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Biophysical characterisation of adducts formed between anticancer metallodrugs and selected proteins: New insights from X-ray diffraction and mass spectrometry studies

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Abstract

There is considerable interest today for the reactions of anticancer metallodrugs with proteins as these interactions might feature processes that are crucial for the biodistribution, the toxicity and even the mechanism of action of this important group of anticancer agents. We survey here the results of research activities carried out in our "Laboratory of Metals in Medicine" (Department of Chemistry, University of Florence) during the last three years, concerning the molecular characterisation of adducts formed between platinum, ruthenium and gold metallodrugs and a few model proteins. Valuable structural and functional information on these adducts could be derived from several biophysical studies mainly relying on the application of X-ray diffraction and ESI MS techniques. The value and the limitations of both approaches are outlined through a number of examples. Remarkably, the structural and functional information achieved on the respective metallodrug–protein adducts allowed us to identify some general trends in the reactivity of anticancer metallodrugs with protein targets.
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1. Introduction

Since the discovery of the antitumor activity of cisplatin (*cis*-$[PtCl_2(NH_3)_2]$; Chart 1), during the 1960s, metal-based drugs have played a major role in anticancer medical treatments [1–6]. As a matter of fact, a few platinum compounds are today among the most widely used anticancer agents in the clinics. Research in this field is still very active and has been extended, in recent years, to include a conspicuous number of non-platinum metallodrugs.

During the last three decades, the interest of the scientific community working on anticancer metal-based compounds has mostly focused on their interactions with DNA, the commonly accepted "primary" target for platinum compounds that were described and analysed through hundreds of research papers [7]. In contrast, rather surprisingly, the reactions of platinum and non-platinum anticancer metallodrugs with proteins have received so far very little attention. Only a few biophysical studies have indeed appeared dealing with the interactions of anticancer metallodrugs with proteins. These studies mostly concerned the two major serum proteins, albumin [8] and transferrin [9–11], as well as metallothioneins [12–14], small, cysteine-rich intracellular proteins, primarily involved in storage and detoxification of soft metal ions [15]. Additional studies were carried out on a few other proteins such as ubiquitin [16], haemoglobin [17,18], myoglobin [19], cytochrome *c* [20] and glutathione-S-transferase [21], serving, in most cases, as model proteins. The most relevant achievements obtained on this issue until 2005 were excellently summarised by Keppler and coworkers in a comprehensive review appeared on *Chem. Rev.* [22].

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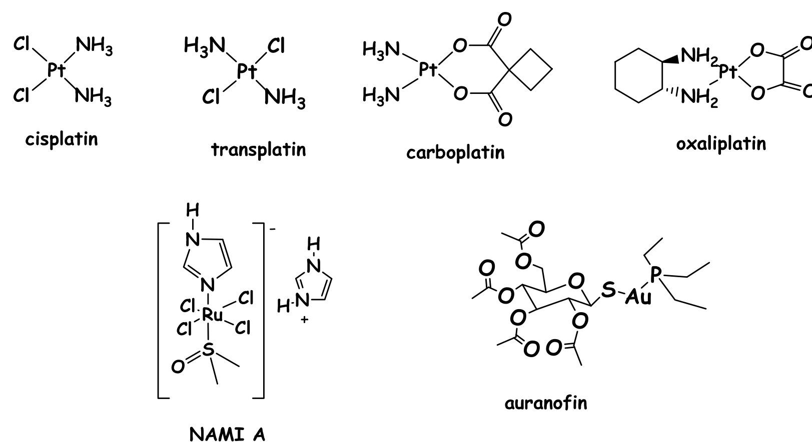

Chart 1. Chemical formula of classical platinum, ruthenium or gold-based pharmacological agents.

We believe that this topic deserves more and more attention as it is increasingly evident that the interactions of anticancer metallodrugs with proteins play crucial roles not only in their uptake and biodistribution processes but also in determining their overall toxicity profile. Even more interestingly, reactions of anticancer metallodrugs with proteins are likely to be involved in some crucial aspects of their mechanism of action. This latter statement is particularly true for non-platinum anticancer metallodrugs such as ruthenium and gold compounds for which DNA-independent mechanisms of action have been proposed and experimentally supported [23,24]. For instance, it was suggested that dimethylsulfoxide ruthenium(III) drugs might either interfere with specific proteins involved in signal transduction pathways or alter cell adhesion processes [25]. Direct antimitochondrial effects were demonstrated for a few cytotoxic gold complexes with gold in the oxidation states +1 and +3 [26]. Thus, further work is absolutely required to analyse the reactions of metallodrugs with proteins at the molecular level, to identify possible common trends in these reactions, to characterise the structure and reactivity of the resulting adducts and identify the most important intracellular protein targets for the various classes of anticancer metallodrugs.

Nowadays, the study of the interactions occurring between metallodrugs and proteins may take new and considerable advantage of the availability of very sophisticated and advanced analytical tools. For instance, a number of papers have highlighted the great potential of modern mass spectrometry ionization methods, in particular ESI and MALDI MS, to characterise metal–protein adducts at the molecular level [27–29]. On the other hand, X-ray diffraction studies of such adducts, although not trivial, may result extremely valuable in providing detailed structural information on the formed metallodrug–protein species. On the whole, these methods have the potential to offer rather exhaustive descriptions of metallodrug/protein interactions when working on the purified components. Conversely, the rapid development of modern proteomic technologies and the use of advanced protein separation techniques, coupled to very sensitive metal detection methods, hold promise for the successful analysis of complex mixtures of metallated proteins and for the identification of those proteins that act as primary "metallodrug receptors" and/or "metallodrug targets". Thus, these latter techniques open the way to the investigation of far more complicated systems such as metallodrug/treated cell populations and/or cell homogenates, that reflect, more closely, the reality of metallic species in the cell world [30,31].

The purpose of this short review is to present the main results of research work mostly carried out in our "Laboratory of Metals in Medicine" in Florence through the years 2005–2007, concerning the formation and characterisation of adducts between various kinds of metal compounds and a few model proteins. Based on a few representative examples, the specific importance of X-ray diffraction and mass spectrometry techniques to identify the main features of metallodrug–protein interactions will be stressed and discussed. Detailed insight is offered on the general modes of interaction of metallodrugs with proteins and on the possible biological relevance of such reactions. The interest of this short review will be intentionally limited to analyse a small number of examples concerning platinum, ruthenium and gold anticancer drugs.

