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Reactivity and Biological Properties of a Series of Cytotoxic $PtI_2(amine)_2$ Complexes, Either *cis* or *trans* Configured

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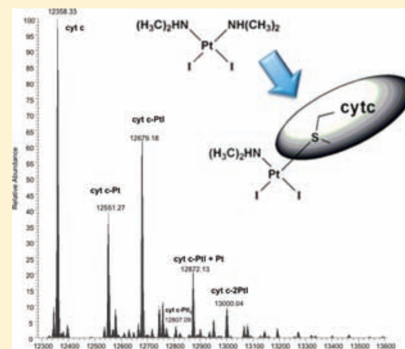
Ⓢ *Supporting Information*

ABSTRACT: Six diiodido−diamine platinum(II) complexes, either *cis* or *trans* configured, were prepared, differing only in the nature of the amine ligand (isopropylamine, dimethylamine, or methylamine), and their antiproliferative properties were evaluated against a panel of human tumor cell lines. Both series of complexes manifested pronounced cytotoxic effects, with the *trans* isomers being, generally, more effective than their *cis* counterparts. Cell cycle analysis revealed different modes of action for these new Pt(II) complexes with respect to cisplatin. The reactivity of these platinum compounds with a number of biomolecules, including cytochrome c, two sulfur containing modified amino acids, 9-ethylguanine, and a single strand oligonucleotide, was analyzed in depth by mass spectrometry and NMR spectroscopy. Interestingly, significant differences in the reactivity of the investigated compounds toward the various model biomolecules were observed: in particular we observed that *trans* complexes preferentially release their iodide ligands upon biomolecule binding, while the *cis* isomers may release the amine ligands with retention of iodides. Such differences in reactivity may have important mechanistic implications and a relevant impact on the respective pharmacological profiles.

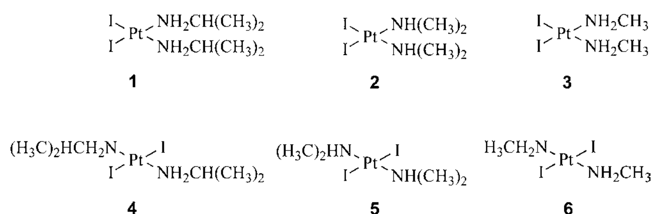


INTRODUCTION

Since the discovery of its outstanding antitumor properties and its introduction in the clinic, cisplatin has been considered a primary anticancer drug lead.[1] Several attempts were made to modify its chemical structure with the goal of obtaining more effective and less toxic antitumor platinum compounds.[2] Rather stringent structural requirements were thus defined for anticancer platinum(II) agents such as the presence of two labile ligands (*leaving groups*) in *cis* position and the need of two inert amine ligands (*nonleaving groups* or *spectator ligands*) in the two remaining coordination positions.[3−5] Nonetheless, several examples of active Pt(II) complexes were reported later that do not obey established structure−activity rules.[6−8] A few of these compounds, bearing aliphatic amines as nonleaving groups, of general formula *trans*-$[PtCl_2LL']$ (where L and L′ = aliphatic amines) were developed by some of us and were shown to overcome cisplatin resistance in tumor cells overexpressing the *ras* oncogene, as well as producing promising effects in tumor xenografts.[9,10]

Within this frame, we recently reconsidered the possibility of replacing chlorides with iodides within classical square planar platinum(II) complexes. In particular, we investigated the *cis*-diiodido-diisopropylamine platinum(II) complex (**1**, Chart 1)

Chart 1. Schematic Representation of *cis* and *trans* Pt(II) Complexes Studied in This Work



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and showed that it manifests an unusual reactivity toward model proteins and peptides, characterized by retention of the iodide ligands and concomitant loss of the amines, while keeping appreciable antiproliferative properties.[11]

