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Focussed review

Exploring the mechanisms of metal-based pharmacological agents via an integrated approach

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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 evidenced 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, the possibility of target elucidation is essential for the identification of new therapeutic applications for metal compounds or as molecular biological tools. Here we present the results of our recent research in the field, which in our opinion constitute the basis of a systematic and interdisciplinary approach to address some of the critical issues in the study of the molecular mechanisms of metallodrugs' action via the implementation of high-resolution biophysical techniques coupled with more pharmacological methods.

1. Introduction

Among the main concepts and strategies of medicinal inorganic chemistry, the introduction of metal ions or metal compounds to the biological system for imaging or therapeutic purposes occupies an important part, and several examples demonstrate the success of this approach. [1-3] Thus, coordination compounds have found their application in the treatment of various diseases (e.g., Ehrlich's ancient salvarsan against syphilis, [4] bismuth compounds as antiulcer drugs, [5] vanadium complexes for the treatment of diabetes) [6] and in medical diagnostics, as contrast agents for magnetic resonance imaging [7,8] and radio-pharmaceuticals. [9,10] Certainly, in the last two decades, research on metal-based drugs has made remarkable progresses with particular achievements in cancer chemotherapy. In fact, since the discovery of the antitumor activity of cisplatin (*cis*-$[PtCl_2(NH_3)_2]$, Fig. 1) [11] in the late 1960's, metal-based compounds have played a major role in anticancer medical treatments. [12-16] The mechanism of action proposed for cisplatin and its structural analogues appears to be related to the formation of hydrolysed species that react mainly with DNA. [17] The effectiveness of Pt(II) complexes is still hindered by clinical problems, including acquired or intrinsic resistance that limits the spectrum of cancers that can be treated, and high toxicity leading to side effects. Therefore, research in this field has been extended to include a conspicuous number of non-platinum metallodrugs. [18,19] Particular attention has been devoted to a few ruthenium compounds and - to a smaller extent - to some iron, titanium, osmium, iridium, tin, copper and palladium complexes. [18,20-22] Notably, two ruthenium(III) compounds, i.e. imidazolium *trans*-[tetrachloro(DMSO) (imidazole)ruthenate(III)] NAMI-A, [23] and indazolium *trans*-[tetrachloro(bisindazole) ruthenate(III) KP 1019 [24] (Fig. 1) have entered Phase I-II clinical trials. Organometallic Ru(II) complexes have been also widely investigated as anticancer drugs, but still remain in the early pre-clinical stage of investigation. [25,26] In spite of the numerous hypotheses raised so far and of several proposed targets, the mode of action of anticancer ruthenium compounds is still largely unknown.

Interestingly, a number of gold coordination compounds have also been taken into consideration as potential anticancer agents due to their availability with increased stability, and relevant antiproliferative activities. [27] It is worth mentioning that gold compounds have a long and important tradition in medicine, the so-called *Chrysotherapy*. In early times of modern pharmacology, gold complexes were widely used for the treatment of several diseases, especially as anti-infective and anti-tubercular agents. [28] Nowadays, gold compounds have found rather limited medical application, and some of them, namely gold(I) complexes with thiolate and phosphine ligands (Fig. 2), are presently used only for the treatment of severe rheumatoid arthritis, [29] although also proved to possess anticancer properties. This is probably the result of the relevant systemic toxicity (e.g. nephrotoxicity) and of the poor chemical stability of many of the initially tested compounds.

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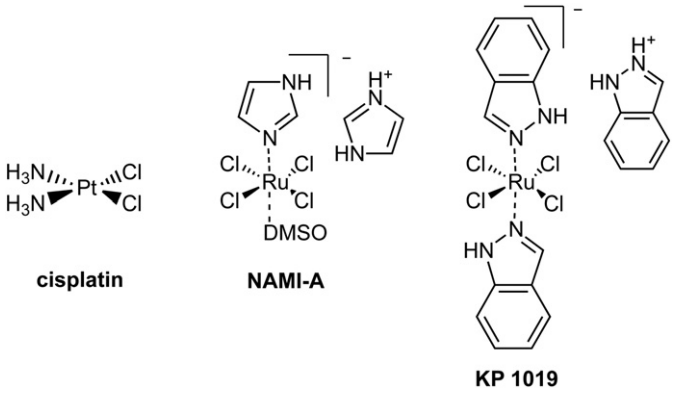

Fig. 1. Structures of anticancer metal-based drugs.

It is worth noting that, in addition to the extensive work on anticancer metal complexes, a few related studies on other therapeutic activities of metal-based compounds (e.g. as anti-viral agents, [30,31] or for the treatment of some asthmatic or parasite diseases, including malaria, trypanosomiasis and leishmaniasis) have been reported. [32]

In spite of their great potential, metal compounds are still poorly developed and studied as therapeutic agents, and only limited mechanistic information is available in the literature. A systematic study conducted at the National Cancer Institute (NCI) in the US few years ago on 1100 metal or metalloid containing compounds with potential anticancer activity, and relying on a clustering analysis aimed at establishing correlations between specific cytotoxic responses and differential gene expression profiles, has confirmed the variegate ensemble of possible mechanisms of action for metal compounds. [33] Practically, the possible molecular pathways could be segregated into four response classes according to metal compound preference for binding to biological sulfhydryl groups, chelation, generation of reactive oxygen species (ROS), and production of lipophilic ions. Such a categorization, although useful, is certainly extremely broad, and far from the identification of specific molecular targets for metallodrugs, necessary to the development of targeted therapies.

In general, for metal-based drugs, it can be stated that interactions with various proteins/enzymes play crucial roles in inducing the observed biological effects. [34] Rather surprisingly, the reactions of metal complexes with proteins have received for several years limited attention, especially if considering the wide variety of compounds reported in the literature which display an extremely variegate ensemble of chemico-physical properties and possible reactivity pathways in biological systems. Moreover, only in limited cases metal compounds have proved to fulfil the need of novel and creative design strategies for specific molecular recognition of single biomacromolecular targets within complex biological systems. [35] Since it is increasingly evident that metal/protein interactions are essential not only to orient metallodrug activity, but also in determining the metal compounds overall pharmacological and toxicological profile, protein target identification for the various families of metal-based compounds is absolutely essential.