2. Basic aspects of metallodrugs/protein interactions

As mentioned above, this focused review will be restricted to a few platinum, ruthenium and gold compounds with reported cytotoxic and/or antitumor activity, whose biologically activity and significance were already established thanks to the work of other research groups. Thus, the most relevant platinum complexes in clinical use will be considered, i.e. cisplatin, carboplatin and oxaliplatin (Chart 1) but also some new experimental platinum compounds bearing different structural motifs such as the platinum iminoethers developed by Giovanni Natile in Bari. Among ruthenium compounds we will focus on the ruthenium(III) complex, imidazolium *trans*-[tetrachloro(DMSO) (imidazole)ruthenate(III)], NAMI A [32] (Chart 1), now in phase II clinical trials [33], but also on a few ruthenium(II)–arene complexes developed in

Lausanne by the group of Paul Dyson [34]. Finally, in regard to gold metallodrugs, we will refer to a few gold(III) compounds developed in our group and also to (triethylphosphine(2,3, 4,6-tetra-*O*-acetyl-β-1-D-thiopyranosato-S) gold(I)), auranofin (Chart 1), a clinically established gold(I) antiarthritic agent, known to produce large cytotoxic effects *in vitro* (*but not in vivo*) [35].

Remarkably, most of the above mentioned compounds are known to behave as *pro-drugs*, in other words an activation step is required before they can react with biomolecular targets and cause their specific biological effects. Usually, this step consists of the release of a weak ligand (the so called "leaving group") from the first coordination sphere of the metal and of its replacement by a water molecule. The resulting "aqua species" usually manifest a high propensity to react with protein side-chains, showing a pronounced preference for histidine, cysteine and methionine residues, but also for carboxylate groups. Alternatively, metallodrug activation may occur through a redox process, for instance metal reduction, as it is the case for newly developed anticancer platinum(IV) compounds. In most cases, the reaction of activated metallodrugs with protein side chains leads to formation of relatively tight metallodrug–protein *complexes* or *adducts* in which metallic fragments are covalently bound to proteins. These adducts usually manifest an appreciable stability. However, a further reactivity may be expected: (i) if the metallic fragments still bears reactive sites; (ii) if the adduct is reacted with other biomolecules showing a higher affinity for the metal itself; (iii) if the protein possesses stronger, but kinetically disfavoured, binding sites for the metallic fragment. Of course, this residual reactivity may be very important in order to assess whether the formed species will keep some biological activity.

Proteins that were selected in our research group as suitable models to test reactivity with metallodrugs are the following: bovine erythrocyte superoxide dismutase (SOD) (EC 1.15.1.1), hen egg white lysozyme (HEWL) (EC 3.2.1.17), horse heart cytochrome *c* (cyt *c*) and bovine erythrocytes ubiquitin (Ub). Schematic drawings for some of these proteins are shown in Fig. 1. For all these proteins high resolution crystal structures are available. Notably, these proteins are of moderate to small size with MW ranging from 34,000 (SOD in the dimeric form) to 6500 Da. Moreover, all these proteins are commercially available, manifest a high stability in solution under physiological-like conditions, are relatively cheap and water soluble. In most cases, they exhibit a basic p*I* and are thus appropriate for ESI MS detection in the positive mode. All these features render experimental work on these model systems rather comfortable.

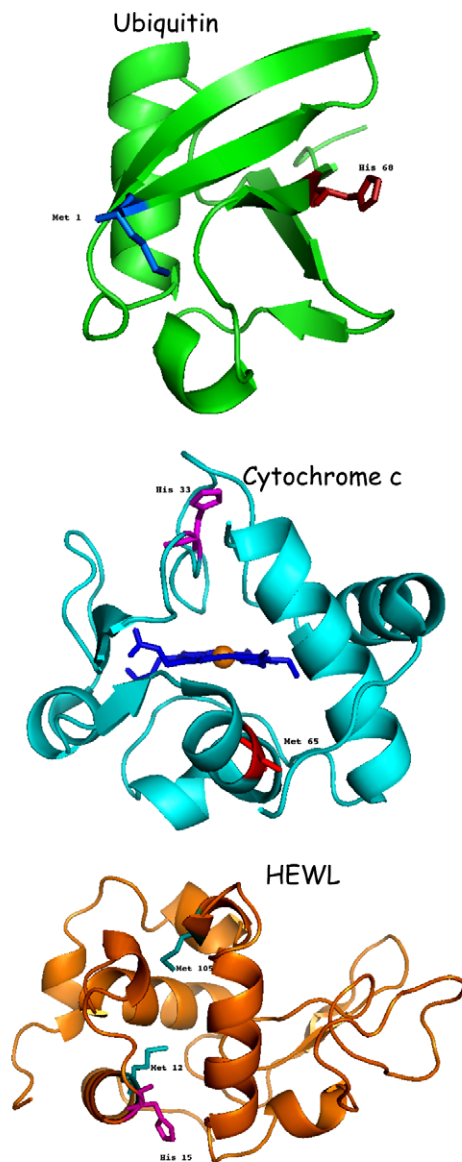

Fig. 1. Molecular structures of ubiquitin, cytochrome *c* and HEWL, with the aminoacidic residues, possible metal binding sites, highlighted as stick graphics. In detail Met 1 (blue) and His 68 (red) are highlighted in ubiquitin; Met 65 (red) and His 33 (magenta), heme (blue) and iron (orange) are reported for cyt *c*; His 15 (magenta) and Met 12 and Met 105 (cyan) are highlighted in HEWL. The PDB files are available at the website www.rcsb.org. The figure was generated using Pymol (DeLano Scientific LLC; http//pymol.sourceforge.net).

3. X-ray crystallography

Although single crystal X-ray diffraction still represents the election tool to obtain high quality structural information on proteins, especially for those of medium to large size, very few crystal structures have been solved, until now, to a high resolution for metallodrug–protein adducts. This situation may be principally ascribed to the intrinsic difficulty in obtaining good quality crystals for metallodrug/protein adducts.

When we started this approach in our laboratory in early 2005, we soon realised that only very few crystal structures had appeared for protein–metallodrug adducts. Just a few additional structures, somehow connected to our topics, had been obtained by Jaouen and coworkers working on specific lysozyme modifications caused by a variety of organometallic agents [36,37]. In contrast, a

quite conspicuous number of crystallographic structures were available in the HAD data bank in relation to the heavy atom replacement methodology [38]. However, these latter structures dealt only marginally with effective metallodrugs; moreover, in several cases, details on the coordination environment of the bound metal are not available.

