Enhanced Cytotoxicity and Reactivity of a Novel Platinum(IV) Family with DNA-Targeting Naphthalimide Ligands

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ABSTRACT

Pt(IV) complexes are known as prodrugs that can potentially overcome cisplatin limitations by slowing down its reactivity, and once reduced, act as the corresponding Pt(II) drugs. We report a new approach towards *trans* Pt(IV) complexes, conceived to afford nonconventional active *trans* Pt(II) complexes with dual targeting properties. The reduction of the complexes has been studied in the presence of ascorbic acid and glutathione, showing that different species are formed in the process. The interaction with DNA after reduction has been also studied and correlated to the formation of Pt(II) species. The cytotoxicity profile of the Pt(IV) complexes corroborated the rationale behind this approach.

INTRODUCTION

Metal complexes have been widely used in diagnosis and therapy, and platinum derivatives, and in particular cisplatin, are the leaders in the chemotherapy of several types of cancer, in spite of their relevant side effects[1, 2]. For a long time researchers have worked towards increasing the activity of those drugs, in order to minimize the unwanted effects[3]. But only the so-called "nonconventional" metallodrugs have reached innovative results, generating potential drugs with higher activity and, in some particular cases, higher specificity[4]. Two of the most successful approaches for nonconventional Pt complexes are exploring the *trans* configuration and the variation on the oxidation state using Platinum(IV) complexes.[5]

Trans Pt(II) complexes were first reported in the late 1980´s [6], and our research group reported more recently active *trans* platinum complexes with aliphatic amines[7]. Based in this particular discovery, we have synthesized several active derivatives, varying the ligands in *trans* configuration to the aliphatic amines. The different approaches took advantage of bioactive ligands such as steroids[8], phosphine groups[9], planar amines[10] and more recently naphthalimides with fluorescent and intercalating properties[11]. Those last derivatives showed DNA targeting abilities, and they can be used not only as probes but also as targeted vehicles in the biological system.

Pt(IV) complexes have been largely accepted by the scientific community as prodrugs, activated *in vivo* via reduction of Pt(IV) to Pt(II) compounds with the loss of the two axial ligands.[12] The hypothesis to explain the mechanism of action of Pt(IV) prodrugs is that the DNA damage is caused by the Pt(II) complex, implying that the reduction of the Pt(IV) to Pt(II) must take place assisted by biological reducing agents or by the reasonably hypoxic conditions in the tumoral microenvironment[12].

There are many reports on the mechanism of reduction of Pt(IV) complexes and it is difficult to establish an unequivocal correlation between the different factors and reduction potential. [13] The axial ligands and the reduction potential seems to have the strongest effect, while other factors like the equatorial ligands show a smaller effect[12]. As it is largely accepted that the mechanism of action of these Pt(IV) complexes is based on its reduction *in vivo*, it is easy to envisage that variations on the ligands should allow the modulation of their pharmacokinetic properties such as: a) reduction potential (E^o), b) water solubility, stability and lipophilicity, and c) predisposition to produce secondary reactions to achieve their biological target. Therefore, the properties of these prodrugs can be controlled by modifying the nature of their axial ligands.

Our group reported the first mononuclear Pt(IV) complexes carrying phosphine groups[14] as equatorial ligands, which proved to be quite active in vitro and showed not an easy reduction versus agents such as glutathione (GSH) and ascorbate. These encouraging results with the mono-phosphine derivatives prompted us to explore a new series of Pt(IV) complexes taking into consideration the naphthalimides Pt(II) derivatives mentioned before. The DNA targeting ability of the new complexes can benefit from the improved features of Pt(IV) prodrugs, which are less prone to afford aquation in the biological media[15]. Moreover, the study of their behavior in solution would shed more light into the mechanism of action of Pt(IV) complexes.

EXPERIMENTAL METHODS

General procedures

Mono-dimensional ^{195}Pt-NMR, ^{13}C-NMR and ^{1}H-NMR and [^{1}H-^{13}C] bi-dimensional experiments were performed in DMSO-d_6 and acetone d_6 using a Bruker AMX-300 (300 MHz) spectrometer at room temperature (25 ºC). HPLC was performed in equipment Agilent Technology 1200 Infinitive Series. Elemental analyses were performed on a Perkin Elmer 2400 Series II

microanalyzer. Mass spectrometry assays were performed in a Hybrid Quad-Tof (QTOF) mass spectrometer: QSTAR (ABSciex) using the samples from the NMR experiments (deuterated and no deuterated solvents).

Synthesis and characterization of complexes

The starting materials *trans*- Pt(II) complexes: **Pt(II)-1a**, **Pt(II)-1b**, **Pt(II)-2a** and **Pt(II)-2b** were synthesized as previously reported[11]. The Pt(IV) complexes were achieved using Cl_2 gas over a solution of the corresponding Pt(II) complex, as indicated:

A Cl_2 gas generator (set up by hydrochloric acid drip method over $KMnO_4$ and placed as a bubbled system) was connected into a reaction flask with a suspension of 30 mg of the corresponding *trans*-Pt(II) complex in methanol. The linked system allowed a continuous Cl_2 gas bubbled into the suspension which was kept continuously from 3 to 5 h until a clear solution was formed. The solvent was removed under reduced pressure to give the final yellow solid, which was dried in an oven. All the complexes were characterized by the usual techniques (see detailed data described below and bi-dimensional NMR spectra collected in the SI: SI3 to SI10). The abbreviation stand for: ipa (isopropylamine); dma (dimethylamnine); abid [2-(3-Aminopropyl)-1H-benz(de)isoquinoline- 1,3 (2H-dione)]; dibibi: [*N*-(3-(1,3-dioxo-1H-benz(de)isoquinolin-2-(3H)-yl)propyl) isonicotinamide]

- *trans*-[$PtCl_4$(abid)(ipa)], **Pt(IV)-1a:** ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 8.50 – 8.43 (m, 4H), 7.86 (t, *J* = 7.7 Hz, 2H), 4.08 – 4.04 (t, *J* = 6.3 Hz, 2H), 3.21 – 3.14 (m, 1H), 2.78–2.72 (m, 2H), 2.18 – 2.13 (m, 2H), 1.24 (d, *J* = 6.4 Hz, 6H). ^{13}C-NMR (DMSO-d_6, 75 MHz) δ 164.0, 134.8, 131.7, 131.4, 127.8, 127.7, 122.5, 49.4, 44.8, 37.5, 27.3, 22.8.^{195}Pt-NMR (DMSO-d_6, 64 MHz): δ -211.7. Elemental Analysis, Found: C, 32.87%; H, 3.40%; N, 5.99%. Calculated $PtC_{18}H_{23}Cl_4N_3O_2$,: C, 33.25%; H, 3.56%; N, 6.46%. Yield: 62%.

- *trans*-[$PtCl_4$(abid)(dma)], **Pt(IV)-1b**: ^{1}H-NMR (acetone-d$_6$, 300 MHz): δ 8.61 – 8.46 (m, 4H), 7.92 (t, *J* = 7.8 Hz, 2H), 4.37 (t, *J* = 6.3 Hz, 2H), 3.20 – 3.14 (m, 2H), 2.75 (d, *J* = 5.7 Hz, 6H), 2.44 – 2.35 (m, 2H). ^{13}C-NMR (acetone-d$_6$, 75 MHz): δ 165.7, 135.9, 133.5, 132.7, 129.7, 128.7, 124.3, 46.4, 38.4, 29.2. ^{195}Pt-NMR (acetone-d$_6$, 64 MHz): δ -302.2. MS (MALDI) m/z: 600.1 [M-Cl]$^+$, 565.1 [M-2Cl]$^+$. Elemental Analysis, Found: C, 33.00%; H, 3.29%; N, 6.47%. Calculated $PtC_{17}H_{21}Cl_4N_3O_2$: C, 33.12%; H, 3.59%; N, 6.47%. Yield: 38%.