Within this frame, several issues need to be addressed. Not only the identification of preferential targets (if any) for metal complexes is crucial, but also establishing the nature of their interactions with biomolecules at a molecular level is important for determining the molecular mechanism of metal activity and providing an insight into potential side effects that may occur. Therefore, knowledge of the species in which each metal complex enters the cells, or whether and how the metabolized complex is already inactivated at this time should be achieved. Thus, information on the chemical speciation and the metal binding sites is essential for an insight into metal-bioligand interactions. Another critical aspect in this research field is the determination of the biodistribution (in space and time) of a certain metal compound, as well as the analysis of the various pathways that contribute to metal uptake and trafficking. [36]

Due to the extreme complexity of the biological systems, it is now widely accepted that innovative and information-rich methods are absolutely needed to afford the above mentioned goals, such as those offered by the so called *Omics sciences*. Recently, both *Proteomic* and *Metallomic* strategies were successfully implemented for the elucidation of specific mechanistic features of anticancer metallodrugs within an innovative “Systems Biology” perspective. [37] While Proteomics is now an established branch of the new omics sciences, Metallomics is a younger discipline, still in the search of a definitive conceptual and methodological organization, that has been defined as the "comprehensive analysis of the entirety of metal and metalloid species within a cell or tissue type". [38] Thus, these new approaches provided an important contribution in terms of target identification for metallodrugs, but up to now limited to a few cases. [39-43] Together with these powerful tools, and in order to tackle the challenging problem of characterizing the behaviour of metallodrugs in complex biological media and tissues *in vitro* and *in vivo*, bioanalytical and biophysical methods are widely employed, including fluorescence microscopy, X-ray based techniques, various spectroscopies, as well as hyphenated separation methods coupled to mass spectrometry techniques. [44-46]

It is also worth mentioning that a main issue in medicinal chemistry is the need of new investigational models able to fill the gap between the classical *in vitro* drug screening cellular assays and the *in vivo* models. In fact, often the anticancer data obtained on cell-based assays are not confirmed in animal models and *vice versa*, resulting in the wrong selection of the drug leads, as well as in the misleading interpretation of the mechanisms of pharmacological action.

In the last years, within this research field, we succeeded at establishing a general methodology to evaluate an ensemble of structurally different and representative metal compounds (e.g. based on Pt, Ru, Os, Au etc.) with antitumor properties, and to obtain detailed information on their cellular and biochemical mechanisms of actions. The selected investigational approach is based on the integration of various strategies, comprising extensive chemical/structural characterisation of the metal compounds, evaluation of their anticancer effects on cell lines *in vitro*, evaluation of the compound cellular uptake, analysis of metal speciation, analysis of the reactivity of metal compounds with biomolecules at a molecular level, and study of their interactions with relevant proteins/enzymes. The studies were carried out mainly through the application of techniques such

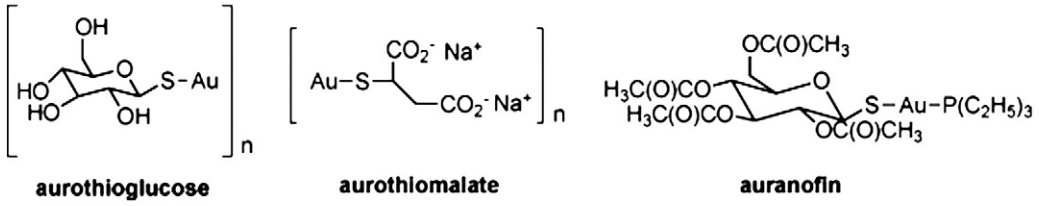

Fig. 2. Gold(I) complexes used in rheumatoid arthritis treatment.

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as X-ray crystallography, [47-49] NMR spectroscopy, [50] and X-ray absorption spectroscopy (XAS) methods, [51,52] aimed at providing information about metal local structure in molecular complexes, speciation of metal compounds, as well as metal biodistribution. Moreover, new experimental protocols and approaches were developed, in particular based on mass spectrometry, that allowed the rapid screening of a wider range of metal complexes for their reactivity with protein targets including elucidation of drug specificity/selectivity. [53-61] The direct monitoring of real-time protein-mediated metabolism of anticancer agents using hyphenated (coupled, tandem) techniques, that combine chromatographic separation with sensitive and element-specific detection techniques, such as inductively coupled plasma-mass spectrometry (ICP-MS), was also effectively used. [61] A part of our research was also dedicated to the development of new *in vitro* screenings of drug cytotoxicity on cancer cell models. Finally, target identification was achieved via enzyme inhibition studies (spectrophotometric or fluorimetric assays) coupled to analytical methods (e.g. mass spectrometry), aimed at evaluating the possible inhibition properties of metal complexes on putative pharmacological target proteins. The selection of the investigational enzyme have been performed on the basis of the relevance of the protein system with respect to cancer, as well as on structural considerations highlighting possible binding affinity of each metal complex for the selected target.

The purpose of this review is to present the main results of our research work carried out through the years 2005–2011, concerning the above mentioned mechanistic studies of different families of anticancer metal compounds. The review will be intentionally limited to analyse three main classes of metal complexes, based on platinum, ruthenium and gold, respectively. Detailed insight is offered on the general modes of interaction of the compounds with proteins and on the possible biological relevance of such reactions.

2. Platinum compounds

At the beginning of our research in 2005, in the group of medicinal inorganic chemistry leaded by Prof. Messori in Firenze, we considered the most relevant anticancer platinum complexes in clinical use *i.e.* cisplatin, carboplatin and oxaliplatin, but also some new experimental platinum compounds bearing different structural motifs such as cytotoxic *trans*-platinum(II) complexes widely investigated as "rule-breaker" anticancer drugs. [62] We soon realized that structural information on Pt complexes/proteins interactions was almost lacking in the field; therefore, we selected X-ray crystallography and high-resolution electrospray ionization mass spectrometry (ESI-MS) to study metal compound/protein adducts in both solid state and solution. Remarkably, cisplatin turned out to be highly reactive with all the investigated proteins, and Pt-protein adducts were detectable in all cases. Thus, we published two of the first X-ray structures of adducts of cisplatin with model proteins, namely bovine erythrocyte superoxide dismutase (SOD), [47] and hen egg white lysozyme (HEWL) [48] (Fig. 3). Interestingly, a common feature of cisplatin reactivity in both protein adducts was the selective binding of Pt(II) to the imidazole group of histidine residues, namely His-19 and His-15, respectively.

