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Cellular trafficking, accumulation and DNA platination of a series of cisplatin-based dicarboxylato Pt(IV) prodrugs

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ABSTRACT

A series of Pt(IV) anticancer prodrug candidates, having the equatorial arrangement of cisplatin and bearing two aliphatic carboxylato axial ligands, has been investigated to prove the relationship between lipophilicity, cellular accumulation, DNA platination and antiproliferative activity on the cisplatin-sensitive A2780 ovarian cancer cell line. Unlike cisplatin, no facilitated influx/efflux mechanism appears to operate in the case of the Pt(IV) complexes under investigation, thus indicating that they enter by passive diffusion. While Pt(IV) complexes having lipophilicity comparable to that of cisplatin (negative values of log $P_{o/w}$) exhibit a cellular accumulation similar to that of cisplatin, the most lipophilic complexes of the series show much higher cellular accumulation (stemming from enhanced passive diffusion), accompanied by greater DNA platination and cell growth inhibition. Even if the Pt(IV) complexes are removed from the culture medium in the recovery process, the level of DNA platination remains very high and persistent in time, indicating efficient storing of the complexes and poor detoxification efficiency.

1. Introduction

It is widely accepted that the antitumor activity of Pt(II) drugs is related to their ability to form adducts with DNA, which generally correlates with their potency [1].

Octahedral Pt(IV) complexes are considered as prodrugs [2]. They are kinetically quite inert and should reach the tumor cells intact. Here they undergo reduction by biomolecules, whose low molecular weight (MW) prototypes are ascorbic acid (AA) and reduced glutathione (GSH) [3–5]. However, the reduction of Pt(IV) complexes by a still unidentified high MW cellular component plays an important role [6]. Such a reduction is usually accompanied by loss of the two axial ligands generating the corresponding cytotoxic Pt(II) species (Fig. 1). Sometimes scrambling between axial ligands (acetates) and equatorial leaving groups (chlorides) may occur during the reduction process, and this phenomenon seems to befall in the presence of bulky carrier groups such as adamantylamine, cyclohexylamine, n-butylamine, isopropylamine [7], and picoline [8]. The equatorial carrier ligands, which always remain coordinated to Pt core during the reduction, should influence the antiproliferative activity on different tumor cell lines in the same manner they do in their Pt(II) counterparts [9]. As a rule of thumb, the axial ligands affect the cellular accumulation of the complex by virtue of their contribution, among the other ligands, to the overall lipophilicity and substantially determine the reduction potential and/or reduction kinetics.

In previous studies on large series of Pt(IV) complexes, a clear relationship between reduction potential and lipophilicity on the one hand and cytotoxicity on the other hand has been established: "the easier the reduction and the higher the lipophilicity, the greater is the cytotoxicity" [10–16]. However, this paradigm was not fully satisfied for other Pt(IV) compounds, indicating the difficulty to simply relate the chemico-physical parameters with the biological activity of such complexes acting with an intricate sequence of reactions [13,17,18].

DNA platination should parallel the trend of cellular Pt accumulation, with a latency time given by the kinetics of Pt(IV) → Pt(II) reduction in addition to that of aquation of Pt(II) metabolites to produce reactive electrophiles. Moreover, removal of Pt-DNA adducts and efflux of the corresponding Pt derivatives can occur more or less efficiently, depending upon the type of cell line and its status. Thus, the final amount of DNA platination at a given time point is a consequence of an intricate sequence of events. For cisplatin, the IC_{50} value (half maximal inhibition concentration) inversely correlates with the amount of Pt-DNA adducts [19], but it is not granted that the same relationship holds also for Pt(IV) complexes. Equimolar concentrations of cisplatin and lipophilic satraplatin (i.e. (*OC*-6-43)-bis(acetato)amminedichlorido(cyclohexylamine)platinum(IV)) resulted in comparable amount of DNA platination, but the cytotoxic potency was different [20]. It is important to recall that the metabolites obtained from reduction of cisplatin and satraplatin are

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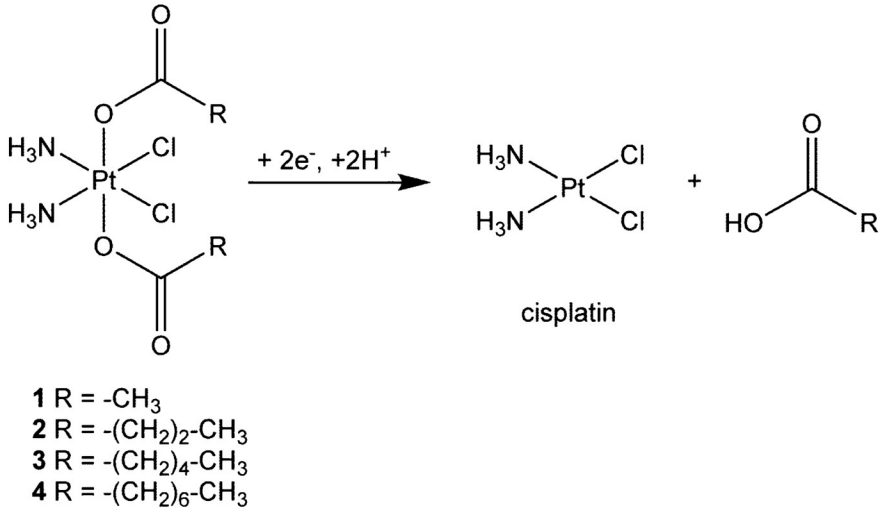

Fig. 1. Sketch of the dicarboxylato Pt(IV) complexes under investigation and their reduction reaction.

structurally different, therefore the comparison between the activities of the two drugs is not straightforward.

