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

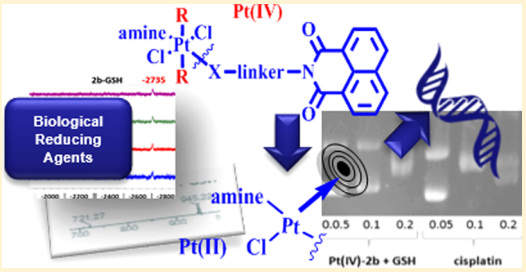
Francisco Navas,[†] Filipa Mendes,*[,‡] Isabel Santos,[‡] Carmen Navarro-Ranninger,[†] Silvia Cabrera,[†] and Adoración G. Quiroga*[,†]

[†]Departamento de Química Inorgánica, Universidad Autónoma de Madrid, ES-28049 Madrid, Spain

[‡]Centro de Ciências e Tecnologias Nucleares, Instituto Superior Técnico, Universidade de Lisboa, 2695-066 Bobadela LRS, Portugal

Supporting Information

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 toward 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, 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 toward increasing the activity of those drugs, in order to minimize the unwanted effects.[3] However, only the so-called "nonconventional" metallodrugs have achieved 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 varying the oxidation state using platinum(IV) complexes.[5]

Trans Pt(II) complexes were first reported in the late 1980s,[6] and our research group reported later on active trans platinum complexes with aliphatic amines.[7] On the basis of this particular discovery, we synthesized several active derivatives, varying the ligands in a configuration trans 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 Pt(IV) to Pt(II) must take place by the assistance of biological reducing agents or by the reasonably hypoxic conditions in the tumoral microenvironment.[12]

Tetraplatin/ormaplatin have been investigated in a clinical trial, but their development was stopped in phase I due to significant neurotoxicity.[13] The possible reason is that the reduction potential in the case of axial chlorido ligands is quite high and therefore these complexes are easily reduced in the blood. 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.[14] The axial ligands and the reduction potential seem to have the strongest effect, while other factors such as 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 their 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 ($E°$), (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[15] as equatorial ligands, which proved to be quite active in vitro and were not easily reduced versus agents such as glutathione (GSH) and ascorbate. These encouraging results with the monophosphine derivatives prompted us to explore a new series of Pt(IV) complexes, taking into consideration the Pt(II) naphthalimide 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 biological

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media.[16] Moreover, the study of their behavior in solution would shed more light on the mechanism of action of Pt(IV) complexes.

EXPERIMENTAL METHODS

General Procedures. One-dimensional ^{195}Pt NMR, ^{13}C NMR, and ^{1}H NMR and [^{1}H−^{13}C] two-dimensional experiments were performed in DMSO-d_6 and acetone-d_6 using a Bruker Avance II-HD Nanobay 300 MHz spectrometer at room temperature (25 °C). HPLC was performed with an Agilent Technology 1200 Infinitive Series instrument. Elemental analyses were performed on a PerkinElmer 2400 Series II microanalyzer. Mass spectrometry assays were performed with a Hybrid Quad-Tof (QTOF) mass spectrometer:Q-STAR (ABSciex) using the samples from the NMR experiments (deuterated and nondeuterated solvents).

