each benzenesulfonamide coordinates to the active site Zn^{2+} ion and, in the case of BR30, the IDA-Cu^{2+} unit binds to His-64 of CAII and His-200 of CAI [156]. However, recent studies with 1,2-dithienylethene-based compounds incorporating benzenesulfonamide and Cu(II)-IDA-, bis-benzenesulfonamide-, bis-Cu(II)-IDA-, and bis-ethyleneglycol-methyl ether moieties, showed not equally promising results in terms of inhibitor selectivity for various isoforms [157].

Other copper(II) complexes of DTPA-, DOTA-, and TETA-tailed sulfonamides targeting the tumour-associated transmembrane isoform CA IX were also recently reported (Fig. 9B) [158]. The new compounds were designed in such a way as to possess high affinity for Cu(II) ions, exploiting four pendant carboxylate moieties in the DTPA derivatives, as well as the cyclen/cyclam macrocyles and three pendant acetate moieties in the DOTA and TETA derivatives. Most importantly, copper complexes presented higher inhibitory potency than the corresponding sulfonamide ligands, and showed membrane impermeability, thus having the possibility to specifically target the transmembrane CA IX, which has an extracellular active site. Incorporation of radioactive copper isotopes in this type of CA inhibitor may lead to interesting diagnostic/therapeutic applications for such compounds, provided their stability in physiological conditions.

The X-ray crystal structures of four metallocene-based CA inhibitors containing triazole–ferrocene or triazole–ruthenocene fragments and a sulfonamide group in complex with CAII have also been recently reported by Salmon et al. [159]. In this study, the

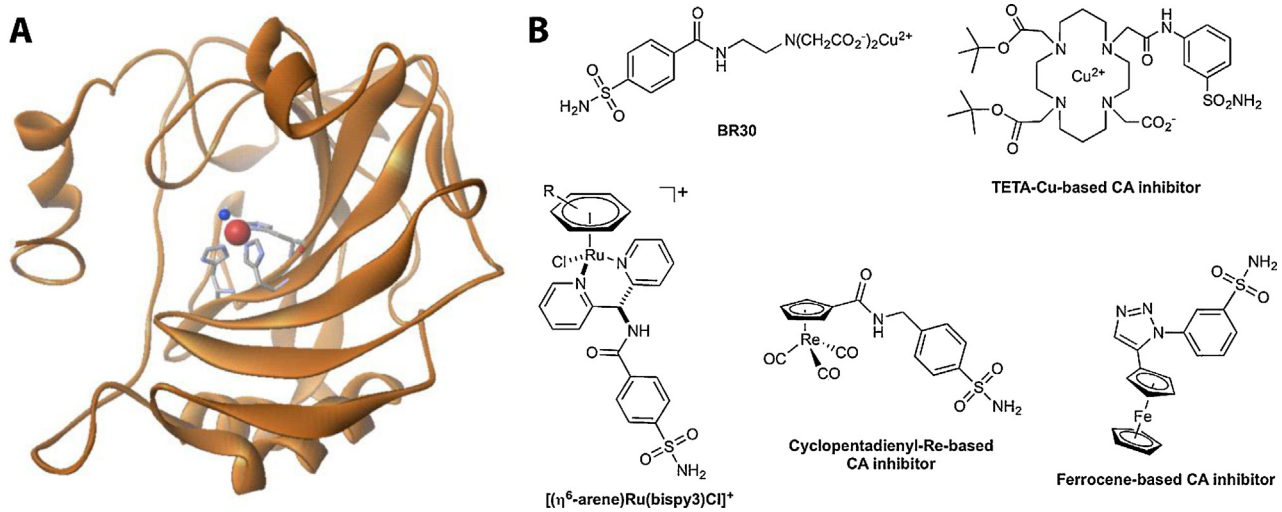


Fig. 9. (A) Carbonic anhydrase II structure. The Zn atom is depicted as a red sphere, the oxygen of the OH^- as a blue sphere, the three catalytic His residues as grey sticks. This figure was generated using the pdb1CA2 and Chimera software (http://www.cgl.ucsf.edu/chimera), (B) Sulfonamide-containing metal-based inhibitors of carbonic anhydrase.