It is worth noting that crystal structures provide only a static vision of the metallodrug-protein derivative in the solid state allowing only an accurate description of the "final species". Thus, information on the early stages of the binding process and on the residual reactivity of the bound metal fragments is usually elusive and it can be inferred from crystallographic data only indirectly. In this respect mass spectrometry may offer an alternative approach to this problem, and provide independent and complementary information on the above mentioned mechanistic features. Therefore, we exploited high-resolution ESI-MS for the characterisation of Pt drug adducts with model proteins and for the description of their formation process in solution. [53,56,60,63] In general, in the case of cisplatin, the observed protein-bound Pt fragments were of the type $[Pt(NH_3)_2(H_2O)_n]^{2+}$ ($n = 0, 1$), as expected according to the classical reactivity of the compound in aqueous solution, with the exception of the above mentioned solid state Pt-SOD adduct in which the chloride ligands appeared to be preserved.

Interestingly, the classical Pt(II) complex carboplatin (CPT), that presents extreme kinetic inertness *in vitro* in buffered solutions, showed a high reactivity with the model protein cytochrome c. [60] Hence, it could be inferred that interactions taking place *in vivo* with biologically occurring components (either low or high molecular mass) will trigger CPT activation within biological fluids. Protein binding for CPT can occur either through loss of the 1,1-cyclobutanedicarboxylate (cbdca) ligand or through a "ring-opening" reaction with retention of the cbdca group and eventual release of ammonia. Further hydrolysis of the cbdca ligand yielded "cisplatin-like" species.

Similarly, cytotoxic *trans*-Pt(II) iminoethers developed by Prof. Natile and co-workers in Bari were analyzed for their reactivity with

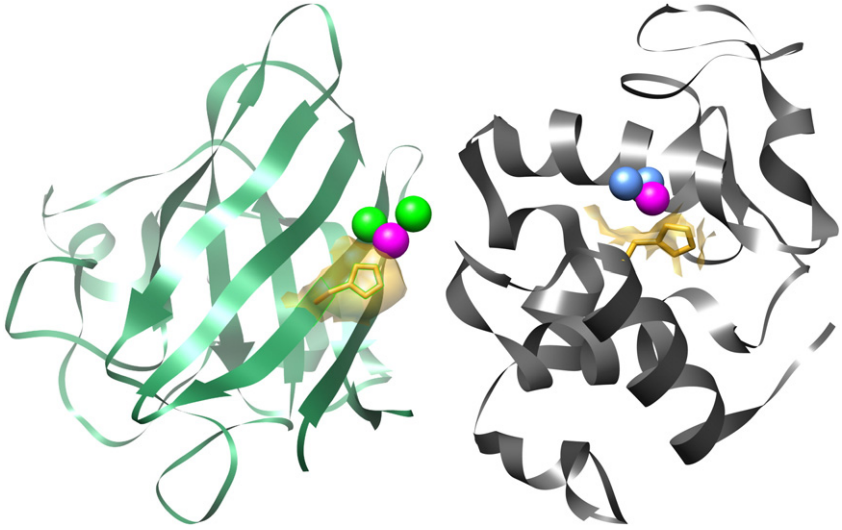

Fig. 3. Left: Ribbon representation of the asymmetric unit containing the physiological monomer of SOD bound to cisplatin (PDB 2AEO); [33] the side chain of His-19 is shown as ball-and-stick model (yellow) along with Pt (magenta) and Cl (green) shown as spheres. Right: Schematic representation of the asymmetric unit describing the surface interaction of cisplatin with HEWL (PDB 2I6Z); [34] the side chain of His-15 is shown as ball-and-stick model along with platinum (magenta) and ammonia ligands (blue) depicted as spheres.

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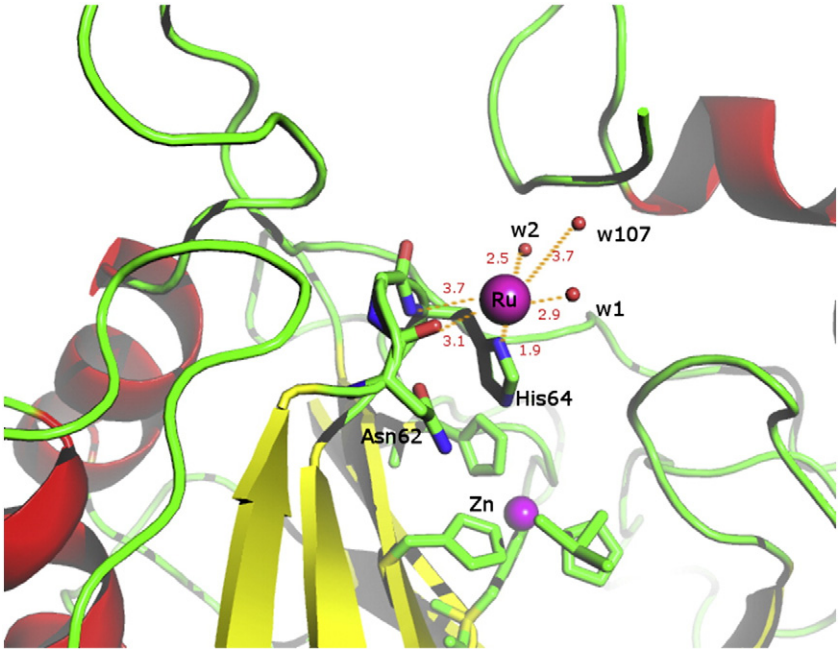

Fig. 4. Detail of the Ru centre interactions with residues His-64 and Asn-62 (stick models) and with three water molecules with the relative bond lengths reported. The oxygen atoms from water molecules are represented as red spheres (PDB 3M1J). Reproduced from ref. [58].

model proteins by ESI-MS, [54] and the results evidenced the hydrolysis of the iminoether ligands upon protein binding, most likely fostered by electrostatic interactions between the metal complex and basic surface areas of the protein. Most importantly, the obtained results implied that platinum drugs dissolved in biological media - thus, in the presence of many chemical components, including macromolecules - can manifest a chemical reactivity that is profoundly distinct from that observed when they are just dissolved in simple buffered solutions. These observations posed us important "caveats" to extrapolating the behaviour observed in solution for metallodrug to that believed to occur inside cells.