- *trans*-[$PtCl_4$(dibibi)(ipa)], **Pt(IV)-2a**: ^{1}H-NMR (DMSO-d$_6$, 300 MHz): δ 9.10 (s, 1H) 9.08 (d, *J* = 6.6 Hz, 2H), 8.46 (m, 4H), 8.01 (d, *J* = 6.6 Hz, 2H), 7.86 (t, *J* = 7.8 Hz, 2H), 6.68 (m, 2H), 4.15 (t, *J* = 7.1 Hz, 2H), 3.36 (m, 2H), 3.30 (t, *J* = 7.1 Hz, 2H), 1.97 (m, 2H), 1.33 (d, *J* = 6.5 Hz, 6H).^{13}C-NMR (DMSO-d$_6$, 75 MHz): δ 163.4, 162.9, 152.6, 146.3, 134.3, 131.2, 130.6, 127.5, 127.1, 123.6, 122.0, 50.6, 37.7, 37.5, 27.3, 22.1. ^{195}Pt-NMR (DMSO-d$_6$, 64 MHz): δ - 155.7. Elemental Analysis, Found: C, 38.07%; H, 3.73%; N, 7.10%. Calculated $PtC_{24}H_{26}Cl_4N_4O_3$: C, 38.16%; H, 3.47%; N, 7.42%. Yield: 67%.

- *trans*-[PtCl4(dibibi)(dma)], **Pt(IV)-2b**: ^{1}H-NMR (DMSO- d$_6$, 300 MHz): δ 9.0 (d, *J* = 5.3 Hz, 2H), 8.46 (m, 4H), 7.98 (d, *J* = 6.6 Hz, 2H), 7.84 (t, *J* = 7.8 Hz, 2H), 4.13 (t, *J* = 7.1 Hz, 2H), 3.37 (t, *J* = 7.1 Hz, 2H), 2.53 (d, *J* = 6.0 Hz, 6H), 1.95 (m, 2H). ^{13}C-NMR (DMSO- d$_6$, 75 MHz): δ 163.4, 162.9, 153.2, 141.7, 134.8, 130.7, 127.4, 127.2, 123.6, 122.0, 46.4, 37.6, 35.1, 27.3. ^{195}Pt-NMR ($CDCl_3$, 64 MHz): δ -162.7. Elemental analysis, Found: C, 36.61%; H, 3.23%; N, 7.24%. Calculated $PtC_{23}H_{24}Cl_4N_4O_3 \cdot 0.5H_2O$: C, 36.82%; H, 3.36%; N, 7.47%. Yield: 53%.

Stability studies by HPLC

Stability studies of the *trans*-Pt(IV) complexes were performed by HPLC analysis using the following solvents: DMSO, saline solution (0.9% NaCl), and PBS. Stock solutions of the complexes were prepared fresh in DMSO (5 mM).

For stability in DMSO, 1 mL of 100 μM solution of the test compound was incubated at 37 ºC temperature and aliquots of 20 μL were taken at various time points (0, 1, 2, 5 and 24 h), and analyzed by RP-HPLC on an Agilent 1200 system using a Zorbax Eclipse Plus C18 column (4.6 × 100 mm, 3.5 μm): flow rate, 1 mL/min; detection, UV 254 nm; gradient solvent system A/B (acetonitrile/water), initial 30% A + 70% B; 10 min linear gradient to 70% A + 30% B; 5 min linear gradient to 100% A. The disappearance of the compound over time was expressed as the remaining percentage compared to the initial amount. For studies in saline or PBS solutions, 1 mL of 100 μM of the test compound in saline solution or PBS was incubated at 37 °C. At different time points aliquots were analyzed by RP-HPLC analysis following the aforementioned conditions. In these cases, the disappearance of the compound was monitored at 210 nm.

NMR studies with reducing agents

A solution of 8 mg of the complex (**Pt(IV)-1a** and **Pt(IV)-2b**) in 50 μL of DMSO-d_6 was slowly added to a 300 μL of a mixture with D_2O: DMSO-d_6 (1:1) at 37 ºC using a thermoshaker. A solution of the equivalent amount of the reducing agent: GSH or Ascorbic acid (AsA) in 150 μL of D_2O was carefully added to the complex solution at 37 ºC. Each sample was monitored by ^{195}Pt-NMR and by ESI from fresh to 24 h. The samples were duplicated, checked by NMR and injected to ESI mass equipment afterwards.

DNA interaction studies

The plasmid pBR322 DNA stock was purchased to Gencust at a concentration of 0.5 μg/μL in phosphate buffer 50 mM (pH = 7.4). Stock solutions of the complexes were prepared in DMSO at 5 mM and then diluted with water until the desired concentration. The DNA interaction studies were performed in a total volume of 20 μL. The 20 μL containing 0.125 μg/μL of DNA in 10 mM

Tris-HCl (pH 7.6) and 1 mM EDTA, were incubated with the platinum compounds at ri values ranging from 0.05 to 0.2 (defined as the molar ratio Pt/nucleotide). The samples were incubated at 37 ºC for 24 h, after which 2 μL of a loading dye buffer containing 50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol was added. The total of the sample (20 μL) was loaded in the agarose gel (1.2% w/v) and the electrophoresis was carried out for a period of 150 min approximately at 70 V in TAE 1x (Tris-acetate/EDTA) buffer. After electrophoresis, the gel was immersed in 200 mL of Millipore water containing 10 μL from a 10 mg/mL stock solution of ethidium bromide for 30 min to stain the DNA. Finally, the stained gel was analyzed with a UVITEC Cambridge with a UVIDOC HD2.

Cell culture and Cytotoxicity assays

The human ovarian cancer cell lines A2780 and A2870cisR (resistant to cisplatin) (ECACC, UK) were grown in RPMI 1640 culture medium (Invitrogen) supplemented with 10% FBS at 37 °C in a humidified atmosphere of 95% of air and 5% CO_2 (Heraeus, Germany). The human non tumoral cell line HEK293 was grown in DMEM culture medium (Invitrogen) supplemented with 10% FBS in similar conditions as above.

The cytotoxicity of the complexes against the different cell lines was evaluated using a colorimetric method based on the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which is reduced by viable cells to yield purple formazan crystals. Cells were seeded in 96-well plates at a density of $1x10^4$ to $1.5x10^4$ cells per well in 200 μL of culture medium and left to incubate overnight for optimal adherence. After careful removal of the medium, 200 μL of a dilution series of the compounds (stock solutions prepared fresh in DMSO) in medium were added and incubation was performed at 37 °C/5% CO_2 for 72 h. The percentage

of DMSO in cell culture medium did not exceed 0.5%. At the end of the incubation period, the compounds were removed and the cells were incubated with 200 µL of MTT solution (500 µg/mL). After 3-4 h at 37 °C/5% CO_2, the medium was removed and the purple formazan crystals were dissolved in 200 µL of DMSO by shaking. The cell viability was evaluated by measurement of the absorbance at 570 nm using a plate spectrophotometer (Power Wave Xs, Bio-Tek). The cell viability was calculated dividing the absorbance of each well by that of the control wells. Each point was determined in at least 4 replicates in 2 independent assays.