Therefore, up to now, the relationship between lipophilicity, cell accumulation, DNA platination and antiproliferative activity of Pt(IV) complexes is not completely assessed. To answer this question, a series of dicarboxylato-platinum(IV) complexes, having the equatorial arrangement of cisplatin and bearing two aliphatic carboxylato axial ligands of different chain lengths, namely *trans*,*cis*,*cis*-[Pt(carboxylato)$_2$Cl$_2$(NH$_3$)$_2$], where carboxylato = $CH_3(CH_2)_nCOO^-$ (n = 0, **1**; n = 2, **2**; n = 4, **3**; n = 6, **4**, Fig. 1) has been investigated having cisplatin as a reference. The interaction with copper transport proteins, the cellular accumulation, the DNA platination, and the antiproliferative activity of these complexes have been studied on the cisplatin-sensitive A2780 ovarian cancer cell line.

2. Experimental section

2.1. Synthesis of the Pt(IV) complexes *1–4*

Compounds **1–4** were prepared according to published procedures [21,22]. Characterization and determination of the purity of the complexes were performed by means of the usual analytical techniques: elemental analysis, determination of Pt content by inductively coupled plasma–optical emission spectroscopy (ICP–OES), HPLC, electrospray ionization-mass spectrometry (ESI-MS) and multinuclear NMR.

2.2. Reduction of *1* and interaction with model proteins

Complex **1** was dissolved in water at 1 mM concentration. The solution was vortexed and sonicated at 40 °C for 15 min and the exact Pt concentration was determined by atomic absorption spectroscopy using a Varian 880Z instrument. Sodium ascorbate stock solution (45 mM) was prepared from ascorbic acid (AA, 80 mg, 0.225 mmol) dissolved in H_2O (10 mL) and neutralized with NaOH until pH 7.1. Mets7 peptide (MTGMKGMS) was purchased from GenScript (USA) and was dissolved, immediately prior to use, in pure water at a 1 mM concentration. Mnk1 protein was recombinantly expressed by standard biotechnological techniques, using *Escherichia coli* BL21DE3-Gold cells (Stratagene, USA) and a pET vector under the control of the IPTG (isopropyl-β-D-1-thiogalactopyranoside) inducible *lac* (lactose) promoter (Novagen, USA). Purification was achieved by combining immobilized metal affinity and size-exclusion chromatography, as previously described [23]. All steps of purification were carried out in the presence of an excess of the reducing agent dithiothreitol (DTT) to preserve the protein in its active form. Protein purity was determined by SDS-PAGE and ESI-MS. To remove the buffer and to reduce the excess of DTT, the apoprotein was extensively washed with deoxygenated H_2O by using Centricon filters (3 kDa molecular weight cutoff) and under strictly anaerobic condition. The freshly prepared protein sample, handled under inert N_2 atmosphere inside a glove-box, was immediately used for the incubations with the Pt complexes.

The reduction of Pt(IV) complexes by GSH (in phosphate buffer, PB) or sodium ascorbate, as well as the reactions between the Pt(IV) or Pt(II) complexes and the Mets7/Mnk1 (1:1 molar ratio) were analyzed with a dual electrospray interface and a 6530 Series Accurate-Mass Quadrupole Time-of-Flight LC/MS (Agilent, USA). ESI-MS spectrometry, due to the soft ionization, is generally accepted as an efficient method for the characterization of metal complexes in solution, albeit care must be used when correlating gas phase ions to species present in solution [24]. The reaction mixtures were kept stirring under anaerobic conditions at 37 or 40 °C and, from time to time, aliquots were removed, five-fold diluted with deoxygenated water, and injected in the mass spectrometer. Acetic acid (1% v/v) was added before injection to obtain a good volatilization. When the reaction was performed in the presence of PB, the samples for spectrometric analysis were desalted by Zip-Tip$_{C18}$ pipette tips (Millipore, USA). Binding and washing of the protein or peptide on the Zip-Tip resin were performed using 0.1% trifluoroacetic acid (TFA) in water, while the elution was performed with 0.1% TFA in acetonitrile/water (50/50 v/v). This sample was further diluted with pure water and then injected. Ionization was achieved in the positive and negative ion modes by application of +3.5 kV at the entrance of the capillary; the pressure of the nebulizer gas was 35 psi. The drying gas was heated to 300 °C at a flow rate of 8 L min^{-1}. Full scan mass spectra were recorded in the mass/charge (m/z) range of 100–3000. For all relevant peaks, it was checked that the experimental isotopic pattern corresponded to the theoretical one.

2.3. Cell culture and viability tests

All the compounds under investigation were tested on the human ovarian carcinoma cell line A2780, purchased from ECACC (European Collection of Cell Cultures, UK). The cells were grown in RPMI1640 medium supplemented with L-glutamine (2 mM), penicillin (100 IU mL^{-1}), streptomycin (100 mg L^{-1}) and 10% fetal bovine serum (FBS). Cell culture and the treatments were carried on at 37 °C in a 5% CO_2 humidified chamber. Cells were treated with the compounds under investigation according to different schedules of contact time (CT, the time for which cells are challenged with the drug

under investigation dissolved in the culture medium) and recovery (R, the time in which the cells are incubated in fresh, drug-free medium) as indicated in the tables. Cisplatin was dissolved in 0.9% w/v NaCl aqueous solution brought to pH 3 with HCl (final stock concentration 1 mM). All Pt(IV) complexes were dissolved in absolute ethanol (final stock concentration 5 mM) and stored at −66 °C. The mother solutions were diluted in complete medium, to the required concentration range. In the case of co-solvent the total absolute ethanol concentration never exceeded 0.2% (this concentration was found to be non-toxic to the tested cell). To assess the growth inhibition of the compounds under investigation, a cell viability test, i.e. the resazurin reduction assay, was used [25]. Briefly, cells were seeded in black sterile tissue-culture treated 96-well plates. At the end of the treatment, viability was assayed by 10 μg mL^{-1} resazurin (Acros Chemicals, France) in fresh medium for 1 h at 37 °C, and the amount of the reduced product, resorufin, was measured by means of fluorescence (excitation λ = 535 nm, emission λ = 595 nm) with a Tecan Infinite F200Pro plate reader (Tecan, Austria). In each experiment, cells were challenged with the drug candidates at different concentrations and the final data were calculated from at least three replicates of the same experiment performed in triplicate. The fluorescence of 8 wells containing the medium without cells was used as blank. Fluorescence data were normalized to 100% cell viability for untreated (NT) cells. Half inhibiting concentration (IC_{50}), defined as the concentration of the drug reducing cell viability by 50%, was obtained from the dose–response sigmoid using Origin Pro (version 8, Microcal Software, Inc., Northampton, MA, USA).

