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Interaction of cisplatin with a CCHC zinc finger motif‡

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The interaction between cisplatin and an 18-residue CCHC zinc finger motif derived from a retroviral nucleocapsid protein (PyrZf18) has been studied using UV–visible, CD and ^{1}H NMR spectroscopies and ESI-MS spectrometry.

Cisplatin irreversibly blocks the cysteine zinc binding groups in the free peptide and is able to slowly eject zinc from the zinc–peptide complex. The observed end product of the reaction with cisplatin is a complex in which only one ammonia molecule is coordinated to platinum. After an initial binding with two cysteine residues and the formation of the (PyrZf18)–platinum–$(NH_3)_2$ complex, a release of one ammonia molecule occurs because of *trans*-labilization, and the third cysteine is coordinated, leading to a mixture of isomers and/or conformers of the (PyrZf18)–platinum–NH_3 complex. The results are discussed with respect to the potential antiretroviral activity of platinum(II) compounds and to the possible interaction of cisplatin with the cellular nucleic acid binding proteins. Copyright © 2013 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cisplatin; CCHC zinc finger; cellular nucleic acid binding proteins; retroviral nucleocapsid protein; ^{1}H NMR; CD spectroscopy; ESI-MS

Introduction

(SP-4-2)-diamminedichloridoplatinum(II) (cisplatin) and many of its analogs are currently used in the treatment of testicular and ovarian cancers and increasingly in other types of solid tumors [1]. The cytotoxic effect of cisplatin is thought to be associated with platination of DNA bases, predominantly guanine, formation of intrastrand cross-links, and induction of DNA bending [2]. Damage to DNA eventually causes apoptotic cell death through a complex cascade of biochemical processes. Although DNA represents the probable primary target for this metallodrug, only a very modest fraction of the administered cisplatin reacts with nuclear DNA producing the critical cytotoxic lesions. The largest amount of cisplatin is known to bind extracellular and intracellular proteins, reacting with methionine and cysteine residues [3]. These interactions, explored only marginally, are probably responsible for the relevant toxic side effects caused by platinum drugs and might also play some role in the anticancer mechanism. Moreover, the reactions with sulfur-containing proteins such as metallothioneins have been associated with cellular resistance against platinum drugs. In this context, a crucial role is played by the interactions between platinum drugs, including cisplatin, and a number of potentially platinum-binding cysteine-rich proteins, such as those containing zinc finger domains, generally indicated as CCCC (steroid receptor), CCHH (cellular or transcription factor), and CCHC (retroviral nucleocapsid proteins) boxes. It is known that cisplatin irreversibly binds human polymerase alpha [4], which contains a CCCC zinc finger domain. Details on the binding mechanism of cisplatin to the DNA binding motif of human polymerase alpha and polymerase kappa, the latter containing a CCHC zinc finger domain, have been recently reported by Volckova *et al.* [5]. These authors demonstrated that, upon binding to cisplatin, CCCC and CCHC zinc finger domains release zinc ions, undergoing dramatic structural modifications. Other studies have shown that *trans*-platinum derivatives are capable of ejecting zinc ion from a CCHC-type zinc finger domain of the HIV-1 nucleocapsid protein (NCp7) [6,7] and exerting antiretroviral activity [6]. It is worth noting that CCHC zinc binding domains with very high amino acid sequence and functional homology to corresponding domains of retroviral nucleocapsid proteins are present in CNBPs, also known as CCHC-type zinc finger CNBPs [8]. This class of highly homologous proteins is found in humans and other vertebrates. Although the biological functions of CNBPs are still under investigation, the striking conservation in various organisms suggests that CNBPs play a fundamental biological role across different species. In fact, the available biological and structural data indicate that CNBPs are implicated in transcriptional and posttranscriptional control of many genes and cell proliferation [9]. In view of the biological and pharmacological relevance of the interactions between platinum drugs and proteins with CCHC-type zinc finger domain,