authors have concluded that the barrel-shaped hydrophobic ferrocene and ruthenocene moieties provide a structure-based avenue to better occupy the hydrophobic binding patch within the enzyme active site, and consequently allows the design of more efficient metallocene-based human CA inhibitors. Based on the knowledge of the structural parameters involved in the interaction enzyme-metallocene, the same authors have prepared a new series of derivatives of the same type with moderate to good inhibitory potency, with various metallocenes displaying significant selectivity for CA IX and CA XII. Indeed, the most potent compound is a ferrocene-based inhibitor that had a K_i of 5.9 nM and 6.8 nM at CA IX and XII, respectively. It is worth mentioning that the activity of one regioisomer of this potent compound towards the same isozymes is significantly lower. Additionally, the *in vitro* ADME properties (*e.g.* Log *P*, Log *D*, solubility, *etc.*) of representative metallocenes have been also determined. Brought together, the results confirmed that the barrel-shaped metallocene moiety provides a means of discriminating the CA isozymes active site when compared to the corresponding non-metallated phenyl analogues, while biopharmaceutical properties were unchanged [159,160].

There are also a few examples of organometallic piano-stool complexes bound to CA II (Fig. 9B). Monnard et al. have reported a series of d^6-piano-stool Ru complexes bearing an arylsulfonamide anchor with only sub-micromolar affinity towards hCA II. The X-ray crystal structure of one of the complexes ($[(\eta^6\text{-}C_6Me_6)Ru(bispy3)Cl]^+$) with hCA II have been determined, highlighting the nature of the host–guest interactions [161].

Aimed at selective targeting of CA IX *in vivo* for diagnostic and/or treatment purposes, Alberto and co-workers have synthesized, following the so-called extended 3D space population concept, four arylsulfonamide, -sulfamide, and -sulfamate based CAIs with the organometallic motif $[(Cp\text{-}R)M(CO)_3]$ (M = Re or ^{99m}Tc) (Fig. 9), and evaluated their affinity to CA isoforms [162]. The enzymatic assays with purified 12 CA isozymes have shown that the compounds presented inhibition constants in the low nanomolar range for some of the isoforms. The values obtained are in the same range as those found for organic inhibitors. One of the compounds displayed superior selectivity for hCA II, IX and XIV, which contrasts with the acetazolamide standard that does not show any distinct preference pattern for any of the isoforms. The binding mode of the aforementioned Re(I) complexes with CA was assessed by X-ray crystallography. The crystal structure of human CA II complexed with one of the compounds is presented in Fig. 10.

Recently, one of us reported on the moderate inhibition of human CA II by the antimetastatic Ru complex [tetrachloro(DMSO)(imidazole)-ruthenate(III)], NAMI-A [163]. The X-ray structure of the adduct formed between NAMI-A and hCAII could be solved at 1.8 Å resolution showing that Ru selectively binds His-64, providing conclusive evidence that none of the original ligands of ruthenium in NAMI-A are conserved upon protein binding and supporting the view that the compound can behave as an "extreme" prodrug. Interestingly, His-64 plays an important role in hCA II catalysis, being situated in the middle of the active site cavity and acting as a proton shuttling residue between the zinc-bound water and the reaction medium, for the generation of the nucleophilic active form of the enzyme. Notably, examination of the electron-density map showed the presence of an imidazole ligand (not bound to Ru) in the active site region, anchored to the Zn(II) ion of the hCAII active site within almost regular tetrahedral coordination geometry (see Fig. 11). A similar binding mode to the zinc ion of hCA II has been previously reported for the competitive inhibitor 1,2,4-triazole [164], polyamines [165], as well as sulfocumarins [166], and it is another interesting aspect of the observed reactivity that would deserve further exploration to exploit other strategies to CA inhibition.

Overall, the above mentioned studies demonstrate that CA inhibition by metal compounds is worth attempting for various therapeutic applications. The development of diagnostic tools based on metal-based CAI is also an attractive future research direction.

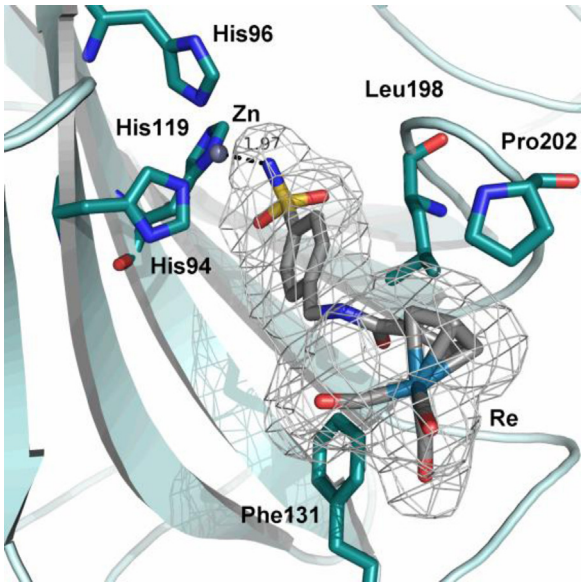


Fig. 10. Crystal structure of a rhenium arylsulfonamide-cyclopentadienyl-based inhibitor bound to hCA II. Zinc atom is shown in grey sphere. The figure was generated using the pdb id 3RJ7 and PyMOL [106].