Thus, if one discards crystal structures of the HAD data bank and those related to the "specialistic" work of Jaouen, only six crystal structures are left for adducts of proteins with platinum, ruthenium or gold metallodrugs (Table 1) [39–42]. Notably PDB coordinates have been deposited only for three of these five adducts. Moreover, it turned out that no crystal structure was available for any adduct of cisplatin with proteins.

This lack of structural information prompted us to start an intense program of metallodrug–protein crystallization trials in order to obtain crystals suitable for X-ray diffraction analysis. Notably, this approach was successful twice with cisplatin, i.e. in the cases of the cisplatin/SOD and of the cisplatin/HEWL adducts [43,44]; however, several other attempts with a variety of other different metallodrugs and proteins failed so that the overall rate of success was rather low.

The two mentioned cisplatin-protein derivatives were obtained in the crystalline form by different crystallisation methods: in fact, the SOD derivative was prepared through "co-crystallisation" while crystals for the cisplatin/HEWL derivative were obtained by the so called "soaking" procedure. Crystal structures for both these derivatives were solved by classical methods of protein crystallography. The overall structures of the two adducts are reported in Figs. 2 and 3, respectively; the coordination environment determined for the platinum ions in these two cases is also shown.

More in detail, crystals of cisplatin-treated bovine erythrocyte SOD, suitable for X-ray diffraction analysis, were obtained after incubation of the protein with a tenfold molar excess of cisplatin for two weeks at 4 °C. X-ray diffraction data were collected at low temperature and the structure solved to 1.8 Å resolutions. Unambiguous evidence was obtained concerning His 19 as the primary anchoring site for cisplatin on SOD. Binding was found to be highly selective: indeed, while occupation of the His

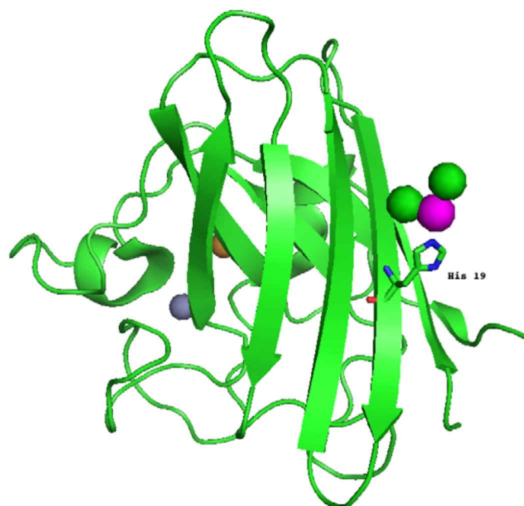

Fig. 2. Schematic representation of the asymmetric unit containing the physiological monomer of SOD bound to cisplatin; the side chain of His 19 is shown along with Cu (orange), Zn (gray, Pt (magenta) and Cl (green).

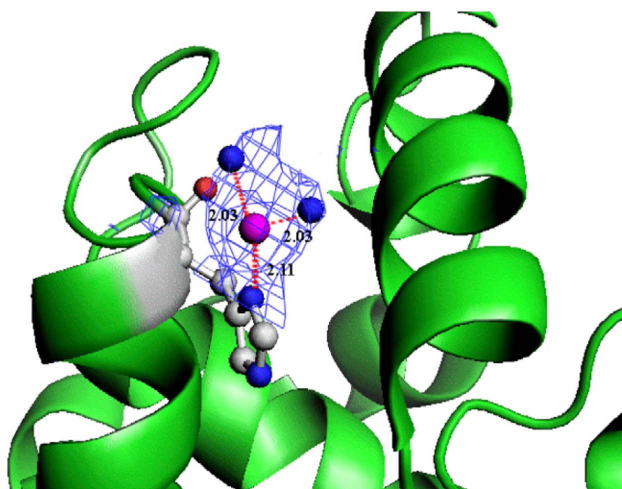

Fig. 3. Fo–Fc map at 1σ of cisplatin/HEWL adduct, covering platinum(II) (magenta) that interacts with Nε of His 15 and with two ammonia ligands (blue) and the relative bond lengths (Å).

site is relevant (>60–70%), no other binding sites could be detected for platinum on the protein surface, even at

Table 1
Crystal structures of pharmacologically relevant metal complexes bound to proteins

Metal complex/protein adduct	Metal	Resolution (Å)	Metal binding-site	PDB	Year of publication
Cisplatin/beSOD	Pt(II)	1.8	His 19	2AEO	2006
Cisplatin/HEWL	Pt(II)	1.9	His 15	2I67	2007
NAMI A/human lactoferrin	Ru(III)	2.6	His 253	–	1996
KP1019/human lactoferrin	Ru(III)	2.2	His 253	–	1996
$HInd_2[RuIndCl_5]$[a]/human lactoferrin	Ru(III)	2.4	His 253	–	1996
$[(\eta^6$-*p*-cymene)$RuCl_2H_2O$/HEWL	Ru(II)	1.6	His 15	1T3P	2004
$Au(PEt_3)Cl$[b]/cyclophilin-3	Au(I)	1.85	His 133	1E3B	2000
Gold thiomalate/human cathepsin K	Au(I)	2.0	Cys 25	2ATO	2007

[a] Ind = indazole.
[b] PEt_3 = triethylphosphine.

very low occupation values. Remarkably, clear evidence for ammonia release from cisplatin was obtained as well (see Fig. 2 for details on the chemical environment of Pt(II)). Such unexpected reactivity found for cisplatin was tentatively explained in terms of the less polarizable environment, which is likely formed when amino acid residues of proteins react with the drug. According to theoretical studies this peculiar condition greatly reduces the thermodynamic *trans* influence and enhances the kinetic *trans* effect at the platinum center, therefore favouring the detachment of at least one ammonia ligand [45–47].

At variance, a more classical chemical environment was found for the platinum(II) center in the cisplatin–HEWL derivative (Fig. 3). Indeed, in this latter structure solved to 1.9 Å resolutions, the platinum atom is bound to the Nε of His 15 and to the nitrogen of two ammonia molecules in *cis*. The fourth ligand is not detectable: it might well correspond to a loosely bound/disordered platinum-coordinated water molecule. No other significant modifications of the electron density map of the protein surface were observed ruling out the presence of additional (secondary) binding sites; for instance the two methionine residues (Met 12 and Met 105), that commonly represent preferred anchoring sites for platinum(II) compounds, turned out to be absolutely unaffected at a careful examination.

It is of interest to observe that, in both reported cases, protein platination takes place predominantly to a single protein site and that occupancy of secondary site is not detected at all within the resolution limits of the technique. Remarkably, in both cases, the Nε of histidines has been found to serve as the primary anchoring site for platinum(II). Unfortunately, several other attempts to obtain crystals suitable for X-ray diffraction were unsuccessful for other platinum derivatives highlighting the intrinsic risks of this approach.