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Abbreviations used: *CNBPs, cellular nucleic acid binding proteins; EDT, 1, 2-ethanedithiol; Fmoc-PAL-PEG-PS resin, Fmoc-(aminomethyl-3,5-dimethoxyphenoxy) valeric acid–polyethylene glycol polystyrene resin; PAR, 4-(pyridyl-2-azo)-resorcinol; TCEP, tris(2-carboxyethyl)phosphine*

we have investigated the interaction between cisplatin and a zinc finger peptide of CCHC type with a general sequence, -Cys-*f*-*X*-Cys-Gly-+-*X*2-Gly-His-*X*3-*d*-Cys-, where *X* is a variable amino acid, *f* is an aromatic residue, + is a charged residue, and *d* is a side-chain carbonyl-containing residue. We have chosen this zinc finger peptide, hereafter denoted as peptide–Zn, as a model peptide. It corresponds to the first zinc knuckle of EIAV NCp11 and shows very high sequence homology with other lentiviral NCps, such as HIV-1 NCp7 and HIV-2 NCp8, and also with zinc finger motifs found in the human CNBPs. The binding properties of this peptide to Co(II) and Zn(II) ions were reported in a previous investigation [10]. ESI-MS and 1D and 2D ^{1}H NMR spectroscopy studies of the reaction between cisplatin and the (PyrZf18)–Zn complex provide evidence for zinc ejection and the formation of the (PyrZf18)–platinum–$(NH_3)_2$ complex that eventually converts into the mono-amino derivative (PyrZf18)–platinum–(NH_3).

Materials and Methods

Peptide Synthesis

Protected Nα-Fmoc amino acid derivatives and coupling reagents were purchased from Inbios srl (Napoli, Italy), and Fmoc-PAL-PEG-PS resin from Applied Biosystems (Foster City, CA, USA). All other chemicals were purchased from Sigma-Aldrich (Bucks, Switzerland) and were used as received, unless otherwise stated. The synthesis of the 18-amino-acid peptide (H-QTCYNCGKPGHLSSQCRA-NH_2) was carried out in solid phase under standard conditions using the Fmoc strategy on Fmoc-PAL-PEG-PS resin (0.73 mmol/g) on a Pioneer flow peptide synthesizer (Applied Biosystems). The scale synthesis was 0.1 mmol. The peptide was cleaved from the solid support by suspending the resin in a TFA (88%), phenol (4%), EDT (2%), thioanisol (3%), and H_2O (3%) mixture for 3 h at room temperature. The mixture was then filtered, and the solution was concentrated under vacuum. The crude product was precipitated by adding cold ether, the liquid phase was removed by filtration, and the solid was suspended in water/0.1% TFA and then was recollected after lyophilization (160 mg). The product was analyzed by RP-HPLC (Shimadzu, Kyoto, Japan; LC-20 AD) on a C_{18} Phenomenex (Torrance, CA, USA) column (10 mm × 250 mm, 300-Å pore size, 5-μm particle size) with a linear gradient of 5–40% (v/v) acetonitrile containing 0.1% (v/v) TFA over 20 min. The spontaneous partial cyclization of the glutamine N-terminal residue was observed to give a pyroglutamic derivative. N-terminal glutamine (Zf18) and pyroglutamic acid (PyrZf18) peptides were separated using RP-HPLC (t_R 6.0 min for Zf18 and t_R 12.0 min for PyrZf18). In a subsequent synthesis, cyclization was completed by treating 13.5 mg of the crude peptide with 13.50 ml of acetic acid for 6 days at room temperature. The desired compound was obtained at HPLC purity higher than 95% with a final 35% yield. The identity was confirmed by ESI [Zf18 [M + 1] = 1954 a.m.u. (calculated 1953.07), PyrZf18 = [M + 1] = 1935 a.m.u. (calculated 1936.07)]

CD Spectroscopy

CD spectra were recorded on a Jasco J-600 spectropolarimeter (Jasco, Easton, MD, USA) using a 0.1-cm-path-length cylindrical quartz cell. A peptide sample (PyrZf18) of 125 μM in a 10 mM sodium phosphate buffer at pH 7.5 was reduced with TCEP. $ZnCl_2$ was dissolved in water at a concentration of 1 mM and added to peptide 30 min before recording the spectra. Data were acquired at 20 °C and were baseline corrected by subtraction of buffer/TCEP and/or buffer/TCEP/$ZnCl_2$. In each case, the final spectrum is the sum of 16 separate spectra with a 0.1-nm step resolution, a 2-s time constant, a 50-nm/min scan speed, a 1-nm bandwidth, and a 20-mdg sensitivity.