2.4. Cellular accumulation and DNA platination

A2780 cells were seeded in 10 mm Petri dishes or 175 cm^2 flasks and treated with the complexes under investigations (10 μM) with the following schedules: 4 h CT, 24 h CT, or 4 h CT followed by 20 h R (4 h CT + 20 h R). At the end of the exposure, cells were washed three times with phosphate buffered saline (PBS), detached from the Petri dishes using 0.05% Trypsin 1X + 2% EDTA (HyClone, Thermo Fisher) and harvested in fresh complete medium. An automatic cell counting device (Countess®, Life Technologies), was used to measure the number and the mean diameter from every cell count. From the same sample, about 5 × 10^6 cells were taken out for cellular accumulation analysis, while about 20 × 10^6 cells were taken out for the DNA platination analysis. Moreover, 100 μL of medium was taken out from each sample at time zero to check the extracellular Pt concentration.

For the cellular Pt accumulation analysis, the cells were transferred into a borosilicate glass tube and centrifuged at 1100 rpm for 5 min at room temperature. The supernatant was carefully removed by aspiration, while about 200 μL of the supernatant was left in order to limit the cellular loss. Cellular pellets were stored at −80 °C until mineralization.

Platinum content determination was performed by ICP-MS (Thermo Optek X Series 2). Instrumental settings were optimized in order to yield maximum sensitivity for platinum. For quantitative determination, the most abundant isotopes of platinum and indium (used as internal standard) were measured at *m/z* 195 and 115, respectively. Mineralization was performed by the addition of 70% w/w HNO_3 to each sample (after defrosting), followed by incubation for 1 h at 60 °C in an ultrasonic bath. Before the ICP-MS measurement, the HNO_3 was diluted to a final 1% concentration.

The cellular Pt accumulation was referred as ng Pt per 10^6 cells. In order to obtain the Pt cellular concentration, the total cellular volume of each sample was obtained considering the mean cell diameter and cell number.

For the DNA platination analysis, the cells were transferred into a plastic tube and centrifuged at 1100 rpm for 5 min at room temperature. The supernatant was carefully removed by aspiration, and the cell pellets were stored at −20 °C until the total genomic DNA was extracted with a commercial kit (PerfectPure Cultured Mammalian Cells, 5Prime-Eppendorf), following the manufacturer's instructions. Briefly, during cell lysis, DNA was purified by RNAse A and proteinase K treatment, then extracted on silica-based centrifugation columns. After washing, DNA was eluted in 300 μL of elution buffer. An amount of 8 μL of sample or elution buffer (used as blank) was diluted in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) to 80 μL, corresponding to a 0.5 cm path length in UV-transparent microplate half-area wells (UV-Star®, Greiner Bio-one). Absorbance at 260 nm (A_{260}, relative to nucleic acids) and 280 nm (A_{280}, relative to proteins) was recorded from triplicate wells with a Tecan Infinite F200Pro plate reader (Tecan, Austria). For each well, A_{260} and A_{280} were corrected by subtraction of the background, then the purity of the samples was verified by means of the A_{260} to A_{280} ratio, that always resulted >2. After the subtraction of mean A_{260} of blank wells, the DNA concentration was computed from the corrected A_{260} by means of a calibration curve obtained with calf thymus DNA. Under these conditions an absorbance of 1 unit at 260 nm corresponds to 100 μg of DNA per mL. The remaining amount of DNA elution buffer was transferred into a borosilicate glass tube and its precise volume determined by means of weight to compute the total amount of DNA, then stored at −20 °C until mineralization. Mineralization and Pt content determination were performed as above reported for the whole cells.

The level of Pt found in cells after drug treatment and normalized upon the cell number (cellular Pt accumulation) was expressed as ng Pt per 10^6 cells. The mean cellular volume, calculated from the actual mean cell diameter measured for every sample, was used to obtain the cellular Pt concentration. The ratio between the cellular and actual extracellular concentrations, is defined as accumulation ratio, AR [11].

The amount of Pt bound to DNA (experimental P_{DNA}) was expressed as pg of Pt per μg of DNA experimentally found. Unfortunately the DNA recovery by the commercial kits is never quantitative especially when a large number of cells is processed (in the actual case the yield was evaluated about 70%). This discrepancy can be overcome by taking into account that one million of human female diploid nuclei contain 6.55 μg of DNA [26] and then calculating the overall amount of DNA-bound Pt per million cells by multiplying experimental P_{DNA} by 6.55:

$$\text{overall } P_{DNA}\left[\frac{\text{pg Pt}}{10^6 \text{ cells}}\right] = \text{experimental } P_{DNA}\left[\frac{\text{pg Pt}}{\mu\text{g DNA}}\right] \times 6.55\left[\frac{\mu\text{g DNA}}{10^6 \text{ cells}}\right].$$

Therefore, the percent ratio of Pt bound to DNA (P_{DNA} %) is given by the ratio between overall P_{DNA} (in pg per 10^6 cells) and cellular Pt accumulation (in ng per 10^6 cells) as follows:

$$P_{DNA}\% = \frac{\text{overall } P_{DNA}\left[\frac{\text{pg Pt}}{10^6 \text{ cells}}\right]}{1000 \times \text{cellular Pt accumulation}\left[\frac{\text{ng Pt}}{10^6 \text{ cells}}\right]} \times 100.$$

3. Results and discussion

3.1. Interaction of *1–4* complexes with copper transporters

Recently several publications have highlighted the role of transporters in the active translocation of Pt(II) drugs across biological membranes [27]. In particular at least 16 membrane transporter proteins belonging to different families have been recognized as possible determinants in the transport of platinum-based anticancer compounds. Copper transporter 1 (Ctr1) is the major copper carrier operating via facilitated diffusion or secondary active transport in mammals with an important role in copper homeostasis. The protein contains two extracellular, N-terminal, domains rich in methionines and one intracellular,

C-terminal, domain rich in histidines which can act as metal binding sites [28–30]. Many recombinant studies have provided strong evidence for the role of Ctr1 in both platinum cellular accumulation and corresponding antiproliferative activity [31–33].

