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Study by HPLC-MS of the interaction of platinum antitumor complexes with potato carboxypeptidase inhibitor (PCI)

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ABSTRACT

The interaction of the well-known antitumor drug cisplatin *cis*-$[PtCl_2(NH_3)_2]$ and the compound *trans*-$[PtCl_2NH_3$(4-hydroxymethylpyridine)] with the small protein potato carboxypeptidase inhibitor (PCI) and a PCI mutant in which glycine-39 was substituted by methionine has been followed by HPLC/mass spectrometry. Our results showed that both Pt drugs were able to bind PCI through Met-39 and histidines in mutated PCI, whereas only the *trans* complex interacted significantly with wild PCI. In the cytotoxic studies, the monofunctional adduct PCI-Met–cisplatin was neither more active nor more selective than cisplatin itself when tested against three tumor cell lines with different number of EGF receptors. Those results suggested that the poor activity of the adduct could be just due to the small fraction of cisplatin which was decoordinated from the adduct and able to penetrate the tumor cells, as well as to the changes in the structure of the platinum drug after the loss of NH_3 groups upon binding PCI-Met.

1. Introduction

Potato carboxypeptidase inhibitor (PCI) is a small, 39 residues, globular protein that competitively inhibits several metallocarboxypeptidases with a K_i in the nanomolar range.[1] PCI belongs to the cysteine-knot or T-knot super-family of proteins, named because of their particular pattern of disulfide bridges.[2] PCI shares this T-knot scaffold, with other plant protease inhibitors and with animal peptidic growth factors, such as the epidermal growth factor (EGF).[3]

EGF is a peptide factor that can induce the cells to advance into the G_1 phase and is required for differentiation of epidermal tissues.[4,5] Both EGF and transforming growth factor TGF-α (highly homologous to EGF) bind to EGF receptor (EGFR) to produce its activation and a concomitant cascade of biological processes necessary for proliferation in many cell types as well as in cancer cells.[6,7] Most human cancers arise in the epithelial component of organs, including the skin, breast, lung, and gastrointestinal and genitourinary tracts. Growth factors EGF and TGF-α, and their receptors seem to play a prominent role in epithelial neoplasia since they are implicated in tumor cell growth, vascularization, invasiveness, and metastasis. Moreover, in many tumors of epithelial origin (carcinomas), EGFR has been found to be overexpressed.[8,9] Due to the importance of EGFR in carcinomas, its wrong or non-activation seems to be an excellent target for cancer therapy.[5,10,11]

In 1998, Blanco-Aparicio et al. reported for first time the ability of PCI to act as an antagonist of human EGF.[12] PCI binds EGFR with less affinity than EGF but it is still capable of competitively inhibiting all steps in ligand-induced activation of the receptor: ligand binding, receptor dimerization, induction of tyrosine kinase activity, and tyrosine transphosphorylation. Therefore, PCI is considered as a cytostatic agent, able to block the cell cycle between G_0 and G_1 phases selectively in cancer cells, without directly inducing apoptosis.

On the other hand, despite the effort to find novel chemotherapeutic agents, cisplatin and its analogous complexes are still the most used inorganic drugs in cancer treatments.[13] However, platinum complexes display an alarming lack of selectivity toward tumor cells, consequently causing severe side effects. In addition, its limited activity only against certain lines and the acquired or natural resistance of some kinds of neoplasms to these compounds have been the origin of a big deal of strategies designed to obtain more efficient drugs. Unfortunately, until now only two more intravenously administrable Pt(II) complexes, besides cisplatin, have been approved for worldwide clinical practice: carboplatin and oxaliplatin.[14–18] The urgent need of new approaches in the fight against cancer is then clear.

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Here, we present an ambitious attempt to combine the selective and cytostatic protein PCI with two highly cytotoxic (but not selective) platinum complexes: cisplatin and the *trans* complex *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)]. This latter drug proved to effectively induce apoptosis of HL-60 tumor cells in a better extent than cisplatin[19,20] and its interaction with DNA was well established[21]; thus, considered by the authors as a good candidate for further studies.

ESI-MS is the softest ionization method available and hence is well suited for analyzing the interactions of metal complexes and protein.[22–24] These interactions have been deeply studied when it comes to metal anticancer drugs and serum proteins in blood[23] in terms of transportation; however, there is little knowledge about the mechanism through which the complex acts after these interactions. Known to the authors, there appeared only two related reviews where the therapeutic potential of protein-bound metal complexes was studied.[25,26]

Since adducts of platinum drugs with proteins may play a crucial role in their uptake, biodistribution, resistance processes and, in general, their pharmacokinetic properties, more recently several research groups have been investigating, mainly by ESI-MS, the type of interactions that take place between different *cis*/*trans* platinum antitumor complexes and proteins such as cytochromes or metalothionenines. Interestingly, some unexpected changes in the identity of the *cis* complexes were found, including the hydrolysis of the amino ligands after an initial binding to the proteins through the loss of the Cl^- ligands.[27,28]

In this work, we study in detail by HPLC-MS how two known strong cytotoxic platinum complexes interact with PCI, and we show some preliminary results about how that binding affects the overall activity and selectivity of the adduct metal–protein. PCI was modified by replacing the last amino acid (glycine-39) in the C-tail for methionine in order to increase its rate of binding to Pt drugs, given the high affinity of platinum for sulfur containing ligands such as methionine and cysteine.[29,30] It was described that, in presence of nucleic acids, Pt–peptide adducts favor the migration of Pt complexes from the peptide to N7 of guanines in single stranded nucleic acids.[31–34] All mentioned facts make PCI act as an appropriate selective carrier or Pt reservoir for the necessary Pt–DNA interaction, besides the activity that PCI might display by itself.