We believe that these latter findings may give new strength to the use of iodide ligands in platinum drug designs. Indeed, development of iodidoplatinum complexes as experimental anticancer agents was largely hampered in early times of platinum medicinal chemistry by the idea that the higher stability and lower reactivity of Pt−I bonds compared to those of Pt−Cl bonds in aqueous solution[12−14] would greatly reduce or even abolish any pharmacological efficacy. Moreover, an initial report on *cis*-$[PtI_2(NH_3)_2]$ documented a substantial lack of activity as an anticancer agent in selected in vivo animal models.[15] Nonetheless, a few sporadic studies in subsequent years highlighted a considerable and unexpected reactivity for iodido Pt(II) and Pt(IV) complexes[16] toward a few important biomolecular targets (e.g., serum albumin and glutathione).[17,18]

In view of the paucity of studies on iodido platinum complexes as potential anticancer agents and of our recently published results, we have expanded here our investigations in this research area. Accordingly, we have prepared two series of iodido−platinum(II) complexes, *cis* and *trans* configured, and investigated some aspects of their possible mode of action in a more systematic manner. In addition to our previous compound, two more $[PtI_2(amine)_2]$ complexes (where amine = dimethylamine (**2**) or methylamine (**3**)) were prepared and characterized in parallel to three *trans* analogues **4**−**6** (Chart 1). For all six compounds, the chemical behavior in solution and the in vitro antiproliferative actions were evaluated on a few human cancer cell lines. In addition, the respective cell cycle effects were explored for complexes **1** and **4** as each representative of *trans* and *cis* configurations. As the mechanism of action of this promising family of Pt complexes is not fully understood and might involve interactions with biomolecules other than DNA, adduct formation with proteins needs to be considered in more depth. Therefore, the reactivity of these platinum(II) compounds with a few representative biomolecules was comparatively analyzed through a variety of physicochemical techniques. An overall picture is eventually achieved to describe the reactivity and the biological actions of this novel class of platinum(II) complexes, opening a new perspective for iodido−platinum complexes as potential and innovative antitumor agents.

■ EXPERIMENTAL SECTION

Synthesis of Compounds. Complex **1** was synthesized as described in the previous communication.[11] Characterization was in agreement with our previous data. Chemical starting materials: K_2PtCl_4, amines and biological molecules were purchased from Sigma-Aldrich. All tested synthesized compounds possess a purity >95%, which is shown by the values of the compounds on the elemental analysis performed with a LECO CHNS-932 elemental analyzer.

General Method for the Synthesis of cis-PtI$_2$*(amine)*$_2$. Dharas method[19] and ref 20 were followed with a few modifications: K_2PtCl_4 (500 mg, 1.2 mmol) was dissolved in approximately 5 mL H_2O, and KI (1 g, 6 mmol) was added to produce a dark solution. After 5 min of stirring (to complete K_2PtI_4 formation), the corresponding amine in its hydrochloride form was added (4.8 mmol). The reaction mixture was treated at room temperature in the dark with a solution of KOH (269 mg; 4.8 mmol). When the addition was finished, the reaction mixture was allowed to stir for 2 h at room temperature in the dark. The yellow precipitate was collected, washed intensively with warm water, and vacuum-dried overnight at 60 °C in a drying oven.

cis-$[PtI_2(dma)_2]$ **2**: Yellow solid (85%). NMR (acetone-d_6): δ(^{1}H, 300.13 MHz) 2.79 (d, *J* = 6.0 Hz, 6H, CH_3), 4.9 (s, 1H, NH). δ(^{13}C, 125.76 MHz; 500 MHz) 43.0. δ(^{195}Pt, 64.53 MHz; Na_2PtCl_6) −3237 ppm. Anal. Calcd for $PtC_4H_{14}N_2I_2K_{0,1}I_{0,1}$: C, 8.64; H, 2.54; N, 5.04%. Found: C, 8.47; H, 2.63; N 5.25%.

cis-$[PtI_2(ma)_2]$ **3**: Yellow solid (60%). NMR (acetone-d6): δ(^{1}H, 300.13 MHz) 2.64 (t, *J* = 6.4 Hz, 3H, CH_3), 4.44 (2H, NH_2). δ(^{13}C, 125.76 MHz) 32.9. δ(^{195}Pt, 64.53 MHz, Na_2PtCl_6) −3334 ppm. Anal. Calcd for $PtC_2H_{10}N_2I_2$: C, 4.70; H, 1.97; N, 5.48%. Found: C, 4.54; H, 2.03; N, 5.47%.