Later on, in collaboration with the group of Prof. Navarro-Ranninger and Dr. Quiroga in Madrid, *trans*-Pt(II) complexes bearing aliphatic amines ligands have also been screened for their interactions with proteins as putative biological targets by a variety of techniques such as ESI-MS, inductively coupled plasma-optical emission spectroscopy (ICP-OES) and 2D [^{1}H,^{15}N], [^{1}H,^{13}C] HSQC, as well as [^{1}H,^{1}H] NOESY NMR spectroscopy. [50,64,65] In order to determine whether Pt complexes can transfer from a peptide to an oligonucleotide, transfer experiments were performed in which a model peptide was pre-incubated with one of the platinum compounds under physiological-type conditions for 24 hours, after which an oligonucleotide was added and the mixture analyzed by ESI-MS in both positive and negative ionization modes. [64] Such an experiment imitates, to some extent, transporter-mediated delivery to DNA nucleobases. In general, significant differences between the *trans*-Pt(II) complexes and cisplatin were observed, indicating the preferential binding of the *trans* compounds towards the peptides, whereas cisplatin showed a preference to bind to oligonucleotides. This selectivity could have relevance to the mode of action of the *trans* compounds. Should adduct formation with nucleic acids (either directly or via transfer from another biomolecule) prove to be inefficient to damage DNA, proteinaceous targets could be involved in inhibiting cellular proliferation, especially for compounds of comparable cytotoxicity.

3. Antimetastatic ruthenium compounds

The ruthenium complex NAMI-A (Fig. 1) was the first Ru(III) compound to enter clinical trials, as it manifested very promising antimestastatic properties in animal models while showing a rather acceptable toxicity profile. [66-68] In spite of the fact that NAMI-A has already reached clinical stage of development, yet, its mechanisms of action are still poorly understood, although some proteins have been proposed as possible targets. [34] Thus, we started analyzing at a molecular level the reaction of NAMI-A with the model proteins lysozyme (HEWL), and cytochrome c (cyt c). [69] Mainly on the basis of NMR spectroscopy and ESI-MS results obtained on NAMI-A/cyt c adducts, we proposed that the compound might form a series of protein-adducts in which its original ligands are progressively lost. In the final NAMI-A/cyt c adduct the Ru(III) ion alone, *i.e. without any of its original ligands*, is found associated to the protein. To gain further insight into the molecular interactions of this intriguing metallodrug with proteins we have studied its reaction with human carbonic anhydrase II (hCAII), a very well known and intensely investigated zinc-enzyme, playing relevant roles in a variety of pathologies. [70,71] In our study hCAII was used primarily as a model protein. The choice of this model system was dictated by its well-characterized X-ray structure, by the ease to obtain high quality crystals and by the favorable ESI-MS behavior in spite of the relatively high molecular weight (about 29 KDa). 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 (Fig. 4), [72] and providing conclusive evidence that none of the original ligands of ruthenium in NAMI-A are conserved upon protein binding supporting the view that NAMI-A can behave as an "extreme" prodrug capable of loosing all its ligands and to serve as a "naked" protein metalating agent. Complementary, MS studies showed that hCAII/Ru adduct formation follows a multi-stage process in which NAMI-A progressively looses all its original ligands upon protein binding.

In the frame of a recent and productive collaboration with the group of Prof. Dyson in Lausanne, we have also widely investigated the family of anticancer organometallic ruthenium(II)-arene compounds of general formula [Ru(η^6-arene)Cl$_2$(PTA)] (PTA = 1,3,5-triaza-7-phosphatricyclo-[3.3.1.1]decane), termed RAPTA (Fig. 5). [25] The properties of RAPTA complexes on tests requiring the interaction of the tumour cells with extra cellular matrix components suggest that their effect on cell surface molecules may be responsible for part of their activity; however, the compounds also accumulate

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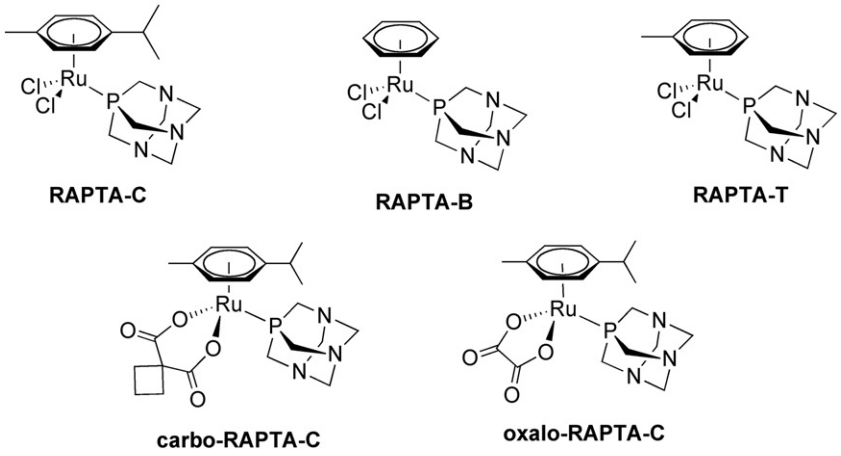

Fig. 5. Organometallic Ru(II)-arene complexes investigated in our studies.

