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Design and Biological Evaluation of New Platinum(II) Complexes Bearing Ligands with DNA-Targeting Ability

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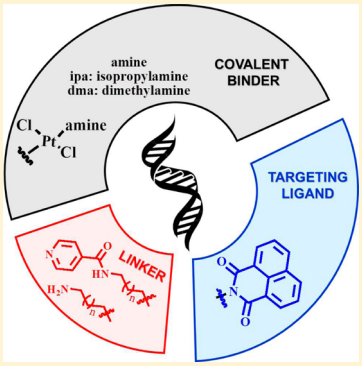
Supporting Information

ABSTRACT: A novel series of platinum(II) complexes bearing aliphatic amines and ligands with DNA-targeting properties was synthesized to achieve more potent and selective metallodrugs. We developed six new platinum-based drugs, which contain methylamine, **1a−c**, and isopropylamine, **2a−c**, both in the trans position to a selected targeting ligand: naphthalimide. The activity of the complexes has been evaluated in order to confirm the improvements from our proposed approach, and the complexes demonstrate better cytotoxic activity on cancer cell lines when compared with the ligands and, importantly, with cisplatin. Further studies were performed to assess their subcellular localization and binding mode to DNA.



INTRODUCTION

Finding anticancer metallodrugs that are more active and specific than the current clinical drugs is a difficult task but also an active area of research. The discovery of new biological targets in the past few years has made this area even more appealing for further investigation,[1−3] as a new diversity of metallodrug targets opens the possibility of obtaining new drugs that circumvent the serious and undesirable side effects of clinical drugs such as cisplatin.[4]

The most used metal-based drug in clinics, cisplatin, shows limited clinical efficiency.[5] For many years, research was conducted with the objective of circumventing cisplatin's limitations using mainly platinum analogues that could cause the same type of damage. Only a few examples from thousands of different attempts such as carboplatin (second generation)[6] or oxaliplatin (third generation)[7] have improved some aspects of the pharmacological profile of cisplatin, but those drugs still cause side effects and show lower potency than cisplatin.[8] Development of unconventional metallodrugs (organometallic complexes with antitumor activity,[9] coordination complexes containing biologically active ligands,[10] or complexes bearing targeting ligands such as mitaplatin[11]) turned around this belief and led the research to a new generation of metallodrugs. The *trans*-platinum complexes are nonclassically structured drugs[12] which have a proven enhanced cytotoxicity compared to cisplatin.[13] In particular, the family of *trans*-platinum complexes bearing aliphatic amines showed extra features such as different values of aquation,[14] selective cytotoxicity,[15] and modulation of the activity by replacing the aliphatic amines[16] or more interestingly replacing the leaving groups.[17] The search for new targeting ligands that might improve the selectivity of those complexes led us to look at naphthalimide compounds (NI), which are considered potential anticancer agents because they interact reversibly with DNA by intercalation.[18] Some of those compounds, for example, amonafide and mitonafide, have even entered into clinical trials.[19] Moreover, use of naphthalene derivatives to enhance the cytotoxic effect of ruthenium−arene complexes has been recently reported. In this study the arene ligand was used to tether the biologically active group, i.e., naphthalimide, with the aim of reaching histone nucleoproteins instead of the DNA helix itself.[20] Some other compounds, mainly Au(I) derivatives, have been reported to be inhibitors of proteins such as human thioredoxin reductase (hTrxR) coordinating to the cysteine site.[21,22] Nowadays, considerable evidence has been gathered that metal-based drugs can target selenocysteine or cysteine residues within proteins through ligand-substitution reactions under physiological conditions.[23]

Herein we disclose a new family of nonconventional platinum metallodrugs with dimethylamine, isopropylamine, and naphthalimide as ligands, the latter with potential fluorescent and intercalating properties, to achieve a novel metallodrug which can be used not only as a probe but also as a targeted vehicle in the biological system (Figure 1). Thus, we

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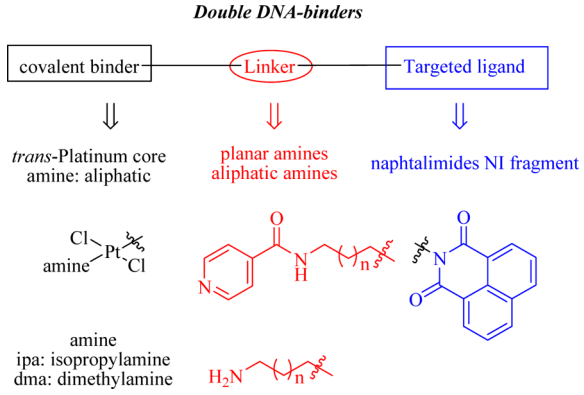

Figure 1. *trans*-Platinum(II) complexes containing DNA-targeted ligands.

hypothesized that the synergistic combination of a metal covalent binder (DNA fixation) and an intercalator would enhance the known antitumor activity of the *trans*-platinum complexes with aliphatic amines in order to further develop the novel compounds toward a higher specificity and cytotoxicity for cancer cell lines.

EXPERIMENTAL SECTION

Materials and Methods. NMR spectra were acquired on a Bruker 300 spectrometer, running at 300, 75, and 64.5 MHz for ^{1}H, ^{13}C, and ^{195}Pt, respectively. Chemical shifts (δ) are reported in ppm relative to residual solvent signals ($CDCl_3$ 7.26 ppm for ^{1}H NMR, 77.0 ppm for ^{13}C NMR; DMSO-d_6 2.50 ppm for ^{1}H NMR, 39.52 for ^{13}C NMR; CD_3OD 3.31 ppm for ^{1}H NMR, 49.00 ppm for ^{13}C NMR; acetone-d_6 2.05 ppm for ^{1}H NMR, 29.84 for ^{13}C NMR). ^{13}C NMR spectra were acquired on a broad-band decoupled mode. ^{195}Pt NMR spectra were obtained with chemical shifts reported in ppm downfield relative to the external reference 1.0 M Na_2PtCl_6 in D_2O. Melting points were measured using a Gallenkamp apparatus in open capillary tubes. Analytical thin layer chromatography (TLC) was performed using precoated aluminum-backed plates (Merck Kieselgel 60-F254) and visualized by ultraviolet irradiation or iodine dip. All reagents and materials were purchased from commercial sources and used without further purification. *cis*-$[PtCl_2(dma)_2]$ was prepared following a procedure previously reported by our research group.[14] The stability of the complexes has been monitored by NMR in DMSO solution over 24 h as a standard procedure. No solvent coordination was observed in the stock solution of every complex.