2.5. Thymidine kinases

Thymidine kinases, namely human cytosolic thymidine kinase (hTK1) and herpes simplex virus thymidine kinase type 1 (HSV1-TK) are key enzymes for metabolisms of viruses and mammals, and have been proposed as suitable targets for non-invasive imaging of gene therapy and cancer [168–171]. Human thymidine kinase is a cytosolic enzyme that catalyzes the γ-phosphate transfer from ATP to the 5′-hydroxyl groups of thymidine (dT) and 2′-deoxyuridine (dUrd). Different series of ^{99m}Tc(I)/Re(I) complexes for specific targeting of human thymidine kinase 1 (hTK1) have been proposed to detect/visualize increased hTK1activity

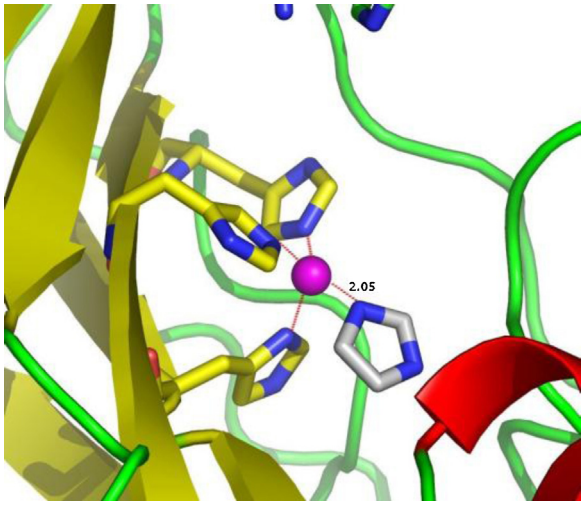


Fig. 11. Detail of the active site of hCAII adduct with NAMI-A highlighting the imidazole from the compound directly coordinated to Zn^{2+} at a distance of 2.05 Å. The residues His-94, His-96, His-119 are shown as yellow sticks; the Zn atom is depicted as a magenta sphere, and the imidazole ring as white sticks. This figure was generated using the pdb3M1J and COOT [167].