Log P determination

The octanol-water partition coefficients for Pt(IV) and Pt(II) compounds were determined by modification of reported shake-flask method.[16] Water (50 mL, distilled after milli-Q purification) and n-octanol (50 mL) were shaken together for 96 h to allow saturation of both phases. Solutions of the complexes were prepared in the water phase and an equal volume of octanol was added. Biphasic solutions were mixed for ten minutes and then centrifuged for five minutes at 6000 rpm to allow separation. The concentration of the complexes in both phases was determined by UV-VIS. Reported logP is defined as log $[\text{complex}]_{oct}/[\text{complex}]_{wat}$.

RESULTS AND DISCUSSION

Chemistry

Chart 1 shows the synthetic routes used to achieve the Pt(IV) complexes. In our previous publication, we used the Pt(II) derivatives with aliphatic amines to react with the coordinating atom (N) bound to the naphthalimides by two different linkers: a flexible linker using a straight chain alkyl spacer and a more rigid using an aromatic ring spacer. This Pt(II) approach proved to afford dual DNA targeting complexes.[11] Based on this successful outcome, we envisaged that their

Pt(IV) complexes with chloride and hydroxy ligands would facilitate the entrance of the molecule into the tumoral cells, allowing a better intracellular accumulation and ultimately better cytotoxic activity, due to the higher amount of active complex that might reach the biological target.

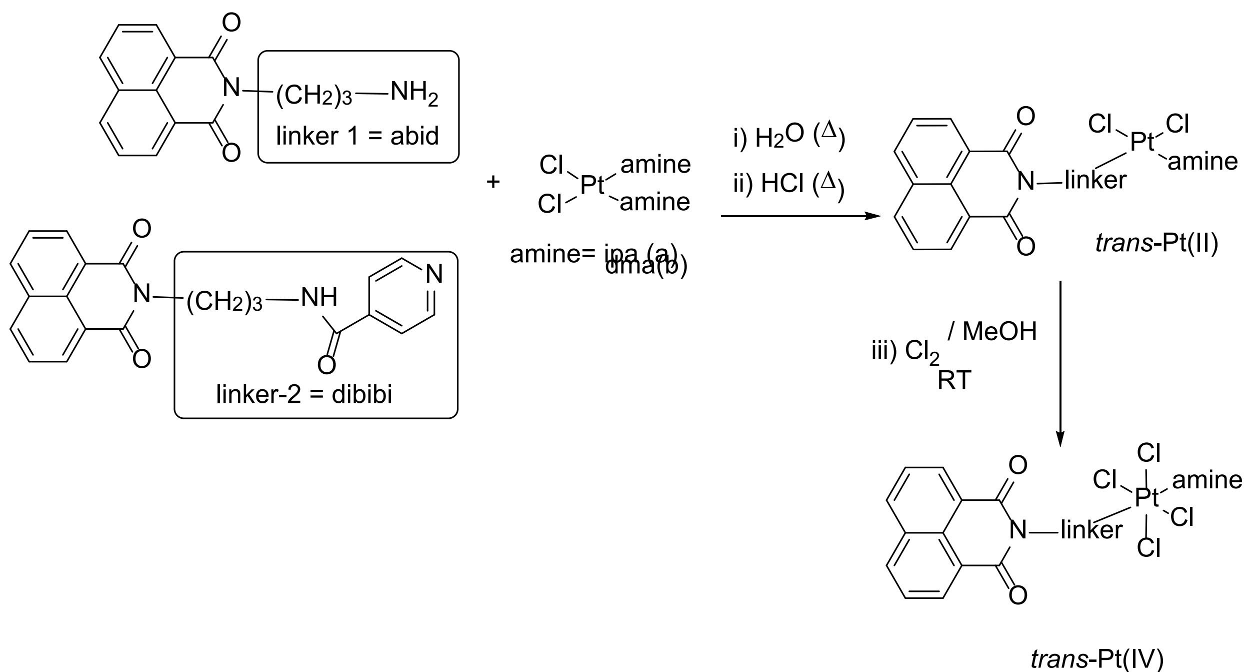

Chart 1. Synthetic routes explored to achieve the Pt(IV) complexes. i) and ii) steps described in reference [11]. Step iii described in this manuscript

The most common strategy to obtain Pt(IV) complexes is the oxidation of the parent Pt(II) complexes using Cl_2 or H_2O_2. After many oxidation attempts with Cl_2 using different solvents, we found that MeOH rendered a higher amount of a cleaner oxidation product compared with water, chloroform, acetone and dichloromethane which are the most commonly used solvents in the literature (Chart 1). Indeed, we were able to achieve the final Pt(IV) complexes: **Pt(IV)-1a**, **Pt(IV)-2a**, **Pt(IV)-1b**, and **Pt(IV)-2b** from *trans* Pt(II) derivatives: **Pt(II)-1a**, **Pt(II)-2a**, **Pt(II)-1b**, and **Pt(II)-2b** with yields from 38 to 67%. Unfortunately, the oxidation of the Pt(II) complexes using H_2O_2 did not lead to the expected results. This oxidation reaction was studied using different

conditions, as it seemed to take place at a very slow rate, generating a mixture, which made the purification process extremely tedious and laborious. After many attempts using stoichiometric amounts up to 20 equivalents of H_2O_2 during 4, 6 and up to 16 hours, we could not isolate the Pt(IV) compounds. We have monitored the reaction using ^{1}H NMR over time and we could observe the coexistence of both species in solution, being the Pt(II) always the major species. Both complexes showed a similar solubility, making the purification not feasible, and longer reaction times and/or higher amounts of oxidant reagent only produced decomposition and speciation of the compounds. Therefore, the OH derivatives were not further evaluated.

The characterization of the obtained complexes was performed by the usual spectroscopy NMR techniques (Figure SI 4-11), elemental analysis, and mass spectrometry (MALDI). ^{195}Pt-NMR was the most useful NMR experiment to confirm that the oxidation of the metal has taken place and thus to confirm the presence of Pt(IV). The $Pt(IV)N_2Cl_4$ moiety of the complexes was ascertained by examining the δ^{195}Pt NMR value around -200 ppm, which was in agreement with the values published in the literature[17]. Moreover, it also produces a change of more than 2000 ppm in the chemical shift compared with their Pt(II) counterpart, proving once more, that the oxidation has taken place.[18]

Stability of Pt(IV) complexes in solution

The stability of the Pt(IV) complexes in solution was studied by ^{195}Pt-NMR spectroscopy in DMSO-d^6. Analysis were performed for all the complexes, with samples freshly prepared and at 24 h, with spectra confirming the stability (Figure SI1). HPLC analysis for all the Pt(IV) complexes was also performed using DMSO (solvent used for the cytotoxicity studies stock solution), aqueous saline solution (administration media) and PBS (mimicking biological media).