Synthesis of Ligands a–c. *N-(3-Aminopropyl)-1,8-naphthalimide (**a**).* Ligand **a** was synthesized following the procedure reported by Lichelli et al.[24] in 77% yield. ^{1}H NMR (CD_3OD, 300 MHz): δ 8.54 (dd, J = 7.5, 1.0 Hz, 2H) 8.34 (dd, J = 8.4, 1.0 Hz, 2H), 7.81 (t, J = 6.9 Hz, 2H), 4.25 (t, J = 6.8 Hz, 2H), 2.73 (t, J = 6.8 Hz, 2H), 1.93 (quint, J = 6.8 Hz, 2H).

*N-(3-Aminohexyl)-1,8-naphthalimide (**b**).*[25] Ligand **b** was synthesized following the procedure reported by Lichelli et al.[24] in 78% yield. ^{1}H NMR (CD_3OD, 300 MHz): δ 8.54 (d, J = 7.3 Hz, 2H), 8.34 (d, J = 8.3 Hz, 2H), 7.81 (t, J = 7.7 Hz, 2H), 4.15 (t, J = 7.6 Hz, 2H), 2.66 (t, J = 6.9 Hz, 2H), 1.79–1.69 (m, 2H), 1.54–1.39 (m, 6H).

*N-(3-Isonicotinamidopropyl)-1,8-naphthalimide (**c**).* Isonicotinoyl chloride hydrochloride (0.50 g, 2.90 mmol) and triethyl amine (1.22 mL, 8.72 mmol) in 10 mL of dry DMF were stirred at room temperature and under argon atmosphere for 15 min. Then ligand **a** (1.11 g, 4.35 mmol) was added to the mixture and stirred at room temperature for 48 h. The reaction mixture was concentrated to dryness under reduced pressure. The solid was triturated with distilled water, filtered, washed thoroughly with cool distilled water, and vacuum dried overnight at 40 °C in a drying oven to yield a pale orange solid (72%). MP: 152–155 °C (decomposed). ^{1}H NMR (CD_3OD, 300 MHz): δ 8.66 (dd, J = 4.5, 1.6 Hz, 2H), 8.50 (d, J = 7.3 Hz, 2H), 8.37 (d, J = 8.4 Hz, 2H), 7.80–7.73 (m, 4H), 4.25 (t, J = 6.9 Hz, 2H), 3.49 (t, J = 6.8 Hz, 2H), 2.06 (pent, J = 6.9 Hz, 2H). ^{13}C NMR (CD_3OD, 100 MHz): δ 167.6, 165.7, 150.9, 144.0, 135.6, 133.1, 132.2, 129.2, 128.1, 123.6, 122.8, 39.0, 38.9, 28.8. MS (EI) m/z: 360.1 $[M + H]^+$. Anal. Calcd for $C_{21}H_{17}N_3O_3$: C, 70.18; H, 4.77; N, 11.69. Found: C, 69.68; H, 4.96; N, 11.30.

General Procedure for Synthesis of *trans*-Platinum(II) Complexes 1a–c. To a solution of *cis*-$[PtCl_2(dma)_2]$ (0.20 g, 0.56 mmol) in a minimum amount of water was added the corresponding ligand **a**, **b**, or **c** (2.25 mmol), and the mixture was stirred at 95 °C for 16 h. After cooling, the mixture was filtrated and the yellow solution was refluxed with hydrogen chloride (11.20 mmol) at 95 °C for 1 (ligand **a**) or 16 h (ligands **b** and **c**) to obtain a solid, which was filtered, washed with water, and purified as indicated in each complex.

*trans-[PtCl$_2$(a)(NH(CH$_3$)$_2$)] (**1a**).* The pale yellow solid was dissolved in 20 mL of chloroform, filtered (0.2 μm filter), and precipitated using hexane to obtain a pale yellow solid, which was vacuum dried for 48 h at 60 °C in a drying oven (31% yield). MP: 242–244 °C (decomp.). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.52–8.42 (m, 4H), 7.87 (t, J = 7.8 Hz, 2H), 5.10–4.98 (m, 1H), 4.47–4.35 (m, 2H), 4.10 (t, J = 6.3. Hz, 2H), 2.58–2.42 (m, 2H), 2.34 (d, J = 5.7 Hz, 6H), 2.15–2.03 (m, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 163.7, 134.3, 131.2, 130.8, 127.4, 127.2, 122.0, 42.9, 42.8, 37.0, 28.6. ^{195}Pt NMR (DMSO-d_6, 64.5 MHz): δ −2192. MS (MALDI) m/z: 530 $[M - Cl]^+$. Anal. Calcd for $C_{17}H_{21}Cl_2N_3O_2Pt$: C, 36.12; H, 3.74; N, 7.43. Found: C, 36.14; H, 3.84; N, 7.31.

*trans-[PtCl$_2$(b)(NH(CH$_3$)$_2$)] (**1b**).* The pale yellow solid was dissolved in 20 mL of chloroform, filtered (0.2 μm filter), and precipitated with hexane to obtain a pale yellow solid, which was vacuum dried for 48 h at 60 °C in a drying oven (41% yield). MP: 169–171 °C (decomp.). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.60 (d, J = 7.3 Hz, 2H), 8.20 (d, J = 8.3 Hz, 2H), 7.75 (t, J = 7.4 Hz, 2H), 4.17 (t, J = 7.3 Hz, 2H), 3.88–3.81 (bs, 1H), 3.38–3.17 (bs, 2H), 2.90–2.73 (m, 2H), 2.66 (d, J = 6.0 Hz, 6H), 1.81–1.60 (m, 4H), 1.46–1.41 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.4, 134.0, 131.7, 131.4, 128.3, 127.1, 122.8, 47.0, 43.6, 40.1, 31.2, 28.0, 26.6, 26.1. ^{195}Pt NMR ($CDCl_3$, 64.5 MHz): δ −2185. MS (ES^+) m/z: 608 $[M]^+$; 572 $[M - Cl]^+$. Anal. Calcd for $C_{20}H_{27}Cl_2N_3O_2Pt$: C, 39.55; H, 4.48; N, 6.92. Found: C, 39.54; H, 4.40; N, 6.68.