On the contrary, ATP7A and ATP7B are membrane transporters that translocate copper into the Trans-Golgi network and are involved in the copper export achieved by relocation of the transporter on the cell membrane [34]. The overexpression of ATP7A/B in tumor cells increases their resistance to Pt(II) drugs [35]. The literature data about the possible involvement of copper transporters in the mechanism of influx/efflux of Pt(IV) complexes are limited to the observation that $[PtCl_6]^{2-}$ ion interacts with Ctr1 only upon reduction to Pt(II) [32].

To test the ability of Ctr1 and ATP7A to react directly with the Pt(IV) complexes under investigation, two model proteins (i.e. Mets7, an octapeptide resembling one of the methionine-rich motifs present on the extracellular N-terminal domain of Ctr1 [36], and Mnk1, the first cytoplasmic N-terminal domain of ATP7A [37]) were incubated with **1**, chosen as model because of its acceptable water solubility. The solution of each protein was incubated with an equimolar amount of **1** (100 μM final concentration) at 37 °C and the reaction course monitored by ESI-MS. Soon after mixing complex **1** with *apo*Mets7 or *apo*Mnk1, the ESI-MS spectra recorded in positive ion mode showed only the *apo* species (peak of doubly charged peptide at *m/z* 442.73 and series of multiply charged states of *apo*Mnk1 at the expected *m/z* values, respectively), whereas the spectra recorded in negative ion mode displayed the peak at *m/z* 417.02 corresponding to unreacted complex $\{\mathbf{1}\text{-H}\}^-$. The reactions were monitored for 5 days and in no case there were changes in the ESI-MS spectra which could witness the formation of adducts between Mets7 or Mnk1 and **1**.

Although *apo*Mets7 and *apo*Mnk1 contain methionine or cysteine residues, which have good coordinating ability towards platinum, they are unable to react with **1** nor they are able to reduce Pt(IV) to Pt(II) complexes, while such a reduction takes place readily with isoelectronic Au(III) complexes [38,39]. Therefore, the interaction with model proteins was repeated in the presence of GSH and AA, the biomolecules usually employed to test the reduction of Pt(IV) prodrugs to Pt(II) species [17,32,40].

AA is known to be the primary, low molecular weight, antioxidant present in living organisms. Considering its two pK_a values (pK_1 = 3.95 and pK_2 = 11.24) at physiological pH the prevailing species (99.9%) is the ascorbate monoanion. This can undergo two one-electron losses leading to the formation of dehydroascorbic acid [41]. Ascorbate is present at lower concentration in the blood plasma (40–80 μM) than in the cytoplasm of human neutrophils (about 1 mM). The tripeptide glutathione, γ-glutamylcysteinylglycine, is the most relevant non-protein, thiol found in all mammalian tissues. Glutathione exists in the thiol-reduced (GSH) and disulfide-oxidized (GSSG) forms. GSH is the predominant form, existing in millimolar concentrations in most cells (0.5–10 mM), whereas GSSG content is less than 1% of GSH [42]. About 85–90% of cellular GSH is in the cytosol, 10% is in mitochondria and a small percent is in the endoplasmic reticulum. GSH, a one electron reducing agent, is one of the primary defenses against toxins and oxidants in the cell, and can deactivate electrophilic drugs, including Pt drugs [43].

Since the octapeptide *apo*Mets7 resembles the methionine-rich motif of the extracellular N-terminal domains of Ctr1, the interaction with **1** (100 μM) under reducing conditions was performed by incubation with a 10-fold excess of sodium ascorbate. On the contrary, in the case of *apo*Mnk1, the interaction with **1** under reducing conditions was performed using a 10-fold excess of GSH. The peptide/protein to complex ratio was always 1:1 and the reactions were performed at 37 °C in 25 mM neutral PB and monitored over time with ESI-MS. Despite the presence of a reducing agent, no reaction took place even after 5 days of incubation. ESI-MS spectra showed only the peaks relative to *apo*Mets7 or *apo*Mnk1 (positive ions mode) and **1** (negative ions mode). Therefore, the reaction conditions were forced by increasing the absolute concentration of the reactants (0.75 mM concentration of **1** and 7.5 mM concentration of reducing agent) and the temperature (40 °C) [44]. The reaction of **1** with sodium ascorbate was monitored by ESI-MS: the intensity of the peaks of the Pt(IV) species decreased with time, until complete disappearance after 24 h, while a rather weak peak at *m/z* 323.94, corresponding to $\{[PtCl_2(NH_3)_2] + Na\}^+$, witnessed the formation of cisplatin. Thus the Pt(IV) → Pt(II) reduction has occurred without any detectable scrambling of axial acetates with equatorial chlorides, as previously reported for cisplatin-based Pt(IV) derivatives [7,18].