General Method for the Synthesis of trans-PtI$_2$*(amine)*$_2$. The synthesis was performed following other methods[20] with significant variations described as follows: *cis*-$PtI_2(amine)_2$ (2.4 mmol) was suspended in water, and the corresponding base (12 mmol) was added to the solution and heated at reflux temperature until it turned to clear solution. The clear solution was concentrated at high temperature (100 °C) until detection of a bright orange solid, which was allowed to stand overnight at 4 °C until complete precipitation. Then the orange/yellow solid was filtered off, washed with warm water, and vacuum-dried overnight at 60 °C in a drying oven. Afterward, recrystallization in chloroform/ether is in some cases required.

trans-$[PtI_2(ipa)_2]$ **4**: Orange solid (69%). NMR (acetone-d^6): δ(^{1}H, 300.13 MHz) 1.30 (d, *J* = 6.5 Hz, 6H, CH_3) 3.40 (sp, *J* = 6.5 Hz, 1H, CH) 3.89 (b.s, 2H, NH_2). δ(^{13}C, 125.76 MHz): 24.5(C1), 51.5(C2). δ(^{195}Pt, 64.53 MHz, Na_2PtCl_6) −3333 ppm. Anal. Calcd for $C_6H_{19}N_2O_{0.5}I_2Pt$: C, 12.5; H, 3.32; N, 4.86%. Found: C, 12.44; H, 3.22; N, 4.88%.

trans-$[PtI_2(dma)_2]$ **5**: Yellow solid (62%). NMR (acetone-d^6): δ(^{1}H, 300.13 MHz) 2.65 (d, *J* = 5.9 Hz, 6H, CH_3), 4.43 (H, NH). δ(^{13}C, 125.76 MHz): 45.5. δ(^{195}Pt, 64.53 MHz, Na_2PtCl_6) −3046 ppm. Anal. Calcd for $C_4H_{14}N_2I_2Pt$: C, 8.91; H, 2.62; N, 5.20%. Found: C, 8.66; H, 2.49; N, 5.48%.

trans-$[PtI_2(ma)_2]$ **6**: Yellow solid (85%). NMR (acetone-d^6): δ(^{1}H, 300.13 MHz) 2.48 (t, *J* =6.3 Hz, 3H, CH_3), 3.9 (2H, NH_2). δ(^{13}C, 125.76 MHz): 33.98. δ(^{195}Pt, 64.53 MHz, Na_2PtCl_6) −3350 ppm. Anal Calcd for $C_2H_{11}N_2I_2PtO_{0,5}$: C, 4.62; H, 2.13; N, 5.38. Found: C, 4.29 ; H, 1.94; N, 5.55%.

Synthesis of 9-Ethylguanine Adducts with Complexes 1 and 4. A concentrated water solution of $AgNO_3$ (126 mg 0.38 mmol) was added to a solution of complex **1** or complex **4** (100 mg 0.18 mmol) in acetone and stirred at room temperature and in the dark for 2 h. The reaction mixture was filtered with Celite over 9-EtG (63 mg. 0.35 mmol). The milky solution was stirred at room temperature for 24 h in the dark for complex **1** adducts and 3 days for complex **4** adducts. A white solid was isolated by filtration, washed with acetone, and dried under vacuum.