*trans-[PtCl$_2$(c)(NH(CH$_3$)$_2$)] (**1c**).* The pale yellow solid was resuspended in 20 mL of water and refluxed at 95 °C for 2 h to obtain a pale yellow solid, which was vacuum dried for 48 h at 60 °C in a drying oven (32% yield). MP: 230–232 °C (decomp.).^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.02 (d, J = 5.3 Hz, 2H), 8.67 (d, J = 7.3 Hz, 2H), 8.30 (d, J = 8.3 Hz, 2H), 7.99–7.91 (m, 1H), 7.88–7.79 (m, 4H), 4.35 (t, J = 6.0 Hz, 2H), 4.26–4.21 (m, 1H), 3.42–3.48 (m, 2H), 2.78 (d, J = 6.0 Hz, 6H), 2.14–2.07 (m, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 165.1, 163.2, 154.3, 143.4, 134.8, 132.0, 131.8, 128.4, 127.3, 122.9, 122.2, 43.7, 37.5, 36.5, 27.6. ^{195}Pt NMR ($CDCl_3$, 64.5 MHz): δ −2046. MS (ES) m/z: 670 $[M]^+$; 635 $[M - Cl]^+$. Anal. Calcd for $C_{23}H_{24}Cl_2N_4O_3Pt$: C, 41.20; H, 3.61; N, 8.36. Found: C, 41.62; H, 3.71; N, 8.30.

General Procedure for Synthesis of *trans*-Platinum(II) Complexes 2a–c. To a solution of *cis*-$[PtCl_2(ipa)_2]$ (0.25 g, 0.65 mmol) in a minimum amount of water was added the corresponding ligand **a**, **b**, or **c** (1.63 mmol), and the mixture was stirred at 95 °C for 16 h. After cooling, the mixture was centrifuged and then filtrated. The yellow solution obtain was refluxed with hydrogen chloride (13.02 mmol) at 95 °C for 16 (ligands **b** and **c**) or 24 h (ligand **a**) to obtain a solid, which was filtered, washed with water, and purified as indicated in each complex.

*trans-[PtCl$_2$(a)(NHCH(CH$_3$)$_2$)] (**2a**).* The pale yellow solid was resuspended in 20 mL of water and refluxed at 95 °C for 2 h to obtain a pale yellow solid, which was washed with methanol and vacuum dried for 72 h at 40 °C in a drying oven (43% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.52–8.40 (m, 4H), 7.86 (t, J = 7.7 Hz, 2H), 4.48 (s, 2H), 4.24–4.08 (m, 4H), 3.08–2.95 (m, 1H), 2.21–2.06 (m, 2H), 1.16 (d, J = 6.4 Hz, 6H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 163.7, 134.4, 131.3, 130.8, 127.4, 127.2, 122.0, 42.0, 42.7, 36.9, 28.6, 23,08. ^{195}Pt NMR (DMSO-d_6, 64.5 MHz): δ −2194. MS (MALDI)

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m/z: 580 $[M]^+$; 544 $[M - Cl]^+$. Anal. Calcd for $C_{18}H_{23}Cl_2N_3O_2Pt \cdot 0.5KCl$: C, 39.68; H, 4.07; N, 7.71. Found: C, 39.29; H, 3.95; N, 7.62.

trans-[PtCl$_2$(b)(NHCH(CH$_3$)$_2$)] (**2b**). The pale yellow solid was dissolved in 20 mL of chloroform, filtered (0.2 μm filter), and precipitated with hexane to obtain a pale yellow solid, which was vacuum dried for 72 h at 60 °C in a drying oven (50% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.61 (d, *J* = 8.1 Hz, 2H), 8.21 (d, *J* = 7.6 Hz, 2H), 7.75 (t, *J* = 7.6 Hz, 2H), 4.18 (t, *J* = 7.3 Hz, 2H), 3.61−3.21 (m, 5H), 2.88−2.78 (m, 2H), 1.77−1.68 (m, 4H), 1.54−1.37 (m, 4H), 1.32 (d, *J* = 6.3 Hz, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.4, 134.0, 131.8, 131.4, 128.3, 127.1, 122.9, 48.6, 46.7, 40.2, 31.0, 28.0, 26.7, 26.3, 24.0. ^{195}Pt NMR ($CDCl_3$, 64.5 MHz): δ −2161. MS (MALDI) *m/z*: 622 $[M]^+$; 585 $[M - Cl]^+$. Anal. Calcd for $C_{21}H_{29}Cl_2N_3O_2Pt$: C, 40.59; H, 4.70; N, 6.76. Found: C, 40.14; H, 4.90; N, 6.44.

trans-[PtCl$_2$(c)(NHCH(CH$_3$)$_2$)] (**2c**). The pale yellow solid was resuspended in 20 mL of water and refluxed at 95 °C for 2 h to obtain a pale yellow solid, which was vacuum dried for 48 h at 60 °C in a drying oven (48% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.97 (t, *J* = 5.5 Hz, 1H), 8.86 (d, *J* = 6.5 Hz, 1H), 8.49 (t, *J* = 7.5 Hz, 4H), 7.87 (t, *J* = 7.8 Hz, 2H), 7.76 (d, *J* = 6.6 Hz, 2H), 4.72−4.70 (m, 2H), 4.13 (t, *J* = 7.1 Hz, 2H), 3.40−3.32 (m, 2H), 3.17−3.05 (m, 1H), 2.04−1.86 (m, 2H), 1.25 (d, *J* = 6.5 Hz, 6H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 163.4, 163.2, 153.4, 143.0, 134.3, 131.3, 130.7, 127.4, 127.2, 123.0, 122.1, 47.7, 37.7, 37.5, 27.4, 23.0. ^{195}Pt NMR (DMSO-d_6, 64.5 MHz): δ −2065. MS (MALDI) *m/z*: 685 $[M]^+$; 649 $[M - Cl]^+$. Anal. Calcd for $C_{24}H_{26}Cl_2N_4O_3Pt \cdot 0.8H_2O$: C, 41.25; H, 3.98; N, 8.02. Found: C, 40.99; H, 4.05; N, 7.76.

Cytotoxicity Assays. The cytotoxic activity of the compounds was tested on human epithelial ovarian carcinoma cells, both sensitive (A2780) and resistant (A2780cisR) to cisplatin (*cis*-DDP), and on nontumoral human embryonic kidney cells (HEK 293). The nontumoral and ovarian cancer cells were treated with decreasing concentrations (200−0.002 μM) of the different compounds and incubated for 72 h at 37 °C. With the exception of *cis*-DDP, which is water soluble, all tested compounds were first solubilized in DMSO and then diluted in the media for cell studies. The percentage of DMSO never exceeded 1%, and this concentration was without cytotoxic effect. After incubation, cellular viability was assessed by the MTT assay (MTT = 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide). Inhibition of growth (%) was calculated by correlation with a control incubated without drug. IC_{50} values were determined and expressed in micromolar concentrations and are summarized in Table 1.