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

A Cl_2 gas generator (set up by a hydrochloric acid drip method over $KMnO_4$ and placed as a bubbled system) was connected to a reaction flask containing a suspension of 30 mg of the corresponding trans Pt(II) complex in methanol. The linked system allowed continuous bubbling of Cl_2 gas into the suspension until a clear solution was formed. This smooth flow of the Cl_2 gas stream was maintained continuously from 3−5 h. The solvent was removed under reduced pressure to give the final yellow solid, which was dried in an oven. All complexes were characterized by the usual techniques (see detailed data described below and two-dimensional NMR spectra collected in Figures SI4−SI11 in the Supporting Information). Abbreviations: ipa, isopropylamine; dma, dimethylamine; abid, 2-(3-aminopropyl)-1*H*-benz(de)isoquinoline-1,3(2*H*-dione); dibibi, *N*-(3-(1,3-dioxo-1*H*-benz(de)isoquinolin-2-(3*H*)-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. Anal. Found: C, 32.87; H, 3.40; N, 5.99. Calcd for $PtC_{18}H_{23}Cl_4N_3O_2$: 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 $[M - 2Cl]^+$. Anal. Found: C, 33.00; H, 3.29; N, 6.47. Calcd for $PtC_{17}H_{21}Cl_4N_3O_2$: 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. Anal. Found: C, 38.07; H, 3.73; N, 7.10. Calcd for $PtC_{24}H_{26}Cl_4N_4O_3$: 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. Anal. Found: C, 36.61; H, 3.23; N, 7.24. Calcd for $PtC_{23}H_{24}Cl_4N_4O_3{\cdot}0.5H_2O$: 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 tests in DMSO, 1 mL of a 100 μM solution of the test compound was incubated at 37 °C 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^{-1}; 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 in comparison 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.

Studies of Interaction with HSA by HPLC. The samples were prepared using a 3:1 Pt:HSA molar ratio, from a 5 mM stock solution of the complexes **Pt(IV)-1a** and **Pt(IV)-2b** in DMSO and a 0.1 mM stock solution of HSA in Milli-Q water, to achieve a final volume of 1 mL with saline solution (0.9% NaCl), with 2% of DMSO. The samples were incubated in a thermoshaker at 37 °C with slight stirring during the entire experiment (24 h). Aliquots of 80 μL of each sample were extracted at various times (0.5, 2, 5, and 24 h). Samples were precipitated by adding 20 μL of MeOH and centrifuging at 0 °C at 5000 rpm for 5 min. The pellet was discarded, and 20 μL of the supernatant was 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^{-1}; 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 concentration of the remaining compound was measured over time and expressed as the percentage of compound in comparison to the initial amount (time 0 h, complex without HSA).

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 °C 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 °C. Each sample was monitored by ^{195}Pt NMR and by ESI from 0 to 24 h. The samples were duplicated, checked by NMR, and injected into the ESI mass equipment afterward.

DNA Interaction Studies. The plasmid pBR322 DNA stock was purchased from Gencust at a concentration of 0.5 μg μL^{-1} 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 to the desired concentration. The DNA interaction studies were performed with a total volume of 20 μL. A 20 μL portion containing 0.125 μg μL^{-1} of DNA in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA was incubated with the platinum compounds at r_i values ranging from 0.05 to 0.2 (defined as the molar ratio Pt:nucleotide). The samples were incubated at 37 °C 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 amount of the sample (20 μL) was loaded in the agarose gel (1.2% w/v) and electrophoresis was carried out for a period of 150 min at approximately 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^{-1} stock solution of ethidium bromide for 30 min to stain the DNA. Finally, the stained gel was analyzed with a UVITEC Cambridge UVIDOC HD2 instrument.

Cell Culture and Cytotoxicity Assays. The human ovarian cancer cell lines A2780 and A2780cisR (resistant to cisplatin) (ECACC, U.K.) were grown in RPMI 1640 culture medium (Invitrogen) supplemented with 10% FBS at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 (Heraeus, Germany). The human nontumoral cell line HEK 293 was grown in DMEM culture medium (Invitrogen) supplemented with 10% FBS under conditions similar to those above.