2. Results

2.1. Wild type-PCI

Initially, the interaction between wild type-PCI (WT-PCI) and platinum complexes, cisplatin and *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)], was studied in order to establish a reference for the posterior interaction of the same complexes with the mutated protein PCI-Met. The TIC chromatogram for WT-PCI in aqueous solution, room temperature, and neutral pH shows a single peak at a retention time of 5.47 corresponding to the pure WT-PCI protein (data shown in supporting information). The mobile phase consisted of acetonitrile and aqueous ammonium acetate 0.01 M, starting from 20% of acetonitrile up to 100%, in a period of 30 min. After injection in the HPLC chromatograph, the different fractions were separated by the column and they are detected in the Mass Spectrometer to give the TIC chromatogram, where the intensity of electric signal is plotted versus time.

2.1.1. HPLC-MS results for WT-PCI–cisplatin

The interaction between WT-PCI and cisplatin (ratio 1:1) was followed by HPCL-MS at 1, 3, 6, 24, and 48 h. Chromatograms in Figure 1 show that, using experimental conditions described in 2.1, cisplatin barely interacts with WT-PCI, even at long times of interaction.

In general, WT-PCI displays low levels of impurities and so the experiment begins close to 100% of free and pure protein. The rate of reaction with cisplatin is markedly slow, as shown by the presence of only a 5% of monofunctional adduct after 48 h (Fig. 2). In Table 1, the data have been summarized.

2.1.2. HPLC-MS results for WT-PCI-(*trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)])

TIC chromatograms and MS-ESP spectra obtained for the interaction of WT-PCI with *trans* platinum complex *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] at 1, 4, 6, 24, 48, and 72 h (ratio 1:1) are shown in Figure 3. Data are summarized in Table 2.

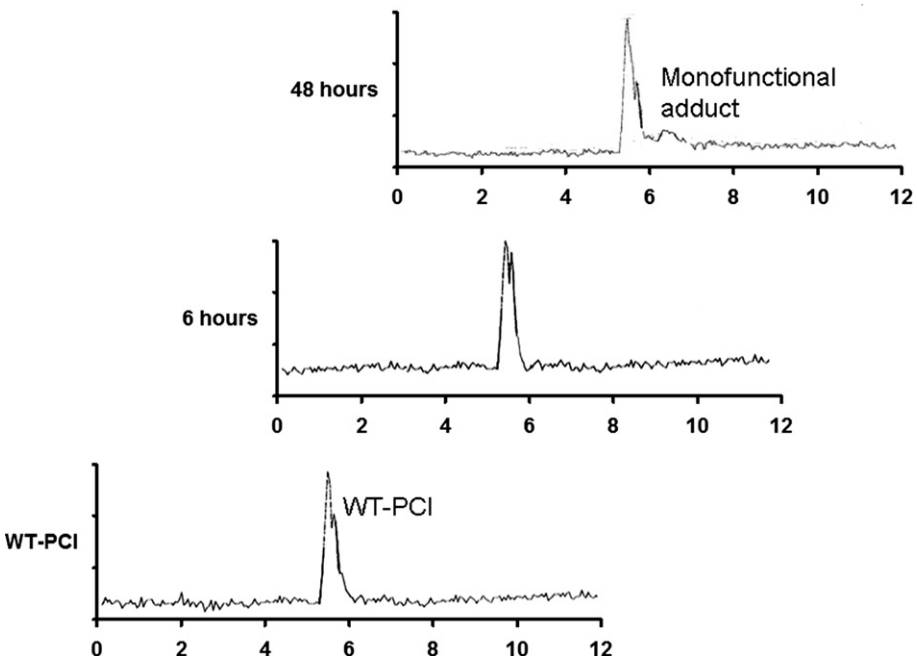

Figure 1. TIC chromatograms for the interaction of WT-PCI and cisplatin.

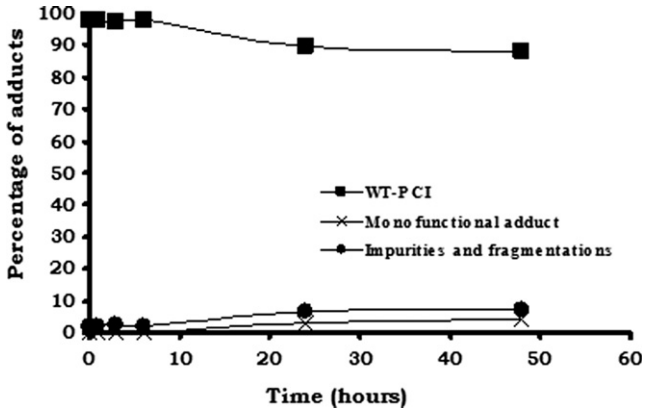

Figure 2. Evolution of adducts versus time when WT-PCI interacts with cisplatin.

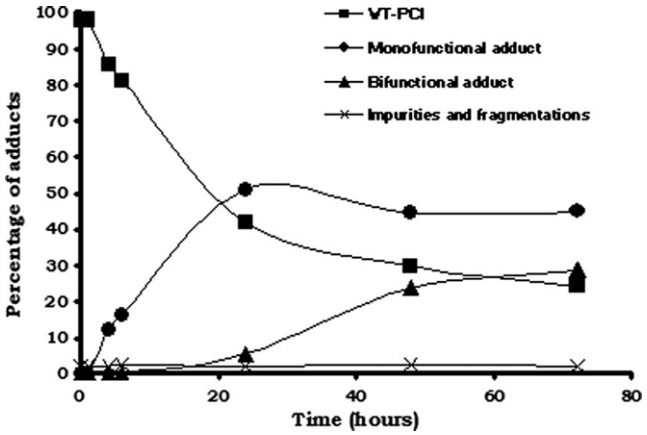

Figure 4. Evolution of adducts versus time when WT-PCI interacts with *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine).