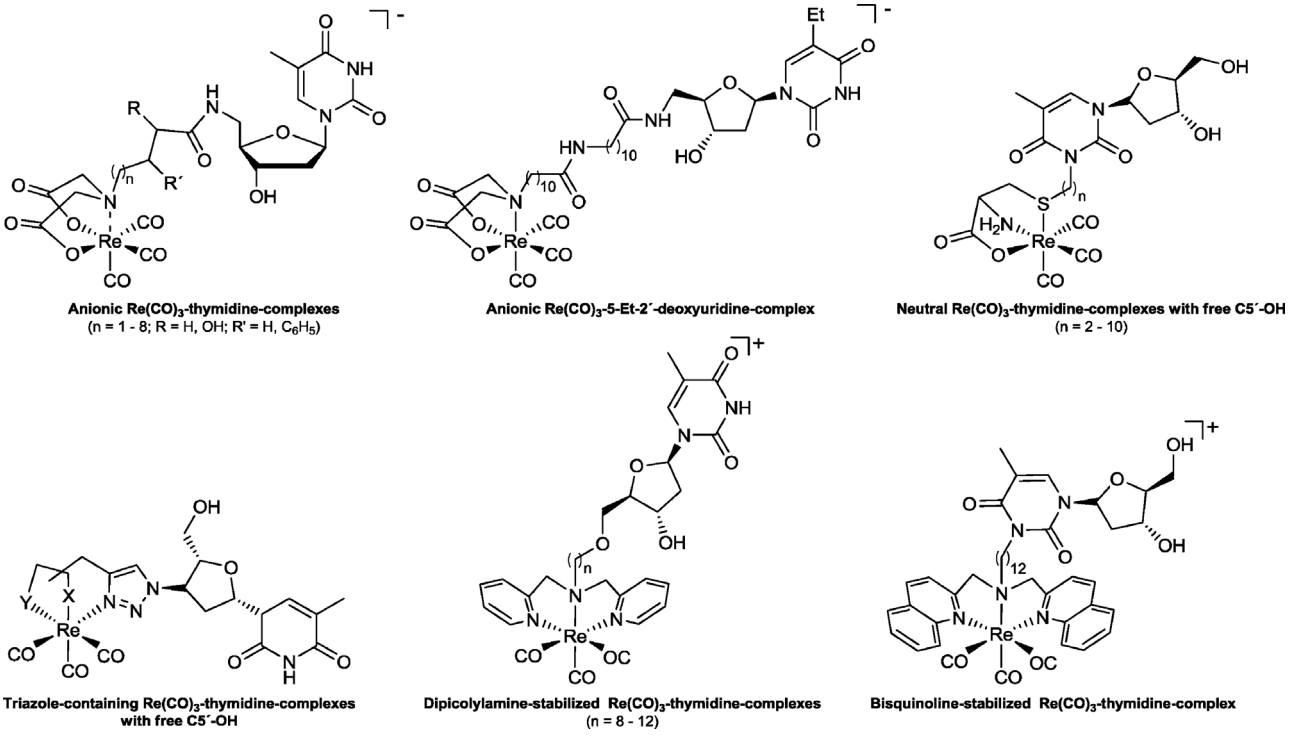


Fig. 12. $Re(CO)_3$-complexes containing pendant thymidine and uridine moieties for targeting hTK1or HSV1-TK.

associated to proliferating cancer cells. In this context, the Re(I) complexes are considered "cold" surrogates of the respective ^{99m}Tc(I)-complexes.

Schibli et al. have introduced the first organometallic inhibitors of hTK1, namely a set of anionic Re(I) tricarbonyl complexes, which, besides the organometallic core stabilized by a tridentate iminodiacetic acid-based chelator, contain pendant 5′-aminothymidine analogues and alkyl chains of various lengths (Fig. 12).

The inhibitory potency of the complexes was tested towards hTK1 and also HSV1-TK, and it has been observed that in the case of hTK1 the inhibition capacity of the complexes improved with increasing spacer length. On the other hand, the complexes showed none or only slight inhibition of the HSV1-TK [172]. Based on the assumption that the lack of activity against HSV1-TK was the result of steric clashes with the enzyme's ternary structure due to inappropriate lengths of the spacers between the metal core and the thymidine moiety, the same group has prepared a new family of thymidine derivatives with either significantly shorter or longer alkyl spacers to avoid potential interferences between the metal core and the protein. The effect of the overall charge of the complex in its inhibitory capacity was also investigated [173]. Brought together, the enzymatic assays revealed mixed inhibition of hTK1 for all thymidine complexes independent of the spacer length. Moderate competitive inhibition of HSV1-TK was only achieved when the pendant thymidine fragment and the metal core were separated by a spacer of *ca.* 30 Å length. These observations were also supported by *in silico* molecular docking and molecular dynamic experiments. Further studies by the same group has shown that selective inhibition of HSV1-TK was only achieved with a new anionic rhenium organometallic complex bearing a pendant 5′-carboxamide 5-ethyl-2′-deoxyuridine derivative [174] (Fig. 12). Indeed, inhibition of the hTK1 previously reported for Re(I) analogue complexes of the 5′-carboxamide thymidine derivative described above was not observed [174].