within cancer cells and interact to a significant extent with proteins in the cytoplasm. [73] Accordingly, we have reported on the *in vitro* inhibitory properties of a series of RAPTA complexes against cathepsin B (cat B), a lysosomal papain-family cysteine protease, located in both the extracellular and intracellular *milieu*, which is involved in cellular metabolism processes and implicated in tumor progression and metastasis, processes that RAPTA compounds have been shown to prevent. [74] Docking studies of the interactions of representative RAPTA compounds with cat B were performed that provided realistic structures for the resulting protein-metallodrug adducts. Good agreement was generally found between the inhibiting potency of the RAPTA compounds and the computed stability of the corresponding cat B/RAPTA adducts. More recently, we have screened a series of organometallic compounds based on Ru(II), Os(II), Rh(III) and Ir(III) for their cytotoxicity and ability to inhibit cat B *in vitro*, in comparison to the antimetastatic compound NAMI-A. [75] The ruthenium complexes were the most active as enzyme inhibitors. Thus, to build up a rational for the observed differences, DFT calculations of the metal complexes adducts with N-acetyl-L-cysteine-N'-methylamide, a mimic for the Cys residue in the cat B active site, were performed to provide insights into binding thermodynamics in solution.

Comparing the interactions of RAPTA complexes and of NAMI-A with proteins it has been possible to evidence that the two ruthenium compounds manifest different reactivity pathways in spite of their similar antimetastatic effects: while NAMI-A has the general tendency to detach all its ligands from the Ru(III) centre upon protein binding, RAPTA complexes most likely maintain the arene and phosphane moieties anchored to Ru(II). [55,59] It is worth mentioning that target identification for NAMI-A and RAPTA compounds has also been recently attempted by proteomic studies on human ovarian cancer cells A2780, following recently reported procedures that couple 2D gel electrophoresis to MS analysis. [40] At the moment detailed analysis of the obtained results is in progress.

Since mechanisms of cell proliferation and invasion inhibited by RAPTA compounds are involved in the development of both metastases and angiogenesis, recent hypothesis formulated the idea of metal-based antimetastatic drugs that could behave also as anti-angiogenic substances. However, up to now only limited studies appeared in the literature to support this hypothesis. [76] Thus, we discovered the interesting anti-angiogenic effects of RAPTA complexes *in vitro* on endothelial cells and *in vivo* on the chicken's chorioallantoic membrane (CAM). [77] In this context, preliminary results of *in vitro* enzyme inhibition of 100 human kinases indicate that RAPTA compounds are selective inhibitors of the fibroblast growth factor receptor (FGF-R1) and of the vascular endothelial growth factor receptor (VEGFR) involved in tumor growth and angiogenesis. [78,79]

As previously mentioned, the *in vitro* cytotoxicity of RAPTA compounds has been evaluated on various human cancer cell lines, and resulted to be very poor and limited with respect to cisplatin, and in any case it does not appear to be relevant to their effect on metastases *in vivo*. It is worth noting that currently, screening for biological and pharmacological activities of new drugs is achieved by growing cells as a 2-dimensional (2D) monolayer culture. Cell monolayers are physiologically different compared to 3-dimensional (3D) human

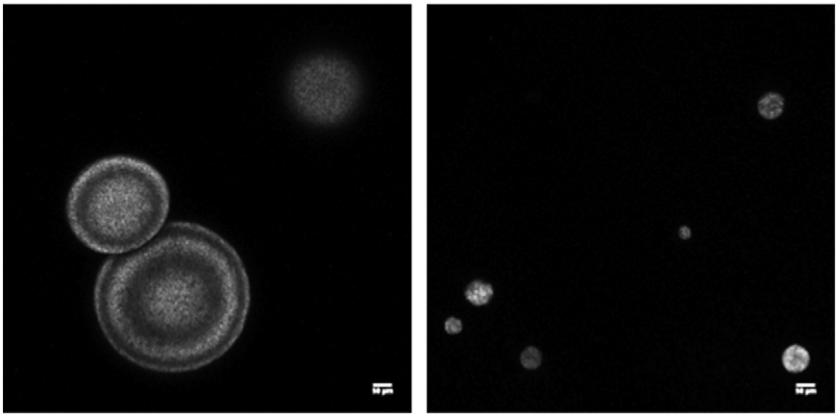

Fig. 6. Differences in spheroid growth in a 3D hydrogel matrix between controls (left) and RAPTA-C treated cells (50 μM) (right).

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tissues or tumors. In fact, although the conventional focus of chemotherapeutic drug development has been on tumor cells, recent studies show that the interactions with the stroma might influence drug sensitivity and development of drug resistance. [80] The extracellular matrix (ECM), which is considered to be part of the stroma, and has been shown to active signalling pathways, is not present in the 2D-cell-based assays. Therefore, several recent studies point out the necessity of a 3D model to screen anticancer activities that closely mimics the *in vivo* behaviour of normal cancerous human tissues. [81-83] In the last years, different biomaterials that mimic key aspects of the ECM have found a number of applications. [84] Protein components of the ECM such as collagen, fibrin, laminin, or elastin, [85,86] as well as mixtures of these materials [87] or other hybrid protein-based matrices such as Matrigel, [88] are widely used as cell-culture substrates due to their inherent resemblance to the natural ECM and complex signaling capabilities. More recently, synthetic hydrogels offered one approach for the generation of bioactive and degradable scaffolds, possess low risk of immune reaction, and provide for straightforward material handling. [89-92] Thus, we screened the Ru(II) compound RAPTA-C (Fig. 5) on a 3D cancer model of cultured human ovarian cancer cells A2780 in a hydrogel system, and compared the potency and efficacy of the metallodrug with respect to the classical 2D-based cytotoxicity assay. Remarkably, the preliminary obtained results show that RAPTA-C is able to inhibit spheroid growth in the 3D cancer model at doses that are well below the IC_{50} out of the 2D cell culture studies (Fig. 6). Further studies are in progress to characterize the observed biological effects in this new model system.