The higher reactivity of *trans* complexes compared to cisplatin allows the formation of monofunctional and bifunctional adducts. At 4 h of reaction, an adduct with deconvoluted mass of 4651 amu starts appearing and it is attributed to the loss of one chloride and the consequent coordination to PCI, likely through one of the histidines. This monofunctional species has a maximum at 24 h and remains constant after that time. The bifunctional adduct also takes place several hours later, though in significant lower percentages. Finally, the system reaches stable values for all adducts and it keeps an interesting 50% of the monofunctional one (Fig. 4).

Interestingly, even after long time of reaction (72 h), there is a remaining free WT-PCI that has not interacted with the platinum drug, probably due to the relatively low affinity of the platinum complex for all different residues of WT-PCI.

2.2. PCI-methionine

WT-PCI was mutated and the terminal glycine was replaced by a methionine to obtain the mutant PCI-methionine (PCI-Met). As previously commented, this modification was motivated by the lack of affinity of cisplatin for the wild type of the protein.

TIC chromatogram and deconvoluted mass for PCI-Met in aqueous solution, room temperature, and neutral pH were first obtained in order to test the purity of the mutant. All the experimental conditions were the same as those previously utilized in the study of WT-PCI. PCI-Met displays a major signal between 5.90 and 6.12 min. When this signal is analyzed by MS-ESP, peaks for m/z, with z = +2, +3, and +4 are observed. Hence, the total deconvoluted mass for PCI-methionine is 4368 amu, also tested by MS-MALDI-TOF (data shown in supporting information).

2.2.1. HPLC-MS results for PCI-Met–cisplatin

TIC chromatograms obtained when PCI-Met interacts with cisplatin for 1, 2, 5, 7, 24, and 72 h at a ratio 1:1 are shown in Figure 5. The same reaction studied at a ratio 1:2 (protein:cisplatin) led to the same results. Data are collected in Table 3.

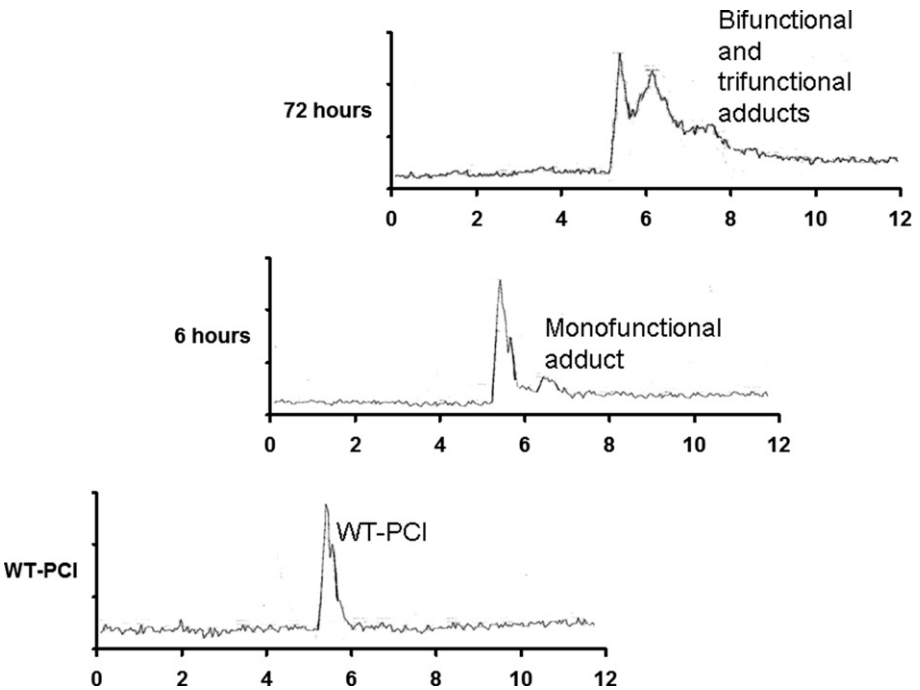

Figure 3. TIC chromatograms for the interaction of WT-PCI and *trans* -[$PtCl_2NH_3$(4-hydroxymethylpyridine)].