Stability Assays. The stability of the compound was performed in DMSO, saline solution (0.9% NaCl), or PBS and determined by HPLC analysis. For stability in DMSO, 1 mL of 1 mM test compound in DMSO (stock solution 10 mM in DMSO) was incubated at rt. At different time points (see Supporting Information for graphs) 30 μL aliquots were taken 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 remaining percentage compared to the initial concentration.

For stabilities in saline or PBS solutions, 1 mL of 100 μM test compound (stock solution 10 mM in DMSO) in saline solution or PBS was incubated at 37 °C. At different time points (see Supporting Information for graphs) 100 μL aliquots were analyzed by RP-HPLC analysis following the aforementioned conditions. In these cases, the disappearance of the compound was monitored at 225 nm.

Uptake and Subcellular Distribution. To explore the uptake of selected complexes and their intracellular distribution, A2780 cells were exposed to each complex (0.5−1 μM) for 24 h at 37 °C, washed with cold PBS, and centrifuged to obtain a cellular pellet following a previously described procedure.[26] Cytosol, nucleus, membrane/particulate, and cytoskeletal fractions were separated using a commercial kit (FractionPREP cell fractionation kit from BioVision) according to the manufacturer's recommendations. The Pt content in the different fractions was measured, after digestion, by ICP-MS on a ICP-MS NexION 300xx PerkinElmer instrument, with ^{187}Rhenium used as internal standard. Analysis was performed in the SIDI department of UAM which implements and maintains the international certification network lQNetISO-900.1.2008 fulfilling all requirements (http://www.uam.es/ss/Satellite/es/1242663063104/contenidoFinal/Calidad.htm). Briefly, samples were digested with ultrapure HNO_3 (65%), H_2O_2, and HCl, evaporated, and resuspended in ultrapure water to obtain a 2.0% (v/v) nitric acid solution.

DNA Electrophoresis. The DNA interaction was evaluated by monitoring the mobility of supercoiled plasmid DNA (covalently closed circular, ccc) and open circular DNA (oc) as previously described.[26] The plasmid DNA used was ϕX174 plasmid DNA (Promega). Each reaction mixture was prepared by adding water (6 μL), supercoiled DNA (2 μL, 200 ng), stock Na_2HPO_4/HCl buffer solution (pH 7.2, 100 mM, 2 μL), and the solution of the complex (10 μL). The final reaction volume was 20 μL, the final buffer concentration was 10 mM, and the final metal concentration varied from 0.5 to 50 μM, corresponding to a r_i (input molar ratio of Pt/nucleotide) of 0.01−1.67. Samples were typically incubated for 24 h at 37 °C in the dark. After incubation, DNA loading buffer (bromophenol blue (0.05%), sucrose (40%), EDTA (0.1 M, pH 8.0), and SDS (0.5%; Sigma, 4 μL) were added to each tube and the sample was loaded onto an agarose gel (0.8%) in TAE buffer [Tris (40 mM), acetic acid (20 mM), EDTA (1 mM), pH 8.0]. Controls of nonincubated plasmid and plasmid incubated with DMSO were loaded on each run. Electrophoresis was carried out for 3.5 h at 90 V. Gels were then stained with TAE buffer containing ethidium bromide 0.5 μg mL^{-1}). Bands were visualized under UV light, and images were captured with an AlphaImagerEP (Alpha Innotech). All samples in each figure were obtained from the same run.

Chloroquine Assay. The interaction of complexes and ligands with ΦX174 plasmid DNA was performed in the presence of chloroquine to evaluate the intercalation/platination effect. Complexes or ligands were incubated with ΦX174 plasmid DNA at 37 °C for 24 h using concentrations of 5 and 10 μM for each compound tested. After addition of DNA loading buffer, samples were loaded onto an agarose gel (0.8%) in TAE buffer containing chloroquine (1.25 μg mL^{-1}) in order to resolve DNA topoisomes, and electrophoresis was performed for 16 h at 20 V in TAE buffer.

DNA Binding Studies. Calf thymus DNA (CT-DNA) sodium salt was purchased from Sigma and used without further purification. DNA concentrations per nucleotide of stock solutions in phosphate buffer (10 mM, pH 7.2) were determined by absorption spectroscopy at 260 nm, after adequate dilution with the buffer and using the reported molar absorptivity of 6600 M^{-1} cm^{-1}.[27] All measurements that

Table 1. Antiproliferative Activity of Platinum Complexes 1a−c, 2a−c, Ligands a−c, and *cis*-DDP in HEK 293, A2780, and A2780cisR Ovarian Cancer Cell Lines[a]

	IC_{50} (μM)[b]			
compound	HEK 293	A2780	A2780cisR	RF[c]
1a	2.9 ± 1.2	0.67 ± 0.3	0.95 ± 0.5	1.4
1b	29 ± 1.8	11.2 ± 4.1	55 ± 3	4.9
1c	30 ± 1.6	13.6 ± 4.9	39 ± 1.2	2.9
2a	0.65 ± 0.2	0.26 ± 0.5	0.84 ± 0.5	3.2
2b	3.39 ± 0.3	2.38 ± 1	6.56 ± 1.3	2.8
2c	3.03 ± 1.0	3.6 ± 1.2	7.18 ± 1.5	2.0
a	79 ± 1.5	29 ± 0.1	28 ± 1.3	0.97
b	96 ± 1.6	65 ± 2	42 ± 1.2	0.65
c	117 ± 2.4	25 ± 1.2	24.6 ± 1.1	0.98
cis-DDP	13 ± 1.4	1.51 ± 0.5	13.3 ± 4.5	8.8

[a]Data were collected after 72 h of exposure to the drug. Values presented are average ± standard deviations of 3 experiments with 4 replicates each. [b]IC_{50} is expressed as the concentration causing 50% cancer cell growth inhibition. [c]RF: resistance factor, defined as the ratio IC_{50} A2780cisR/IC_{50} A2780.

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involved DNA and the different tested compounds were carried out in phosphate buffer (10 mM, pH 7.2). Absorption and fluorescence titrations were performed by keeping the concentrations of the probe constant while varying the concentrations of the DNA. To obtain the intrinsic binding constant (K) all calculations were done by considering the DNA concentration in base pairs, and data were corrected for volume changes.

