Interactions between anticancer *trans*-Pt compounds and proteins: Crystal structures and ESI mass spectra of two protein adducts of -*trans*-dimethylamine methylamine dichlorido platinum(II)

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ABSTRACT: *The adducts formed between trans-dimethylamine methylamine dichlorido platinum(II), [t-$PtCl_2$(dma)(ma)], and two model proteins i.e. hen egg white lysozyme (HEWL) and bovine pancreatic ribonuclease (RNase A), were independently characterized by X-ray crystallography and ESI mass spectrometry. In these adducts the Pt(II) center, upon chloride release, coordinates either to His or Asp residues while both alkyl amino ligands remain bound to the metal. Comparison with the cisplatin derivatives of the same proteins highlights for [t-$PtCl_2$(dma)(ma)] a kind of biomolecular metalation remarkably different from cisplatin. The implications of these results are briefly discussed.*

Since the discovery of the antitumor activity of cisplatin and the establishment of the so called "Hoeschele's rules" defining structure-activity relationships in cytotoxic platinum(II) complexes, the presence of a *cis* configuration with two adjacent leaving ligands has been considered an absolute requirement for the anticancer action. For this reason, *trans*-Pt compounds have long been reputed as uninteresting and largely neglected afterward. This trend was reversed in more recent times when it was realised that a number of *trans*-Pt compounds, including *trans*-[$PtCl_2${EHN=C(OCH_3)CH_3}$_2$], *trans*-[$PtCl_2$(NH_3) (thiazole)], *trans*-dimethylamine methylamine dichloro Pt(II) [*t*-$PtCl_2$(dma)(ma)] and *trans*-isopropylamine dimethylamine dichlorido platinum(II) [*t*-$PtCl_2$(ipa)(dma)] manifest remarkable cytotoxic properties towards several human tumor cell lines[1-3]. It was found that the presence of bulky amino ligands within *trans* square planar platinum(II) configuration leads to a net decrease in reactivity with biomolecules, commonly associated with a higher antitumor activity in vitro[3].

Interactions of Pt-containing drugs with proteins are believed to play key roles in drug resistance processes and also in the mechanism of action of this class of compounds[4-6]. However, the formation of adducts between *trans*-Pt(II) compounds and proteins and their biological consequences have been poorly investigated so far[4-6].

To elucidate this issue in more depth, we describe here the X-ray structure of the HEWL/[*t*-$PtCl_2$(dma)(ma)] derivative and that of the adduct formed between the same Pt compound and RNase A. We have chosen these two proteins since they have been used for more than half a century as model systems for studies on protein chemistry, including metalation caused by metal-based bioactive agents[7,8], like cisplatin[7a,8a.] The structural characterization is supported and completed by independent ESI mass spectrometry measurements.

Crystals of the HEWL/[*t*-$PtCl_2$(dma)(ma)] adduct were obtained by cocrystallization experiments, using 0.6 M $NaNO_3$, 20% ethylene glycole and 0.1 M sodium acetate, at pH 4.2, as reservoir solution. Details of the crystallization procedure, data collection, structure determination and refinement statistics are given in the SI, Table S1 and S2. The structure, which has been solved at 2.5 Å resolution, refines to R factor = 17.9 (Rfree = 25.9). The overall HEWL structure in the adduct is very similar to that of the ligand-free enzyme (PDB code 193L)[9]: the root mean square deviation in positions of CA atoms is as low as 0.3 Å. The inspection of the electron density maps revealed platination of the only HEWL histidine residue, *i.e.* His15, at imidazole NE2 atom (at the so-called left-handed site)[7a] (Figure 1). Pt(II) has an occupancy equal to 0.8 and is also bound to other three light atoms, which have been interpreted as two nitrogens of two amine moieties and to a water molecule. The His(N)-Pt distance is 2.3 Å, whereas Pt-N distances are close to 2.0 Å. The geometry of this site is significantly distorted.

The X-ray structure also reveals the presence of an additional binding site close to Asp101 (Figure 1). In this site, Pt(II) has an occupancy of 0.35. The Asp(OD1)-Pt distance is 2.1 Å, whereas Pt-N distances are close to 2.0 Å.