cis-$Pt(ipa)_2(9\text{-EtG})_2(NO_3)_2$ **b1**: White solid (55%). NMR (D_2O): δ(^{1}H, 300.13 MHz) 1.34 (m, 18H, CH_3-ipa and CH_3-EtG), 2.69 (sp, 2H, CH-ipa), 4.09 (q, 4H, CH_2-EtG), 5.11 (bs, 4H, NH_2), 8.16 (s, 2H, H_8-EtG). δ(^{13}C, 125.76 MHz) 13.6 (CH_3-EtG), 22.0 (CH_3-ipa) 39.2 (CH_2, 9-EtG), 49.0 (CH-ipa), 113.2 (C1, 9-EtG), 156.2 (C2, 9-EtG), 153.7 (C4, 9-EtG), 150.1 (C6, 9-EtG), 140.4 (C8, 9-EtG). δ(^{195}Pt, 64.53 MHz, Na_2PtCl_6) −2476.4 ppm. MS (EI) *m/z*: $[Pt(ipa)_2(9\text{-EtG})_2]^{2+}$ = 670.26 [M^{2+}]. Calcd [$PtC_{20}H_{35}N_{12}O_2$]: 670.27. Anal. Calcd for $PtC_{20}H_{36}N_{14}O_8.H_2O$: C, 29.50; H, 4.70; N, 24.64%. Found: C, 29.18; H, 5.02; N, 24.28%.

cis-$Pt(ipa)_2(9\text{-EtG})_2(SbF_6)_2$ **b1·SbF**$_6$: This antimony hexafluoride salt was prepared by dissolving **b1** in water and subsequent addition of a stoichiometric amount of $NaSbF_6$. Slow addition of acetone allows crystal growing by slow diffusion. After several days, **b1·SbF**$_6$ could be isolated as crystals. White solid (90%). NMR (D_2O): δ(^{1}H, 300.13 MHz) 1.35 (m, 18H, CH_3.ipa and CH_3-EtG), 2.70 (sp, 2H, CH-ipa), 4.11 (q, 4H, CH_2-EtG), 5.11 (bs, 4H, NH_2), 8.14 (s, 2H, H_8-EtG). δ(^{13}C, 125.76 MHz) 14.2 (CH_3-EtG), 22.0 (CH_3-ipa), 39.9 (CH_2,9-EtG), 48.5 (CH-ipa), 113.8 (C1, 9-EtG), 156.7 (C2, 9-EtG), 154.3 (C4, 9-EtG), 150.7 (C6, 9-EtG), 141.0 (C8, 9-EtG). δ(^{195}Pt, 64.53 MHz, Na_2PtCl_6) −2478.6 ppm. MS(EI) *m/z*: $[Pt(ipa)_2(9\text{-EtG})_2]$-$(SbF_6)^+$ = 905.17. [M^+] Calcd [$PtC_{20}H_{35}N_{12}O_2SbF_6$]: 905.15; $[Pt(ipa)_2(9\text{-EtG})_2]^{2+}$ = 670.26 [M^{2+}]. Calcd [$PtC_{20}H_{35}N_{12}O_2$] =

670.27. Anal. Calcd for $PtC_{20}H_{36}N_{12}O_{2}.Sb_{2}F_{12}\cdot 2\ H_{2}O$: C, 20.37; H, 3.41; N, 14.25%. Found: C, 19.98; H, 3.27; N, 14.60.

trans-Pt(ipa)$_2$(9-EtG)$_2$(NO$_3$)$_2$ **b4**: White solid (35%). NMR (D_2O): δ(^{1}H, 300.13 MHz) 1.07 (d, J = 6.2 Hz 6H, CH_3-EtG), 1.51(dd, J = 7.25 and 7.29 Hz 12H, CH_3.ipa) 2.42 (sp J = 6.2 Hz 2H, CH-ipa), 4.23 (q, J = 7.29 Hz 4H, CH_2-EtG), 5.56 (4H, NH_2), 8.62 (s, 2H, H_8-EtG). δ (^{13}C, 125.76 MHz) 14.1 (CH_3-EtG), 22.3 (CH_3-ipa) 40.1 (CH_2,9-EtG), 48.4 (CH-ipa), 114.0 (C1, 9-EtG), 156.9 (C2, 9-EtG), 154.6 (C4, 9-EtG), 151.0 (C6, 9-EtG), 141.3 (C8, 9-EtG) δ (^{195}Pt, 64.53 MHz, Na_2PtCl_6) −2491.0 ppm. MS (EI) m/z: [Pt(ipa)(9-EtG)] = 431 [M^{2+}].