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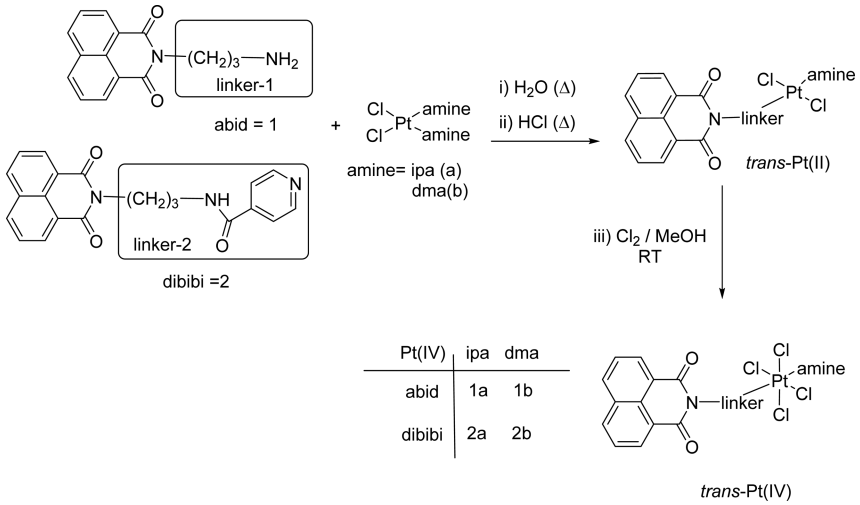
Chart 1. Synthetic Routes Explored To Obtain the Pt(IV) Complexes[a]



[a]Legend: (i, ii) steps described in ref 11; (iii) step described in this paper.

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 (1−1.5) × 10^4 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 the medium was added and incubation was performed at 37 °C/5% CO_2 for 72 h. The percentage of DMSO in the 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^{-1}). 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 by dividing the absorbance of each well by that of the control wells. Each point was determined in at least four replicates in two independent assays.

log *P* Determination. The octanol−water partition coefficients for Pt(IV) and Pt(II) compounds were determined by modification of the reported shake-flask method.[17] 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 10 min and then centrifuged for 5 min at 6000 rpm to allow separation. The concentrations in both phases were determined by UV−vis. The reported log *P* is defined as log $[complex]_{oct}/[complex]_{wat}$.

RESULTS AND DISCUSSION

Chemistry. Chart 1 shows the synthetic routes used to obtain the Pt(IV) complexes. In a 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 linker using an aromatic ring spacer. This Pt(II) approach afforded dual-DNA-targeting complexes.[11] On the basis of 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.

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 in comparison with water, chloroform, acetone, and dichloromethane, which are the most commonly used solvents in the literature (Chart 1). Indeed, we were able to obtain 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 equiv of H_2O_2 over 4, 6, and up to 16 h, we could not isolate the Pt(IV) compounds. We monitored the reaction using ^{1}H NMR over time and could observe the coexistence of both species in solution, Pt(II) always being 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 evaluated further.

The characterization of the obtained complexes was performed by the usual NMR spectroscopy techniques (Figures SI6−SI13 in the Supporting Information), elemental analysis, and mass spectrometry (MALDI). ^{195}Pt NMR was the most useful NMR experiment to confirm that the oxidation of the metal had 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 $\delta(^{195}Pt)$ NMR value at around −200 ppm, which was in agreement with the values published in the literature.[18] Moreover, the moiety also produces a change of more than 2000 ppm in the chemical shift in comparison with its Pt(II) counterpart, proving once more that the oxidation had taken place.[19]

Stability of Pt(IV) Complexes in Solution. The stability of the Pt(IV) complexes in solution was studied by ^{195}Pt NMR

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spectroscopy. Analyses were performed for all of the complexes, with freshly prepared samples and samples at 24 h, with spectra confirming the stability (Figure SI1 in the Supporting Information). 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** are shown in Figure 1. The data corresponding to the

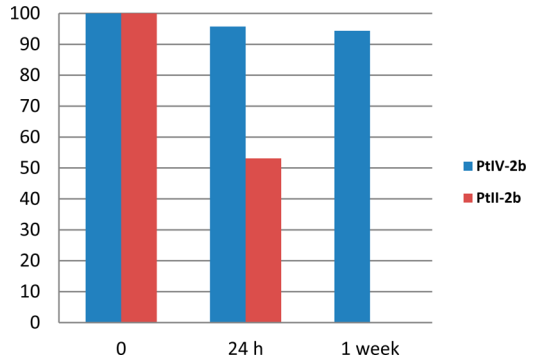

Figure 1. Stability values of complexes **Pt(IV)-2b** and **Pt(II)-2b** in DMSO.

other complex stabilities in DMSO are presented in Figure SI2 in the Supporting Information. 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 1 week of incubation. For all analyzed Pt(IV) complexes the stability values are higher than those of their parent Pt(II) complexes,[11] as can be seen clearly in Figure 1 for compounds **Pt(II)-2b** and **Pt(IV)-2b** and Figure SI2 for the other complexes, corroborating our first assumption of greater stability for the Pt(IV) prodrugs.