Also, it is worth noting that crystal structures provide only a static vision of the metallodrug–protein derivative in the solid state, in practice 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 difficult to extract, as it can be inferred from crystallographic data only indirectly. In this respect, mass spectrometry may offer an alternative approach to this problem and give independent and complementary information on the above mentioned mechanistic features as shown below. Thus, we exploited, in a number of papers, the feasibility of an MS-based strategy for the characterisation of metallodrug adducts with model proteins and for the description of their formation process.

4. ESI mass spectrometry

4.1. Platinum(II) complexes

Today, thanks to the latest technological improvements, ESI MS represents a very powerful method for the molecular characterisation of metallodrug–protein adducts. A series of pioneering studies, carried out by Dani Gibson and coworkers during the 1990s and early 2000s, highlighted the advantages of this method and defined the experimental conditions for its application to simple metallodrug/protein systems. Most of Gibson's studies focused on the reactivity of cisplatin and analogues with ubiquitin, taken as the reference model protein. The careful interpretation of a great deal of ESI MS results, collected under various experimental conditions, allowed Gibson and coworkers to assign the two main platinum binding sites in ubiquitin, to describe the time dependent evolution of the resulting platinum–protein adducts and also to monitor the reactivity of the platinum protein adducts with other relevant biomolecules that are present intracellularly, *e.g.* glutathione and various nucleobases [19].

The high content of structural and functional information that could be derived from those pioneering ESI MS studies prompted us to use a similar approach for the characterisation of metallodrug–protein systems.

In a first study, we analysed the interactions of four classical platinum(II) drugs – namely cisplatin, its inactive isomer transplatin, carboplatin and oxaliplatin – with horse heart cytochrome *c* [48]. Under the applied experimental conditions, the four compounds turned out to exhibit a roughly similar pattern of reactivity with cyt *c*. This finding soon appeared of particular interest and novelty, being in striking contrast with current opinions concerning the comparative reactivity of the investigated platinum drugs. Indeed, the four platinum compounds that were selected for our study, are known to exhibit greatly different stability and reactivity patterns under physiological-like conditions [49–53]. For instance, carboplatin and oxaliplatin were reported to hydrolyse about 100-fold less rapidly than cisplatin [54,55]. The far higher stabilities of carboplatin and oxaliplatin are reflected in a lower reactivity with DNA and with other proteins [56–61]. Thus, the finding that all platinum compounds tested in our study produced substantially similar levels of cyt *c* platination looked very surprising. Accordingly, we hypothesized that cyt *c* should play a major role in enhancing the reactivity of the kinetically stable carboplatin and oxaliplatin compared to cisplatin and transplatin.

Representative ESI MS spectra of cyt *c* adducts with cisplatin or carboplatin are reported in Fig. 4. Samples were prepared in 25 mM tetramethyl ammonium acetate buffer (pH 7.4) by adding freshly prepared buffered solutions of the complexes to cyt *c* solutions. In all cases, the spectra are dominated by peaks of comparable intensity, corresponding to 1:1 Pt–cyt *c* adducts. Remarkably, a residual peak, of low intensity, corresponding to the free protein could still be observed at ~12368 Da; pointing out that nearly complete protein platination has truly occurred.

Afterward, we applied the same investigative approach to platinum adducts of HEWL [44]. Even in this case ESI MS measurements turned out extremely useful to monitor the process of metallodrug–lysozyme adduct formation

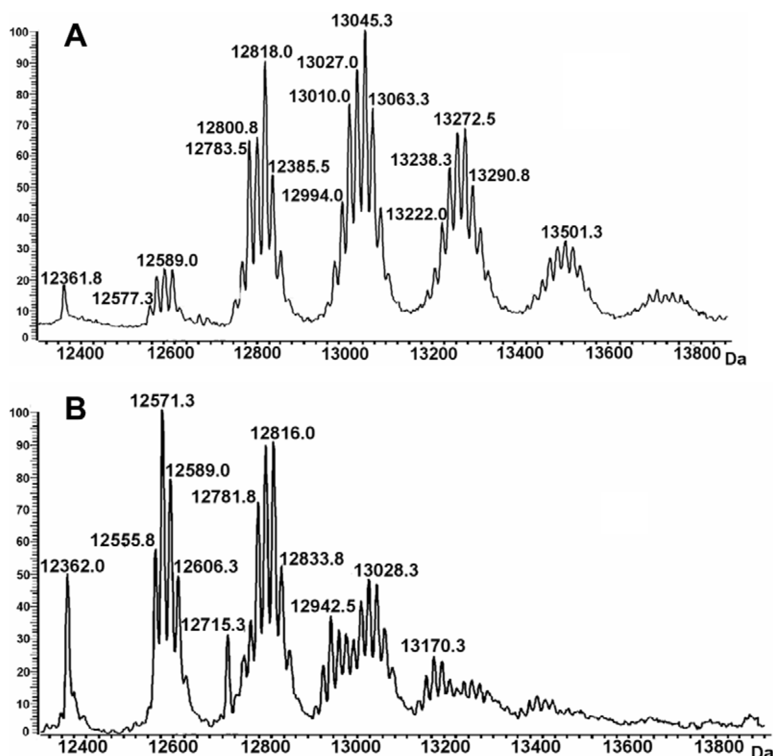

Fig. 4. Deconvoluted ESI MS spectra of the cisplatin (A) or carboplatin (B) adducts with cyt *c* in a 10:1 ratio in 25 mM tetramethyl-ammonium acetate buffer (pH 7.4), incubation time 168 h at 37 °C.

and elucidate the exact nature of the protein bound metallic fragments. We have learned that, under the employed solution conditions: (i) platinum–HEWL adduct formation is rather slow; (ii) that cisplatin is by far the most efficient platinum compound in producing HEWL platination; (iii) that mono-platinated species are by far the predominant ones, suggesting the presence of a highly preferential platinum binding site. Based on the crystal structure of cisplatin HEWL, reported above, this primary binding site was straightforwardly assigned to the imidazole ring of His 15. In addition, it is worth noting that the ESI MS spectrum of the HEWL cisplatin derivative (Fig. 5) shows two peaks of similar intensity at 14,569 and 14,605 Da that formally correspond to either $[Pt(NH_3)_2Cl]^+$ or *intact cisplatin* bound to the native protein. A similar situation was formerly described by Dyson and coworkers in the case of the cisplatin-transferrin system and interpreted in terms of a two-step cisplatin to protein binding process [9].