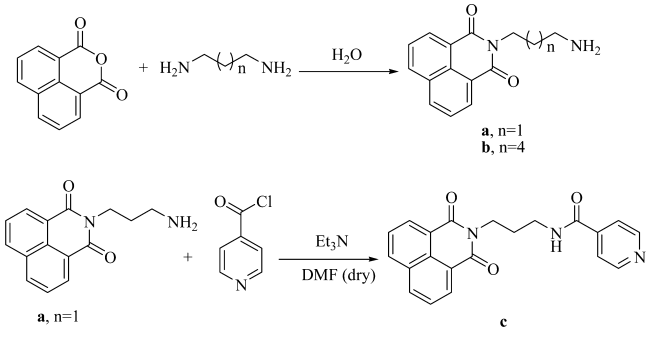
Absorption Spectroscopy Studies. UV−vis absorption spectra were recorded on a PerkinElmer Lambda 35 UV/vis spectrometer using 1 cm path-length quartz cells. In order to eliminate any interference of the DNA absorbance in the region of absorbance of the chromophores an equal amount of CT-DNA in phosphate buffer was added to the sample and reference cells. After each addition of CT-DNA, the solution was allowed to equilibrate and the absorption spectrum was recorded until there were no further changes in the absorbance.

Fluorescence Spectroscopy Studies. Fluorescence spectra were recorded in a PerkinElmer LS 55 fluorescence spectrometer using a quartz cuvette of 1 cm. After each addition of CT-DNA in phosphate buffer, the solution was allowed to equilibrate and the absorption at the excitation wavelength was recorded. Fluorescence spectra were then recorded until there were no further changes in the fluorescence intensity. Complexes **1a** and **2a** were excited at 350 nm, and emission spectra were recorded from λ = 360 to 530 nm with a scan speed of 300 nm min^{-1}. Emission and excitation slits were chosen in order to maximize the fluorescence intensity.

RESULTS AND DISCUSSION

Chemistry. Figure 1 shows the design used to achieve the novel *trans*-platinum(II) complexes. The series of complexes, named **1a−c** and **2a−c**, contain the naphthalimide targeting ligand bound to the platinum atom by two different linkers: aliphatic amine (**a** and **b**, Scheme 1) or isonicotinamide (**c**,

Scheme 1. Synthesis of Ligands a−c

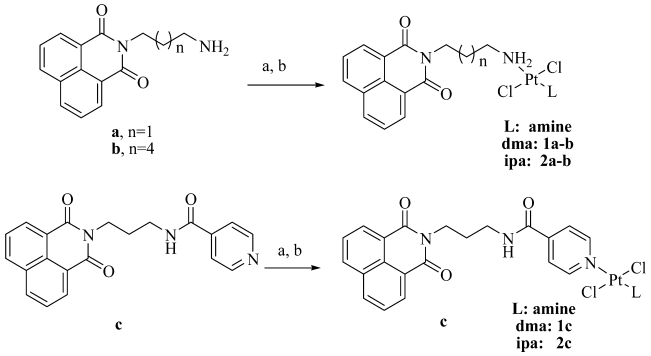


Scheme 1). The first three complexes of the series contain a small aliphatic amine, dimethylamine, as the ligand (L = amine) trans to the corresponding naphthalimide ligands, **1a−c** (Scheme 2). In order to complete this new series of compounds, we selected a different aliphatic amine for ligand L isopropylamine, **2a−c**, based on our previous experience and the observation of better results in specific *trans*-platinum(II) complexes.[13]

First, synthesis of ligands **a−c** in which the naphthalimide intercalator is tethered with the corresponding spacer was easily achieved in high purity on large scale (Scheme 1). Ligands **a** and **b** were prepared following procedures reported in the literature,[24,25] while ligand **c** was obtained in 72% yield by reacting amine from ligand **a** and isonicotinoyl chloride in dry DMF.

In the next step, ligands **a−c** were coordinated to platinum to afford the desired *trans*-platinum(II) complexes **1a−c** (Scheme 2). More concretely, amines **a** and **b** or pyridyl ligand **c** reacted

Scheme 2. Synthesis of *trans*-Platinum(II) Complexes 1a−c and 2a−c[a]



[a](a) *cis*-$[PtCl_2(dma)_2]$ or *cis*-$[PtCl_2(ipa)_2]$, H_2O, 95 °C; (b) HCl conc, 95 °C.

with *cis*-$[PtCl_2(dma)_2]$ in water at 95 °C to form the corresponding cationic tetramine−platinum complex that underwent trans isomerization after treatment with hydrochloric acid at the same temperature. Following this one-pot procedure, all platinum complexes were isolated by filtration and purified by trituration in good yields (31−41%). Finally, the series was completed with complexes **2a−c** (Scheme 2) and achieved using *cis*-$[PtCl_2(ipa)_2]$ as starting material with ligands **a−c**.

Cytotoxic Activity and Cell Uptake. *In Vitro Cytotoxic Activity.* To explore the potential as antitumoral agents of the new series of complexes (Figure 1), the cytotoxic activity toward human epithelial ovarian carcinoma cells, sensitive (A2780) and resistant (A2780cisR) to cisplatin, was tested. Moreover, the cytotoxic activity was also studied in human nontumoral cells (HEK 293). For comparison purposes the cytotoxicity of *cis*-DDP and ligands **a−c** was evaluated under the same experimental conditions.

Cells were treated with increasing concentrations of the different Pt complexes for 72 h continuous incubation at 37 °C, after which the cellular viability was determined by the MTT assay. The viability of cells in the presence of the tested compounds was compared to that observed in controls (no drug), and inhibition of growth (%) was calculated. IC_{50} (i.e., concentration which reduces the growth by 50%) was determined and expressed in micromolar concentration (Table 1).

IC_{50} values of the ligands in Table 1 showed no cytotoxicity, but the coordination of the platinum to these ligands caused an obvious effect in the IC_{50} values, affording better activity. Within the methylamine series, **1a−c**, complex **1a** is a particularly active compound, exhibiting submicromolar IC_{50} values in both cell lines after 72 h of incubation. Under these conditions, **1a** showed a cytotoxic activity 15−20 times higher than the other dimethylamine Pt(II) complexes (**1b** and **1c**), being also more cytotoxic than *cis*-DDP, especially in the A2780cisR cell line. As we envisioned in the design of the series, isopropylamine complexes (**2a−c**) presented a much higher cytotoxicity, generally over 10 times more active than the dimethylamine series. The trend observed so far would indicate **2a** as the most active compound, as it is corroborated by the value in Table 1.