UV–visible Spectroscopic Investigation

A solution containing PAR (50 μM) [11], $ZnCl_2$ (50 μM), and reduced peptide (PyrZf18; 50 μM) was prepared and used to record the absorption spectra in the 340–600-nm range using a Varian CARY-5E UV-Vis-NIR spectrophotometer (Palo Alto, CA, USA). Identical conditions were adopted when cisplatin (to a final concentration of 100 μM) was added to the PAR–(PyrZf18)–zinc complex.

Mass Spectrometry

ESI-MS data were obtained using a Finnigan Surveyor MSQ single quadrupole ESI mass spectrometer coupled with a Finnigan Surveyor (Finnigan/Thermo Electron Corporation, San Jose, CA, USA). One milligram of PyrZf18 (5.0×10^{-4} mol) was dissolved in 1.0 ml of phosphate buffer, pH 7.4. The solution was kept in inert atmosphere, and 400 μl of TCEP (0.1 mM) in NaOH (1.0 M) was added. The mixture was stirred overnight, and the reduction of a sulfur bridge was tested by MALDI-TOF MS: calculated $[M + H]^+$ *m/z* 1935.8, found *m/z* 1936.7. One hundred twenty-five microliters of cisplatin, previously dissolved in water (concentration 3.3 mM), was added to the buffer. After 48 h at room temperature, the sample was analyzed by ESI-MS: calculated $[M + 3H]^{3+}$ 722.6, found *m/z* 721.0. The mixture was divided into two vials. One portion was stirred at room temperature for 7 days. The coordination was checked again by ESI-MS: calculated $[M + 3H]^{3+} = 715.6$, found *m/z* 716.0. Six microliters of aqueous solution of $ZnCl_2$ (1 mM) was added to the other portion. The mixture was stirred for 48 h, and then ESI-MS spectra were taken.

NMR Spectroscopy

(Zf18)–Zn was prepared by dissolving the peptide at 2.5 mM in 80% H_2O–20% D_2O. The pH was adjusted to 7.0, and 1.2 equivalents of $ZnCl_2$ were added to form the complex. The non-thiol-reducing agent TCEP was added to prevent the oxidation of cysteine residues.

The (PyrZf18) platinum complex was prepared by dissolving the peptide in 80% H_2O–20% D_2O (buffer sodium phosphate, pH 7) and then adding cisplatin in slight excess (1.2 equivalents) with respect to the PyrZf18 concentration of 1.8 mM. TCEP was added also to this sample. A tenfold diluted sample was used to check possible aggregation.

NMR data were collected using a VARIAN Unity Inova 500-MHz spectrometer (Palo Alto, CA, USA) with sample at 298 K. Chemical shifts were referenced to the methyl resonance of 4,4-dimethyl-4-silapentane-1-sulfonic acid used as the internal standard. Proton chemical shift assignments were obtained from TOCSY [12,13] and NOESY [14] experiments. In TOCSY and NOESY experiments, the water signal was suppressed by a 2.5 presaturation pulse. 2D experiments were recorded in phase-sensitive mode using the States method [15]. Mixing times were 50 and 80 ms for the TOCSY spectra and 200 ms for the NOESY spectrum.

Journal of PeptideScience

Results

A nucleocapsid zinc finger peptide with sequence H-QT**C**YN**C**GKP GHLSSQ**C**RA-NH_2, corresponding to the first zinc knuckle of EIAV NCp11, was used as the model peptide. The synthesis was carried out on solid phase using the standard Fmoc protocol. The N-terminal glutamine residue of peptide undergoes partially nonenzymatic spontaneous cyclization, resulting in the formation of pyroglutamic acid during the cleavage process. The HPLC purification allowed us to isolate both PyrZf18 and Zf18 products. To obtain only pyroglutamic acid at the N-terminal position, the conversion was completed by dissolving the peptide in acetic acid for 6 days. PyrZf18 was used in all the experimental investigations except for measuring the ^{1}H NMR spectra of the zinc–peptide complex in which Zf18 was used.

We used PAR to qualitatively demonstrate zinc ejection from this CCHC nucleocapsid zinc finger as previously reported [6,16]. PAR is a tridentate ligand forming ML_2-type complexes with Zn(II) and other transition metals and is used to measure micromolar concentrations of these metals [11]. Free PAR absorbs at ~410 nm, whereas the PAR_2–Zn^{2+} complex shows an absorption maximum at ~500 nm.