The stability results in DMSO of complexes **Pt(IV)-2b** and **Pt(II)-2b** is shown in Figure 1. The data corresponding to the other complexes stability in DMSO are presented in figure SI2. The stabilities are plotted graphically where the stability value is expressed as the amount of complex that remains intact considering the initial amount (time t_0) as 100%. The stability of the complexes in DMSO is clearly high, as all values are between 55 and 95%, even after one week of incubation. For all analyzed Pt(IV) complexes the stability values are higher than their parent Pt(II) complexes,[11] as it can be seen clearly in Figure 1 for compounds **2b** (**Pt(II)** and **Pt(IV)**) and Figure SI1 for the other complexes, corroborating our first assumption of greater stability for the Pt(IV) prodrugs.

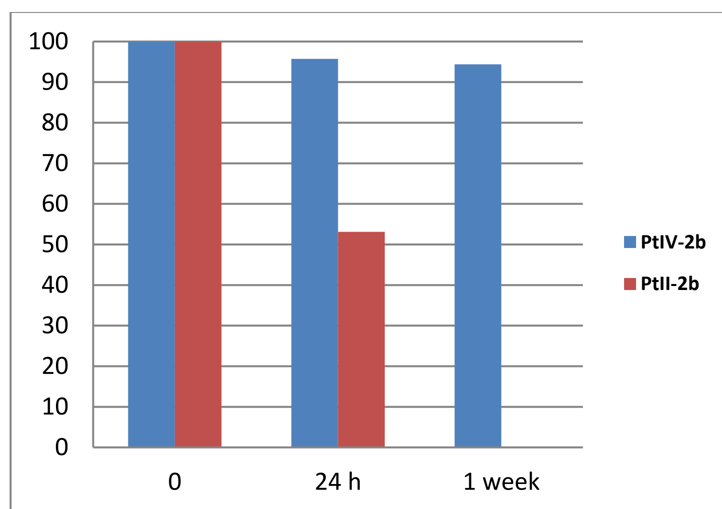

Figure 1. Stability values of **Pt(IV)-2b** and **Pt(II)-2b** complexes in DMSO**.**

In solutions such as PBS (pH = 7.4) or NaCl (0.9 %), these complexes are also stable, although the stability values slightly vary from PBS to NaCl (Figure 2). In PBS, the four Pt(IV) complexes are quite stable (up to 5 h), and the stability pattern is very similar; only after 24 h the amount of intact complexes starts to decrease to values from 44 to 55% (Figure 2, striped-square columns). With saline solution, the complexes´ stability is also high, but the values showed a higher variation,

with amounts of intact complex after 24 hours incubation ranging from 28% (in the worst case) to 65%. As it can be seen in Figure 2, all complexes are stable in both buffers, with only complex **Pt(IV)-1b** presenting a low stability in NaCl solution (28%) after 24 hours, being nevertheless two times more stable in PBS (46% of intact complex).

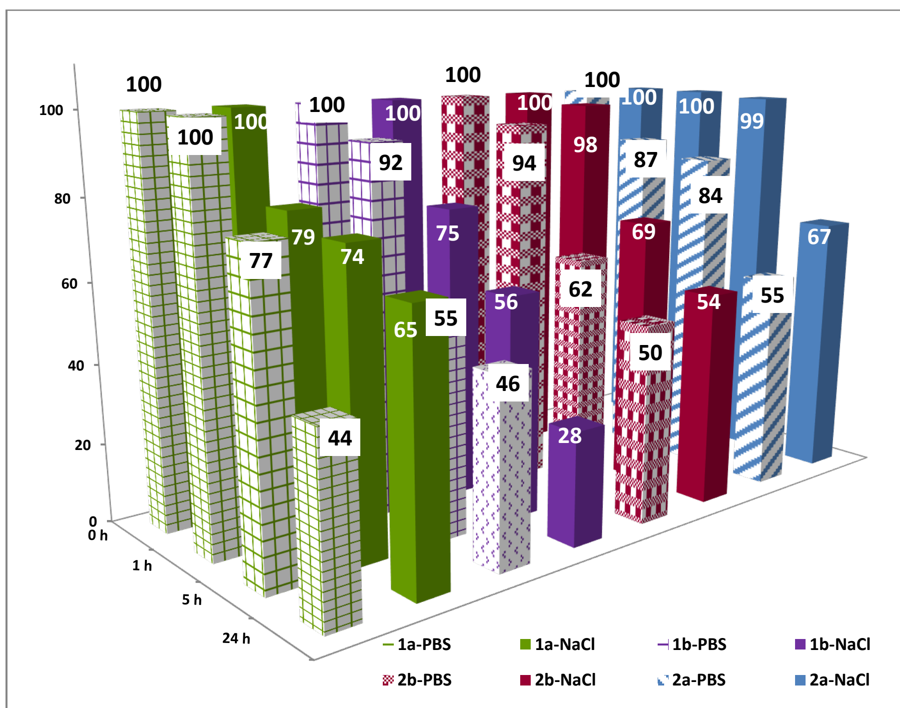

Figure 2. Stability values in PBS and NaCl of Pt(IV) complexes.

Taking together all the data, we can consider that the new Pt(IV) complexes have improved their pharmacological stability versus the corresponding parent Pt(II) complexes. For example fter 24 h, complexes The general trend of the stability is a decrease with time in every condition, but NaCl seems to have a positive effect. Moreover, it is important to point out that the reduction of the Pt(IV) complexes to the parent Pt(II) complexes has not been immediately detected neither in NaCl nor in PBS by HPLC nor in the early times by NMR. For this reason, the reduction process to afford the Pt(II) complexes is taken into consideration further in this manuscript and studied using other experiments and methods.

trans-Pt(IV) complexes interaction with DNA

After the evaluation of the stability of the Pt(IV) complexes, the following step was the study of their interaction with a model DNA molecule (plasmid pBR322). The reduction of Pt(IV) complexes usually takes place affording the parent Pt(II) complexes, although, recently other species have been also detected in biological media, showing a different interaction with plasmid DNA[19, 20]. In previous studies, we showed that Pt(II) complexes are able to form adducts with of the supercoiled ϕX174 using gel electrophoresis analysis, even in the presence of chloroquine and furthermore the cellular uptake showed accumulation in the nucleus[11]. In order to verify if the reduction of the Pt(IV) complexes under analysis affords the parent Pt(II) complexes, we have studied their interaction with a plasmid model, pBR322, in the absence and presence of biological reductants AsA and GSH[20]. Figures 3 and SI11 to SI14 present the electrophoretic mobility of the plasmid incubated with Pt(IV) and Pt(II) complexes in the presence and absence of the reductants.

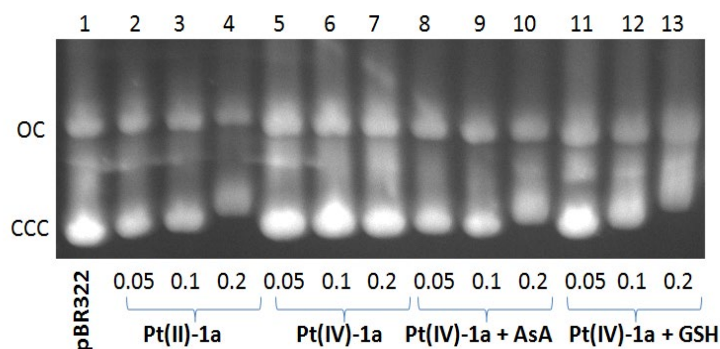

Figure 3. Agarose gel electrophoresis of pBR322 plasmid treated with **Pt(IV)-1a** complex and **Pt(IV)-1a** with AsA (ratio 1:1) and with GSH (ratio 1:2). Lane 1: DNA of control plasmid; Lanes 2-4: **Pt(II)-1a** and DNA incubated at ri = 0.05 to 0.2; Lanes 5-7: **Pt(IV)-1a** and DNA incubated at ri = 0.05 to 0.2; Lanes 8-10: **Pt(IV)-1a** with AsA (ratio 1:1) and DNA incubated at ri = 0.05 to 0.2; and Lanes 11-13: **Pt(IV)-1a** with GSH (ratio 1:2) and DNA incubated at ri = 0.05 to 0.2.