A part of our research work has also been devoted to the design and screening of new metal compounds as therapeutic agents. It is worth noting that in the field of metallodrugs development various tactics, and some new approaches have been employed to improve the physico-chemical and biological properties of metal complexes. Among the possible strategies the concept of *multinuclearity* has led to innovative chemical and biological activities for a number of bi- and poly-metallic complexes, either homo- or hetero-metallic as possible anticancer agents, including polynuclear platinum, ruthenium and gold compounds. [93-101] Thus, we reported on the synthesis and biological characterization of bimetallic Ti-Ru complexes based on a titanocene-phosphine backbone anchored to a Ru(II)-arene scaffold, which showed to have improved antiproliferative effects on cancer cells in comparison to their mononuclear Ti and Ru organometallic precursors (Fig. 7). [102]

Moreover, the dinuclear Ti-Ru complexes showed relevant cat B inhibition properties *in vitro* that correlate to some extent with the observed antiproliferative effects. Enzyme inhibition by the bimetallic complexes appeared also to be influenced by the length of the alkyl chain in between the metal centres. However, it should be noted that inhibition of cathepsins might occur either extracellularly or intracellularly and that other studies are necessary to clarify all the relevant targets of these compounds.

4. Gold compounds

Nowadays, gold compounds constitute a variegate family of very promising experimental agents for cancer treatment. Indeed, several gold(I) and gold(III) complexes were shown to manifest outstanding antiproliferative properties *in vitro* against selected human tumor cell lines, and some of them performed remarkably well even in tumor models *in vivo*. [27,103,104] As previously mentioned, investigations on the cytotoxicity scores of gold complexes have been initially focused mainly on auranofin (1-thio-β-D-glucopyranose-2,3,4,6-tetraacetato-S)(triethylphosphine) gold(I), Fig. 2) and its analogues, which present linear gold phosphane thiolate structures. [105] More recently, a variety of gold derivatives have also been tested as potential antitumor agents, including organogold derivatives, complexes with polydentate nitrogen donor ligands, gold porphyrins, gold dithiocarbamates, and gold N-heterocyclic carbene (NHC) carbenes. [106-110] Within this frame, our research has considered the synthesis and biological characterization of new anticancer gold compounds of different families, also in collaboration with other research centres, including cytotoxic mono- and di-nuclear Au(III) complexes with bidentate nitrogen donor ligands; [98,99,111] organogold complexes, [112] Au(I) complexes containing water soluble phosphane ligands, [113], as well as 1-thio-β-D-glucose 2,3,4,6-tetraacetate moieties, [114] known to act as a true substrate for the glucose active-transport system, and, therefore, used to increase the uptake of metal compounds (Fig. 8). Mono-, di- and trinuclear organometallic alkynyl Au(I) complexes containing water soluble phosphane ligands were also reported, [101,115] some of them showing luminescence properties which allowed to detect the compounds' cellular uptake via fluorescence microscopy. [101]

Following our promising results obtained on heteronuclear Ti(IV)-Ru(II) complexes mentioned above, and in collaboration with the group of Prof. Le Gendre and Dr. Piquet in Dijon, we developed new di-and trinuclear organometallic Ti(IV)-Au(I) complexes of the type reported in Fig. 8, in which the two metal centres are linked via a phosphine ligand directly anchored to the arene moiety. [116] A major challenge in this approach was the achievement of the stability of the compounds in aqueous environment, normally hampered by the presence of the titanocene fragment. Interestingly, all the compounds were found to be considerably more cytotoxic on cancer cell lines than their parent mononuclear titanocene-phosphine precursors, and even cisplatin, especially in the Pt resistant cell lines. Additional protein binding MS studies together with the cytotoxicity profiles supported the idea that the Au centre in the heterometallic scaffold plays a fundamental role in determining the biological

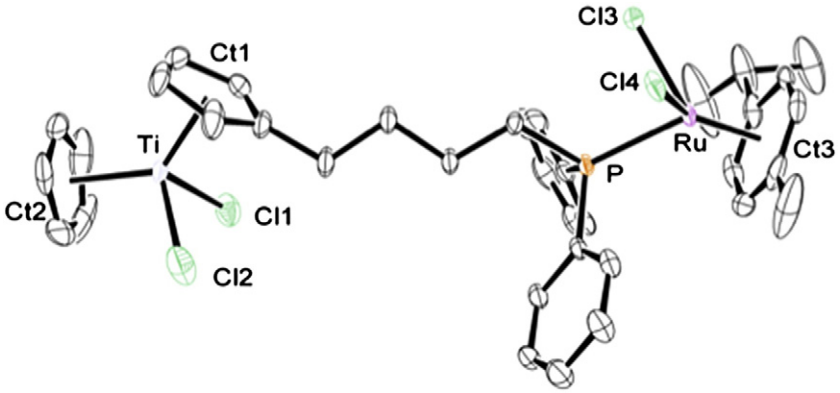

Fig. 7. ORTEP view of $[(\eta^6\text{-}p\text{-cymene})[(\eta^5\text{-}C_5H_5)(\mu\text{-}\eta^5{:}\kappa^1\text{-}C_5H_4(CH_2)_4PPh_2)TiCl_2]RuCl_2]$.

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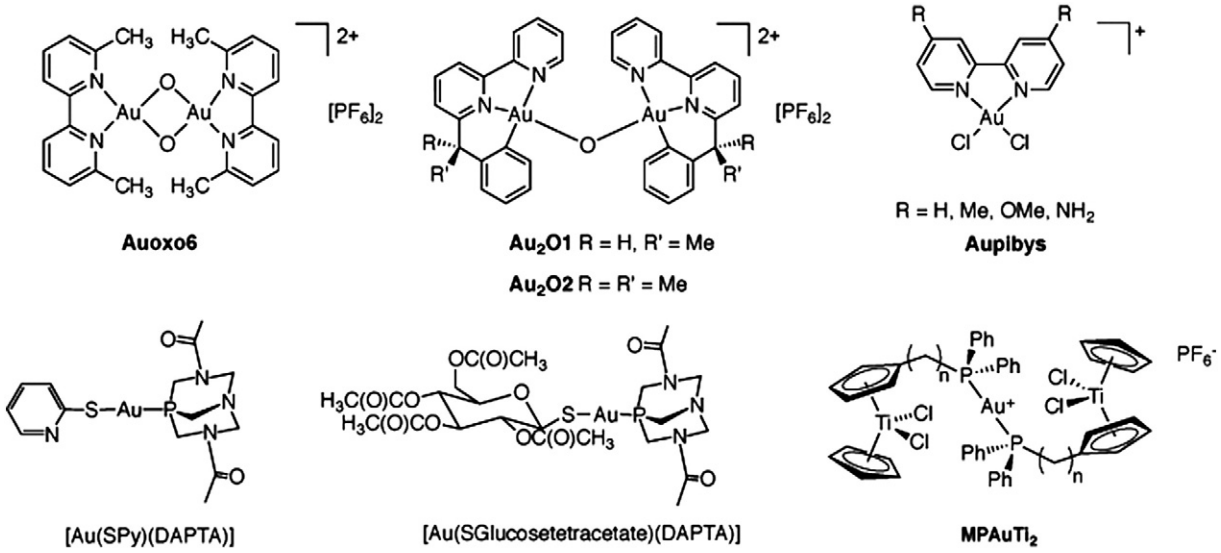