The functionalization at position C5′ in all the previously described M(I) compounds (M = Re, ^{99m}Tc) prevents phosphorylation and, consequently, such complexes were considered unattractive as potential radiotracers for noninvasive imaging of tumour progression as the mechanism by which they would be trapped inside cancer cells relies on phosphorylation of the complex. Therefore, new M(I) complexes (M = Re, ^{99m}Tc) containing thymidine derivatives with free C5′-hydroxyl group which are still recognized as substrates by hTK1 have been proposed. Schibli and co-workers synthesized and characterized a set of N3-functionalized Re and ^{99m}Tc organometallic thymidine analogues with neutral, cationic or anionic overall charge and spacers of various lengths between the organometallic core and the thymine base [175]. The phosphorylation of the metal complexes in the presence of hTK1 and ATP was assessed quantitatively relative to thymidine. Despite being all substrates of recombinant hTK1, it has been concluded that the neutral complexes were phosphorylated to a greater extent than the charged complexes and that the extent of phosphorylation was further improved by increasing the spacer length (Fig. 12). A molecular dynamics simulation study performed with a modified hTK1 structure supported the experimental observations. Additionally, *in vitro* cell internalization experiments performed in a human neuroblastoma cell line (SKNMC) showed significant uptake for the neutral, lipophilic complexes. Further work by the same group has shown that neutral $[Re(CO)_2(NO)]_2^+$-labelled thymidine derivatives presented substrate activity towards hTK1 comparable to that of the structurally analogous anionic $[Re(CO)_3]^+$-labelled thymidine derivatives [176]. It is also worth mentioning that Struthers et al. have introduced an elegant strategy based on "click chemistry" for the preparation of neutral and cationic Re/^{99m}Tc organometallic complexes containing thymidine derivatives with free C5′ hydroxyl group [177]. Most recently, using this synthetic strategy, the same authors introduced Re/^{99m}Tc tricarbonyl complexes with different structures and overall charges from a common precursor, and the first organometallic hTK1 substrates in which thymidine is modified at the C3′ position were identified (Fig. 12). Evaluation of substrate activity towards this enzyme has shown that the influence of the overall charge of the derivatives is dependent on the position of functionalization. In the case of the C3′-functionalized

derivatives, neutral and anionic substrates were most readily phosphorylated, whereas for the N3-functionalized derivatives, cationic and neutral complexes were apparently better substrates for the enzyme than anionic derivatives [178].

With the aim of introducing rhenium tricarbonyl complexes bearing nucleosides for chemo- and/or radiotherapy, profiting from both the potential antiproliferative properties of Re(I) complexes and/or β^--emission of the radioisotopes ^{186}Re and ^{188}Re, Zubieta and co-workers have introduced a set of $Re(CO)_3$ complexes stabilized by various neutral tridentate chelators (*e.g.* dipicolylamine or bisquinoline type) with spacers of different lengths attached to pendant thymidine or uridine moieties at different positions (Fig. 12). Among the cationic dipicolylamine-containing complexes, the one with a dodecylene spacer at C5′ exhibited the highest toxicity against the A549 lung carcinoma cell line [179,180]. However, despite this result, the toxicity of this complex could not be assigned solely and directly to the inhibition of hTK-1. Most recently, the same group reported a series of N3-conjugated $Re(CO)_3$–thymidine complexes stabilized by a bisquinoline bifunctional chelator. The complex carrying a dodecylene spacer presented again the highest cytotoxicity against A549 lung carcinoma cell line. Cellular uptake studies with the same compound have been performed by fluorescence microscopy, showing that it was clearly internalized into A549 cells. However, also in this family of compounds, the cytotoxic effect could not be correlated with the inhibitory ability of the complexes towards hTK-1 [181].

2.6. Parasite enzymes as targets

Malaria, trypanosomiasis and leishmaniasis are tropical diseases caused by parasitic protozoans. The malaria causative agent, transmitted by the mosquito vector (female mosquito of the *Anopheles* genus), is a unicellular eukaryote belonging to the *Apicomplexa* phylum and named *Plasmodium* spp. The protozoan parasites *Trypanosoma cruzi* and *Trypanosoma brucei* are the etiologic agents of american trypanosomiasis (Chagas disease) and human african trypanosomiasis (sleeping sickness), respectively. Both are transmitted to the mammalian host by insects: *T. Brucei* by the tsetse fly through saliva, and *T. Cruzi* by hematophagus triatomine bugs through the insect faeces near the site of the bite wound. Leishmaniasis is a disease with extensive morbidity and mortality with various forms that are caused by protozoa of the genus *Leishmania*.

Metal compounds have already found extensive application in the treatment of parasitic diseases in the pioneering times of modern pharmacology, mostly based on an empirical use [182,183]. Various inorganic salts were thus administered against the major tropical diseases, sometimes with very good results. Notably, as a consequence of those ancient observations, a few antimony compounds (see Fig. 1) still constitute the treatment of choice for some forms of leishmaniasis [183]. Bismuth is still used sporadically in the prophylaxis of malaria. Conversely, arsenicals, although effective, were withdrawn completely because of their recognized toxicity. However, no detailed structure/function studies were ever performed on antiparasitic metal-based compounds.