Preliminary tests were performed to verify if PAR could be efficiently used in the studied system. The zinc finger PyrZf18 was able to displace zinc ions from the PAR_2–Zn complex although to a lesser extent than EDTA. In fact, it lowered the maximum at 500 nm when added (1 : 1 peptide/zinc molar ratio) to a solution containing the PAR_2–Zn complex. The observed smaller efficiency in the displacement is in agreement with the measured lower affinity constant of the peptide–zinc complex (10^{12} M^{-1}) compared with EDTA–zinc ($10^{13.65}$ M^{-1}). No interferences were observed in the PAR_2–Zn absorption spectrum because of cisplatin and/or TCEP. In addition, the absorption spectrum of the (PyrZf18)–Zn complex solution in the presence of PAR and TCEP remained unchanged for more than 24 h under our experimental conditions.

Upon addition of cisplatin to a solution containing the peptide–Zn complex and PAR, a slow growth of the typical PAR_2–Zn absorption band at ~500 nm was observed (Figure 1). Furthermore, the peptide that had reacted with cisplatin in a 1 : 1 molar ratio for 48 h, when added to the PAR_2–Zn solution, was unable to displace zinc from the PAR_2–Zn complex. On the contrary, the free peptide immediately lowered the PAR_2–Zn absorption at 500 nm as previously mentioned. To confirm our findings, we used ESI-MS analysis that provided clear evidence for the formation of the (PyrZf18)–platinum–$(NH_3)_2$ complex (48 h reaction time), showing a typical Pt isotopic pattern. Moreover, ESI-MS spectra taken after 7 days revealed that it converts into the (PyrZf18)–platinum–NH_3 complex (Figure 2). The addition of a zinc chloride solution to the (PyrZf18)–platinum–$(NH_3)_2$ and to the (PyrZf18)–platinum–NH_3 complex and MS analyses after 48 h indicated that the complexes are stable in the presence of equimolar zinc ion concentrations.

The effect of Zn^{2+} and Pt^{2+} on peptide secondary structures was investigated by CD and NMR spectroscopies. Figure 3 shows the CD spectra of the (PyrZf18), the (PyrZf18)–zinc complex, and the (PyrZf18)–platinum complex obtained after the reaction of cisplatin and peptide for 7 days. The CD spectrum of the

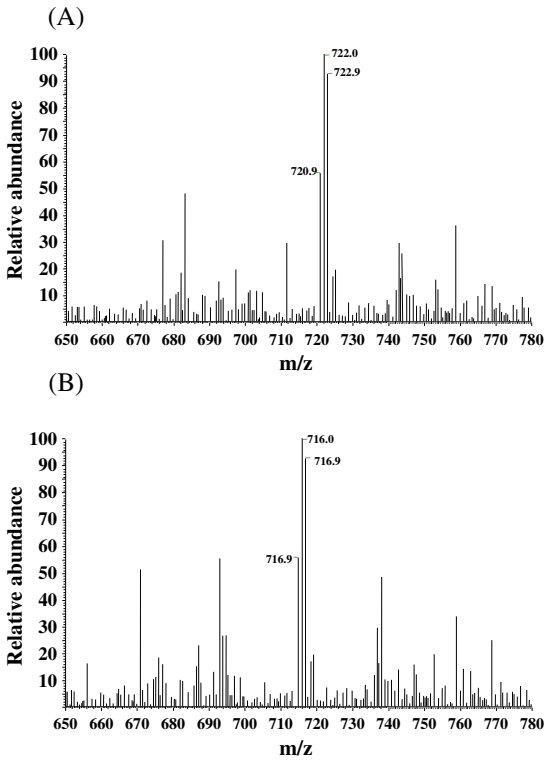

Figure 2. ESI mass spectra of (A) (PyrZf18)–platinum–$(NH_3)_2$ and (B) (PyrZf18)–platinum–(NH_3).