The activity of these complexes nicely supports our approach of including a targeting ligand in order to achieve better and more selective drugs, as for example, the covalent binder

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contained in ligand **c** by itself showed no cytotoxicity in previous studies (Figure 1).[28,29]

Although more cytotoxic than the corresponding ligands, the Pt complexes present different activity profiles. Comparing both series, we observed that **1a** and **2a** are able to overcome significant cisplatin cross-resistance with resistance factors ranging from 1.4 to 3.2. The *trans*-platinum(II) complex with the longer aliphatic linker **1b** and the *trans*-platinum(II) complex with the isonicotinamide linker **1c** have shown similar cytotoxic activities in the micromolar range. Notably, they seem to be less efficient in the cisplatin-resistant cell line. On the other hand, the isopropylamine derivatives **2b**–**c** are quite efficient in the resistant cell line, albeit the values are not as good as **2a**. Interestingly, **1a**, which is the most cytotoxic in cancer cell lines, is also the most selective one. Complex **1a** is approximately 4- and 5.6-times more toxic on the sensitive A2780 cells. This trend is not so clear in the isopropylamine derivatives where all complexes are in general around the same RF value.

In order to assess the selectivity of these new Pt complexes for tumoral versus nontumoral cells, their antiproliferative effects on human embryonic kidney cells HEK293 were also screened. In general, we observed higher activity of the isopopylamine complexes in nontumoral cell lines, while complexes **1a**–**c** showed better selectivity, being less active in nontumoral cells. In particular, compounds **1b** and **1c** are even more selective than cisplatin.

In summary, the *trans*-platinum complexes **1a** and **2a** with the intercalator bound via the shorter aliphatic amine linker showed the highest cytotoxic activity within both series for the sensitive and resistant cell lines, and they are also particularly active when compared with cisplatin.

Cellular Uptake. Uptake studies in A2780 cells were performed to evaluate cell permeability/entrance of selected complexes. Furthermore and to ascertain if the cellular toxicity is related to DNA cleavage, subcellular fractionation was also performed. Complexes **1a** and **2a** were chosen because they are the most cytotoxic of each family, and **2b** was also studied as it has the same amine (ipa) as **2a** but different linker length.

Cells were incubated for 24 h with complexes **1a**, **2a**, and **2b** at 1 μM, and then the different subcellular fractions were prepared and analyzed by ICP-MS. From the % of total Pt applied (Figure 2a) we can see that using the same concentration the total uptake after 24 h of incubation of **1a** and **2a** is similar but ca. 50% higher than that of **2b**. When the distribution of Pt in the different subcellular fractions is quantified (Figure 2b), results show that complexes **1a** and **2a** present similar profiles, i.e., low retention in the cytosol and a high amount in the membrane and cytoskeleton. Complex **2b** is the compound that shows the highest nuclear uptake when compared to the others. However, the higher nuclear uptake of **2b** compared to **1a** and **2a** is not correlated with the cytotoxicity, as **2b** is less cytotoxic than both **1a** and **2a**.

When A2780 cells were incubated with **1a**, **2a**, and **2b** at IC_{50} concentrations found at 24 h (Table 2) it can be seen that complex **2a** is the compound that shows highest uptake, which agrees with the cytotoxicity data that present **2a** as the most active member of this novel family.

Stability Assays. We selected complexes **1a** and **2a** to study the stability, as those complexes turned out to be two of the most promising candidates from the series. The stability studies were performed in (a) DMSO, as this is the solvent used to make the stock solutions, (b) saline solution (0.9% NaCl solution), which is a component of the pharmaceutical dosage forms for platinum complexes, and (c) PBS, which is a regular solvent used to mimic the extracellular fluid. The stability of the complexes in the different solutions was analyzed and quantified by reverse phase HPLC.[30] The graphs represent the amount of drug that remains intact in solution along time, calculated using the initial amount (t = 0 h) of the compound as 100% value.

Solutions of the complexes in DMSO (1 mM) were stable after 24 h at room temperature, in the expected range for platinum complexes (see Graphics S1 and S2, Supporting Information, and Experimental Section for methodology details). In addition, both complexes showed high stability in saline solution, with almost 100% remaining unaltered after 24 h of incubation. The stability in PBS after 24 h was complete for **1a** and almost complete (86%) for **2a**. These results revealed the high stability of complexes **1a** and **2a** in solution in the different media evaluated in this study.

DNA Interaction. In order to shed light into the mode of action of these complexes, we proceed to study their potential interaction with DNA through a set of different assays.

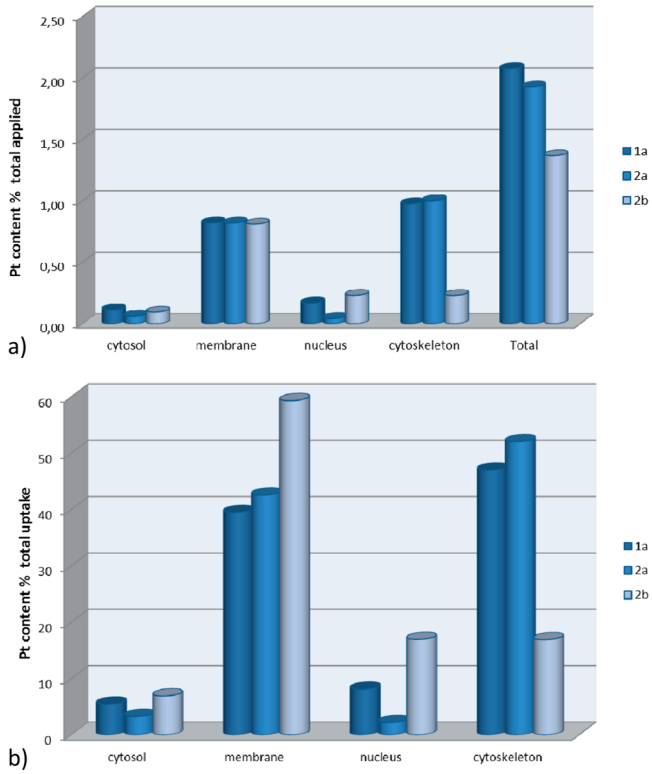

Figure 2. Subcellular distribution of **1a**, **2a**, and **2b** in A2780 cells after 24 h of incubation with 1 μM concentration: (a) % of total applied (amount in each fraction vs the total amount applied in the medium) and (b) % of total uptake in the different fractions (amount in each fraction vs the total amount that entered the cells).