Cisplatin has been also included in this experiment (Figure SI12 and SI13) as a model of clinical metallodrug for comparison purposes. Cisplatin has been widely reported to produce changes in both plasmid DNA isoforms: reducing the covalently closed circular (ccc) mobility (via unwinding) and increasing the open circular (oc) mobility until both reach a co-migration point.[21]

After 24 hours of incubation, none of the Pt(IV) complexes showed interaction with pBR322 at any of the concentrations assayed. The concentration is represented as ri, defined as the molar ratio Pt/nucleotide (r_i up to 0.2 at figure 3 and 4: lanes 5-7 and figure SI12 and SI13: lanes 2-4), where the electrophoretic mobility of both DNA isoforms remains unchanged. These results were expected, as to our knowledge, Pt(IV) complexes have not been described to interact with plasmid DNA[20, 22, 23], contrary to Pt(II) complexes. Only complex **Pt(IV)-2a** showed a slight interaction with the ccc form at the highest ri assayed. The incubation of the different Pt(IV) complexes with AsA and GSH induces alterations in the mobility of plasmid DNA, which indicates that there is reduction, affording the Pt(II) species.

From the entire series, this change is more evident when DNA is incubated with complexes **Pt(IV)-1b**, **Pt(IV)-2a**, and **Pt(IV)-2b** (Figures SI11, SI12 and SI13); because their Pt(II) counterparts interact more strongly with the plasmid showing a pattern similar to cisplatin (Figure SI.3. for **Pt(II)-2a** and **Pt(II)-2b**). In particular, Figure 3 shows a small change in the mobility of the supercoiled form ccc when complex **Pt(IV)-1a** is incubated with the reductants. Nevertheless, reduction is probably occurring, as this phenomenon is in agreement with the smaller DNA interaction of its counterpart **Pt(II)-1a** (Figure 3: lanes 2 to 4). Comparing the incubation with AsA (line 8 to 10) to GSH (line 11 to 13), it is quite surprising to see that complex **Pt(IV)-1a** shows a higher interaction with the ccc form in the presence of GSH than with AsA, as the other three Pt^{IV} compounds seemed to be reduced faster in the presence of AsA.

The changes observed in the DNA mobility after incubation with **Pt(IV)-1b**, **Pt(IV)-2a** and **Pt(IV)-2b** with both reductants (Figure SI11, SI12 and SI13) is clearly stronger. At the maximum concentration assayed (lines 10 and 13 for figure SM1 and lines 7 and 10 for figures SI12 and SI13), the gel electrophoresis showed that the mobility of the ccc isoform is delayed, in such a way, that it migrates at the same position than oc. This effect has been typically observed for the parent Pt(II) derivatives (Figure 3 and SI11 and in SI14 for **Pt(II)-2a** and **Pt(II)-2b**). The Pt(II) complexes pattern of interaction is somewhat different to the one produced by cisplatin in which both forms of the plasmid DNA have altered their mobility until reaching co-migration[21], such interaction is quite similar to the one produced with ϕX174 in our previous publication [11].

In summary, these results indicate that the incubation of the Pt(IV) complexes with the biological reductants AsA and GSH, affords the corresponding Pt(II) complexes with the loss of the axial chloride ligands. When comparing all the experiments, it is clear that there are some differences; for example complex **Pt(IV)-2a** seems more prone to be reduced in the presence of AsA (changes in DNA mobility at $r_i = 0.1$) while the other Pt(IV) complexes need a higher concentration (changes in DNA mobility only at $r_i = 0.2$). In the incubations with GSH, there seem to be two complexes (**Pt(IV)-2a** and **Pt(IV)-2b)** more sensitive to reduction, for which, in lanes with a lower r_i there is already a change in the mobility of the ccc form of pBR322 (lanes 12 and 13, figures SI12 and SI13), possibly produced by the Pt(II) species formed.

NMR studies of *trans*-Pt(IV) complexes in the presence of biological reducing agents

In order to further assess the effect of these biological reductants on the Pt(IV) complexes reactivity, we have monitored their reaction with the biological reductants by ^{195}Pt-NMR (Figure 4). The ^{195}Pt-NMR allows easily the differentiation of the two oxidation states as their chemical

shifts are situated in very different areas and also allows the differentiation in the chemical shift of the Pt atom provoked by ligand substitution.[18] The motorization of the Pt(IV) complexes in DMSO and D_2O: DMSO-d_6 solution showed no speciation along 24h, and setting the conditions for further studies (see experimental).

The reaction of complex **Pt(IV)-2b** with AsA slowly affords the Pt(II) counterpart (signal previously reported at δ^{195}Pt= -2049 ppm). Furthermore, **Pt(IV)-2b** complex not only reduces in the presence of GSH but also, once it is reduced, coordinates to GSH. The indication of this phenomena is clear from the NMR spectra at Figure 4, where the signal raised at -2735 ppm is typical of Pt(II) species of PtN2SX moiety.[24] Complex **Pt(IV)-1a** versus GSH is reduced to the Pt(II) parent complex (at δ^{195}Pt= -2201 ppm, as previously reported) with no coordination to GSH, but on the contrary, versus AsA not only affords the parent complex but also a new species at δ^{195}Pt= -3160 ppm. This difference is possibly caused by a different reduction pathway which might be related with the higher drug cytotoxicity observed (see following section).