The stability of the complexes was also assessed in PBS and NaCl, and the data have been compiled in Figure SI3 in the Supporting Information. Taking all the data together, we can consider that the new Pt(IV) complexes have improved their pharmacological stability versus the corresponding parent Pt(II) complexes. The general trend of the series is a higher stability in NaCl solution in comparison with PBS, although both assays showed satisfactory values for pharmacological agents. 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 in the early stages by NMR neither in NaCl nor in PBS.

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 to afford the parent Pt(II) complexes, although recently other species have been also detected in biological media, showing a different interaction with plasmid DNA.[20,21] In previous studies, we showed that Pt(II) complexes are able to form adducts with 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 the biological reductants AsA and GSH.[21] Figure 2 and Figures SI12–SI15

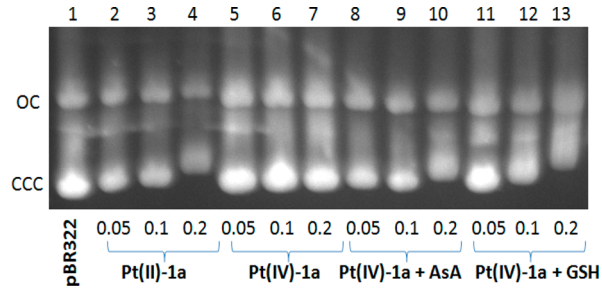

Figure 2. Agarose gel electrophoresis of pBR322 plasmid treated with complexes **Pt(IV)-1a** and **Pt(IV)-1a** with AsA (ratio 1:1) and with GSH (ratio 1:2): (lane 1) DNA of control plasmid; (lanes 2–4) **Pt(II)-1a** and DNA incubated at r_i = 0.05–0.2; (lanes 5–7) **Pt(IV)-1a** and DNA incubated at r_i = 0.05–0.2; (lanes 8–10) **Pt(IV)-1a** with AsA (ratio 1:1) and DNA incubated at r_i = 0.05–0.2; (lanes 11–13) **Pt(IV)-1a** with GSH (ratio 1:2) and DNA incubated at r_i = 0.05–0.2.

in the Supporting Information present the electrophoretic mobility of the plasmid incubated with Pt(IV) and Pt(II) complexes in the presence and absence of the reductants.

Cisplatin has been also included in this experiment (Figures SI13 and SI14 in the Supporting Information) as a model of a 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 comigration point.[22]

After 24 h of incubation, none of the Pt(IV) complexes showed interaction with pBR322 at any of the concentrations assayed. The concentration is represented as r_i, defined as the molar ratio Pt/nucleotide (r_i up to 0.2 at Figure 2, lanes 4 and 7, Figure SI12 in the Supporting Information, lane 7, and Figures SI13 and SI14 in the Supporting Information, lane 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,[21,23,24] in contrast to Pt(II) complexes. Only complex **Pt(IV)-2a** showed a slight interaction with the ccc form at the highest r_i value 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 SI12–SI14 in the Supporting Information), because their Pt(II) counterparts interact more strongly with the plasmid, showing a pattern similar to that of cisplatin (Figure SI15 in the Supporting Information for **Pt(II)-2a** and **Pt(II)-2b**). In particular, Figure 2 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 2, lanes 2–4). On comparison of the incubation with AsA (lines 8–10) to GSH (lines 11–13), 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