After reduction of **1**, the solutions were treated with *apo*Mets7 or *apo*Mnk1 to reach a Pt complex to protein ratio of 1:1 and a concentration of 100 μM for each reactant. The reaction solutions were kept at 37 °C and monitored by ESI-MS. After the addition of *apo*Mets7, a new peak corresponding to the doubly charged species $\{Mets7H^+ + PtCl^+\}$ (*m/z* 557.69) was observed right from the beginning. After 24 h, such a species became prevalent together with the $\{Mets7 + Pt^{2+}\}$ cation (*m/z* 539.71), meanwhile the intensity of the *apo* peptide peak greatly decreased (Fig. 2). It is noteworthy that the Pt(II) species loses from the early beginning both ammine ligands, which are replaced by methionine residues of Mets7. The final adducts are identical to those observed when Mets7 was reacted directly with cisplatin [32,45].

When *apo*Mnk1 was incubated with a solution of **1** previously reduced with sodium ascorbate, it started to precipitate, causing the

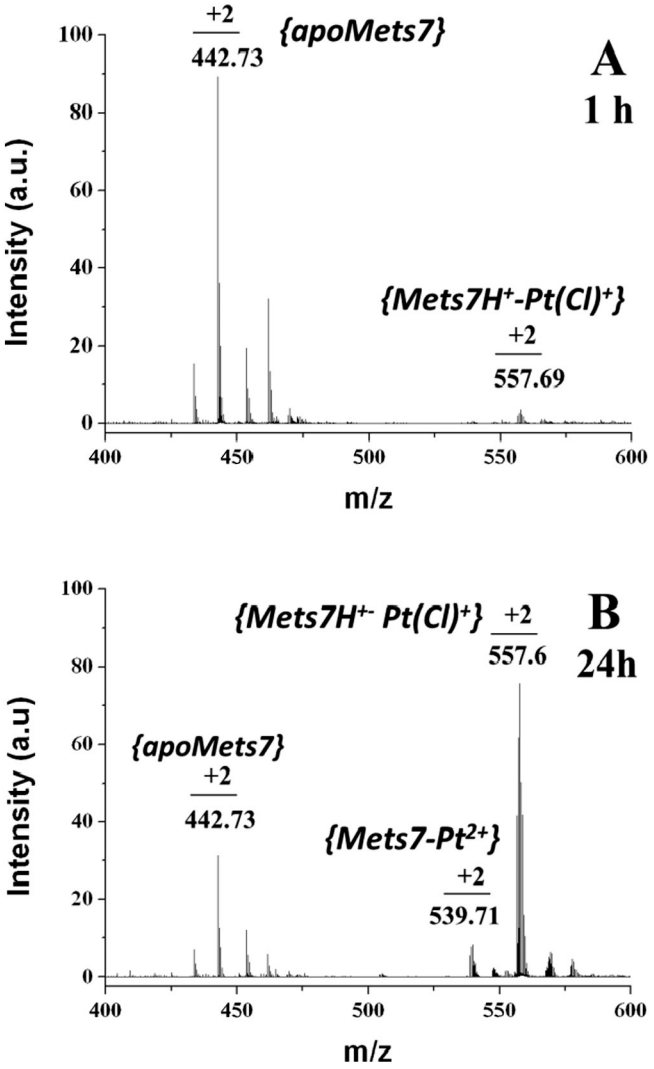

Fig. 2. ESI-MS spectra of *apo*Mets7 treated with **1** (previously reduced with sodium ascorbate) recorded after 1 h (A) and 24 h (B) of incubation.

progressive disappearance of its ESI-MS peaks, without the appearance of new peaks indicative of the formation of soluble adducts with Pt.

The formation of adducts with "naked" Pt was already observed in the case of direct reaction between cisplatin and *apo*Mets7 or *apo*Mnk1 [32,46]. In contrast, in the case of oxaliplatin, that contains the more stable chelating cyclohexane-1*R*,2*R*-diamine (dach) ligand, the "Pt(dach)" moiety was maintained intact after interaction with the model proteins [47]. For this reason a complex similar to **1**, but containing the dach ligand in place of the two ammine molecules (namely *trans*,*cis*,*cis*-[Pt(acetato)$_2$Cl$_2$(dach)]) was tested in a similar experiment. Similarly to the case of **1**, no direct reaction took place with both model proteins. However, after reduction with sodium ascorbate, the reaction with *apo*Mets7 showed decrease of the starting *apo* protein (complete disappearance after 48 h) and concomitant increase of peaks corresponding to {Mets7 + Pt(dach)$^{2+}$} (doubly charged adduct, *m*/*z* 596.71) and {Mets7H^{+} + Pt(dach)$^{2+}$} (triply charged adduct, *m*/*z* 398.15) (Fig. S1, see the Supplementary data). When Mnk1 was reacted with *trans*,*cis*,*cis*-[Pt(acetato)$_2$Cl$_2$(dach)], previously reduced with sodium ascorbate, a slight precipitation of protein occurred. However, in this case, it was possible to observe, already after about 3 h of incubation, the appearance of new signals corresponding to adduct formation between the protein and {Pt(dach)Cl}$^{+}$ or {Pt(dach)}$^{2+}$ moieties. With time the intensity of signals corresponding to the monodentate adduct {Mnk1–[Pt(dach)Cl]$^{+}$} {Mnk1–[Pt(dach)Cl]$^{+}$} (*m*/*z* 1097.55) decreased while the signals corresponding to the bidentate adduct {Mnk1–[Pt(dach)]$^{2+}$} (*m*/*z* 1101.78) increased (Fig. S2, see the Supplementary data). This two-step reaction mechanism is peculiar of Mnk1 [46].

In conclusion, the reported experiments suggest that Pt(IV) complexes, differently from Pt(II) complexes, are not substrates for copper transporters strengthening the idea that they enter the cells by passive diffusion.

3.2. Lipophilicity and cellular accumulation of *1*–*4* complexes on A2780 cell line

Cellular accumulation represents the balance at a given time between cellular influx and efflux and is an important factor determining the biological activity of any drug [48]. The lipophilicity of a drug is a key feature directly related to its ability to passively cross cellular membranes [49,50]. Generally, the shake-flask log $P_{o/w}$, in which *n*-octanol is taken as a model of the lipid bilayer of a cell membrane and water mimics the extracellular environment, is used to represent the lipophilicity of a molecule. The log $P_{o/w}$ values of complexes **1**–**4** are reported in Table 1, along with that of cisplatin. Even for such a limited number of compounds, it is possible to observe a linear relationship between log $P_{o/w}$ and the number of carbon atoms in the chain of axial ligands [16,21].