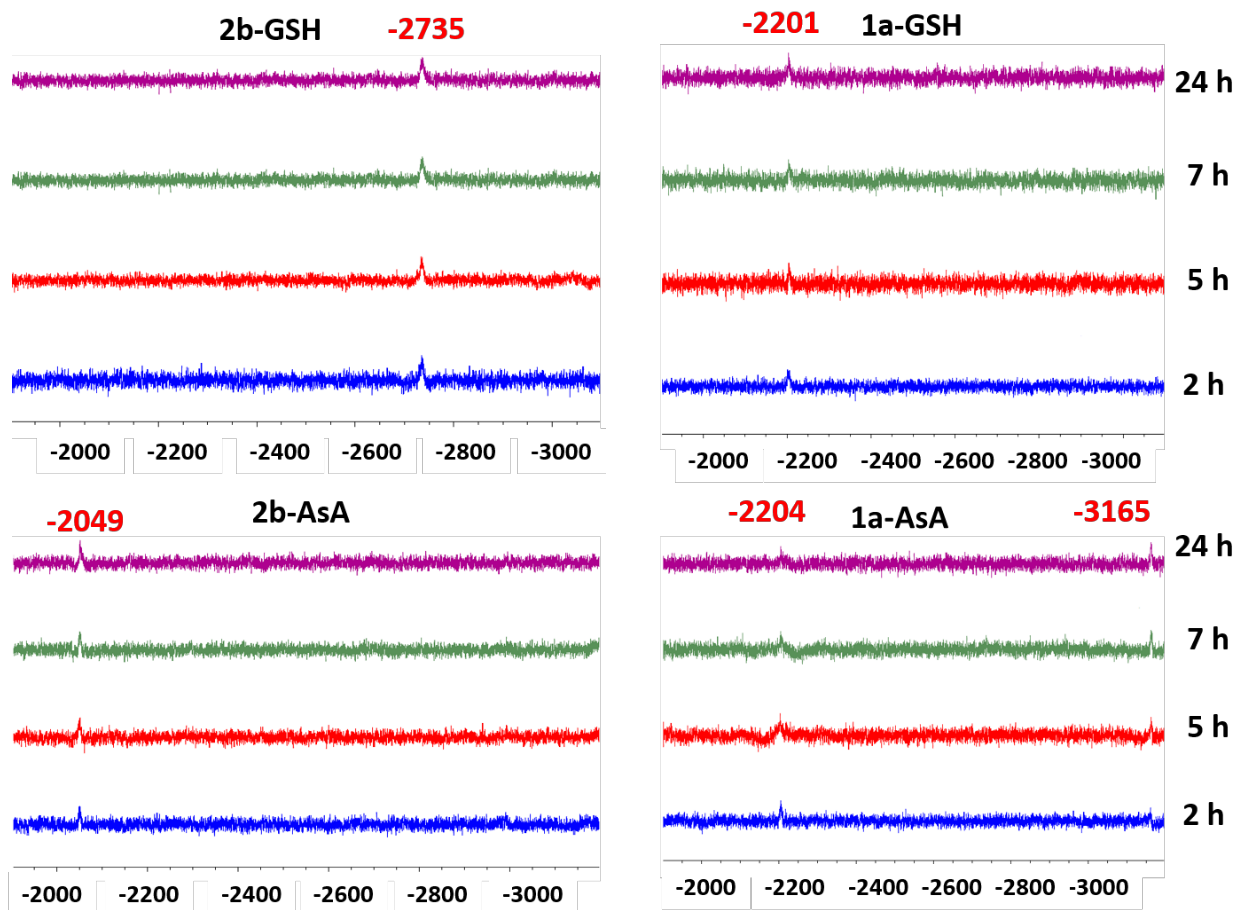

Figure 4. ^{195}Pt-NMR spectroscopy of the progress (2 to 24h) of the reaction of complexes **Pt(IV)-1a** and **Pt(IV)-2b** with GSH and ASA.

This behavior of **Pt(IV)-1a** prompted us to study the species by HPLC, which usually shows the speciation (if any) and it is often used to assess purity and stability. We have compared the species produced in such solutions with the original species detected for the Pt(II) solution previously reported. The amount of the Pt(IV) complexes remaining in solution over time and in the presence of the reducing agents has been calculated, and the amount of the Pt(IV) complexes at the initial time (t_0) measured before the addition of the reductants was considered100%.

As expected, the reduction of complex **Pt(IV)-1a** with both reductants is so rapid that after 5 h versus GSH, the amount of Pt(IV) complex detected is only 3%. Furthermore, the amount of Pt(IV)

was not detected by ^{195}Pt-NMR (data not shown). After 24 h, the amount of Pt(IV) is minimal (0.6 and 0.1%). The speciation detected in the chromatogram is quite higher than the species detected by ^{195}Pt-NMR, but we could detect the peak at the same retention time than **Pt(II)-1a,** which correlates with the mayor signal in the ^{195}Pt-NMR spectra.

Table 1. Amount of intact Pt(IV) species in the presence of GSH and AsA determined by HPLC.

	Pt(IV)-1a (%)		**Pt(IV)-2b (%)**	
Time (h)	GSH	AsA	GSH	AsA
0	100	100	100	100
5	3	0,4	1,3	15
24	0,6	0,1	1	11

As we have mentioned previously, the reduction of complex **Pt(IV)-1a** with AsA is also very rapid, moreover the chromatogram at t_0 (approximately after 3 minutes of mixing time) already showed one Pt(II) species, in a ratio of 1:1 (**Pt(II)-1a: Pt(IV)-1a)**. The longer the reaction time, the higher the amount of species detected in the chromatogram, which does not correlate with the only two species detected by ^{195}Pt NMR. Using the same sample, we could not detect in the chromatogram the two major species (equally intense) detected by NMR (Figure 4. **Pt(IV)-1a** after 5, 7 and 24 h). We performed many attempts to find the same species than those detected by NMR, trying different conditions reported in the literature to run the analysis[25-27], but we did not succeed. In fact, most of the conditions described for cisplatin and similar complexes use formic acid in the mobile phase, but we found formic acid to have a very strong impact in the integrity of our complexes.

We undertook the challenge to further characterize these species in solution, and the samples from the NMR studies were prepared fresh and used immediately afterwards in a ESI spectrometry

study. The results from ESI will help to detect the species in solution with a high ionization efficiency, as it can be seen in the following results, but we must be cautious not to determine species abundance in the ESI spectra.

The ESI spectra of the **Pt(IV)2b-AsA** sample corresponding to the NMR spectra in Figure 4 is shown in Figure 5. The molecular peak corresponds with the expected Pt(II) complex as a result of the reduction with AsA. The **Pt(IV)2b-GSH** sample corresponding to Figure 4 showed by ESI-MS the same results: coordination of GSH which in this case is clearly represented in the specie at 945.22, which correspond to the molecular peak $[Pt(dibibi)(dma)(GSH)]^{+}$