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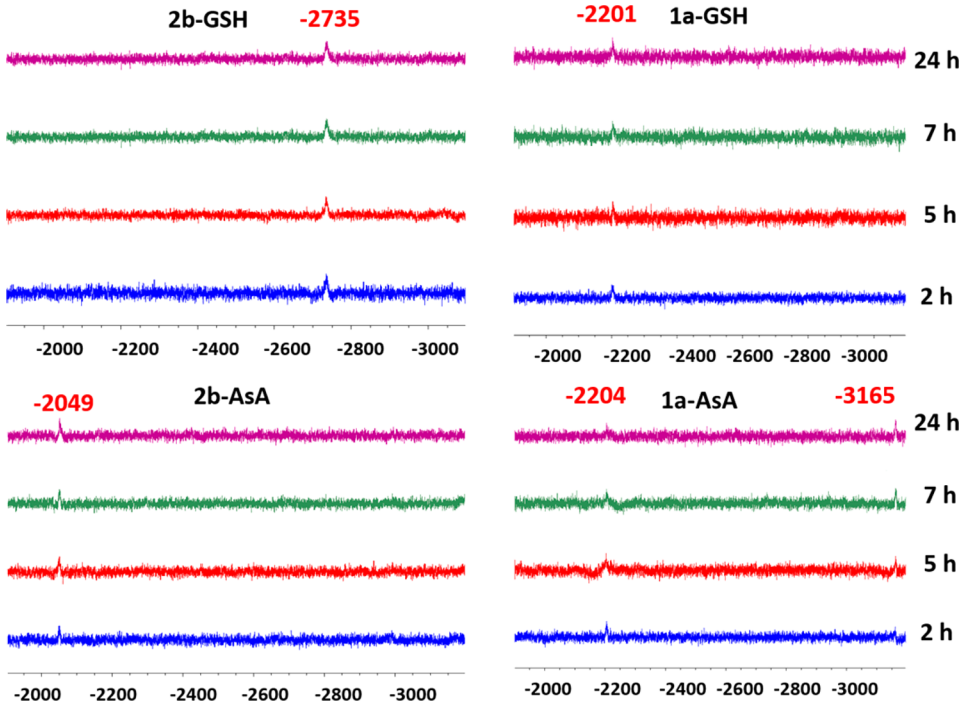

Figure 3. ^{195}Pt NMR spectroscopy of the progress (2–24 h) of the reaction of complexes **Pt(IV)-1a** and **Pt(IV)-2b** with GSH and ASA.

other three Pt^{IV} compounds seemed to be reduced more quickly 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 (Figures SI12–SI14 in the Supporting Information) is clearly stronger. At the maximum concentration assayed (lines 7, 10, and 13 for Figure SI12 and lines 4, 7, and 10 for Figures SI13 and SI14), gel electrophoresis showed that the mobility of the ccc isoform is delayed in such a way that it migrates at the same position as that for oc. This effect has been typically observed for the parent Pt(II) derivatives (Figure 2 and Figures SI12 and SI15 for **Pt(II)-2a** and **Pt(II)-2b**). The Pt(II) complex pattern of interaction is somewhat different from that produced by cisplatin, in which both forms of the plasmid DNA have altered their mobility until reaching comigration;[22] such an interaction is quite similar to that 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 all of the experiments are compared, 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 8 and 9, Figures SI13 and SI14 in the Supporting Information), 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) complex reactivity, we have monitored their reaction with the biological reductants by ^{195}Pt NMR (Figure 3). ^{195}Pt NMR allows easy differentiation of the two oxidation states, as their chemical shifts are situated in very different areas, and also allows differentiation in the chemical shift of the Pt atom provoked by ligand substitution.[19] The monitoring of the Pt(IV) complexes in DMSO and D_2O:DMSO-d_6 solution showed no speciation over 24 h. This experiment allowed setting the conditions for further studies (see Experimental Methods).

The reaction of complex **Pt(IV)-2b** with AsA slowly affords the Pt(II) counterpart (signal previously reported at $\delta(^{195}Pt)$ −2049 ppm). Furthermore, **Pt(IV)-2b** complex is not only reduced in the presence of GSH but also, once it is reduced, coordinates to GSH. The indication of this phenomenon is clear from the NMR spectra in Figure 3, where the signal appearing at −2735 ppm is typical of Pt(II) species of a PtN_2SX moiety.[25] Complex **Pt(IV)-1a** versus GSH is reduced to the Pt(II) parent complex (at $\delta(^{195}Pt)$ −2201 ppm, as previously reported) with no coordination to GSH, but in contrast, the complex versus AsA affords not only the parent complex but also a new species at $\delta(^{195}Pt)$ −3160 ppm. This difference is possibly caused by a different reduction pathway, which might be related to the higher drug cytotoxicity observed (see a following section).