Fig. 8. Structures of cytotoxic gold(III) and gold(I) complexes, and of the heteronuclear compound **MPAuTi$_2$** $[[(\eta^5\text{-}C_5H_5)TiCl_2(\mu\text{-}\eta^5{:}\kappa^1\text{-}C_5H_4PPh_2)]_2Au]PF_6$.

activity of the reported compounds, possibly due to its known high affinity for binding to amino acid residues in proteins/enzymes.

It has emerged that the mechanisms of action cytotoxic gold complexes are rather distinct from cisplatin, for which DNA is thought to be the major target. [117] Indeed, various protein targets have been reported for cytotoxic gold complexes, including thioredoxin reductase (TrxR), a seleno-enzyme critically involved in the regulation of the intracellular redox state and of mitochondrial functions, [110] the proteasome, [118] cysteine proteases, [119] as well as serum albumin (HSA), human glutathione reductase (hGR), and protein tyrosine phosphatases (PTPs). [120]

Among the possible protein targets for the new gold complexes developed by us, the seleno-enzyme TrxR was widely investigated, and the interactions of gold compounds with mammalian TrxR isoforms were characterized via biochemical and mass spectrometry methods. [100,113,121] According to various hypotheses, enzyme inhibition should be achieved by direct binding of the Au ions to the TrxR selenolate in the active site; however, still no structural information has been reported so far to support this mechanism. [110] Thus, we studied the mechanism of inhibition of TrxR by a series of structurally diverse gold(III) compounds in comparison to the anti-arthritic gold(I) drugs auranofin and aurothiomalate. The tested compounds - either gold(III) or gold(I) - were found to produce potent enzyme inhibition only after pre-reduction of the enzyme with NADPH, indicating that TrxR inhibition is the result of protein structural changes occurring upon cofactor binding. Matrix assisted laser desorption ionization-time of flight (MALDI-ToF) MS experiments on the intact enzyme provided evidence for extensive enzyme metalation, while experiments on trypsinized gold(III)-protein adducts identified a specific protein fragment, distant from the selenocysteine residue, bearing an attached gold(I) ion deprived of its organic ligands. Independent mechanistic information on the system was derived from biotin-conjugated iodoacetamide (BIAM) assays capable of monitoring selective metal binding to cysteine and/or selenocysteine residues. Interestingly, while the effects produced by auranofin could be essentially ascribed to gold(I) coordination to the active site selenol, the effects caused by the various gold(III) compounds were better interpreted in terms of oxidative protein damage.

The inhibition of TrxR by gold compounds might explain our results on the potent antiplasmodial effects by auranofin and a few related compounds on the malaria parasite *P. falciparum in vitro*. [122,123] *P. falciparum* growth inhibition is probably the consequence of direct interactions of the metal center with specific parasite's biomolecular targets (e.g. thioredoxin reductase and falcipain). Alternatively, the gold compounds might act by inducing severe oxidative stress to which *P. falciparum* is extremely sensitive.

In the search of protein targets for gold compounds, a systematic investigation of the cell growth inhibition properties of a representative ensemble of gold complexes on a panel of 36 cancer cell lines, and subsequent *COMPARE* analysis, highlighted, for the first time, two well-known biomolecular systems such as histone deacetylase (HDAC) and protein kinase C (PKC), as probable targets for gold compounds. [124] The *COMPARE* algorithm uses *in vitro* activity data to obtain clues as to the mechanism of action of a test compound correlated to those of 110 standard cytotoxic agents. Further experimental work is now warranted to validate these hypotheses on the isolated enzymes; however these findings open the way to a more direct evaluation of gold compounds toward specific and relevant targets.

As previously mentioned, the identification of possible targets for metallodrugs is not trivial, and may be achieved through different approaches that often take advantage of the existing knowledge on protein systems relevant to cancer. For example, we recently considered the poly-(adenosine diphosphate (ADP)-ribose) polymerases (PARPs) which are essential proteins involved in DNA repair mechanism and in cancer resistance to chemotherapies. [125,126] Notably, PARP-1, the most studied member of the PARP family, is characterized by the presence of two long zinc finger domains (ZF-PARPs, also termed as nick-sensors), that are positioned upstream of the catalytic domain, [127] and mediate specific nicked DNA recognition. [128] We proposed a new mechanistic hypothesis in which PARP-1 is a pharmacological target of metal-based drugs. Such a hypothesis is based on the study of PARP-1 inhibition by a series of metal-based compounds (based on platinum, ruthenium and gold) on purified enzyme, and on protein extracts from human cancer cells. [129] The selected series of anticancer metal complexes were found to inhibit PARP-1 activity to various extents, with the Au(III) complexes being the most effective (nM range). Considering that the PARP structure contains two zinc-finger (ZF) motifs that may be altered by the metal complexes, we have also studied the nature of the possible metal-ZF-PARP adducts by high-resolution ESI-Fourier Transformion cyclotron mass spectrometry (ESI-FT-ICR MS). Remarkably, excellent correlation between PARP-1 inhibition and the mode of metal binding to the ZF domain was observed. In fact, gold complexes were shown to efficiently react with ZF-PARP domains with substitution by Au(III) of the Zn ion and the formation of so called "gold fingers" (Fig. 9).

