VIP Anticancer Agents

DOI: 10.1002/anie.200703271

Interaction between Platinum Complexes and a Methionine Motif Found in Copper Transport Proteins**

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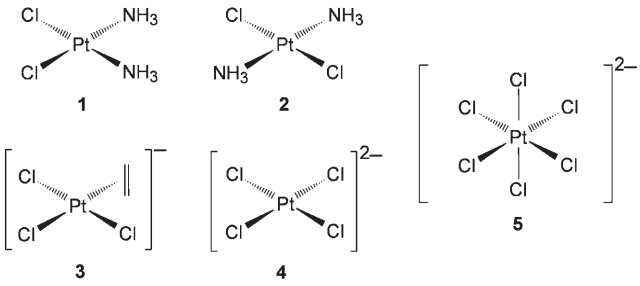
Cisplatin (*cis*-$[PtCl_2(NH_3)_2]$, *cis*-DDP) and other platinum complexes used in anticancer therapy, such as carboplatin and oxaliplatin, are able to cross the cell plasma membrane and eventually reach the nucleus, where they form adducts with DNA that are at the basis of their antitumor activity.[1,2] Early studies suggested that *cis*-DDP enters the cell mainly by passive diffusion.[3] However, more recently, a growing body of evidence has revealed an alternative route for *cis*-DDP uptake involving the high-affinity copper transporter Ctr1, a permease located on the plasma membrane and constituted by three transmembrane helices, an extracellular N-terminal domain, and a cytosolic C-terminal domain.[4] Three Ctr1 molecules form a symmetrical trimer with a channel-like architecture, as revealed by electron microscopy.[5] Methionine (Met)-rich motifs located in the N-terminal domain and in the inner side of the channel pore are critical for the binding of copper[6] (Supporting Information). In the present work, we tested whether the same Met-rich motifs are also able to bind platinum complexes, as a first step in understanding the chemical mechanisms of cellular transport of platinum anticancer drugs by Ctr1.

For this purpose we used the octapeptide MTGMKGMS (Mets7 hereafter), highly similar to the seventh Met-rich motif of yeast Ctr1 (MSGMSGM), for which the interaction with Cu^{I} has already been characterized by electrospray mass spectrometry (ESI-MS) and UV/Vis spectroscopy.[7] We investigated the coordination properties of Mets7 towards the platinum complexes shown in Scheme 1.

The stoichiometry of the adducts was determined by ESI-MS. When *cis*-DDP is added to Mets7, a peak at *m*/*z* 557 is observed soon after mixing, corresponding to the doubly charged species $[Mets7H^{+} + PtCl^{+}]$. After 24 h, no apopeptide peak (*m*/*z* 442) is left, and the prevalent species in solution is $[Mets7 + Pt(OH_2)^{2+}]$ (*m*/*z* 548), which eventually converts into $[Mets7 + Pt^{2+}]$ (*m*/*z* 539). Therefore, the Pt^{II} ion loses all the original ligands, which are replaced by Mets7 residues (Figure 1a). When *trans*-DDP is added to Mets7, a peak at *m*/*z* 556, corresponding to $[Mets7 + Pt(NH_3)_2^{2+}]$, is observed. The intensity of this peak increases with time at the expense of the apopeptide peak up to complete conversion and then no further changes are observed over a period of days (Figure 1b). Isotopic distributions of the peaks at 557



Scheme 1. Platinum complexes used in this investigation.