HPLC and NMR Correlation Studies of *trans*-Pt(IV) Complexes in the Presence of Biological Reducing Agents. This behavior of **Pt(IV)-1a** prompted us to study the species by HPLC, which usually shows the speciation (if any) and 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 considered to be 100% (Table 1)

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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
2	50	50	18	20
5	3	0,4	1,3	15
24	0,6	0,1	1	11

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 that of the species detected by ^{195}Pt NMR, but we could detect the peak at the same retention time as for **Pt(II)-1a,** which correlates with the mayor signal in the ^{195}Pt NMR spectra (see Table 1, **Pt(IV)1a**, t = 2 h).

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 3, **Pt(IV)-1a** after 5, 7, and 24 h). We performed many attempts to find the same species as those detected by NMR, trying different conditions reported in the literature to run the analysis,[26−28] 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 effect on 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 afterward in a ESI spectrometry study. The results from ESI help to detect the species in solution with a high ionization efficiency, as 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 3 are shown in Figure 4. The molecular peak corresponds to the expected Pt(II) complex as a result of the reduction with AsA. The **Pt(IV)2b-GSH** sample corresponding to Figure 3 showed by ESI-MS the same results: coordination of GSH, which in this case is clearly represented in the species at 945.22, which corresponds to the molecular peak [Pt(dibibi)(dma)(GSH)]$^+$.

All the ESI spectra of sample of **Pt(IV)1a** are compiled in the Supporting Information. We can see clearly the molecular peaks of the two species detected by NMR with AsA (Figure SI16 in the Supporting Information): the parent complex **Pt(II)-1a** (also detected by HPLC) and the species at −3160 ppm that most likely corresponds to DMSO coordination (Figure SI16 peak at 627.16) resulting from the solvent used for NMR experiment. Most clarifying was an ESI mass analysis performed with the Pt(IV) **1a-GSH** sample used in the NMR experiment (Figure SI17). 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 into the ESI spectrometer, and the results are collected in Figure SI17 in the Supporting Information. The solid sample (Figure SI17, left) showed a molecular peak corresponding to [PtCl(abid)(ipa)dmso]$^+$. This phenomenon can be interpreted by the slow tendency of **Pt(II)-1a** complex to coordinate to DMSO when it becomes 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 SI17, right) does not coordinate to GSH (nor when it is reduced).

To assert the improvement of these complexes, we studied the behavior of **Pt(IV)-1a** and **Pt(IV)-2b** versus one of the main protein targets for platinum drugs: HSA (human serum albumin) (Table 2). HSA in known to be one of the proteins with greater affinity for platinum compounds in blood.[29] Complex **Pt(IV)-1a** shows stability versus HAS along the first 5 h similar to that of Pt(IV) complexes reported in the literature,[29] though at longer times it is not as stable as the best example.[30] **Pt(IV)-2b**, on the other hand, has a more rapid