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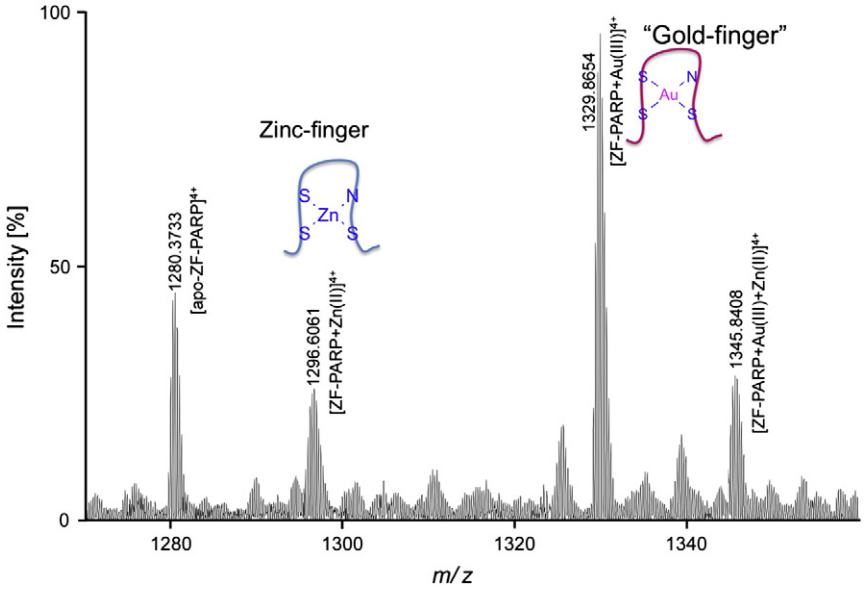

Fig. 9. Representative ESI FT-ICR MS spectrum of ZF-PARP incubated with a gold(III) compound for 5 min at room temperature.

5. Concluding remarks

The research described herein is in the frame of the emerging field of Metallomics, a transdisciplinary research area with an impact on geochemistry, clinical biology and pharmacology, plant and animal physiology and nutrition, with an ultimate goal to provide a global and systematic understanding of the metal uptake, trafficking, function and excretion in biological systems. [130] Specifically, the proposed project belongs to the area of medicinal inorganic chemistry, where the intentional introduction of metal ions into a biological system has proved to be useful for either therapeutic or diagnostic purposes. [2] Indeed, metal-based chemotherapeutics, such as anticancer agents, metal-mediated antibiotics, antibacterials, antivirals, antiparasitics, antiarthritics, and radiosensitizing agents, appear, as do radiopharmaceuticals. Notably, the importance of medicinal inorganic chemistry and the potential of metals to contribute more widely to the treatment of diseases are recognized by the National Institutes of Health Metals in Medicine program. However, metal-based therapeutics remain as a minority of all drugs in the market today. This is partially due to the prejudice against metal compounds based on their perceived toxicity and scarce selectivity towards specific molecular targets. [3]

However, the possibility offered by metal compounds to design innovative chemical scaffolds for tackling the challenge of specific molecular recognition in complex biological systems is very appealing and worth exploring. Within this frame, our study is aimed at establishing a general strategy for metallodrug development with the implementation of high-resolution biophysical techniques coupled with pharmacological methods. An important task of our research is to discover the unique properties of metal compounds as modulators (inhibitors or activators) of proteins/enzyme activities, and to exploit them for different therapeutic purposes or as molecular biological tools. This task is strictly linked to the identification of new protein targets for metal complexes, which might suggest novel applications such as the detection of protein activities in biological systems. As an example, the diverse range of biological functions of zinc-fingers and zinc-finger-like proteins targeted by gold complexes, including DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly, will allow to exploit new strategies to regulate fundamental cellular processes for therapeutic or diagnostic purposes. It must be noted that recently we have also identified the aquaporins (AQPs), membrane water channels with crucial roles in normal human physiology and pathophysiology, [131] as possible target systems for metal compounds (not published data). Certainly, there is considerable potential for translating knowledge of AQP structure, function and physiology to the clinic, and there is great translational potential in aquaporin-based therapeutics.

In conclusion, while we have been able to establish the basis of an integrated strategy to metallodrug development, and even identified gold complexes with promising biological properties, further efforts will be necessary to direct our studies towards other fundamental aspects of drug action, such as the understanding of metal compounds toxicological and pharmacokinetic properties, as well as the integration of our data with *in vivo* studies. It is worth mentioning that, in the case of cancer, development of new therapeutic treatments focuses increasingly on the relation of the cancer tissue with its microenvironment. However, a major obstacle for the development of new anticancer therapies has been the lack of relevant animal models that would reproduce all the events involved in disease progression from the early-stage primary tumor until the development of mature metastatic tissue. Therefore, innovative investigational models and new possible applications for metal compounds should be exploited in collaboration within different centres and research networks at the highest possible level of interdisciplinarity.

Abbreviations

BIAM	biotin-conjugated iodoacetamide
CPT	carboplatin
cat B	cathepsin B
CAM	chicken's chorioallantoic membrane
cbdca	1,1-cyclobutanedicarboxylate
cyt c	cytochrome c
ECM	extracellular matrix
ESI-MS	electrospray ionization mass spectrometry
ESI-FT-ICR	
ESI-Fourier Transform-ion cyclotron	
hCAII	human carbonic anhydrase II
HEWL	hen egg white lysozyme
HDAC	histone deacetylase
ICP-MS	inductively coupled plasma-mass spectrometry
ICP-OES	inductively coupled plasma-optical emission spectroscopy
MALDI-ToF	
matrix assisted laser desorption ionization-time of flight	

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PARP	poly-(adenosine diphosphate (ADP)-ribose) polymerase
PKC	protein kinase C
PTA	1,3,5-triaza-7-phosphaadamantane
SOD	bovine erythrocyte superoxide dismutase
RAPTA-C	$Ru(\eta^6\text{-p-cymene})(PTA)Cl_2$
TrxR	thioredoxin reductase
ZF	zinc finger

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