In both Pt binding sites, the quality of the electron density maps does not permit to gain conclusive information on the

nature of Pt ligands. Therefore, electrospray ionization (ESI) mass spectra were registered to obtain further, independent information on the same adduct. In particular, HEWL was dissolved in a suitable buffer (20 mM ammonium acetate buffer, pH 6.8) and treated with a 3:1 excess of a fresh solution of the Pt compound. Then, ESI mass spectra were recorded after increasing time intervals (Figure 2a). Formation of metallodrug-protein adducts is well witnessed by the appearance of two main peaks of greater molecular mass than the native protein. Interestingly, these two peaks -that may be attributed to the same platinum adduct- show values of 14609.83 and 14633.87 Da (i.e. the previous peak plus a Na^+ ion), suggesting the formation of an adduct where the Pt compound loses one Cl ligand, retaining the dma and ma ligands. Adduct formation is nearly complete after the first 24 h; no relevant further changes are detected at 72 h.

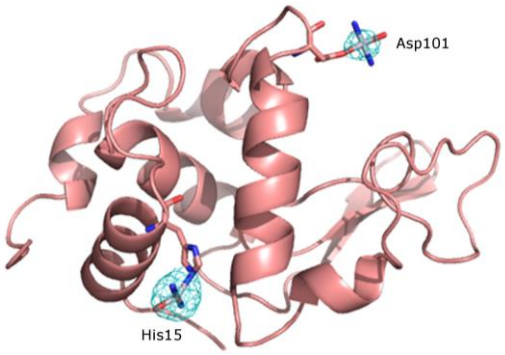


Figure 1. Cartoon representation of the adduct between HEWL and *t*-$PtCl_2$(dma)(ma). The side chains of His15 and Asp101, which are exposed to the solvent, are shown along with Pt and its ligands. 2Fo-Fc electron density maps are contoured at 3σ level. The structure has been deposited in the Protein Data Bank under the accession code 4QGZ.

A similar approach was used to monitor the interactions of [*t*-$PtCl_2$(dma)(ma)] with RNase A. Crystals of the metallodrug-protein complex were obtained by soaking experiments. Ligand-free RNase A crystals grown by hanging drop vapour diffusion method using 20% PEG4000, 10 mM sodium citrate pH 5.0 and protein concentration 20 mg mL^{-1} were incubated for five days with an excess of the Pt compound at 1:10 protein to metal molar ratio. X-ray diffraction data were collected on these crystals at 2.00 Å resolution. Details of crystallization, data collection and structure refinement are given in the SI and in Tables S1 and S2.

The structure of this adduct, which contains two molecules in the asymmetric unit (Figure S1), refines to Rfactor = 21.4 (Rfree = 27.5). The overall structure of the RNase A molecules in the complex is very similar to that of the ligand-free protein (PDB code 1JVT)[10]: the root mean square deviations in CA atom positions are in the range 0.4-0.6 Å. Platination just induces some conformational disorder of residues 16-22 in molecules A and B.

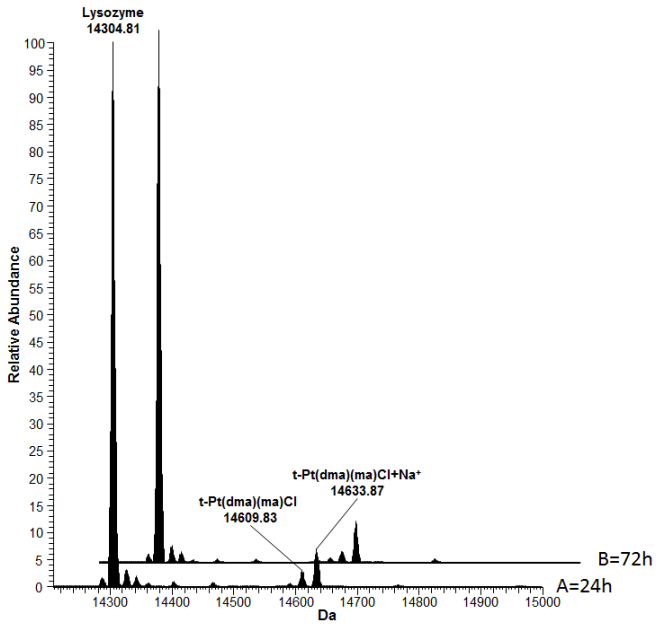
In both molecules, platination occurs at the NE2 atom of the imidazole ring of His105 (occupancy = 1.0) and at the ND1 atom of His48 (occupancy = 0.3 and 0.4 for molecule A and B, respectively). Moreover, platination also occurs at His119 *i.e.* at the enzyme active site (Figure 3A). In molecule A, platination occurs at ND1 and NE2 atoms of imidazole ring of His119 (occupancy 0.3 and 0.4, respectively), which adopts two conformations. Also in molecule B platinated His119 adopts two distinct conformations and in both cases Pt binds NE2 atom (Pt occupancy 0.5 and 0.5, respectively).