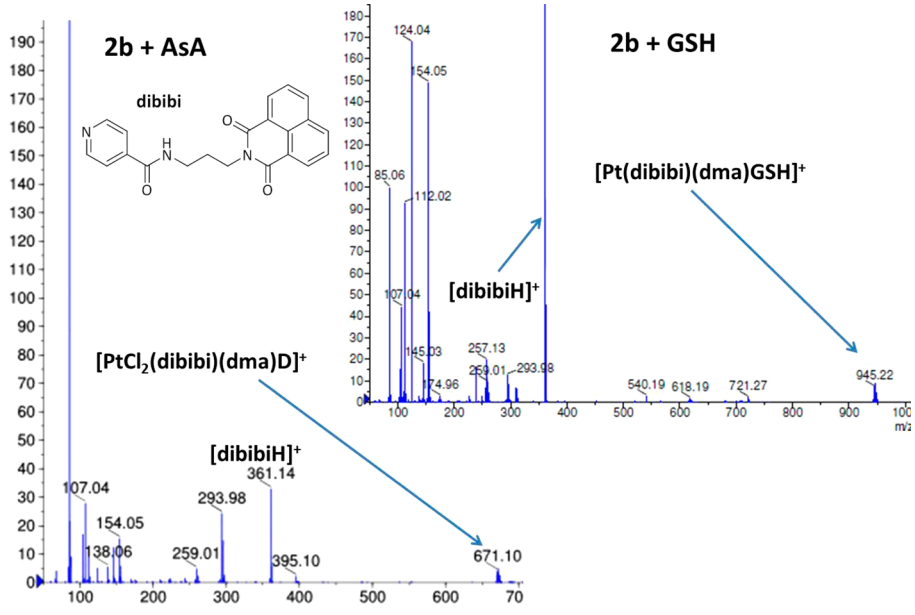

Figure 4. Mass spectra of a solution of complex **Pt(IV)-2b** with AsA (left) and GSH (right) using the same conditions as in Figure 3.

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Table 2. Amount of Intact Pt(IV) Species in the Presence of HSA Determined by HPLC

t (h)	Pt(IV)-1a (%)	Pt(IV)-2b (%)
0	100	100
0.5	59.2	13.2
2	42.3	11.8
5	39.5	8.9
24	1.7	3.63

affinity for HSA, which correlates with a greater tendency to coordinate to a sulfur donor such as GSH.

Cytotoxicity. To explore the potential of the new series of complexes as antitumoral agents, their antiproliferative properties were assayed by monitoring their 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 nontumoral cell line HEK293, by a colorimetric method (MTT assay). A comparison between the activity of the 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} values for the compounds, presented in Table 3.

Table 3. IC_{50} Values for 72 h Treatment of Different Human Cell Lines

	IC_{50} (μM)			
compound	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 in Table 3 showed that they do not present cytotoxicity, but the coordination of the platinum to these ligands caused an obvious effect on 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 in the values of their parent Pt(II).

The higher activity of the Pt(IV) series might be related to their higher lipophilicity, which obviously will increase the uptake in comparison to the Pt(II) counterparts. Shake-flask log $P_{o/w}$ experiments on all compounds were performed, and the values are compiled in Table 4. The higher lipophilic character of the Pt(IV) series from 1.90 to 1.00 correlated with a better uptake and a higher cytotoxicity.

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 significantly overcome cisplatin cross-resistance, with resistance factors ranging from 6 to 8. Interestingly, complex **Pt(IV)-2b**, which clearly binds to GSH once it is reduced (see Figure 4), showed lower cytotoxicity in the resistant cell line (in which one of the resistance mechanisms is overexpression of GSH), in comparison 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 on the basis of 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.

Table 4. log P Values for Pt(IV) and Pt(II) Series[a]

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

[a]cDPP: −2,44 ref 31.

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 that of their Pt(II) complexes. The novel Pt(IV) series shows 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 affords not only the Pt(II) parent complex but also other species whose nature could be determined by ESI-MS. The Pt(II) species formed from these prodrugs interact with DNA (plasmid pBR322), while the Pt(IV) species 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 in comparison with the parent Pt(II) complexes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.7b00136.

Additional ^{195}Pt NMR spectra at 0 and 24 h in DMSO, HPLC, [^{1}H−^{13}C] two-dimensional NMR spectra, and Pt(II) electrophoresis in agarose gel (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail for F.M.: fmendes@ctn.tecnico.ulisboa.pt.

*E-mail for A.G.Q.: adoracion.gomez@uam.es.

ORCID

Filipa Mendes: 0000-0003-0646-1687

Silvia Cabrera: 0000-0002-4907-2932

Adoración G. Quiroga: 0000-0002-9261-9542

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Notes

The authors declare no competing financial interest.

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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

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