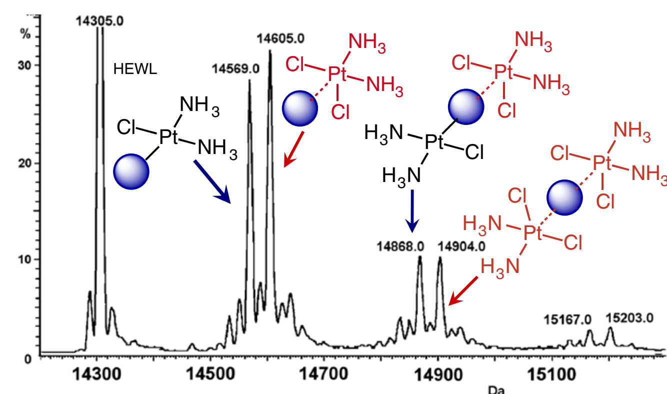

Fig. 5. Deconvoluted ESI MS spectrum of adducts formed between HEWL and cisplatin, after 48 h incubation at 37 °C. The initial ruthenium/protein stoichiometry of each sample is 3:1.

Very recently, we have extended the ESI MS approach to monitor the reactions of some novel anticancer platinum(II) iminoether complexes, namely *trans*- and *cis-EE* (*trans*- and *cis*-$[PtCl_2\{(E)\text{-}HN{=}C(OCH_3)CH_3\}_2]$, respectively) and *trans*- and *cis-Z* (*trans*- and *cis*-$[PtCl_2(NH_3)\{(Z)\text{-}HN{=}C(OCH_3)CH_3\}]$, respectively) (Chart 2), with

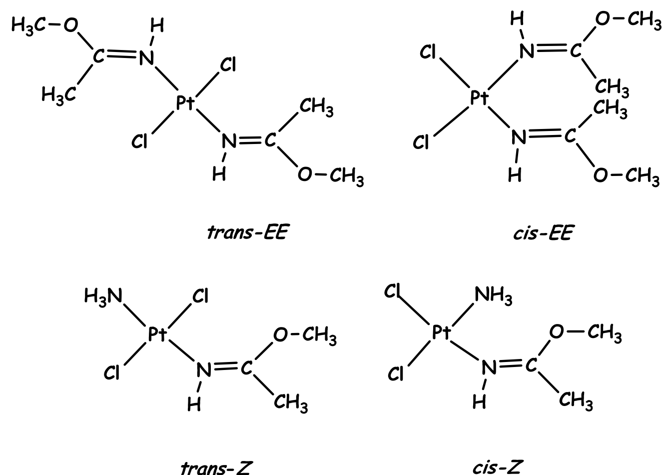

Chart 2. Schematic drawing of the selected platinum(II) iminoether complexes.

horse heart cytochrome *c* [62]. Our investigation was independently supported by NMR, ICP OES, and absorption electronic spectroscopies.

From this study, it has emerged very clearly that interactions with cyt *c* do profoundly alter the intrinsic reactivity of the various platinum iminoethers, leading to the observation of rather unexpected chemical reactions at the level of the platinum ligands. In addition, the kinetics of degradation of the platinum complexes could be measured and found to be largely affected by the interactions with this protein. Remarkably, a profoundly different pattern of reactivity was identified for the *trans* isomers with respect to the *cis* ones. Valuable information for the platinum binding site assignment was also achieved through a partial proteolysis experiment by using the endoproteinase Asp–N. The comparative analysis of the obtained results with those previously reported for classical platinum(II) anticancer drugs made us confident that Met 65 is the major binding site for platinum(II) iminoethers on cyt *c*.

Recently, in collaboration with the research groups of Paul Dyson in Lausanne and Bernhard Keppler in Vienna, we have carried out a comparative study on the applicability of nESI and MALDI techniques to the platinum ubiquitin system. In detail, this study analysed the interactions of oxaliplatin, cisplatin and transplatin with ubiquitin [63]. The advantages and disadvantages of the two alternative methods for studying metallodrug–protein interactions were comparatively assessed. For the first time, MALDI MS was found to be suitable to characterise platinum–protein adducts even though ESI MS still turned out to be the preferred method, especially in terms of a better preservation of intact metal-peptide species.

4.2. Ruthenium complexes

As the ESI MS method produced so valuable and informative results in the case of platinum–protein adducts, we thought that it might be pairwise effective for the characterisation of adducts formed between proteins and a few important non-platinum metallodrugs. Within this frame, we analysed the behaviour of a new class of Ru(II)–arene complexes, the so called RAPTA compounds, developed by Dyson and coworkers (see Chart 3). All these compounds share a common structural motif consisting of a ruthenium(II) center bound to both an arene (cymene in this case) and a 1,3,5-triaza-7-phosphaadamantane (PTA) ligand. They only differ in the nature of the ligands located at the two remaining coordination positions. The first representative member of this family is [Ru(η^6-cymene)(pta)Cl_2] (RAPTA-C). Notably, replacement of the two chloride groups with a variety of bidentate ligands [for instance, either oxalate – to form Ru(η^6-cymene)(pta)(C_2O_4) (oxalo-RAPTA) – or cyclobutane dicarboxylate – to give Ru(η^6-cymene)(pta)($C_6H_6O_4$) (carbo-RAPTA)] – greatly reduces the rate of aquation, thus modifying their overall solution behaviour, without affecting cytotoxicity [64]. The three investigated complexes essentially manifested a similar cell-growth inhibition activity against a number of representative cancer cell lines. The binding of a wide range of RAPTA derivatives to oligonucleotides was formerly studied but no direct correlation between oligonucleotide binding and cytotoxicity could be established [65,66]. This finding might suggest that protein targets are of great importance in producing the observed cytotoxic effects.

In our study the three "RAPTA" complexes were challenged with either cyt *c* or HEWL and the resulting reaction products analysed by ESI MS [67].

Remarkably, the obtained results could be subsequently confirmed by high resolution mass spectrometry measurements, carried out on an LTQ Orbitrap instrument (Thermo, San Jose, CA) equipped with a conventional ESI source. In Fig. 6 the observed and theoretical spectra of 8^+ charged state are shown for cyt *c* (I), cyt $c + [(\eta^6$-cymene)Ru] fragment (II) and cyt $c + [(\eta^6$-cymene)(pta)Ru] fragment (III). The obtained experimental data perfectly match theoretical expectations, thus confirming our hypotheses on the chemical nature of the resulting protein bound fragments.