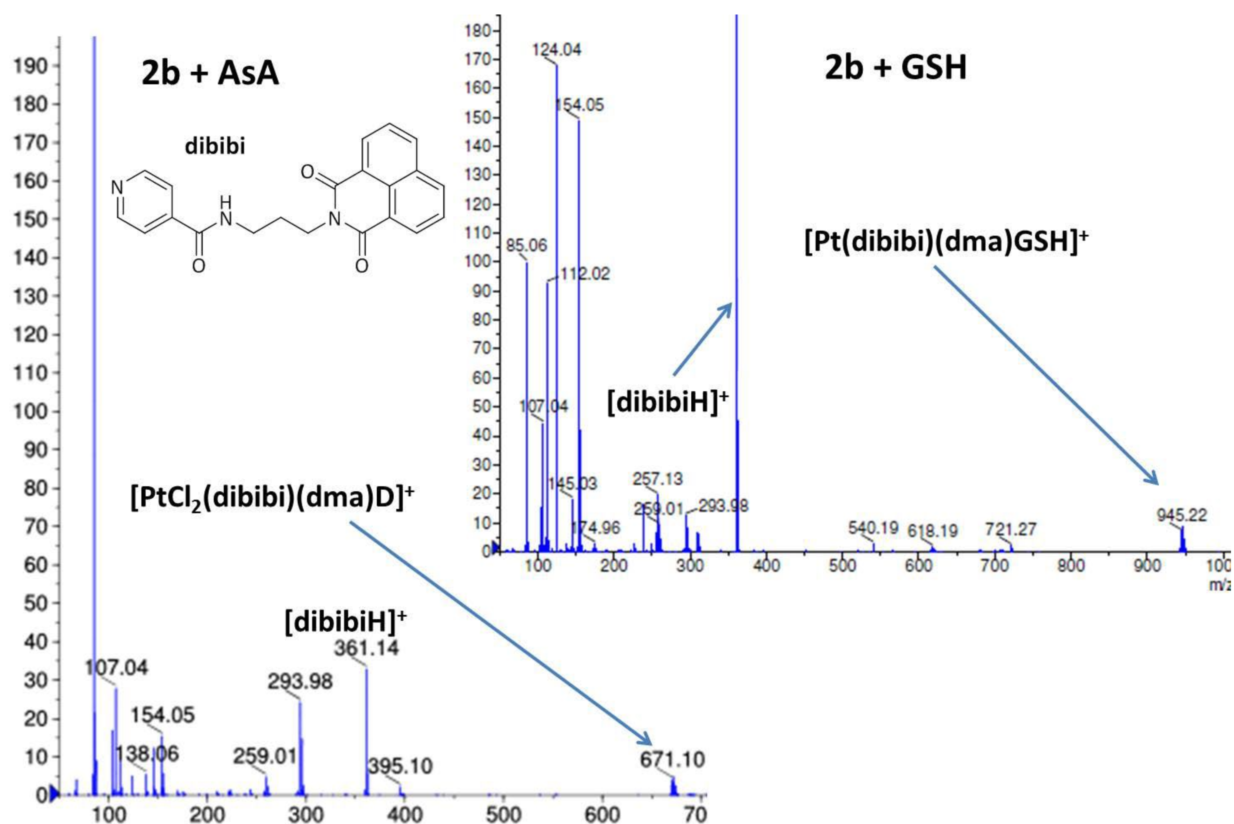

Figure 5. Mass spectra of a solution of complex **Pt(IV)-2b** with AsA (left) and GSH (right) using the same conditions as in Figure 4.

The ESI spectra of sample of Pt(IV)**1a-AsA**is shown in figure 6. We can see clearly the molecular peaks of the two species detected by NMR: the parent complex **Pt(II)-1a** (also detected by HPLC) and the species at -3160 ppm that most likely corresponds to DMSO coordination (Figure 6 peak at 627.16) resulting from the solvent used for NMR experiment.

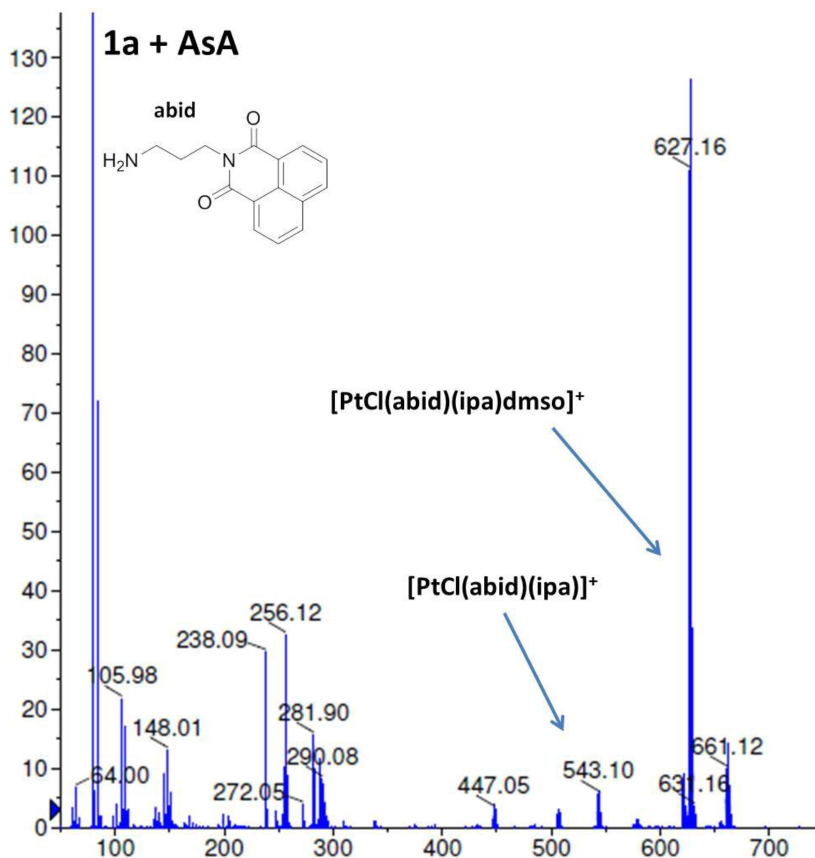

Figure 6. Mass spectra of a solution of complex **Pt(IV)-1a** with AsA using the same conditions as in Figure 4.

Most clarifying was the analysis performed with the Pt(IV) **1a-GSH** sample after the NMR experiment. At that time the sample was an orange solution, which became slowly cloudy and allowed the separation of a very fine solid. Both samples, solid and liquid, were injected separately in the ESI spectrometer and the results are collected in Figure 7. The solid sample (left) showed a

molecular peak corresponding to [PtCl(abid)(ipa)dmso]$^{+}$. This phenomenon can be interpreted with the slow tendency of **Pt(II)-1a** complex to coordinate to DMSO when it gets reduced from its **Pt(IV)-1a** counterpart, as previously no coordination was detected for this complex using several techniques. More importantly, the complex **Pt(IV)-1a** in solution (Figure 7 right) does not coordinate to GSH (nor when it is reduced).