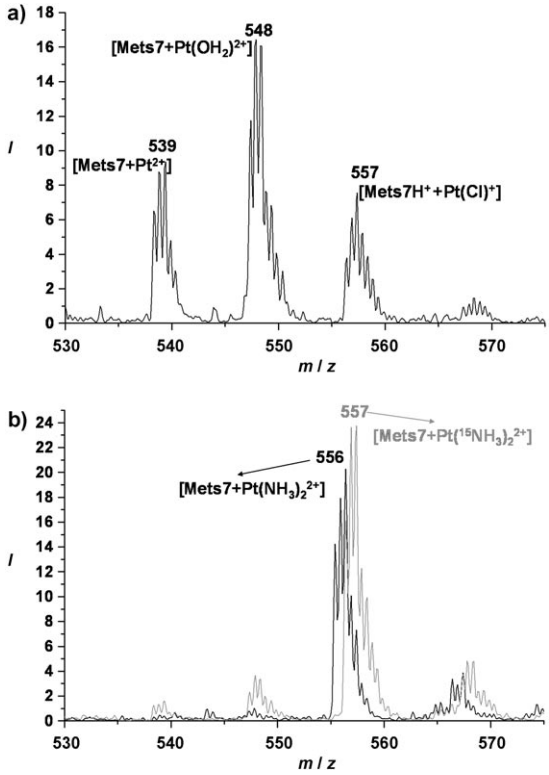


Figure 1. ESI-MS spectra of the reaction mixtures of Mets7 with Pt^{II} complexes 24 h after mixing: a) *cis*-DDP/Mets7 1:1; b) *trans*-DDP/Mets7 1:1 (in black) and ^{15}N-enriched *trans*-DDP/Mets7 1:1 (in light gray). All doubly charged peaks are shown.

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[**] We thank Dr. F. P. Intini for providing ^{15}N-labeled *cis*-DDP and *trans*-DDP, Dr. C. R. Barone and Prof. L. Maresca for providing the Zeise's salt, and G. Di Pinto for recording mass spectra. We thank the University of Bari, the Consorzio Interuniversitario di Ricerca in Chimica dei Metalli nei Sistemi Biologici (CIRCMSB), the Italian Ministero dell'Università e della Ricerca (PRIN 2005 032730), and the European Commission (COST Action D39) for support.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

(*cis*-DDP) and 556 (*trans*-DDP) confirm their different composition (Supporting Information).

The reaction of Mets7 with $[PtCl_3(\eta^2\text{-}C_2H_4)]^-$ (Zeise's anion) or $[PtCl_4]^{2-}$ proceeds fast, as expected for Pt^{II} complexes with good *trans*-labilizing ligands (i.e. C_2H_4 and Cl^-, respectively). The apopeptide completely disappears after around 12 h, and the final adduct is [Mets7 + Pt^{2+}], as in the case of *cis*-DDP. In contrast, the $[PtCl_6]^{2-}$ complex does not form significant adducts with Mets7, and after one week only a very weak peak at *m*/*z* 574 corresponding to [Mets7 + $PtCl_2^{2+}$] can be detected. However, when ascorbate is added to the mixture, Pt^{IV} is reduced to Pt^{II} and the reaction proceeds fast as in the case of $[PtCl_4]^{2-}$ (data not shown).

The peptide conformation was revealed by circular dichroism (CD) spectroscopy. In the far-UV region, free Mets7 shows a negative band at 195 nm which is characteristic of an unstructured peptide. Addition of one mol equivalent of *cis*-DDP produces a substantial change, namely the sudden appearance of a positive band at 200 nm, corresponding to a pure β-turn-type structure[8] (dotted/dashed line in Figure 2).

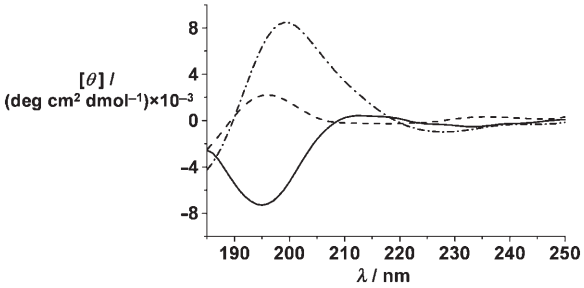

Figure 2. Far-UV CD spectra of Mets7 in the absence of platinum complex (solid line) and in the presence of *cis*-DDP (dashed/dotted line) and *trans*-DDP (dashed line) 24 h after mixing.

In contrast, when *trans*-DDP reacts with Mets7, the structuring effect induced by the metal ion is moderate, as inferred from the appearance of a weak positive band at 195 nm, which indicates only a slight conformational modification of the peptide (Figure 2). Therefore, the CD data appear to reflect the different reactivity of *cis*-DDP and *trans*-DDP towards Mets7: *trans*-DDP, which retains its N-donor ligands, causes a moderate conformational change, whereas *cis*-DDP, whose ligands are completely replaced by Mets7 residues, influences more deeply the peptide conformation.