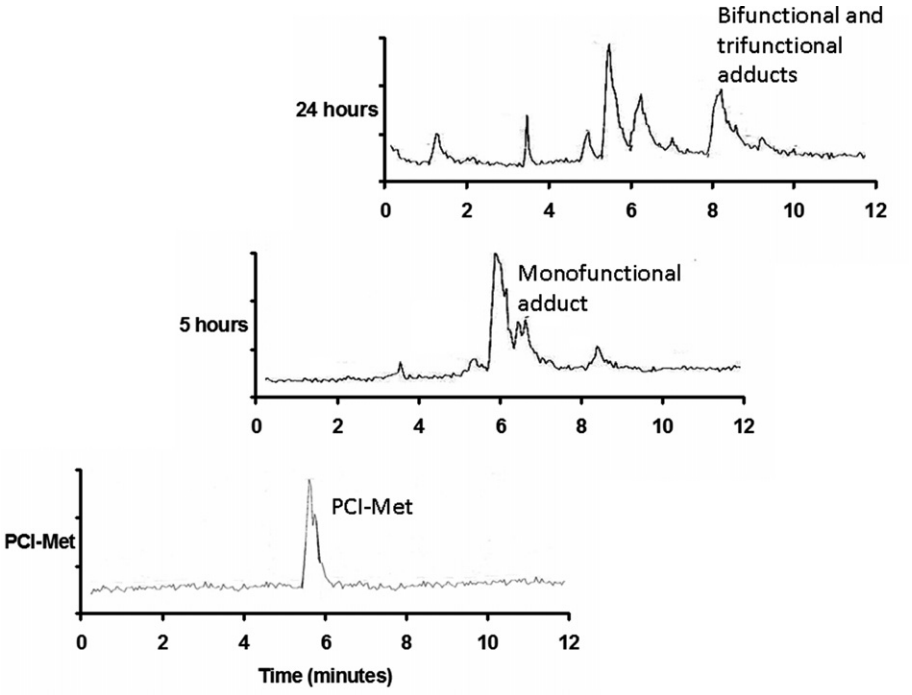

Figure 5. Evolution of TIC chromatograms at several times of interaction between PCI-Met and cisplatin.

The immediate comparison of the results summarized in Table 1 and Table 3 demonstrates the importance that sulfur containing Met-39 has in the interaction of cisplatin with PCI-Met, as expected.

As seen in data from TIC chromatograms and MS spectra, monofunctional adduct (t_r = 6.64 min), in which one of the labile ligands of cisplatin has been replaced by the sulfur atom of the thioether in Met-39 of PCI, appears at relatively short times of reaction (between 1 and 2 h of reaction). However, the highest percentage of monofunctional adduct, 22% of the overall protein, takes place at about 21 h of reaction. At long times of reaction, this adduct evolves to other species named as bifunctional and trifunctional adducts. Both of them start appearing at the same retention time (t_r = 8.42 min) after 5 h, showing an increasing proportion along the interaction. Different conditions were tried unsuccessfully in order to separate them (Fig. 6).

According to our MS results, bifunctional adduct corresponds to the loss of both labile ligands in cisplatin and concomitant binding to two aminoacidic residues of the protein, probably methionine and the imidazolic nitrogen of one of the histidines. Trifunctional adduct has a mass of 4580 amu. This value can be obtained by the displacement of the two chlorides in cisplatin and one NH_3 group to bind through three aminoacidic sites in the protein.

$$(PtNH_3)^{2+} + (PCI - Met) = 212 + 4368 = 4580$$

Impurities and fragmentations are all those signals that were already present in PCI-Met and besides the fragmentations that occur because of the decomposition of PCI in time. In any case, either impurities or fragmentations keep an approximately constant value

Table 1
Data for TIC chromatograms and MS-ESP spectra of the adducts WT-PCI–cisplatin

Adduct	Retention time (t_r, min)	Peaks m/z (m/+2, m/+3, m/+4)	Mass of the adduct	Time of reaction (h)
WT-PCI	5.47	2149, 1433, 1075	4294	0
Monofunctional	6.62	2280, 1520, 1140	4559	24

Table 2
Data for TIC chromatograms and MS-ESP spectra of the adducts WT-PCI-*trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)]

Adduct	Retention time (t_r, min)	Peaks m/z (m/+2, m/+3, m/+4)	Mass of the adduct	Time of reaction (h)
WT-PCI	5.47	2149, 1433, 1075	4294	0
Monofunctional	6.59	2326, 1551, 1164	4651	4
Bifunctional	7.90	2309, 1539, 1154	4616	24

Table 3
Data for TIC chromatograms and MS-ESP spectra of the adducts PCI-Met–cisplatin

Adduct	Retention time (t_r, min)	Peaks m/z (m/+2, m/+3, m/+4)	Mass of the adduct	Time of reaction (h)
PCI-Met	6.12	2185, 1457, 1093	4368	0
Monofunctional	6.64	2317, 1545, 1155	4632	2
Bifunctional	8.42	2298, 1533, 1146	4597	5
Trifunctional	8.42	—, 1527, 1141	4580	5

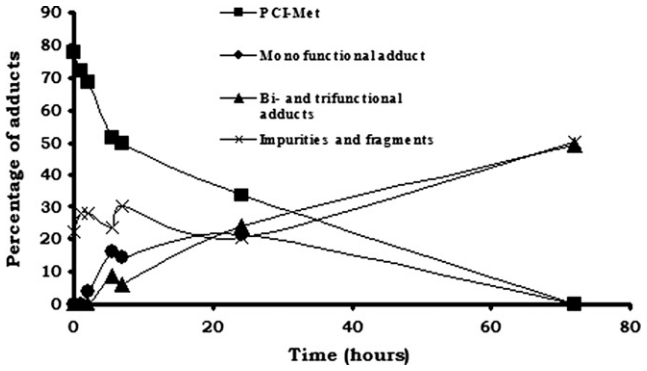

Figure 6. Evolution of adducts versus time when PCI-Met interacts with cisplatin.

(around 20% of the total, also in the free protein) until long times of interaction.

At 72 h, all signals in chromatograms belong to either bi- and trifunctional adducts or fragmentations. Interestingly, PCI-Met keeps the structure with three disulfide bridges in all adducts that take place, since otherwise mass changes were not observed in any case.

2.2.2. HPLC-MS results for PCI-Met-(*trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)])

The same reaction was carried out with the *trans* complex *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] (ratio 1:1) and PCI-Met for 1, 5, 22, and 46 h (Fig. 7).