Very recently, we have extended our ESI MS approach to the well known antimetastatic ruthenium(III) complex, NAMI A, developed by Mestroni, Alessio and Sava in Trieste. Some previous spectroscopic work had been devoted to the analysis of the interactions of NAMI A with typical serum proteins such as serum albumin and serum transferrin [68,69]. Although the main features of NAMI A/serum protein interactions were determined in those initial studies, molecular details of the binding processes could not be fully elucidated due to the relatively high molecular weight of the mentioned proteins and to failure in obtaining high resolution X-ray crystal structures for the resulting ruthenium–protein adducts.

Thus, we made reacting NAMI A either with cyt *c* or HEWL and monitored the resulting products through

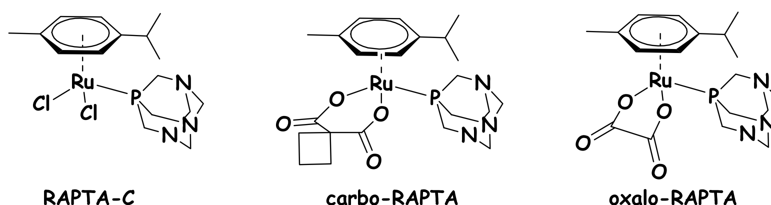

Chart 3. Schematic drawing of the selected ruthenium(II)-arene capped complexes.

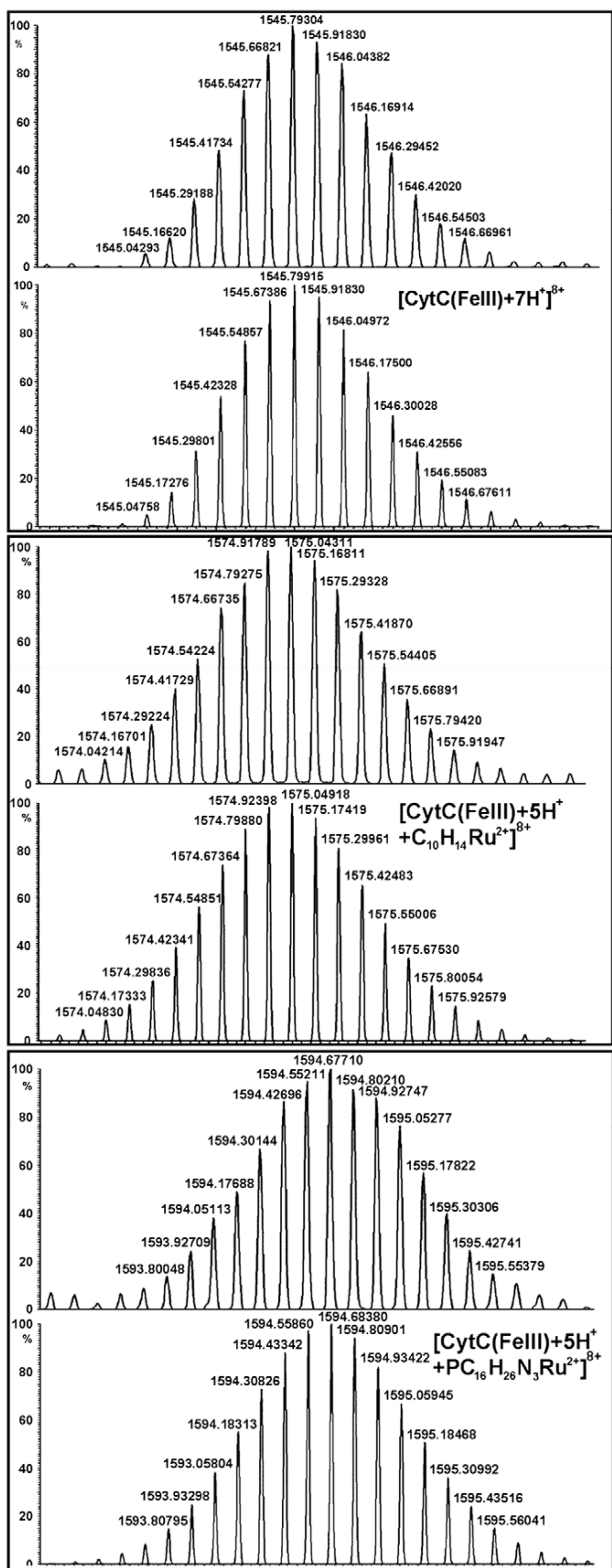

Fig. 6. Comparison between the observed (upper) and theoretical (lower) spectra of 8^+ charge state of cyt *c* (I) cyt *c* + [(η^6-cymene)Ru] fragment (II) and cyt *c* + [(η^6-cymene)(pta)Ru] fragment (III). Data were recorded with an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA).

ESI MS [70]. Quite unexpectedly, two substantially different modes of metallodrug–protein interaction clearly emerged in the two cases. In fact, lysozyme gave rise, predominantly, to non-covalent binding with either intact or mono-hydrolyzed NAMI A, most likely mediated by electrostatic interactions. Protein binding appeared to be largely reversible. On the whole, these interactions with lysozyme greatly slowed down the intrinsic degradation processes of NAMI A.

In contrast, cyt *c* was found to enhance appreciably NAMI A degradation by facilitating the progressive detachment of the various ligands from the ruthenium center. Most likely, this process is favoured by an initial electrostatic interaction between the negatively charged NAMI A "core" and this small basic protein (indeed, similarly to lysozyme, cyt *c* is a highly cationic protein at physiological pH with a p*I* of ~9.59 which is very prone to interact with anions) [71]. Such initial interaction is then progressively replaced by coordinative binding of the ruthenium(III) center to the protein. Eventually, a highly degraded ruthenium containing species, in which most of the original ligands have been lost, was found to remain attached to the protein. The masses of the various protein-bound ruthenium-containing fragments could be determined, in most cases, with high resolution, and the fragments tentatively assigned to specific molecular structures.

It is worth mentioning that we also monitored the reactivity of NAMI A with the above proteins through a variety of independent physico-chemical methods including optical spectroscopy, ^{1}H NMR and ICP OES. The combined use of the mentioned analytical techniques complemented and essentially confirmed the information obtained through ESI MS.

In addition, we found that NAMI A facilitates progressive reduction of ferric cytochrome *c* to the corresponding ferrous species. This finding was quite unexpected being NAMI A a ruthenium(III) compound. The mechanistic details of this redox process are still unclear, but it is reasonable to assume that reduction is mediated by ruthenium binding to a specific protein binding site. Gray and coworkers had previously reported on the formation of a stable pentaammineruthenium(III)-histidine-33 complex as the main product resulting from the reaction of aquopentaammineruthenium(II) with horse heart ferricytochrome *c* [72,73]. In view of the known affinity of ruthenium(III) for histidine residues it is tempting to propose His 33 represent the most likely anchoring site for NAMI A on cyt *c*.