The peptide amino acids involved in platinum coordination were investigated by 2D ^1H,^{15}N- and ^1H,^{13}C-edited HSQC (Supporting Information), ^1H,^1H NOESY, and ^1H,^{13}C HSQC-TOCSY NMR spectroscopy. Binding of *cis*-DDP and *trans*-DDP to Mets7 causes a large downfield shift of the methionine ε-CH_3 and γ-CH_2 signals and affects, to a much smaller extent, a few resonances of Thr2 and Lys5 (Supporting Information). The shifts are similar but not identical for the three methionines; therefore, the ε-CH_3 signals, which are overlapping in apoMets7, split into various signals upon metal coordination. One cross-peak appears at $\delta = 2.4$ ppm, and the other cross-peaks are clustered around $\delta = 2.5$ ppm.

The fate of the ammine ligands in the reaction of ^{15}N-enriched *cis*-DDP or *trans*-DDP with Mets7 was monitored by ^1H,^{15}N HSQC NMR spectroscopy.[9] Addition of Mets7 to a solution of *cis*-DDP causes disappearance of the signal of the dichlorido species (almost complete after around 24 h) and, initially, appearance of cross-peaks relative to intermediate species with $^{15}NH_3$ *trans* to S of methionines ($\delta^{15}N = -43$ and -45 ppm, Supporting Information). The intensities of these latter peaks decrease with time in accord with the complete loss of the $^{15}NH_3$ ligands from the complex. In the case of ^{15}N-enriched *trans*-DDP, the ^{15}N NMR chemical shift remains practically unchanged as expected for substitution reactions involving only the chlorido ligands. However, the amminic ^1H signals undergoes quite a large downfield shift.

^{195}Pt NMR chemical shifts are very sensitive to the nature, number, and geometric arrangement of the coordinated ligands.[9,10] The 2D ^1H,^{195}Pt HMQC spectrum recorded 48 h after mixing Mets7 with ^{15}N-enriched *cis*-DDP (1:1 molar ratio) exhibits a $^{195}Pt/^1H$ cross-peak ($-4155/2.5$ ppm, Figure 3 a) stemming from coupling between the Pt^{II} ion and the

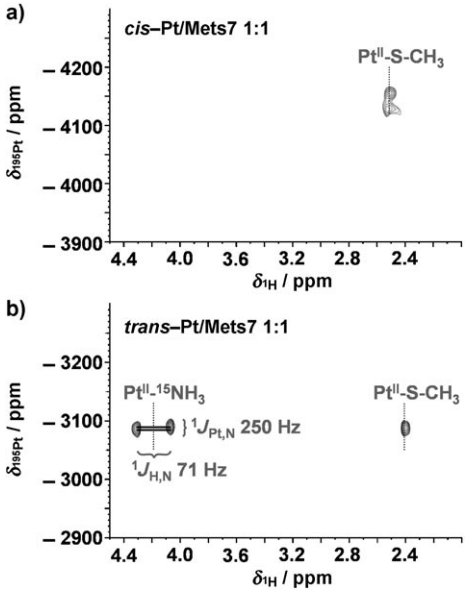

Figure 3. 2D ^1H,^{195}Pt HMQC NMR spectra of the reaction mixtures of Mets7 with Pt^{II} complexes 48 h after mixing: a) ^{15}N-enriched *cis*-DDP/Mets7 1:1; b) ^{15}N-enriched *trans*-DDP/Mets7 1:1. Resonance assignment and $^1J_{Pt,N}$ and $^1J_{H,N}$ coupling constants are indicated.

methyl protons of Mets7 methionines. Moreover, the chemical shift of ^{195}Pt is consistent with a $(N/O)S_3$ set of donor atoms.[10] An analogous cross-peak is observed in the case of *trans*-DDP ($^{195}Pt/^1H$ $-3090/2.4$ ppm, Figure 3 b). However, in this latter case there is also a correlation peak between ^{195}Pt and $^{15}NH_3$ protons (doublet centered at $-3090/4.2$ ppm). Moreover, the chemical shift of ^{195}Pt is typical of a platinum atom in a N_2S_2 environment.[9,10]

These findings provide a proof-of-principle of the very different chemical reactivities of *cis* and *trans* isomers against Met-rich motifs.