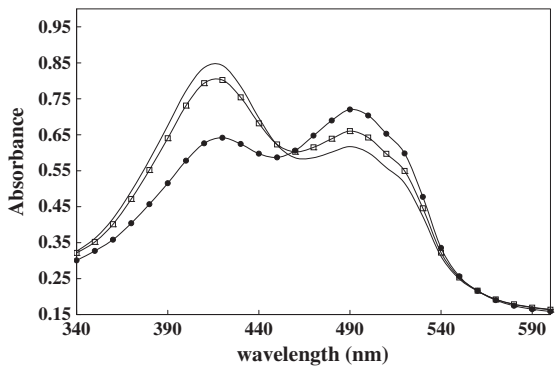

Figure 1. Absorption spectrum of 50 μM PAR, 10 μM Zn^{2+}, and 50 μM peptide in phosphate buffer, pH 7.4, at 25 °C. Spectra taken 1 h (□) and 24 h (●) after the addition of 100 μM cisplatin.

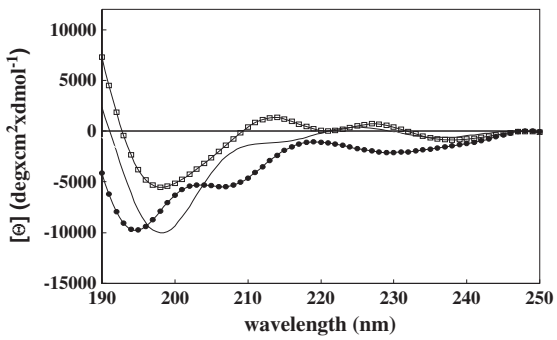

Figure 3. CD spectra of peptide alone (—) and in the presence of 2 equivalents of *cis*-Pt (●) or $ZnCl_2$/Zn finger (□). The data were expressed in terms of [θ], the molar ellipticity.

peptide in the absence of zinc is compatible with random coil conformations. In the presence of zinc (molar ratio zinc/(PyrZf18) 2 : 1), the CD spectrum of the (PyrZf18)–Zn complex shows a pronounced, less negative ellipticity with respect to the free peptide spectrum and a positive ellipticity between 190 and 195 nm, but no shift of the minimum. These features are indicative of the onset of more ordered structures. The effects of zinc on peptide were completely reversed by the addition of 10 mol of EDTA per mole of zinc. The CD spectrum of the peptide–platinum complex undergoes a blueshift of the ellipticity minimum with respect to both the previously described spectra; therefore, the synthetic peptide is partially folded upon reaction with cisplatin although it apparently shows more random conformation with respect to the zinc complex.

The ^{1}H 1D NMR spectrum was measured to confirm the platinum binding to the peptide. The spectrum in the absence of the ion shows poor dispersion of the amide proton resonances, indicating that the peptide does not adopt an ordered conformation in these experimental conditions. After 7 days upon the addition of 1.2 equivalents of cisplatin, a more marked dispersion of the resonances of the amide protons was observed in the NMR spectrum. The sequential resonance assignment for the cisplatin–peptide complex was performed according to the standard method established by Wuthrich [17]. From the amide protons, TOCSY (Figure 4) experiments allowed identification of the α and β protons of almost all the amino acids. NOESY cross-peaks connecting HN of residue *i* with HN and/or H_α and H_β of residue $i+1$ were used for sequential assignment. As already observed in the zinc–(Zf18) complex and in the whole EIAV NCp11 [10], Cys^{3} shows a medium-range NOE between its C_βH and NH of Cys^{6} (position $i+3$), but different from the zinc complex, it did not present NOEs with residues at positions $i+4$ and $i+5$. The number of the cross-peaks in the TOCSY fingerprint region was greater than the expected 17 peaks (one residue has no amide proton because it is a Pro). This could be ascribed neither to the peptide, because MS and HPLC analysis confirmed peptide purity, nor to aggregation as we had no evidence of NMR line-width variations, at least in the concentration range of 0.18–1.8 mM. Furthermore, we could also exclude that the extra peaks in the spectra were due to the presence of *cis–trans* equilibrium around the Lys^{8}-Pro^{9} peptide bond because we observed a strong sequential NOE between the Lys^{8} C_αH and the Pro^{9} $C_\delta H_2$, thus indicating that proline adopts the *trans*-conformation. Almost all the assigned residues in the platinum–(PyrZf18) peptide complex have chemical shifts very similar to those observed for the corresponding residues in the zinc–(Zf18) complex (Table 1) [10], thus suggesting that peptide local conformations in the two metal complexes are quite similar at least for certain residues. An evident and interesting exception is His^{11}, which in the zinc complex is involved in the metal coordination through its N_ε atom, so its H2 and H4 protons experience upfield chemical shifts, whereas in the platinum complex, this residue is not involved in metal binding, so the histidine aromatic protons have chemical shifts close to their random coil values. The other three metal binding residues, the cysteines, appear to be involved in the coordination through their sulfur atoms. This is supported by the fact that in CCHC zinc finger domains, the chemical shifts of Cys residues involved in metal binding are quite typical and well conserved. We did not try to perform the assignment of the extra peaks observed in the NMR spectra.