4.3. Gold complexes

The renaissance of interest for gold compounds as potential anticancer metallodrugs has resulted, in the course of the last decade, in the synthesis of a number of structurally diverse gold(I) and gold(III) species, endowed with sufficient chemical stability and with relevant antiproliferative activities. Most of the mechanistic studies carried

out on cytotoxic gold compounds were generally referred and compared to the behaviour of cisplatin, for which DNA is thought to be the major target.

However, it has emerged quite clearly from the experimental results collected so far that the respective molecular mechanisms are rather distinct. Overall, these mechanistic studies suggest that alternative biochemical processes must be operative, most likely associated to selective modification of some crucial proteins. In this respect, it is worth noting that gold(I) and gold(III) compounds are known to target, rather strongly and selectively, thiol and imidazole groups of proteins (as well as selenol groups) [74].

Specifically, in the case of gold(III) compounds, we have obtained interesting ESI MS results for a series of six dinuclear oxo gold(III) complexes with bipyridyl ligands (Chart 4), of general formula $[Au_2(N,N)_2(\mu\text{-}O)_2][PF_6]_2$ [where N,N = 2,2′-bipyridine (Auoxo1), 4,4′-di-*tert*-butyl-(Auoxo2), 6-methyl-(Auoxo3), 6-neopentyl-(Auoxo4), 6-(2,6-dimethylphenyl)-(Auoxo5), 6,6′-dimethyl-2,2′-bipyridine-(Auoxo 6)], that had earlier been reported to exhibit cytotoxic properties against the A2780 human ovarian carcinoma cell line [75].

ESI MS spectra were recorded after reacting cyt *c* with the various Auoxo complexes (Fig. 7), working at 1:1 Auoxo/cyt *c* ratios. After 12 h incubation, cyt *c* was extensively ultrafiltered against the ammonium carbonate buffer, thus removing unreacted gold species, and the ESI MS spectra of the upper fractions recorded. In all cases, the final deconvoluted ESI MS spectra provided clear evidence of adduct formation. Remarkably, a number of peaks were observed corresponding to formal binding to the protein of a number of Au^+ ions (ranging from 1 to 4). A similar behaviour had previously been reported by Sadler and coworkers for the adducts of gold(I) triethylphosphine chloride with cyclophilin [41].

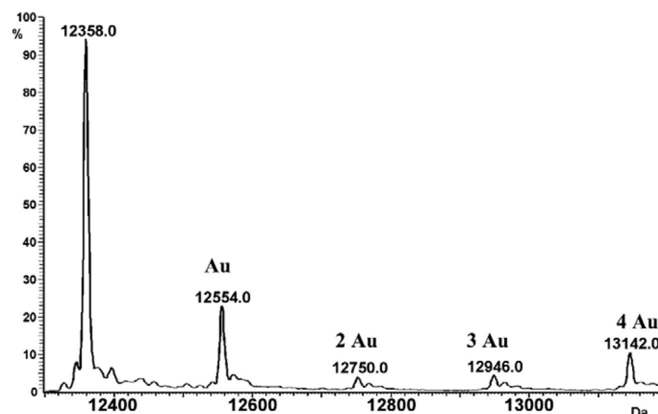

Fig. 7. Representative deconvoluted ESI MS spectral profile for cytochrome *c* adducts with Auoxo1.

It is remarkable that no sign of the bipiridyl ligand coordinated to gold was found anymore implying that the reduction process causes complete disruption of the starting dinuclear compound with cleavage of the oxo-bridges, release of the bipyridyl ligand and protein binding of the isolated gold ions.

Similar studies are now in progress on the interactions of auranofin with another model protein, the zinc–enzyme carbonic anhydrase and with the target enzyme thioredoxin reductase.

5. Final remarks: what we have learned

The application of X-ray diffraction and ESI MS techniques for the characterisation of metallodrug–protein

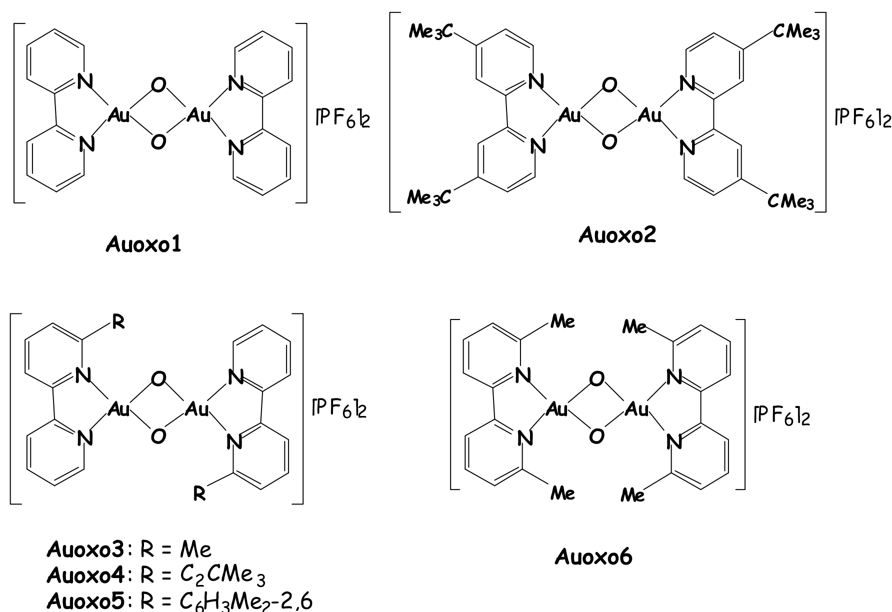

Chart 4. Schematic drawings of the dinuclear gold(III) complexes Auoxo. Auoxo3 is a ca. 1:1 mixture of the *cis* and *trans* isomer while Auoxo4 and Auoxo5 are, as depicted, only *trans* isomers.

adducts has undoubtedly allowed a significant progress in this specific research area. Indeed, thanks to the results provided by these two potent physicochemical methods, the molecular mechanisms of the reactions of metallodrugs with protein targets could be elucidated at least in selected cases with a high accuracy.