The involvement of Cu^I-transporting proteins in the cellular uptake and efflux of Pt^{II} is rather intriguing in view

of the different charge and chemical nature of the two metal ions. There are, however, some similarities worth noting. One similarity concerns the redox process that precedes the uptake. Cu^{II} is reduced on the plasma membrane by the cupric reductase Fre1[11] before being taken up by the Met-rich motifs of Ctr1, which help to stabilize the soft Cu^{I} ion in the oxidizing extracellular environment.[6] Our results on the Pt^{IV} complex indicate that, similarly to Cu^{II}, also Pt^{IV} binding to Met-rich motifs requires metal reduction. Another similarity between copper and *cis*-DDP is the complete loss of previous ligands so that the two metal ions bind to Met-rich motifs in the naked form. This, however, is not the case for *trans*-DDP, which retains its *trans*-ammine ligands. The different behavior of *cis*-DDP and *trans*-DDP can easily be accounted for on the basis of their different geometries, which direct differently the *trans*-labilizing effect of an S-donor ligand (see Supporting Information).

After the interaction with Met-rich motifs of different Ctr1 subunits, *cis*-DDP has been shown to stabilize the trimeric form of the protein[12] and to create a pore through the cell membrane. The next step could be diffusion of platinum through the pore down a concentration gradient, as in the case of copper. However, the analogy with copper would imply that a naked platinum atom reaches the cytoplasm and is delivered to various chaperones by mechanisms similar to those hypothesized for copper.[13–15] This possibility, however, contrasts with fluorescence microscopy studies, which have identified the subcellular location of fluorescein-labeled *cis*-DDP in cancer cells.[16] The platinum drug was not detectable in the cytoplasm, but did accumulate in lysosomes, in the Golgi, and in vesicles of the secretory export pathway expressing ATP7A and ATP7B (two Cu^{I}-transporting ATPases, also known as the Menkes and Wilson proteins, which are involved in Cu^{I} as well as *cis*-DDP efflux[17]). On the basis of our observation that interaction of *cis*-DDP with Mets7 leads to complete loss of the carrier ligands, we propose that, after the initial interaction of some *cis*-DDP with Met-rich motifs of Ctr1 and stabilization of the trimeric form of the protein, an endocytotic process incorporates a portion of extracellular medium (containing undegraded *cis*-DDP) into vesicles which are delivered to subcellular compartments, including the nucleus (Supporting Information). Endocytic vesicles of Ctr1 have already been proposed as a secondary system for delivery of copper to intracellular organelles.[18] Therefore, *cis*-DDP would follow a transport pathway that can be activated by sacrificing some *cis*-DDP units and can guarantee transport of intact *cis*-DDP inside the cell.

Experimental Section

Materials: The peptide Mets7, acetylated at its N-terminus, was purchased from GenScript Corp. The purity was validated to be greater than 98% by analytical HPLC, and the mass was confirmed by ESI-MS. Calcd for $C_{34}H_{61}N_9O_{12}S_3$: 883.36; found: $[Mets7+H^+]$ 883.90. The complexes *cis*-DDP and *trans*-DDP were prepared from K_2PtCl_4 as previously reported.[19,20] The same procedure was adopted for preparing the ^{15}N-enriched complexes ($^{15}NH_3$ was obtained from $^{15}NH_4Cl$ by treatment with a stoichiometric amount of KOH). The Zeise's salt $K[PtCl_3(\eta^2\text{-}C_2H_4)]$ was prepared as previously described.[21] Stock solutions at mM concentration were prepared by dissolving the lyophilized peptide and the platinum complex in nanopure water. All reaction mixtures were obtained with equimolar amounts of Mets7 and platinum complex.

Mass spectrometry: ESI-MS was performed with an electrospray interface and an ion trap mass spectrometer (1100 Series LC/MSD Trap system Agilent, Palo Alto, CA) as described in the Supporting Information. Ammonium acetate (5 mM) was added before injection to obtain a good volatilization in mildly acidic conditions (pH 6).

Circular dichroism: CD spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD, USA) as described in the Supporting Information.

NMR spectroscopy: NMR spectra were recorded at 298 K on a Bruker Avance 600 UltraShield Plus magnet using a triple resonance (TXI) probehead equipped with pulsed field gradients along the *z* axis. 2D $^{1}H,^{15}N$- and $^{1}H,^{13}C$-edited HSQC, $^{1}H,^{1}H$ NOESY, and $^{1}H,^{13}C$ TOCSY NMR spectra were used to assign peptide resonances both in the absence and in the presence of selected platinum complex. Further details are reported in the Supporting Information.

Received: July 21, 2007
Published online: September 20, 2007

Keywords: antitumor agents · copper · methionine · NMR spectroscopy · platinum

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