In recent years, the potential use of inorganic and/or organometallic compounds for treating parasitic illnesses, considering different types of drug targets, and having different mechanisms of actions, has been the subject of various articles [184–187]. Remarkably, the knowledge of the biology of these parasites in our *postgenomic* era has greatly increased, and several protein targets have been identified against which novel metal compounds might be specifically developed and tested. Herein, we will briefly address the most interesting results and the most potent compounds when protein targets, mainly enzymes, are considered.

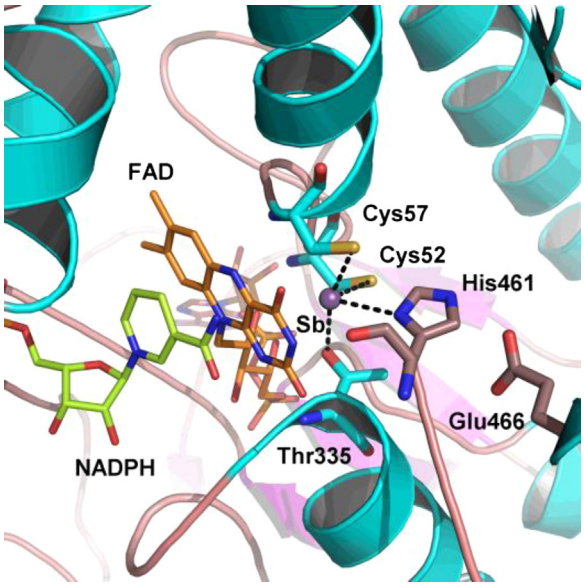


Fig. 13. X-ray structure of the Sb(III) binding site of trypanothione reductase. Antimonial atom is shown in violet. The Sb(III) coordinating residues (Cys-52, Cys-57, His-461 of chain B, and Thr335) are indicated as sticks. NADPH and FAD are coloured light green and orange, respectively. The Glu466 of chain B, which is involved in trypanothione reduction, is depicted in pink sticks. This figure was generated using the pdb id 2W0H and PyMOL [106].

As far as it concerns malaria treatment, metal complexation (*e.g.* Ru, Au, Ir or Fe) of existing antimalarial drugs (*e.g.* chloroquine) has been proposed as an effective option for drug improvement, namely towards higher activities against resistant parasites [185]. Recently, selected metallodrugs such as auranofin, aurothiomalate, triethylphosphinegold(I) chloride, cisplatin, the ruthenium(III) complex NAMI-A, mononuclear and dinuclear gold(III) complexes, as well as bismuth and antimony compounds were evaluated for their antiplasmodial properties [188,189]. All tested metal compounds, although with different potencies, effectively reduced *Plasmodium falciparum* growth *in vitro*, implying high and broad parasite sensitivity to these metals. Good candidate molecular targets for these metal compounds are parasite biomolecules containing functionally relevant thiol and selenol groups such as the previously mentioned thioredoxin reductase, an ubiquitous protein involved in intracellular redox balance, and falcipain, a cysteine protease typical of *P. Falciparum*.

Indeed, since cysteine proteases play key roles in parasitic life cycles including *Schistosoma*, *Plasmodium*, *T. Brucei*, *T. Cruzi*, and *Leishmania*, they have also been considered promising parasite targets for drug development. Colotti et al. recently reported the crystal structure for Leishmania trypanothione reductase disclosing the actual mechanism of enzyme inhibition by antimonials [190]. It was shown that trivalent antimony binds to the protein active site with high affinity, strongly inhibiting enzyme activity. The metal binds directly to Cys-52, Cys-57, Thr-335 and His-461, thereby blocking hydride transfer and trypanothione reduction. Details of the structure are shown in Fig. 13.

Following a strategy similar to that established for anti-malarial agents, one of the approaches that is being explored for treating trypanosomiasis is the use of metallated anti-trypanosomal compounds. The most successful metal-complexes proposed were $[Au_2(mpo\text{-}H)_2(PPh_3)_2]$, $[Pt(mpo\text{-}H)_2]$, $[Pd(mpo\text{-}H)_2]$ and $[VO(mpo\text{-}H)_2]$, which bear 2-mercapto-pyridine *N*-oxide (mpo), a strong and selective inhibitor of NADH–fumarate reductase [191]. The complexes showed significantly increased activities compared to mpo on epimastigotes of different *T. Cruzi* strains. A direct correlation between parasite inhibition and NADH-fumarate reductase

inhibition was found, highlighting this enzyme as the most likely target [192].