According to the data obtained by HPLC-MS (Table 4), after 1 h of interaction, *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] already binds the protein and some peaks start showing up at 8.65 and 9.82 min. Beyond 5 h there is barely a 0.5% of free protein. In the case of this *trans* platinum complex, bifunctional adduct is predominant at all times of reaction; trifunctional adducts roughly take a 6% of the total and only after long periods of time. Monofunctional adduct reaches a peak of 30% at about 5 h and then keeps this value constant. Bifunctional adducts start appearing from the very beginning and are the major products at 5 h (more than 60% of total PCI-Met), as it can be seen in Figure 8 (see Table 5).

The fact that different retention times for bifunctional adducts can be observed might be explained in terms of the two histidines in the protein, so Pt could first bind methionine and later one of the those histidines to give two different bifunctional adducts of same mass.

Table 4
Data for TIC chromatograms and MS-ESP spectra of the adducts PCI-Met-*trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)]

Adduct	Retention time (t_r, min)	Peaks m/z (m/+2, m/+3, m/+4)	Mass of the adduct	Time of reaction (h)
PCI-Met	5.94	2185, 1457, 1093	4368	0
Monofunctional	8.65	—, 1576, 1184	4724	1
Bifunctional	8.65; 9.82	2346, 1564, 1173	4690	1
Trifunctional	10.55	—, 1557, 1168	4672	46

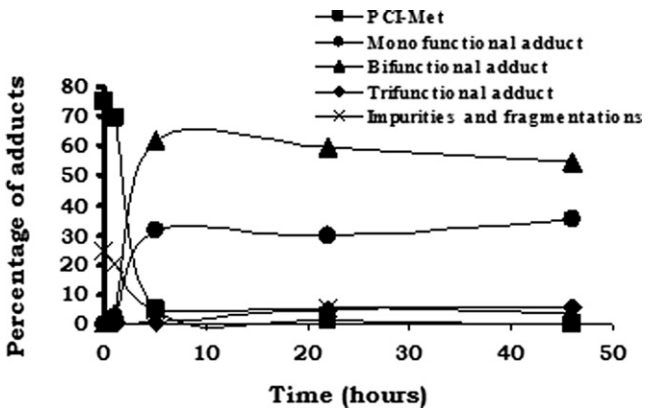

Figure 8. Evolution of adducts versus time when PCI-Met interacts with *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)].

2.3. Preliminary studies of cytotoxicity

The effect of cisplatin and the monofunctional adduct PCI-Met–cisplatin was examined on HL-60 (human leukemia), Capan-1 (human pancreas adenocarcinoma), and A431 (human epithelial carcinoma) cells, by using the MTT assay. The assay, developed as an initial stage of drug screening, measures the amount of MTT reduction by mitochondrial dehydrogenase and assumes that cell viability (corresponding to the reductive activity) is proportional to the production of purple formazan that is measured spectrophotometrically.

Cells were exposed to each compound continuously for a 72 h period at 37 °C and then assayed for growth using the MTT endpoint. Figure 9 shows the dose–response curves of these drugs in terms of the drug effect on the growth of the HL-60, Capan-1,

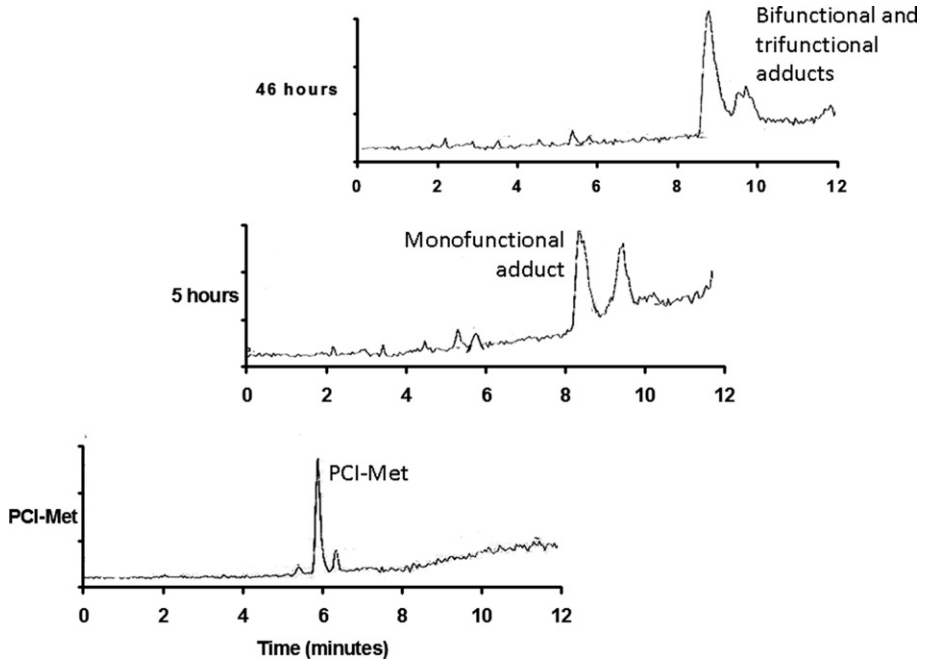

Figure 7. Evolution of TIC chromatograms versus time of the major adducts in the interaction between PCI-Met and the complex *trans*-[$PtCl_2$ NH_3(4-hydroxymethylpyridine)].