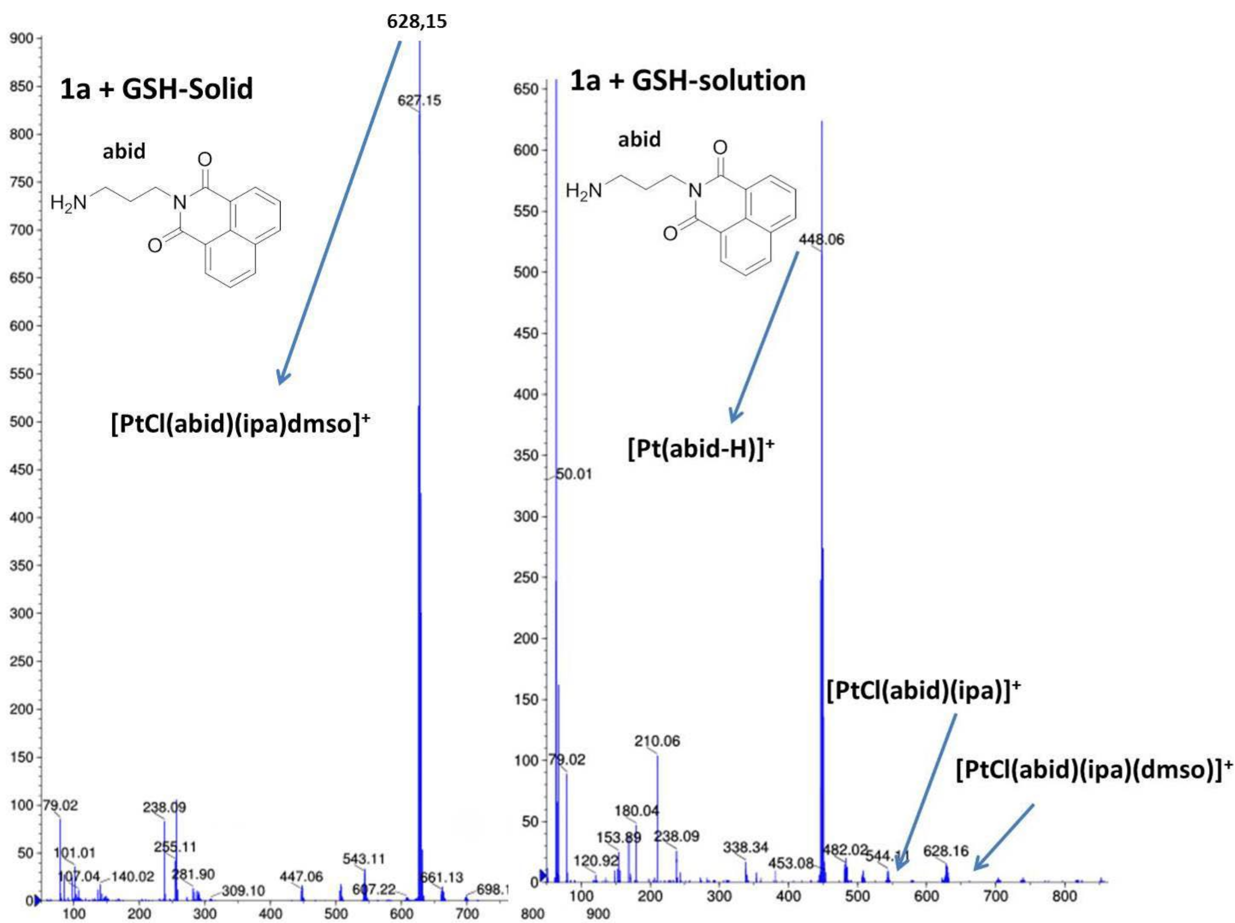

Figure 7. Mass spectra of a solution of complex **Pt(IV)-1a** with GSH at the same conditions as in Figure 4.

Cytotoxicity

To explore the potential as antitumoral agents of the new series of complexes, their antiproliferative properties were assayed by monitoring the ability to inhibit cell growth. Cytotoxic

activity was determined on the human ovarian cancer A2780 cell line, its cisplatin-resistant variant A2780cisR (both recently acquired) and on the human non tumoral cell line HEK293, by a colorimetric method (MTT assay). A comparison between the activity of reference drug cisplatin and the activity of these new metal compounds was performed in these cell models. Using an appropriate range of concentrations (200-0.001 μM), dose-response curves after long-term (72 h) exposures were obtained. From the experimental values, we have calculated the IC_{50} for the compounds, presented in table 2.

Table 2. IC_{50} values for 72 h treatment of different human cell lines.

Compound	**IC_{50} (μM)**				
	A2780	A2780cisR	HEK	RF	
Pt(IV)-1a	0.06±0.04	0.4± 0.1	0.19± 0.2	6.7	
Pt(IV)-1b	0.06±0.05	0.5± 0.2	0.68± 0.3	8.3	
Pt(IV)-2a	1.7±1.0	2.6± 1.0	2.80± 0.1	1.5	
Pt(IV)-2b	2.5±1.2	3.9± 1.4	4.44± 1.4	1.6	
Cisplatin	0.88± 0.2	17.9± 1.2	2.91± 1.5	20	
1 (ligand) [11]	29 ± 0.1	28 ± 1.3	79 ± 1.5	0.97	
2 (ligand) [11]	25 ± 1.2	24.6 ± 1.1	117 ± 2.4	0.98	
Pt(II)-1a [11]	0.26 ± 0.5	0.8 ± 0.5	0.65 ± 0.2	3.2	
Pt(II)-1b [11]	0.67 ± 0.3	0.9 ± 0.5	2.9 ± 1.2	1.4	
Pt(II)-2a [11]	3.6 ± 1.2	7.2 ± 1.5	3.0 ± 1.0	2.0	
Pt(II)-2b [11]	13.6 ± 4.9	39 ± 1.2	30 ± 1.6	2.9	

The IC_{50} values of the ligands showed in Table 2 that they do not present cytotoxicity, but the coordination of the platinum to these ligands caused an obvious effect in the IC_{50} values, affording better activity. The platination of the ligands greatly enhances the cytotoxicity and for this particular case, the oxidation of the Pt(II) complexes afforded novel Pt(IV) drugs of special cytotoxicity, as there is improvement of the values of their parent Pt(II).

The higher activity of the Pt(IV) series might be related to their higher lipophilicity which could increase the cellular uptake compared to the Pt(II) counterparts. Therefore the lipophilicity of the complexes was assessed by shake-flask log Po/w measurements, and the values compiled in table 3. The higher lipophilic character of the Pt(IV) series from 1.90 to 1.00 correlated with a better uptake and a higher cytotoxicity.

	Pt(IV)-1a	**Pt(IV)-1b**	**Pt(IV)-2a**	**Pt(IV)-2b**	**Pt(II)-1a**	**Pt(II)-1b**	**Pt(II)-2a**	**Pt(II)-2b**
Log P	1.90	1.81	1.71	1.00	0.89	0.95	0.40	0.32

The cytotoxicity of the Pt(IV) complexes follows an interesting trend. **Pt(IV)-1a** is clearly the most active compound. Of particular relevance, we observed that **Pt(IV)-1a** and **Pt(IV)-1b** are able to overcome significantly cisplatin cross-resistance with resistance factors ranging from 6 to 8. Interestingly, complex **Pt(IV)-2b** which clearly binds to GSH once is reduced (see Figure 4), showed lower cytotoxicity in the resistant cell line (in which one of the resistance mechanisms is overexpression of GSH), compared to the other tumoral cell line, in which a different biological reductant can potentially act on the prodrug with no further coordination.

The cytotoxicity of the four Pt(IV) complexes is quite high, and based on the differences observed in their speciation in solution versus reducing agents, the active species can be slightly different from their parent Pt(II) and/or a synergistic combination of different species that only occurs in the biological media.

Conclusions

We report new *trans*-Pt(IV) complexes of nonconventional structure bearing aliphatic amines and naphthalimides which afford dual DNA targeted Pt(II) drugs in the presence of biological reductants. Their cytotoxic activity in cancer cell lines proved to be higher than their Pt(II) complexes. The novel Pt(IV) series show a robust integrity in solution, DMSO, PBS and NaCl.

The species formed in the reduction process differ for each complex; therefore, it is reasonable to think that different reduction pathways are taking place. Moreover, the reduction not only affords the Pt(II) parent complex, but also other species which nature could be determined by ESI-MS. The Pt(II) species formed from these prodrugs interact with DNA (plasmid pBR322), while the Pt(IV) do not show direct interaction.

The combination of the different species that only occurs in the biological media seems to be one important feature of these complexes, as a synergistic effect could be the reason for their improved cytotoxicity compared with the parental Pt(II) complexes.

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ASSOCIATED CONTENT

Brief description: extra data of ^{195}Pt-NMR spectra at 0 h and 24h in DMSO, HPLC, [^{1}H-^{13}C] Bidimensional NMR spectra and Pt(II) electrophoresis in agarose gel.

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ABBREVIATIONS

ipa: isopropylamine, dma: dimethylamine, abid: 2-(3-aminopropyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione, dibibi: *N*-(3-(1,3-dioxo-1*H*-benzo[*de*]isoquinoline-2-(3*H*)-il)propyl)isonicotinamide, AsA: Ascorbic Acid, GSH: Glutathione.

SYNOPSIS. By oxidation of dual-targeting compounds, a novel Pt(IV) series shows enhanced cytotoxicity and selectivity towards cancer cell lines, which moreover it presented different and selective reactivity versus biological reducing agents.

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