The validation of parasite cysteine proteases as relevant drug targets, as mentioned before, stimulated the assessment of the inhibitory activity of a series of mixed-ligand oxorhenium(V) complexes of the so-called [3 + 1] type, and cyclometallated organo Au(III) and Pd(II) complexes against parasite cysteine proteases cruzain from *T. Cruzi* and cathepsin B-like cysteine protease (cpB) from *Leishmania major* [186]. Additionally, the inhibitory effectiveness of some of the complexes against *in vitro* models of parasite growth and infectivity was also examined (*T. Cruzi*, *L. Major*, *L. Mexicana* and *L. Donovani*) [186]. The enzymatic assays revealed that the inhibitory potency of some of the complexes is comparable to that of mammalian cathepsin B, also determined in a parallel experiment. The cell studies have shown that the only gold complex tested was both inactive and non-toxic, whereas the only palladium compound assayed, despite being toxic at the highest concentration, extended the life cycle of *T. Cruzi* amastigotes in J774 macrophages at lower concentrations. In the case of the rhenium compounds, the one with the highest inhibitory potency towards purified cruzain was inactive against *T. Cruzi*. Conversely, the complex with the lowest inhibitory potency, was active against the parasite. These results could be ascribed to the different abilities of the compounds to cross the cell membrane.

Nitrogen-containing bisphosphonates (N-BPs) are a class of drugs used in the treatment of bone-related diseases, namely osteoporosis, Paget's disease and tumour bone metastasis, among others. The clinical success of BP's is also associated with their use in radiopharmaceuticals for bone imaging (^{99m}Tc-labelled BPs) or for bone-pain palliation (*e.g.* ^{153}Sm- or $^{186/188}$Re-labelled BPs) [27]. N-BPs decrease bone resorption by inhibiting farnesyl pyrophosphate synthase (FPPS), a key regulatory enzyme in the mevalonate pathway, and thereby preventing the prenylation of small GTPases, which are essential for osteoclast function. Interestingly, besides mammals, FPPS exists also in parasites such as *T. Cruzi*, and various organic bisphosphonates inhibit the proliferation of *T. Cruzi* both *in vitro* and *in vivo* without toxicity for the host cells [193]. In spite of the promising results, bisphosphonates in general are known to present a poor oral bioavailability, mainly due to the high ionization of phosphonate groups at physiological pH. With the aim of overcoming this issue, bisphosphonate–metal complexes have been proposed to improve the pharmacological properties of the bisphosphonates and improve their anti-parasitic action. For example, Demoro et al. have studied a series of complexes of anti-resorptive drugs in clinical use such as alendronate (Ale), pamidronate (Pam) and risedronate (Ris) with Cu, Co, Mn and Ni [187,194]. Some of the complexes presented an improved antiproliferative effect against *T. Cruzi* compared to the free non-coordinated bisphosphonate. In most cases the anti-*T. Cruzi* activity could be correlated with the inhibition of parasitic farnesyl diphosphate synthase (*Tc*FPPS) *in vitro*. It is also worth mentioning that all metal–bisphosphonate complexes are selective inhibitors of *Tc*FPPS, showing no or little inhibition of human FPPS.

With respect to the use of metal compounds for treating parasitic diseases through inhibition of parasite enzymes, namely cysteine proteases or farnesyl diphosphate synthase, special attention should be given to selectivity issues. Indeed, the compounds should not be recognized by the respective mammalian enzymes in order to avoid toxicity and unwanted side effects. Although challenging, this goal can be attainable in the near future by structural studies combining X-ray crystallography, computational chemistry and modern analytic techniques based on NMR or mass spectrometry (MS). Moreover, based on proteomic techniques, novel specific targets in parasites should be identified and validated as relevant drug targets for metal complexes.