Table 5
Approximate IC_{50} values in μM for cisplatin and PCI-Met–cisplatin after 72 h of incubation in HL-60, Capan-1, and A431 cells

	IC_{50} in HL-60	IC_{50} in Capan-1	IC_{50} in A431
Cisplatin	3.3	4.4	5.5
PCI-Met–cisplatin	30.3	68.0	74.1

and A431 cells. Cell lines were chosen according to their number of EGF receptors: HL-60 cells do not show EGF receptors,[35] Capan-1 cells have an average of 4.0×10^4 receptors per cell,[36] and A431 cells display 2.6×10^6 receptors per cell.[37] Bearing this fact in mind, the sensitivity of cells to the drug with PCI should be expected to be different.

Cisplatin was made to react with PCI-Met for a period of 24 h at room temperature in an aqueous solution of PBS buffer. The solution was filtered over Centriplus 3000 filters to remove the excess of non-reacted cisplatin and the final solution was used to treat the different cell lines. The results (Fig. 9) show no selectivity against cells with high number of EGF receptors.

In all cases, cisplatin displays a better behavior than the adduct PCI-Met–cisplatin. The platinum drug by itself shows usual values for IC_{50}[18] and, at least, 10-fold higher than the adduct with PCI-Met in all three tumor cell lines. On the other hand, the adduct is twice more active against HL-60 tumor cells than against A431, which clearly suggests that PCI is not acting as a specific carrier to cells with different number of EGF receptors.

3. Discussion

In view of the fact that a vast majority of cytotoxic metal-containing compounds are administered intravenously, special consideration has been given to interactions of the metal drug with macromolecular blood components. In this context, binding toward serum proteins (albumin or transferrin) that may perform a transport function for metal complexes appears to be the most important issue, because such interactions determine also the overall distribution, excretion and differences in efficacy, activity and toxicity.[38,39] These studies have been performed with ESI-MS[22] which is currently considered as, probably, the best analytical method to do. However, considerably little efforts have been devoted to the study of the interaction with other types of proteins and how those interactions could affect the overall activity of the metal drug. In addition to that fact and due to the general high cytotoxic activity of platinum drugs, a big deal of effort is still nowadays devoted to find new specific carriers to make Pt complexes selectively target tumor cells over sane cells. Therefore, the main idea behind this work was, first, to study the way in which platinum drugs interact with PCI protein and, thus, try to combine the cytostatic activity and selectivity toward epithelial tumor cells of PCI and the high cytotoxic activity of two known platinum drugs.

According to our results, WT-PCI barely interacts with cisplatin, even at high times of reaction (more than 2 days). Therefore, it is interesting to highlight that when PCI does not contain methionine, cisplatin shows much less affinity for histidines than in the case of PCI-Met, which suggests that the first coordination to methionine in the mutant is crucial in order to "activate" the metal complex. Once platinum is coordinated to the sulfur residue, other positions seem to be more accessible to the metal atom.

On the other hand, when the complex *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] is made to interact with WT-PCI the results are significantly different; a relatively high percentage of monofunctional adduct appears and maintains a constant value (about 50% of the total of PCI) even at long times of interaction. From a cytotoxic point of view, this monofunctional adduct is the one that, in principle, could display a better activity, since it is coordinated to the selective "carrier" and it still keeps one labile position to bind nucleobases in DNA.

More interesting is the case of PCI-Met. Both studied drugs display a good rate of interaction with mutated PCI-Met, thanks to the "extra" sulfur coordination site that thioethers in methionine offer. However, and as it was expected, the *trans* platinum drug performs with higher kinetics due to the known superior lability of the *trans* geometry. Bifunctional adducts in both cases are justified by the loss of both Cl^- ligands and coordination to imidazolic nitrogen in one of the histidines in PCI (His-3 or His-15). Taking a look at

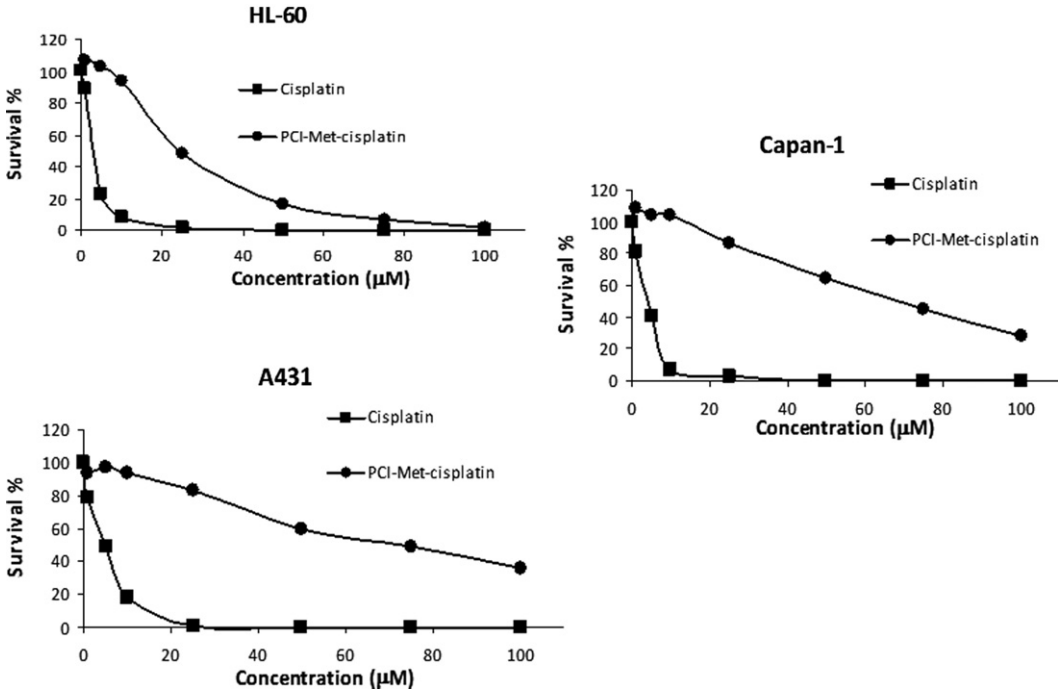

Figure 9. Cytotoxicity experiments in tumor cell lines HL-60, Capan-1, and A431 treated with cisplatin and PCI-Met–cisplatin.