The mechanism by which the prototype cisplatin permeates cells is certainly multifactorial, as a result of a series of different processes such as passive diffusion, carrier-mediated transport and, possibly, pinocytosis. Cellular accumulation affects the activity of cisplatin: a decrease in Pt accumulation, due either to decrease in uptake or increase in efflux, is one of the main causes of chemoresistance [51,52].

Instead of using the cellular Pt accumulation (ng of Pt per 10^6 cells), the accumulation ratio (AR), a concept similar to the "accumulation grade of factor" previously defined by Gust et al. [53], was employed in the present paper. AR is the dimensionless ratio between the cellular Pt concentration (taking into account the experimentally measured cell volume) and the extracellular Pt concentration (i.e. the actual concentration of Pt in the culture medium) [11].

The AR value of **1**–**4** and cisplatin (all employed at 10 μM concentration) was measured in A2780 cells after *i*) 4 h CT, *ii*) 24 h CT, and *iii*) 4 h CT followed by 20 h R in fresh, drug-free complete medium. In the case of Pt(IV) complexes, all AR values increase with log $P_{o/w}$, as well as with the length of the axial carbon chains, as expected for passively diffusing molecules [40].

As shown in Table 1, the AR of cisplatin increased from 4 h CT to 24 h CT. However, cisplatin accumulation significantly dropped when, after 4 h CT, the incubation was prolonged in drug-free medium for further 20 h (4 h CT + 20 h R), indicating an extensive, albeit incomplete, efflux process.

Also in the case of complexes **1**–**3**, the AR progressively increased when the CT was prolonged up to 24 h. On the contrary, the AR of **4** was quite similar ($p > 0.05$, two-sample t-test) in the three experimental conditions (i.e. AR ≅ 50). This saturation plateau suggests a limit in the storing of **4** within the viable cells.

The effect of recovery is different for the four Pt(IV) complexes. Similarly to cisplatin, the less lipophilic compounds **1** and **2** show a significant decrease in AR upon incubation in drug-free medium, whereas the AR value is substantially maintained for **3** and **4**.

Data in Table 1 also show that, for a given incubation time, the AR increases in the following order: **1** < cisplatin ≤ **2** < **3** < **4**. The diacetato **1** and the dibutanoato **2** exhibit an AR lower than and equal to that of cisplatin, respectively. It is possible to hypothesize that for **1** and **2** the lack of influx protein-mediated transport (e.g. Ctr1, operating for cisplatin [32], but not for intact Pt(IV) complexes) is not fully compensated by the increase in passive diffusion (the only uptake process occurring for Pt(IV) derivatives) due to their modest lipophilicity, whereas the opposite is true for **3** and, especially, for **4**.

The decrease in AR observed for cisplatin during recovery is related to the already described efflux transporters. Previous studies demonstrated that intact cisplatin is the predominant species in the cytosolic low molecular weight fraction, but it is nearly completely cleared upon 1 h of recovery by means of extrusion through the P-type copper-transporters [54]. The remaining fraction is mainly sequestered by the sulfur proteins, GSH [55], and by other high MW molecules (i.e. RNA). However, since platinum adducts are slowly extruded from the cells [56,57], the total amount of accumulated Pt increases with the duration of the contact time (from 4 to 24 h CT). Moreover, a significant amount of Pt remains inside the cells also during the recovery period.

Since Pt(IV) complexes act as prodrugs for cisplatin [17], it is intended that reduction products undergo the same fate as cisplatin itself. As far as Pt(IV) complexes are not a substrate for the extruding copper transporter, the accumulated total Pt should be the net result between the Pt(IV) uptake and the efflux of the Pt(II) metabolites. According to previous ^{13}C NMR and X-ray absorption near edge spectroscopy (XANES) studies performed on A2780 cells for **1** [6,58], Pt(IV) complexes are significantly reduced to cisplatin within 4 h and completely reduced after 24 h. Complexes **2**–**4** showed similar reduction kinetics than **1** when challenged with AA at physiological pH [40]. Therefore, it is conceivable that during the recovery period, platinum detoxification mainly involves the Pt(II) metabolites. Unfortunately, ICP-MS determines only the total amount of Pt, without distinguishing between its oxidation states. Noteworthy, when the initial AR of Pt(IV) compounds was similar to that of cisplatin, also the efflux had a similar efficiency; on the contrary, for compounds with high AR (i.e. **3**

Table 1
AR of A2780 cells treated with 10 μM concentration of the platinum complexes. Data are means ± standard deviations of at least 3 independent replicates and were compared to those obtained for each drug after 4 h CT by means of the two sample t-test (*$p < 0.05$;**$p < 0.01$; ***$p < 0.001$; NS = value not statistically different from control sample).

Compound	log $P_{o/w}$	Accumulation ratio, AR		
		4 h CT	4 h CT + 20 h R	24 h CT
Cisplatin	−2.27[a]	1.4 ± 0.6	0.7 ± 0.2 (*)	4.5 ± 1.1 (***)
1	−1.92[b]	0.3 ± 0.1	0.08 ± 0.02 (**)	0.7 ± 0.2 (**)
2	−0.39[b]	2.3 ± 0.8	1.8 ± 0.2 (*)	16.3 ± 5.6 (***)
3	1.14[b]	20.7 ± 4.6	15.2 ± 3.0 (NS)	53.0 ± 16.9 (***)
4	4.1[b]	41.0 ± 8.5	46.8 ± 9.1 (NS)	59.6 ± 14.7 (NS)

[a] From Ref. [22].
[b] From Ref. [40].

and **4**) the detoxification process became less effective. Thus, unlike complexes **1** and **2** for which the excretion from A2780 cells during the recovery period was around 70% and 20%, respectively, the overall efflux was almost negligible in the case of compounds **3** and **4**.