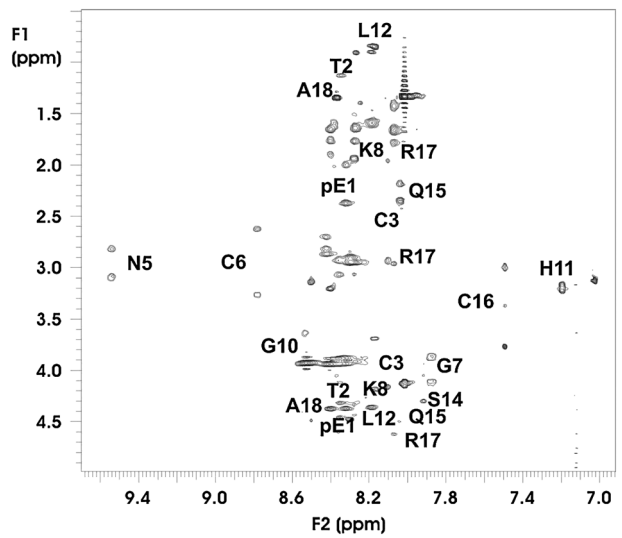

Figure 4. Fingerprint region of the ^{1}H NMR 500-MHz TOCSY spectrum of the platinum–(PyrZf18) complex (mixing time, 80 ms).

Discussion

The obtained results allow us to conclude that cisplatin irreversibly blocks the zinc binding groups in the free peptide and is able to slowly eject zinc from the zinc–peptide complex. The observed end product of the reaction with cisplatin is a complex in which only one ammonia is coordinated to platinum. Very likely, after an initial binding with two cysteine residues and the formation of the (PyrZf18)–platinum–$(NH_3)_2$ complex, a release of one ammonia molecule occurs because of *trans*-labilization, and then the third cysteine is coordinated, leading to a mixture of isomers and/or conformers of the (PyrZf18)–platinum–NH_3 complex, which could explain the observed TOCSY extra peaks.

We have not tried to fully characterize the (PyrZf18)–platinum–NH_3 and the (PyrZf18)–platinum–$(NH_3)_2$ square planar complexes; in particular, we have not investigated the isomers of these complexes. However, it is clear from the NMR study that the His residue is not involved in the platinum coordination; therefore, the only isomers that can be predicted for (PyrZf18)–platinum–NH_3 and (PyrZf18)–platinum–$(NH_3)_2$ are those reported in Figure 5. On the other hand, it is possible to speculate that the isomeric composition of the complexes should depend on the relative reactivity (nucleophilicity/accessibility) of thiol/thiolate groups in the zinc finger coordination site. For example, it is known that in the case of HIV-1 NCp7, the CCHC cores are reactive to soft electrophiles. Although each of the two conserved NCp7 zinc fingers share the same retroviral zinc finger motif, the core of the C-terminal finger is substantially more reactive than the N-terminal finger, and Cys^{49} is the most labile site of NCp7 [18,19]. With this taken into account, it is reasonable to assume that both the chemical composition of the CCHC zinc finger core and its local protein environment can tune the reactivity of these zinc finger motifs toward cisplatin and determine the type and abundance of the isomers formed. However, the stoichiometry of the complexes reported here should be common to different CCHC zinc proteins. In this context, it is worth noting that in the case of a 31-amino-acid CCCC zinc finger [20], the complete displacement of zinc ion was only observed when two platinum ions were linked

Journal of PeptideScience

Table 1. ^{1}H NMR chemical shifts of the studied peptides at 298 K, in 90% H_2O–10% D_2O, pH 7