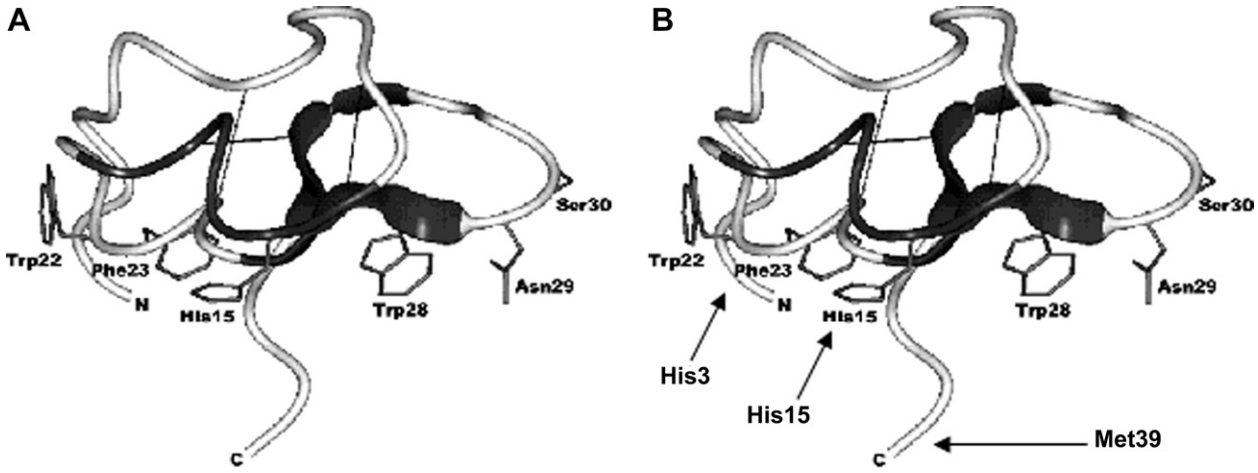

Figure 10. Tridimensional draw of the protein WT-PCI (A) from the crystal structure[40] and PCI-Met (B).

the tridimensional structure of the protein (Fig. 10), and bearing in mind that Pt drugs are first bound to the Met-39, the most probable site of binding could be His-15, since it is the one geometrically more available. According to the MS spectra, trifunctional adducts appear when the metal complex loses not only both Cl^-, but also one of the NH_3 to tri-coordinate the protein, likely through methionine and both histidines. One interesting difference between both complexes is the type of adducts that take place: while cisplatin finally evolves to mentioned trifunctional adducts, *trans*-[$PtCl_2NH_3$(-hydroxymethylpyridine)] forms mono- and bifunctional adducts. Similar behaviors have been described when *cis* and *trans* platinum drugs interact with cytochromes or metallothioneins, and it is justified by the strong *trans* effect of protein sulfur when it is bound to the platinum drug.[27,28] Consequently, in the *cis* geometry that fact implies that after the first coordination through the classic hydrolysis of the labile Cl^- ligands, the Pt drug undergoes hydrolysis of one of the *trans* positions, which in the case of cisplatin are occupied by NH_3 groups, leading to the formation of the trifunctional adducts. This would not occur in the case of *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)], since the first coordination upon loss of one Cl^- leads to the hydrolysis of the second *trans* Cl^-. Therefore, only monofunctional and bifunctional adducts are in this case observed.

Surprisingly and despite the high reactivity of the *trans* complex, the quantity of monofunctional adduct remains constant until long times of reaction. One plausible explanation for that phenomenon is that as a consequence of the trans geometry, after the first binding the Pt drug may not be in the appropriate orientation to bind PCI through the histidines. In addition to that, some other stabilizing interactions may play an important role, despite there is no further evidence to prove it.

Several conclusions can be extracted from the study of the *in vitro* cytotoxic activity of the monofunctional adduct PCI-Met–cisplatin. First of all, A431 cells are expected to be the most sensitive to the drug since they contain an average of 2.6×10^6 EGF receptors per cell, followed by Capan-1 with 4.0×10^4 EGF receptors per cell and HL-60 with no receptors. However, results show that IC_{50} is the lowest for HL-60 when cells are treated with both the adduct PCI-Met–cisplatin and cisplatin. It is also interesting to observe that cytotoxic activity is significantly higher for cisplatin itself than for the adduct with PCI. These effects suggest that the coordination of the platinum drug to the protein may affect the tertiary structure of the macromolecule, changing the conformation and concomitantly disfavoring the recognition by EGF receptors. As it was previously studied, in the superposition of PCI and TGF-α tridimensional structures, according to the disulfide-bridge topology, it is noticed that between the residues of PCI that have a topological similitude with TGF-α are His3 and His15.[41] As commented, these histidines are probably the binding residues that form bifunctional and trifunctional adducts and this species appear at long time periods of incubation, corresponding to those used in cytotoxic assays, so they must be crucial in the EGF receptor recognition. Therefore, it can be concluded that the activity of the adduct might be only due to the partial decoordination of cisplatin that can enter the cells by its regular mechanisms displaying a poor activity since not all cisplatin is released, whereas the species PCI-Met–cisplatin cannot penetrate the membrane. That fact would also explain the lack of selectivity toward cell lines with different numbers of receptors. In addition, the effect of the alterations in cisplatin structure upon coordination to PCI-Met should also be considered. The presence of trifunctional adducts indicates that NH_3 ligands are hydrolyzed after binding to the protein; therefore, even if the adduct PCI-Met–cisplatin is able to keep the affinity for EGF receptors, these important modifications in cisplatin structure might also explain the overall inactivity of the new drug.