3.3. DNA platination of A2780 cells treated with complexes 1–4

To determine the level of DNA platination (P_{DNA}) after treatment of A2780 cells with compounds **1**–**4** and cisplatin, each sample was divided into two aliquots: one was used for AR determination and the other for DNA extraction. P_{DNA} was measured by means of ICP-MS and expressed as pg of Pt per μg of DNA (Table 2). Statistically significant values of P_{DNA} were not obtained in the case of **1** for which the Pt amount was found similar to that of the untreated control (NT), in spite of using a large number of cells. Cisplatin gave the same P_{DNA} ($p > 0.05$) at 4 and 24 h CT, but the P_{DNA} dropped to lower values when, after incubation with the drug for 4 h, cells were incubated in drug-free medium for further 20 h (4 h CT + 20 h R).

It has been previously shown that P_{DNA} of cisplatin is proportional to the external concentration, but not to the duration of the incubation [59]. Accordingly, the present data confirm that P_{DNA} caused by cisplatin was similar for 4 and 24 h CT (Table 2); the found value is similar to that previously reported for ovarian cancer cells treated with 7 μM cisplatin (241 Pt atoms per 10^6 nucleotides correspond to 16 pg of Pt per μg of DNA) [55]. Thus, P_{DNA} represents the result, at a given time, of the equilibrium between DNA platination and DNA repair. The removal of Pt-DNA adducts starts within 2 h [55], a time-lapse comparable to the first aquation reaction, which is the rate-delimiting step for platination by cisplatin [60]. Moreover, as previously reported [61], an efficient removal of the Pt-DNA adducts is observed after post-incubation in drug-free medium for 20 h (Table 2). Pt(IV) complexes generate the same metabolites as cisplatin, however their formation is delayed by the reduction kinetics. In general, the trend of P_{DNA} parallels that of AR (Tables 1 and 2). Accordingly, P_{DNA} by **2**–**4** significantly increased from 4 to 24 h CT. The recovery period in drug-free medium had different effects, P_{DNA} was significantly lowered for complex **2** (probably as a result of the combination between Pt efflux and DNA repair); on the contrary, P_{DNA} was unchanged in the case of **3** and even increased in the case of **4**.

The percent ratio between overall P_{DNA} and cellular Pt accumulation (both referred to one million cells, see Experimental section) is reported as P_{DNA} % (Table 3). P_{DNA} % strongly depends on the cell line employed and its status, the compound under study, its concentration, and the time scale of the experiment. Therefore, for a meaningful comparison all the experiments were performed in strictly analogous conditions.

For cisplatin, the amount of DNA-bound platinum remains almost unchanged, while the cellular Pt content increases with the treatment time, mainly for sequestration of cisplatin by off-target biomolecules such as S-proteins and RNA, and consequently P_{DNA} % decreases with time. In our experimental conditions (i.e. 24 h CT), P_{DNA} % for cisplatin was around 1%, in agreement with the common belief that approximately 1% of the cellular Pt reacts with genomic DNA [62–64]. As far as the Pt(IV) complexes are concerned, **2** and **3** exhibited values of P_{DNA} % again around 1%. In contrast, the most lipophilic compound **4** exhibited the highest P_{DNA} %, (more than 10%). Interestingly, **4** is poorly detoxified by the cells and maintains a high platination level even after drug removal from the culture medium (P_{DNA} % = 8%).

Table 2
Overall Pt bound to DNA (P_{DNA}) of A2780 cells treated with 10 μM concentration of the platinum complexes for 4 h CT, 4 h CT + 20 h R and 24 h CT. Data are means ± standard deviations of at least 3 independent replicates and were compared to those obtained for each drug after 4 h CT by means of the two sample t-test (*$p < 0.05$;**$p < 0.01$; ***$p < 0.001$; NS = value not statistically different from control sample).

Compound	P_{DNA} [pg of Pt per μg of DNA]		
	4 h CT	4 h CT + 20 h R	24 h CT
Cisplatin	21.4 ± 10.9	4.8 ± 1.1 (**)	15.1 ± 3.3 (NS)
2	4.9 ± 1.1	1.9 ± 0.1 (**)	22.3 ± 10.9 (NS)
3	38.5 ± 21.7	36.4 ± 0.5 (NS)	156.3 ± 81.0 (**)
4	113.0 ± 41.4	506.1 ± 114.4 (***)	1697.6 ± 571.8 (***)

Table 3
% of Pt bound to DNA (P_{DNA} %, i.e. the ratio between P_{DNA} and cellular Pt accumulation multiplied by 100). Data are means ± standard deviations of at least 3 independent replicates.

Compound	P_{DNA} %		
	4 h CT	4 h CT + 20 h R	24 h CT
Cisplatin	4.6 ± 1.4	1.9 ± 0.2	1.4 ± 0.1
2	1.0 ± 0.3	ND	0.6 ± 0.2
3	0.7 ± 0.3	0.8 ± 0.1	1.4 ± 0.6
4	1.6 ± 0.4	8.0 ± 4.0	10.9 ± 3.7

ND = not determined since P_{DNA} for this sample is not statistically different from the untreated control.

3.4. In vitro antiproliferative activity

In the attempt to correlate the biological effects with the AR and P_{DNA} values, the residual cell viability was evaluated under strictly analogous conditions. A2780 cells were treated with cisplatin and **1**–**4** for 4 h CT, 4 h CT + 20 h R, or 24 h CT and the residual viability was measured at the end of the experiments by means of a cell viability assay (Fig. 3). After 4 h CT no significant changes were observed in terms of viability as expected for genotoxic agents, that require considerable amount of time to manifest their action. On the contrary, the two longer treatment schedules lowered the viability.