Notably, the whole Pt-compound has been modelled at the His105 binding site (Figure 3b). In this site, His(N)-Pt distance is equal to 2.3 Å, whereas Pt-N distances are close to 1.9 Å. The fourth loosely coordinated ligand of the Pt, placed at 2.6 Å, has been modelled as a water molecule, suggesting that the reactive species, in the conditions used to obtain RNase A/[*t*-$PtCl_2$(dma)(ma)] adduct, is the product of the hydrolysis of *t*-$PtCl_2$(dma)(ma). In the other binding sites, a water molecule is found 2.8-3.0 Å far apart from the metal.

A direct comparison between the structure of the RNase A/[*t*-$PtCl_2$(dma)(ma)] derivative and that of the same protein in complex with cisplatin[8a] demonstrates that *trans* Pt-compounds are able to induce a type of protein metalation remarkably distinct from that inferred by cisplatin. In fact, we have shown that cisplatin binds RNase A through Met29 coordination.[8a] It seems that [*t*-$PtCl_2$(dma)(ma)] is not able to bind Met29 probably due to the greater steric hindrance of the alkyl amine groups compared to amino groups. These results are in line with previous studies showing that transplatin and cisplatin have distinct binding sites upon reaction with ubiquitin.[11]

Even in the case of RNase A, ESI-MS analyses have been carried out. The ESI mass spectra show two main peaks at 14011.31 and 14340.38 Da (Figure 2b) that were attributed to mono and bis coordination, respectively, of the same metallic fragment where the Pt center loses a single chloride ligand (in both cases these peaks are accompanied by the respective Na^+ species).

a



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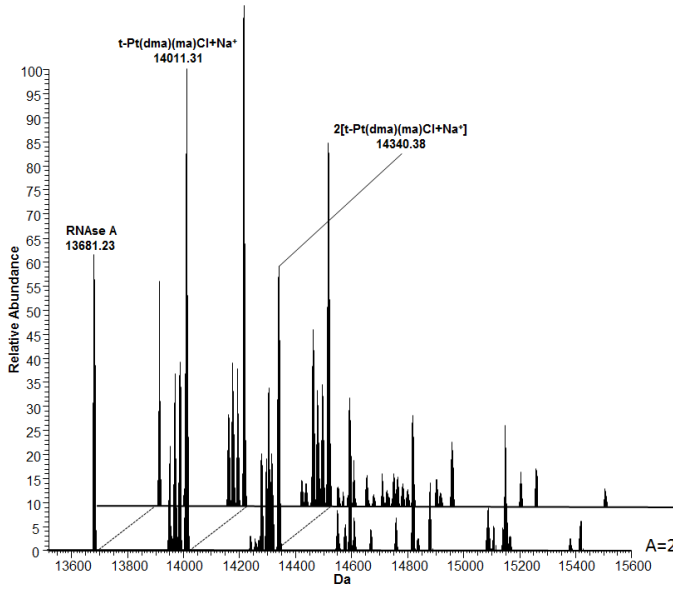
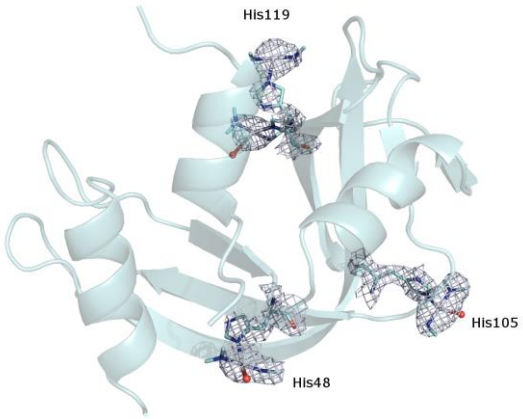


Figure 2. Deconvoluted ESI mass spectra of 10^{-4} M HEWL (a) and RNase (b) treated with [*t*-$PtCl_2$(dma)(ma)] (metal:protein ratio = 3:1) in 20 mM ammonium acetate (pH 6.8) buffer recorded after 24 (A) and 72h (B) of incubation at 37°C.