	Residue						
Q1	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	$C_\delta H$	$C_\varepsilon H$	Others
pE1	***8.32***	***4.38***	***2.15***	***2.35***			
T2	8.68	4.35	4.16	1.25			
	8.38	***4.30***	***4.10***	***1.19***			
C3	8.11	4.18	2.98/2.55				
	8.10	***4.17***	***2.94/1.97***				
Y4	8.74	4.90	3.10/2.50				7.13δ/6.82ε
	8.75	***4.80***	***3.20***				***7.12***δ/***6.84***ε
N5	9.54	4.70	3.10/2.85				
	9.56	***4.62***	***3.10/2.82***				
C6	8.77	4.80	3.31/2.68				
	8.78	***4.90***	***3.22/2.63***				
G7	7.95	3.85/3.65					
	7.87	***4.13/3.87***					
K8	8.22	4.36	1.90/1.78	1.65	1.41	3.00	
	8.27	***4.40***	***1.95***	***1.65***	***1.55***	***2.90***	
P9		4.42	2.32/2.05	1.92	3.78/3.60		
		4.39	***2.30/2.03***	***1.90***	***3.85/3.68***		
G10	8.62	4.15/3.70					
	8.53	***3.94/3.63***					
H11	7.17	4.49	3.17/3.07				7.45ε/6.96δ
	7.18	***4.38***	***3.27***				***8.04***ε***/7.06***δ
L12	8.27	4.62	1.65		0.90		
	8.19	***4.34***	***1.60***	***1.45***	***0.90/0.85***		
S13	9.02	4.47	3.91				
	8.99	***4.40***	***3.99***				
S14	7.89	4.12	3.87				
	7.92	***4.27***	***4.03/3.90***				
Q15	8.03	4.47	2.15	2.44			7.41/6.90
	8.05	***4.50***	***2.29***	***2.40/2.32***			***7.59/6.96***
C16	7.62	3.78	3.10/2.91				
	7.48	***3.75***	***3.39/3.00***				
R17	7.93	4.07	1.97	1.32/1.21	2.95		6.87
	8.06	***4.02***	***1.78***	***1.45***	***2.92***		
A18	8.42	4.22	1.40				
	8.38	***4.37***	***1.30***				

For each residue, the first line refers to the zinc complex: [$ZnCl_2$] = 2.2 mM and [Zf18] = 2.5 mM; the second line, in bold italics, reports data for the platinum complex: [cisplatin] = 2.2 mM and [PyrZf18] = 1.8 mM.

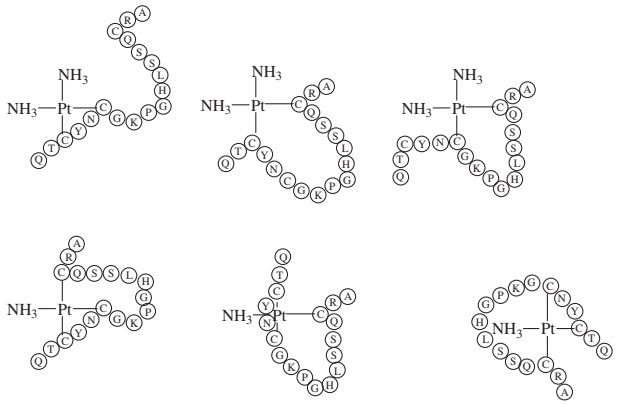

Figure 5. Scheme of all available isomers of (PyrZf18)–platinum–$(NH_3)_2$ and (PyrZf18)–platinum–(NH_3) complexes.

to the peptide; in another case, all the four cysteines of a 35-amino-acid CCCC zinc finger peptide were coordinated to a single Pt^{2+} ion with the release of two ammonia molecules of cisplatin [21]. As reported earlier, *trans*-platin compounds have been studied as potential antiretroviral agents targeting the nucleocapsid protein NCP7 of HIV-1, whereas cisplatin derivatives have not been considered. This is very likely due to a few anecdotal reports on cisplatin cancer treatment of HIV-1-infected patients. In certain cases, no effect of cisplatin on HIV-1 progression has been reported [22], whereas in other circumstances, with a combination chemotherapy with cisplatin, a progressive HIV disease has been experienced after therapy [23]; this could be due to a cisplatin stimulation of HIV replication [24–26]. However, carboplatin, a platinum(II) derivative with *cis*-geometry as opposed to cisplatin, does not stimulate the expression of the HIV long terminal repeat sequences [27]. Therefore, considering these results, we suggest

that other platinum(II) derivatives with *cis*-geometry different from cisplatin could eventually be investigated as antiretroviral agents. Finally, our findings indicate a potential interference of cisplatin with the CNBP functions.

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