Finally, it is also interesting to highlight that in all the studied conditions, PCI and PCI-Met maintain the core structure with the three disulfide bridges, since no changes in mass coming from the cleavage of the S–S bonds are observed.

4. Conclusions

In summary, we were able to study by HPLC-MS the kind of interactions that take place when two platinum complexes with *cis* and *trans* geometries were reacted with two varieties of a carboxipeptidase which has been proved to be a cytostatic agent when utilized against tumor cell lines, PCI. Our results showed that both Pt drugs were able to bind PCI through Met-39 and histidines in mutated PCI, whereas only the *trans* complex interacted significantly with wild PCI. In the cytotoxic studies, the monofunctional adduct PCI-Met–cisplatin was neither more active nor more selective than cisplatin itself when tested against three tumor cell lines with different number of EGF receptors. These results suggested that the poor activity of the adduct could be just due to the small fraction of cisplatin which is decoordinated from the adduct and able to penetrate the tumor cells, as well as to the changes in the structure of the platinum drug after the loss of NH_3 groups upon binding PCI-Met.

5. Methods and materials

5.1. Synthesis of metal complexes

Cisplatin was purchased from Sigma–Aldrich, while complex *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine) was synthesized as previously described by our group.[19,21]

5.2. PCI-Met

PCI was mutated in order to substitute the last residue glycine by a methionine. The PCI mutant was obtained by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit from Stratagene according to the procedure recommended by the manufacturer. Recombinant PCI and PCI-Met were obtained by heterologous expression in *Escherichia coli* strain BL21(DE3). Proteins were purified from the culture medium using a Sep Pak C_{18} cartridge (Waters), followed by anion exchange chromatography on a TSK-DEAE 5PW column (Tosohaas) and by gel filtration chromatography on a Superdex Peptide column (GE). Molecular masses were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The concentration of the purified solutions of recombinant PCI was determined from the A_{280} of the final solution (PCI extinction coefficient $E_{0.1\%} = 3.0$).

5.3. Adducts drug-PCI

In all cases, 70 μl of a metal complex solution (0.01 M) in Milli-Qwater were added to a 1 ml solution 7.00×10^{-5} M of PCI (wild type and mutated with methionine) also prepared in Milli-Q water, to reach a final ratio drug–protein of 1:1. The mixture was shacked and aliquots of 10 μl were injected in the HPLC chromatograph at the described times in each experiment.

5.4. HPLC-MS measurements

All chromatograms were obtained in a Shimadzu HPLC system using a mobile phase of ammonia acetate 0.01 M and acetonitrile HPLC grade, and using a gradient from 20% of acetonitrile to 100% in 30 min. The different species were separated in a Nucleosil C18 (250 mm × 4 mm × 10 μm) chromatographic column.

Mass spectra were recorded in an API 150 EX Applied Biosystems device, with ions source Turboionspray and simple quadrupole analyzer. Only positive polarity was studied in a range of 600–2500 amu.

5.5. Cell lines and growth conditions

The human vulvar epidermoid cell line A431, the pancreatic adenocarcinoma cell line Capan-1, and HL-60 human promyelocytic leukemia cells were obtained from the American Type Culture Collection (Rockville, MD, USA). A431 and Capan-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), in a humidified atmosphere of 5% CO_2 at 37 °C (Gibco). HL-60 cells were cultured in RPM-1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS, life Technologies) in a humidified atmosphere of 5% CO_2 at 37 °C.

5.6. Cytotoxic studies

The adduct PCI-Met–cisplatin for the cytotoxic studies was prepared by mixing 136 μL of a 3.33 mM solution of cisplatin in PBS buffer with 1 ml of a 455 μM solution of PCI-Met in the same buffer, to give a final ratio protein:drug of 1:1. The mixture was reacting for 24 h at room temperature. After that time, the solution is filtered by centrifuging in a Centriplus 3000 filter to concentrate and remove the excess of cisplatin. The concentration of the final solution of PCI-Met–cisplatin was measured by UV–visible spectroscopy, and this solution was finally utilized to treat tumor cells of HL-60, Capan-1, and A431 lines in a range of concentrations between 0 and 75 μM.

Growth inhibitory effect of platinum complexes on cells lines was evaluated using the MTT method ([3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]), which quantifies the number of surviving cells at a given time after exposure to cytotoxic drugs. Cells were plated into 96-well sterile plates in 100 μl of culture medium at a density of 1×10^4 cells per well. Plates of A431 and Capan-1 cells were incubated for 24 h to allow cells to attach. Compounds dissolved in phosphate-buffered saline (PBS) were added in final concentrations ranging from 0 to 75 μM to a volume of 100 μl/well. Twenty-four and 72 h later, 20 μl of MTT solution was added to each well and the plate was incubated for 2–3 h at 37 °C in a humidified 10% CO_2 atmosphere. Cell viability was determined by measuring the absorbance at 495 nm using a Microplate Reader. All cytotoxicity experiments were performed three times in quadruplicate.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.05.064.

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