As anticipated above, the two considered approaches are independent but highly complementary in that X-ray crystallography provides a full description of the "*static*" structure of the metallodrug–protein adducts while ESI MS offers valuable information on the temporal evolution of these adducts. Putting together these two pieces information usually results in an exhaustive structural and functional picture of the analysed systems and of the underneath reactions. Notably, one major limitation of the described approach resides in the intrinsic difficulty to obtain good quality crystals suitable for X-ray diffraction analysis. Indeed, crystal structures of metallodrug–protein have been solved until now only in a very limited number of cases. In these cases, i.e. in the absence of crystallographic data, ESI MS may be complemented by independent information achieved by application of other physico-chemical techniques such as NMR. Another limitation is represented by the fact that the two mentioned techniques have rather different solution requirements that might affect in some cases the resulting reactivity and the nature of the adducts themselves.

In any case, on the ground of the few examples reported above, a rather satisfactory and comprehensive description of the typical reactivity of metallodrugs with protein targets can be truly achieved that might be of broader validity. The main features of such reactivity are outlined below.

Metallodrugs behave very often as *pro-drugs*. This means that they must necessarily undergo an activation step, in most cases a simple aquation reaction, before they can react with protein targets. Generally, this activation step represents the rate limiting step for reactions with biomolecules and has been exploited accordingly; however, we have also shown that the kinetics of this activation step may be greatly influenced by a direct interaction of the metallodrug with the protein itself. Apparently, this is the reason for the unexpectedly similar kinetic profiles found in the reactions of carboplatin and cisplatin with cytochrome *c*, as earlier mentioned.

The resulting "*activated metallodrugs*" usually contain a weakly coordinated water molecule that is easily removed and replaced by a stronger ligand provided by the protein itself. Only a few protein sidechains commonly perform this function in the presence of the rather "soft" metal ions here considered; they are mainly histidines, methionines, cysteines through a nitrogen or a sulphur donor. This second ligand substitution event leads to the formation of the so called metallodrug/protein complex or adduct, the main object of our investigations. Notably, both X-ray diffraction and ESI MS studies converge in showing that, in spite of the large number of potential donors on the protein surface, adduct formation takes place preferentially only in a few positions, implying a rather high selectivity in metal binding. In all cases the preferential choice of some amino acidic residues with respect to others, could be determined by different parameters. A crucial factor might be the accessibility of the residue on the protein surface and steric/electronic effects ascribable to either the metal's donor sets or the protein side-chains. In this respect, it is worth mentioning that the reactivity of a certain metal complex is modulated not only by the physiochemical properties of the metal itself, but also by the protein microenvironment (see for example the above mentioned results on the cisplatin/SOD adduct).

The functional characterisation of the newly formed entities – *i.e* the metallodrug protein adducts – is of extreme importance in relation to their possible biological roles. If the adduct is shown to be devoid of any further reactivity, we can assess quite safely that the reaction has indeed led to inactivation of the metallodrug (provided that the protein itself is not an important biological target). Conversely, if the adduct conserves the capacity of further reacting with other biomolecules and/or of transferring the metallic fragment to other species, one can state that the formed adduct is still a biologically active species and that it might also serve as a reservoir of the metallodrug itself. The ESI MS method is very appropriate for this specific purpose. Notably, the pioneering ESI MS studies by Dani Gibson and coworkers unambiguously showed that the platinum ubiquitin system retains an appreciable capacity of reacting with either glutathione or nucleobases, pointing out that formation of such adduct does not necessarily imply metallodrug inactivation. On turn, we have shown, in our study, a residual important reactivity for NAMI A after binding to cytochrome *c*.

Overall, the studies we have carried out so far on metallodrugs and model proteins permit a rather accurate characterisation of their interaction modes at a molecular level. Of course, when carrying out this type of investigations, one must never forget that just very simplified systems were selected and analysed. Indeed, in most cases, the various biophysical studies were conducted on simple two component systems containing a *single metallodrug* and *a single protein*. Moreover, the investigated protein was, in most cases, a model protein, usually being of moderate to low molecular weight, water soluble, stable and easy to manipulate.

Real cellular systems are of course extremely more complicated as they may contain thousand of different proteins, at highly variable concentrations, and a also conspicuous number of low molecular weight components, most of them in relatively high concentration. In addition, these various components are highly organised and compartmentalised within the various intracellular structures thus offering a peculiar spatial distribution inside cells. It is obvious that studying the interaction of metallodrugs with proteins inside cells represents today a formidable challenge for researchers. Nonetheless, we believe that the reactivity patterns that we have formulated for the simplified

systems may represent a good starting point for the study of cellular systems. In our opinion it is mandatory to extend as soon a possible the biophysical studies we have described above to systems of higher complexity in order to ascertain the extent to which the reactivity models defined in purified and simple systems conserve their validity within the real cellular world. A possible compromise between these two extremes might be given by the investigation of the behaviour of metallodrugs within cell homogenates; the new and potent analytical methods offered by the so called "omic sciences", for instance the methods of metallomics may be of great advantage for these further advancements.

6. Abbreviations

carbo-RAPTA Ru(η^6-cymene)(1,3,5-triaza-7-phosphaadamantane)($C_6H_6O_4$)
cis-EE *cis*-[$PtCl_2${(*E*)-HN=C(OCH_3)CH_3}$_2$]
cis-Z *cis*-[$PtCl_2$(NH_3){(*Z*)-HN=C(OCH_3)CH_3}]
cyt *c* horse heart cytochrome *c*
ESI electrospray ionization
HAD Heavy Atom Databank
HEWL hen egg white lysozyme
ICP OES inductively coupled plasma optical emission spectroscopy
Ind indazole
KP1019 indazolium *trans*-[tetrachlorobis(1H-indazole) ruthenate(III)]
MALDI matrix-assisted laser desorption/ionization
MS mass spectrometry
NAMI A (H2im)[*trans*-RuCl4(Him)(dmso)]
nESI nano electrospray ionization
oxalo-RAPTA Ru(η^6-cymene)(1,3,5-triaza-7-phosphaadamantane)(C_2O_4)
PEt_3 triethylphosphine
p*I* isoelectric point
pta 1,3,5-triaza-7-phosphaadamantane
RAPTA ruthenium(II)-cymene-based complex
RAPTA-C Ru(η^6-cymene)(1,3,5-triaza-7-phosphaadamantane)Cl_2
SOD superoxide dismutase
trans-EE *trans*-[$PtCl_2${(*E*)-HN=C(OCH_3)CH_3}$_2$]
trans-Z *trans*-[$PtCl_2$(NH_3){(*Z*)-HN=C(OCH_3)CH_3}]
Ub ubiquitin

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