Table 2. Cellular Uptake of 1a, 2a, and 2b in A2780 Cells after 24 h of Incubation at IC_{50} Concentrations

	IC_{50} concentration (μM)	Pt content (nanograms/10^6 cells)
1a	2.5	6.0
2a	0.8	10.8
2b	8.0	60.0
cisplatin	16.5	32.8

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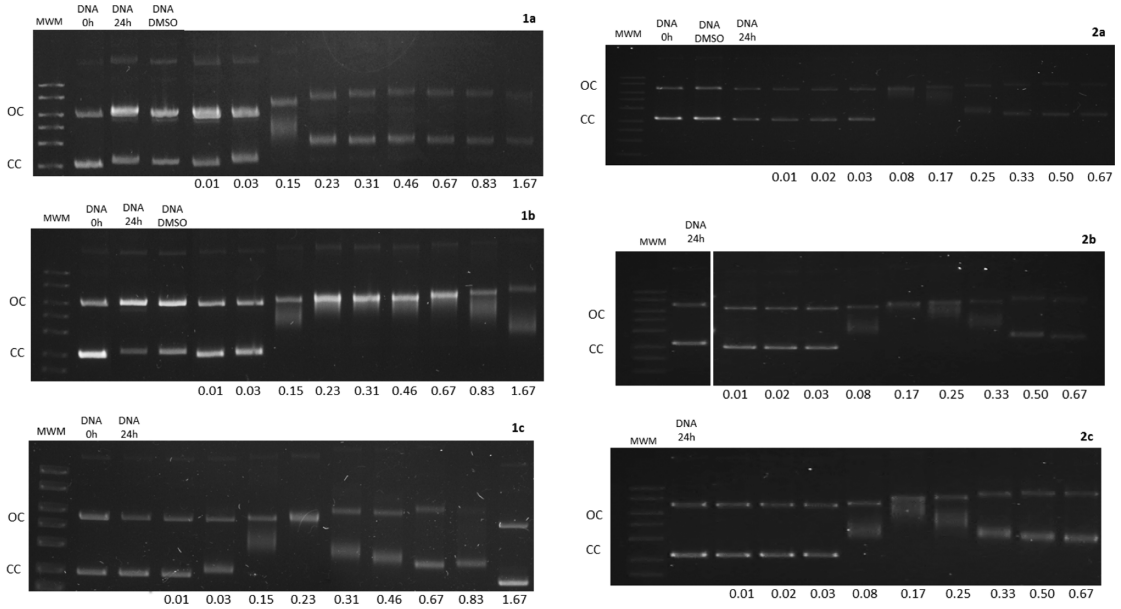

Figure 3. Gel mobility assay of supercoiled ϕX174 plasmid DNA after 24 h incubation at 37 °C in phosphate buffer (pH 7.2) with complexes **1a**–**c** (first column) and **2a**–**c** (second column). Forms cc and oc are closed circular and open circular forms of DNA, respectively.

Plasmid–DNA Studies. The effect of the interaction of Pt complexes **1a**–**c** and **2a**–**c** with supercoiled DNA was determined by the ability of the compounds to modify the electrophoretic mobility of the covalently closed circular (ccc) and open circular (oc) forms of ϕX174 plasmid DNA (Figure 3). Moreover, the same type of experiments were run using the free ligands to gain further insight into the interaction of the respective Pt(II) complexes with DNA.

The electrophoretic mobility of native ϕX174 plasmid DNA and ϕX174 DNA incubated with different concentrations of the Pt(II) complexes was also used to assess the DNA unwinding angle, ϕ, induced by the Pt complexes (Table 3).

Table 3. Unwinding Angles (Φ) of pPlasmid DNA Induced by the Complexes

compound	unwinding angle (deg)[a]
1a	17.4
1b	10.9
1c	9.1
2a	25.1
2b	13
2c	13

[a]Unwinding angle determined assuming the value of 13° for *cis*-DDP.[38,39]

In this type of analysis, a compound that unwinds the DNA double helix reduces the number of supercoils with a consequent decrease of the superhelical density of closed circular DNA and reduction of the rate of migration through the agarose gel.[26] The increase of r_i, defined as the molar ratio of added platinum complex per nucleotide, decreases the rate of migration of cc DNA until it comigrates with oc DNA. In this way, a coalescence point ($r_i = r_b(c)$) is achieved, which corresponds to the amount of platinum needed for complete removal of all supercoils from DNA.

The coalescent points are reached at different r_i values for the different complexes, with increasing r_i from **1a** to **1c** (Figure 3, first column) and from **2a** to **2c** (Figure 3, second column). Complex **1b** showed a r_i value of 0.19, and **1c** has a r_i of 0.23. These two values are higher than the value for **1a** (0.12). The same trend is observed when we compare the r_i values produced by complexes **2a**–**c**.

This effect can be evaluated quantitatively by the DNA unwinding angle, ϕ, determined from the equation $\phi = 18\sigma/r_b(c)$, where σ is the plasmid superhelical density and $r_b(c)$ is the molar ratio of platinum bound per nucleotide at the coalescence point, as mentioned above. The unwinding angles are presented for the Pt complexes under study in Table 3. We found that the ϕ value of the **1a** complex is higher than that of *cis*-DDP (17.4° vs 13°). For **1b** and **1c** the values are similar (10.9° and 9.1°). Once more, we observed the same trend for the isopropylamine series **2a**–**c**. Complex **2a** produced the highest change in the DNA mobility, resulting in the highest ϕ value (25°), which indicates a more dramatic DNA distortion than the rest of the complexes of both series. In fact, this value is even higher than the one (ϕ = 15°) that we previously reported for the congener *trans*-[$PtCl_2$(dimethylamine)-(isopropylamine)] without the naphthalimide moiety.[31]

The comparison of the ϕ values of **1**–**2a** with that of *cis*-DDP (17.4° and 25.1° vs 13°) might indicate the possibility of a contribution of binding modes different from DNA platination for **1**–**2a**, such as an intercalative binding allowed by introduction of the targeting ligand. Moreover, comparison of complexes with different linkers showed that the shorter aliphatic amine linker seems to be the most appropriate for efficient intercalation/platination binding. As mentioned before, the same type of experiments were run using the free ligands to gain further insight into the interaction of the respective Pt(II) complexes with DNA showing no modification in the mobility of the plasmid–DNA (data not shown).