In spite of the fact that the same type of Pt coordination is afforded with the two proteins, a greater amount of adduct is formed in the case of RNase A (see Figure 2).



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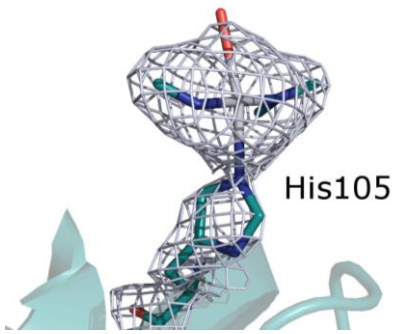

b

Figure 3. a) Cartoon representation of the adduct between RNase A and *t*-$PtCl_2$(dma)(ma). The side chains of His48, His105 and His119 are shown along with Pt and its ligands. The structure has been deposited in the Protein Data Bank under the accession 4QH3. b) A zoom on His105 of molecule A. 2Fo-Fc electron density maps are shown at 1.5 σ level. A scheme showing the coordination around central Pt ion after coordination to His105 is reported in SI (Figure S2).

Although some differences are found upon comparing data obtained from ESI mass spectra with those from X-ray diffraction experiments (which may be easily accounted for upon considering the different conditions of the two experiments); the obtained results demonstrate that alkyl amine ligands are invariantly retained upon reacting the platinum drug with proteins.

In conclusion, we have offered here unambiguous structural evidence that the *trans*-dimethylamine methylamine dichloro Pt(II) complex gives rise to stable adducts with two different model proteins. These adducts are formed through His or Asp binding of a [Pt $(RNH_2)_2Cl]^+$ or [Pt $(RNH_2)_2OH_2]^{2+}$ fragment (Figure S2), *i.e.* the products of the hydrolysys of the original compound in water. These structures grow the crystallographic repertoire of Pt adducts with proteins and represent the first high resolution characterization for adducts formed between *trans*-Pt compounds and proteins. Our data in combination with previous ones[3] reveal that *trans*-Pt compounds do bind proteins with full retention of the alkyl amine Pt ligands. The retention of nitrogen ligands in the reaction of *trans*-Pt complexes with proteins is in agreement with a recent study describing the interactions of other *trans*-Pt compounds with the Atox-1 protein[12].

As expected, based on results from our previous studies of interactions between this Pt complex with oligonucleotides [13], this kind of biomolecular metalation may result in different patterns of DNA platination when compared to cisplatin and its analogues and, accordingly, in a different pharmacological profile.

ASSOCIATED CONTENT

Supporting Information

Materials and analytical techniques, crystallization, X-ray data collection, structure solution and refinement, ESI-MS, Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors; they have approved the final version of the manuscript.

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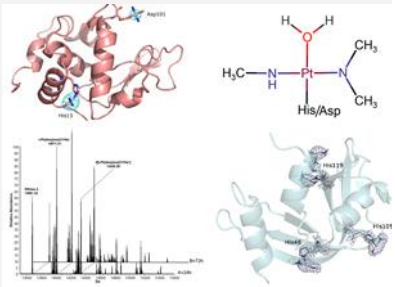
Metal Based Drugs

Luigi Messori, Tiziano Marzo, Elena Michelucci, Irene Russo Krauss, Carmen Navarro-Ranninger, Adoracion G. Quiroga, and Antonello Merlino**

Interactions between anticancer *trans*-Pt compounds and proteins: Crystal structures and ESI mass spectra of two protein adducts of -trans-dimethylamine methylamine dichlorido platinum(II).



Reactivity of *trans*-$PtCl_2$(dma)(ma) with HEWL and RNase A was investigated by X-ray crystallography and ESI-MS. In the resulting adducts the Pt(II) center selectively coordinates to His or Asp residues, retaining alkyl amine ligands.