trans-Pt(ipa)$_2$(9-EtG)$_2$(SbF$_6$)$_2$ **b4·SbF$_6$**: White solid (80%). NMR (D_2O): δ(^{1}H, 300.13 MHz) 1.07 (d, J = 6.4 Hz 6H, CH_3-EtG), 1.51(dd, J = 7.35 and 7.26 Hz 12H, CH_3.ipa) 2.42 (sp, J = 6.4 Hz 2H, CH-ipa), 4.23 (q, J = 7.35 Hz 4H, CH_2-EtG), 5.56 (bs, 4H, NH_2), 8.62 (s, 2H, H_8-EtG). δ(^{13}C, 125.76 MHz) 14.1 (CH_3-EtG), 22.3 (CH_3-ipa) 40.1 (CH_2,9-EtG), 48.4 (CH-ipa), 114.0 (C1, 9-EtG), 156.9 (C2, 9-EtG), 154.6 (C4, 9-EtG), 151.0 (C6, 9-EtG), 141.3 (C8, 9-EtG). δ(^{195}Pt, 64.53 MHz, Na_2PtCl_6) −2491.0 ppm. MS (EI) m/z: [Pt(ipa)(9-EtG)] = 431 [M^{2+}]. Anal. Calcd for $PtC_{20}H_{36}N_{12}O_{2}.Sb_{2}F_{12}\cdot H_{2}O$: C, 20.68; H, 3.29 ; N, 14.47%. Found: C, 20.39; H, 3.27; N, 14.69.

Mono- and Bis-Adducts of 1 and 4 with 9-EtG. Samples of complex **1** and **4** were prepared by mixing 0.5 mL of complex solution (50 mg, 0.09 mmol) in D_2O:acetone-d$_6$ (1:2) with 9-EtG (4 mg, 22.5 x10^{-3}mmol) into a NMR tube. The tube was maintained at 37 °C under slight stirring during the entire experiment (72 h). ^{1}H, ^{13}C, 2D [HSQC], and ^{195}Pt NMR spectra were acquired at various times to monitor the reactivity.

Reactivity with Biomolecules. A mixture of 150 mg (0.26 mmol) of complex **4** and 258 mg (1.58 mmol) or 47.4 mg (0.29 mmol) of N-acetyl-L-cysteine (Pt:N-AcCys ratio 1:1.1 or 1:6, respectively) in 2 mL of acetone-d$_6$ was prepared. ^{1}H, ^{13}C, 2D [^{1}H, ^{13}C] HSQC, and ^{195}Pt NMR spectra were recorded at various times (2, 4, 12, and 24 h).

To afford the metathesis of the iodide ligands and force the formation of N-acetyl-L-cysteine adducts, an additional sample was prepared as follows: a solution of $AgNO_3$ (16.5 mg, 0.097 mmol) in 0.2 mL of D_2O was added to a solution of complex **4** (50 mg, 0.088 mmol) in acetone-d$_6$ and stirred at room temperature in the dark for 10 min. The mixture was filtered and mixed with a freshly prepared solution of N-acetyl-L-cysteine (15.82 mg, 0.097 mmol) in 0.2 mL of acetone-d$_6$. Then ^{1}H, ^{13}C, 2D [^{1}H, ^{13}C] HSQC, and ^{195}Pt NMR spectra were acquired after 4 and 24 h.

UV−Visible Absorption Spectrophotometry. The absorption spectra of **1**−**6** in the UV−visible region were recorded on a Perkin-Elmer Lambda 20 Bio spectrophotometer. Solutions of **1**−**6** (10^{-4} M) MiliQ water were monitored, collecting the electronic spectra over 24 h at room temperature.