3. Concluding remarks and outlook

Incorporation of metals into drug scaffolds offers vast potential for creating promising metal-based drug candidates with unique chemistry and biological activity of clinical significance. As demonstrated by the numerous examples of metal-based complexes with enzyme inhibitory activity (or more in general able to modulate protein activities) cited in the literature, and partly covered by this review, developing research in medicinal inorganic chemistry, as well as in the investigation of metal compound–protein interactions, is entirely beneficial. As an example, the possibility for metal compounds to alter zinc-finger domains is very attractive in drug development. In fact, ZF motives are relevant to the above mentioned protein targets (*e.g.* PARP-1), and zinc-binding domains are structural components of other "hot" proteins involved in cancer development and progression. Of note, the tumour suppressor protein p53, acting also as a transcription factor activating genes involved in cell cycle arrest and apoptosis, is a tetramer composed by four identical monomers, each one containing DNA binding domain and an important structural zinc ion [66,195]. In this case, the Zn^{2+} is coordinated by three Cys and a His residues, in a pseudo-tetrahedral coordination geometry, and it is responsible for connecting two loops inside the domain stabilizing the structure. In response to cellular stress p53 binds to DNA in its tetramer form as a dimer of two identical dimers. The stability of such a tridimensional structure is maintained by the Zn ions in each monomer. Thus, analysis of the role of zinc in p53 function may help the development of new therapeutic strategies, as it is already known that chelation and addition of Zn^{2+} can modulate p53 conformation and DNA binding [195].

In the case of gold compounds as aquaglyceroporin inhibitors, new gold-based scaffolds could be designed also as molecular tools to detect AQP activities *in vivo*, and their use may help gaining an insight into the function of these still not fully understood membrane proteins in biological systems. Most importantly, nowadays, the possibility to exploit protein modulation (by inhibition or activation) by metal compounds for different therapeutic and/or imaging purposes is an intriguing research topic. As a matter of fact, the integration of chemistry and molecular biology with imaging is providing some of the most exciting opportunities in the early detection and treatment of different pathologies. Indeed, the field of *theranostics*, where diagnosis is combined with therapy, is well suited, for example, for a disease as complex as cancer, especially now that genomic and proteomic profiling can provide an extensive 'fingerprint' of each tumour type. Using this information, the ranostic agents can be shaped for personalized treatment to target specific compartments, such as the tumour microenvironment, whilst minimizing damage to normal tissue. In this respect, the visualization of enzyme activity in humans using nuclear imaging techniques may not only be useful for diagnostic purposes but also for therapeutic monitoring of the effects of existing drugs or of those in development. Probing those low-capacity enzyme systems *in vivo* is a challenging task, being much dependent not only on the target selective uptake, but also on the high affinity of the radioprobe to the enzyme. Thus, the use of highly potent inhibitors and/or substrates of enzymes is always mandatory for probing enzyme levels *in vivo*.

Overall, we are convinced that in the future the field of medicinal inorganic chemistry will be a key part of drug development for personalized medicine, allowing also considerable advances in predictive medicine. However, we should also fight against the prejudice associated to metal-based drugs, mostly in terms of *in vivo* toxicity. Thus, as bioinorganic chemists, we should be able to address the difference between the toxicity related to the "naked", non-coordinated metal ion, and that of the corresponding metal stabilized by the coordinating ligand(s). Moreover, the toxicity of

metal complexes is a multifaceted subject during the development of metal-based drugs as it primarily depends on the type of selected application. In the case of metal-based radiopharmaceuticals for diagnosis or therapy toxicity is not the main matter of concern due to the low concentrations of metal-complexes administered to the patients. Instead, in the case of Gd-based MRI contrast agents, due to the high toxicity of Gd metal ion, the complexes must present high thermodynamic stability and kinetic inertness, and, consequently, the study and understanding of the coordination chemistry of the chelating ligands is a key issue. In relation to non-radioactive therapeutic metallodrugs these are often *pro-drugs* in need to undergo activation processes in order to exert their pharmacological effects (*e.g.* cisplatin is activated by hydrolysis inside cancer cells), however, further studies are necessary to "fine tune" the stability metal complexes while maintaining their biological activity and reduce their side-effects. In all cases, the toxicity of a metal compound should be carefully evaluated in various appropriate investigational models, including *in vitro* (*e.g.* cell cultures), but also *ex vivo* (*e.g.* tissue slices), and, eventually, *in vivo*, also taking advantage of recent high-resolution biophysical and analytical techniques aimed at the detection of metal ions in biological samples.

Nevertheless, one should keep in mind that the therapeutic or diagnostic action of all medicines, pure organic and biological included, incorporates a certain degree of risk, and the approval of any new drug is always a matter of risk–benefit assessment. Therefore, it is up to us, medicinal inorganic chemists, to design and prepare exquisite chemical inorganic/organometallic entities with improved safety and efficacy, in order to propose innovative drugs with higher benefits than side effects. Additionally, when foreseeing this field in the coming years, we should always remind the enormous contribution that "old" metal-complexes such as cisplatin, sestamibi or gadolinium-based compounds gave to healthcare improvement in the past.

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