The biological effect (growth inhibition) of Pt-based drugs depends both on the amount of DNA platination and activation of DNA repair systems [65], that lead to different cellular fates [66,67]. It is found that for different Pt(IV) complexes and different treatment times the residual viability of A2780 cells decreases exponentially with the increase of DNA platination (Fig. 4).

4. Conclusions

In the present work, a direct relationship between lipophilicity, cellular accumulation, DNA platination and antiproliferative activity in a series of Pt(IV) candidate prodrugs has been found. Unlike cisplatin, Pt(IV) complexes are not substrates for copper transporter systems, therefore Pt(IV) prodrugs (**1**–**4**) are internalized by cells through passive diffusion and their efflux is unaided by transport proteins [32,68,69]. For complexes **1** and **2**, having log $P_{o/w}$ similar to cisplatin, the lack of protein-mediated transport is not fully compensated by the

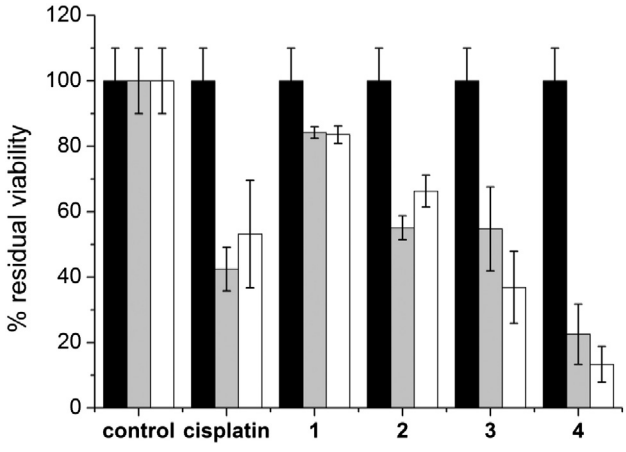

Fig. 3. Viability (%) of A2780 cells treated with 10 μM of the platinum complexes. The residual cell number was recorded after 4 h CT (black bars), 4 h CT + 20 h R (gray bars); and 24 h CT (white bars). Data are means ± standard deviations of at least 3 independent replicates.

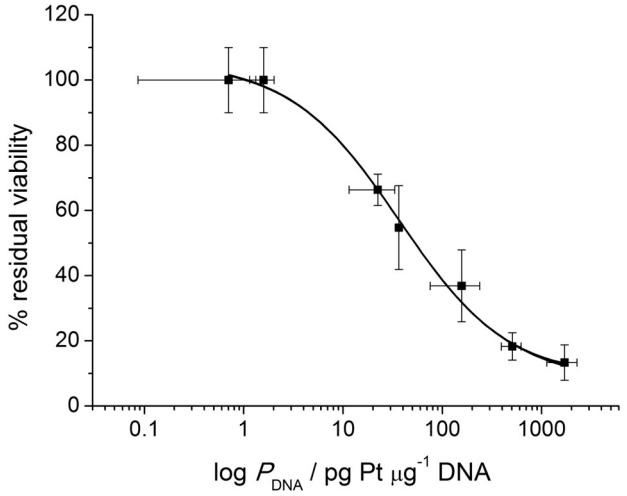

Fig. 4. Viability (%) of A2780 cells versus log P_{DNA} of **2** (24 h CT), **3** and **4** (4 h CT + 20 h R and 24 h CT). Four parameter logistic dose–response curve fit, $R^2 = 0.995$, data are means ± standard deviations of at least 3 independent replicates.

increase in passive diffusion. This results in lower/similar AR and hence lower/similar biological activity with respect to cisplatin. On the contrary, for **3** and, especially, for **4** the high lipophilicity enormously favors the passive diffusion resulting in very high AR, P_{DNA} and cell growth inhibition.

Interestingly, for compound **4** P_{DNA} increases steeply with the contact time and remains almost constant with post-incubation in drug-free medium, indicating efficient storing and poor detoxification. A similar result was recently obtained during the treatment of multicellular tumor spheroids with complexes **1**–**4** [40]. This suggests that sequestration inside cells, possibly in some lipophilic subcellular compartments, may be not per se a negative effect on the antiproliferative activity [70,71]. Thus, the most lipophilic complexes of the title series appear to be ideally suited for bypassing cisplatin resistance, when it relies on reduced cellular accumulation [72].

This study confirms the feasibility of obtaining highly lipophilic Pt(IV) anticancer prodrug candidates by using long-chain aliphatic carboxylates as axial ligands. On the contrary, in satraplatin and LA-12, the Pt(IV) prodrugs under clinical trials at the moment, is the equatorial amine (i.e. cyclohexylamine and adamantylamine, respectively) that guarantees a high lipophilicity.

Abbreviations

AA	ascorbic acid
AR	accumulation ratio
BSA	bovine serum albumin
CT	contact time with the drug
dach	cyclohexane-1*R*,2*R*-diamine
DTT	dithiothreitol
ESI-MS	electrospray ionization-mass spectrometry
FBS	fetal bovine serum
GSH	reduced glutathione, γ-glutamylcysteinylglycine
GSSG	disulfide-oxidized form of GSH
IC_{50}	half maximal inhibition concentration
ICP-MS	inductively coupled plasma-mass spectrometry
ICP-OES	inductively coupled plasma–optical emission spectroscopy
$P_{o/w}$	*n*-octanol/water partition coefficient
NT	untreated cells
PB	phosphate buffer
PBS	phosphate buffered saline
P_{DNA}	Pt bound to DNA (platination)
P_{DNA} %	percentage of Pt bound to DNA
R	recovery in fresh, drug-free medium
RPMI1640	Roswell Park Memorial Institute medium
TE buffer	Tris–HCl/EDTA buffer
TFA	trifluoroacetic acid

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.jinorgbio.2015.05.012. These data include: ESI-MS spectra of *apo*Mets7 and *apo*Mnk1 treated with [Pt(acetato)$_2$Cl$_2$(dach)].

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