We proceed studying the trends observed in the DNA unwinding angle evaluating the intercalation/platination effect in the presence of chloroquine. In these studies, ligands and complexes at 5 mM concentration (r_i = 0.17) were incubated with ϕX174 DNA at 37 °C for 24 h. Afterward, samples were analyzed by agarose gel electrophoresis in the presence of chloroquine (Figure 4). Chloroquine induces positive super-

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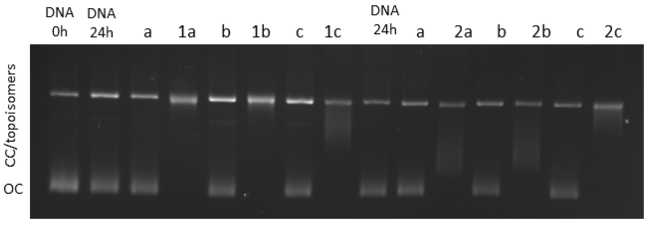

Figure 4. Resolution of DNA topoisomers of ϕX174 DNA incubated with ligands (**a**–**c**) and corresponding platinum complexes (**1a**–**c** and **2a**–**c**) (5 μM) for 24 h at 37 °C in phosphate buffer (pH 7.2) after agarose gel electrophoresis with chloroquine (1.25 μg mL^{-1}).

coiling of plasmid DNA and promotes the resolution of topoisomeric forms of the plasmid: the slowest moving band of each lane represents the open circular DNA (oc form), while the other bands of each lane are all covalently closed circular DNA (cc forms or topoisomers).

Figure 4 shows that the bands of DNA topoisomers of the plasmid incubated with ligands **a**–**c** migrate similarly to those of control plasmid in the presence of chloroquine. This indicates that DNA–naphthalimide adducts are readily displaced by chloroquine. On the other hand, both series of complexes **1a**–**c** and **2a**–**c** produced adducts that in the same experiment are not displaced by chloroquine. In all cases we observed the same pattern of changes in the electrophoretic mobility produced by the complexes compared to the controls, although in a different extent.

The effect produced by the methylamine complexes **1a** and **1b** (lines 4 and 6) is similar to each other and different from complex **1c.** The same trend is observed for the isopropylamine series, with **2a** and **2b** showing similar changes in mobility while **2c** is different. Looking at the structure of the ligands, the main difference observed comparing **a** and **b** to **c** is the planar fragment: isonicotinic amide which is more rigid than the aliphatic chains.

Spectroscopy Titration Studies. We also studied the reaction of complexes **1a** and **2a** with CT-DNA by UV–vis titration experiments. These complexes were selected because they displayed the highest unwinding angles and the best cytotoxicity data.

The UV–vis spectra presented in Figure 5 clearly showed how increasing amounts of DNA led to hypochromism in the spectra of complexes **1a** and **2a**: 40% and 58% for the band at 235 nm and 36% and 54% for the band at 344 nm, respectively.

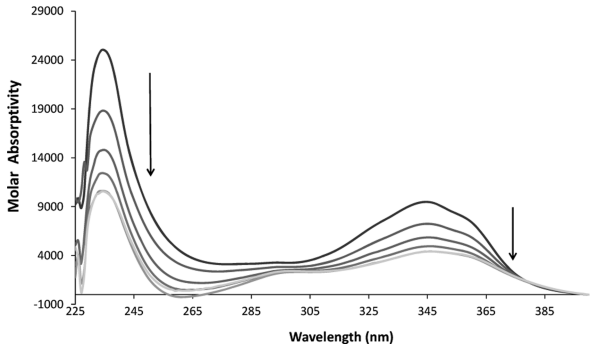

Figure 5. Absorption spectra of complex **2a** (40 μM) in the presence of increasing concentrations of CT-DNA (0–200 μM) in PBS buffer (pH = 7.4). Arrows indicate the changes of the bands upon addition of CT-DNA.

These experiments support the interaction of the naphthalimide part of the complexes with DNA, which can be partially due to intercalation, as DNA intercalators, in general, leads to a hypochromic effect.[32,33]

The emission titration experiments have shown quenching of the fluorescence upon incremental addition of CT-DNA to solutions of complexes **1a** and **2a** (Figure S3, Supporting Information). The decrease is slightly more evident in the case of the dimethylamine derivative, in agreement with the value of the calculated Stern–Volmer constant (K_{sv}) (7.1×10^4 vs 2.3×10^4 M^{-1}).[18]

The data presented here suggest that the complexes do interact/bind to DNA and that this interaction plays an important role in their mechanism of action and can partly explain their different cytotoxic potencies. Nevertheless, in agreement with the data observed in the subcellular distribution experiments, in which the highest nuclear uptake was observed for **2b**, a compound that has a low interaction with DNA, we cannot discard the possibility that tumoral cell death could be induced by other means, for example, by protein interaction. Metal drugs have been reported to bind to proteins, and in particular, platinum compounds have been shown to bind to particular sites of proteins, such a as methionine,[34,35] histidine,[36] cysteine, and selenocysteine[35] residues. These important properties together with recent works describing organometallic Ru compounds with intercalating ligands capable of activating p53 pathways via kinase inhibition[37] open up even more possibilities for the mechanism of action of the complexes described in this study.

CONCLUSIONS

We prepared six Pt^{II}-based complexes that contain naphthalimide ligands to combine the ligand biological properties and DNA covalent binding mode to achieve novel, unconventional, and potent antitumoral complexes. The results showed that we improved the cytotoxicity of the ligands using the nonconventional platinum structure core and also demonstrated that the distance of the polyaromatic DNA-binding group to the metal center is determinant for the cytotoxic activity of these complexes. Complexes **1a** and **2a** showed the best cytotoxicity in both cancer cell lines (cisplatin resistant and nonresistant). These two complexes are the most cytotoxic of the series, with **2a** being the most active and **1a** the most selective toward cancer cell lines compared to nontumoral cells.

The results of the cytotoxicity and DNA-interaction studies together showed that these complexes might act through a combination of platination and naphthalimide–DNA interaction. Moreover, the (sub)cellular uptake studies suggest that in addition to DNA these complexes might also interact with other cellular targets, widening even more the range of possible targets of these new complexes.

ASSOCIATED CONTENT

Supporting Information

Stability studies; absorption and emission spectra; experimental procedures, characterization, and 2D NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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