Cells, Culture, and Plating. The human solid tumor cell lines HBL-100, HeLa, SW1573, T-47D, and WiDr were used in this study. These cell lines were a kind gift from Prof. G. J. Peters (VU Medical Center, Amsterdam, The Netherlands). Cells were maintained in 25 cm^2 culture flasks in RPMI 1640 supplemented with 5% heat inactivated fetal calf serum and 2 mM L-glutamine in a 37 °C, 5% CO_2, 95% humidified air incubator. Exponentially growing cells were trypsinized and resuspended in an antibiotic-containing medium (100 units penicillin G and 0.1 mg of streptomycin per mL). Single cell suspensions displaying >97% viability by trypan blue dye exclusion were subsequently counted. After counting, dilutions were made to give the appropriate cell densities for inoculation onto 96 well microtiter plates. Cells were inoculated in a volume of 100 μL per well at densities of 10 000 (SW1573 and HBL-100), 15 000 (HeLa and T-47D), and 20 000 (WiDr) cells per well, on the basis of their doubling times.

Chemosensitivity Testing. Compounds were initially dissolved in DMSO at 400 times the desired final maximum test concentration. Control cells were exposed to an equivalent concentration of DMSO (0.25% v/v, negative control). Each agent was tested in triplicate at different dilutions in the range of 1−100 μM. The drug treatment was started on day 1 after plating. Drug incubation times were 48 h, after which time cells were precipitated with 25 μL ice-cold TCA (50% w/v) and fixed for 60 min at 4 °C. Then the SRB assay was performed.[21] The optical density (OD) of each well was measured at 492 nm, using BioTek's PowerWave XS Absorbance Microplate Reader. Values were corrected for background OD from wells only containing medium.

Cell Cycle Analysis. Cells were seeded in six well plates at a density of 2.5−5 × 10^5 cells/well. After 24 h, the products were added to the respective well and incubated for an additional period of 24 h. Cells were trypsinized, harvested, transferred to test tubes (12 × 75 mm), and centrifuged at 1500 rpm for 10 min. The supernatant was discarded, and the cell pellets were resuspended in 200 μL of cold PBS and fixed by the addition of 1 mL ice-cold 70% EtOH. Fixed cells were incubated overnight at −20 °C, after which time were centrifuged at 1500 rpm for 10 min. The cell pellets were resuspended in 500 μL of PBS, and 5 μL of DNase-free RNase solution (10 mg/mL) was added. The mixture was incubated at 37 °C for 30 min. Finally, 5 μL of PI (0.5 mg/mL) was added. Flow cytometric determination of DNA content (20 000 cells/sample) was analyzed on a LRSII Flow Cytometer (Becton Dickinson, San José, CA, U.S.A.). The fractions of the cells in G_0/G_1, S, and G_2/M phase were analyzed using FACS Diva 6.0 (BD Software San José, CA, U.S.A.) software.

Annexin V Binding. Cells were seeded in six well plates at a density of 0.25−0.5 × 10^6 cells. After 24 h, the test drug was added, and the cells were incubated for period of 24 h. Then, cells were trypsinized, harvested, transferred to test tubes (12 × 75 mm), and centrifuged at 400 g for 10 min. The supernatant was discarded, and the cells pellets were resuspended in 100 μL of ice-cold 1x Binding Buffer (0.1 m Hepes/NaOH (pH 7.4) 1.4 M NaCl, 25 mM $CaCl_2$).

ESI MS Analysis. ESI-MS spectra of modified amino acids samples were recorded on an QSTAR (Applied Biosystems) mass spectrometer operating in positive ion mode. Experimental parameters were set as follows: ion spray voltage, 5.5 kV; declustering potential, 50 V; focusing potential 210 V, ion source gas, 10 psi; acquisition window, m/z 50−2000; and flow rate, 10 μL/min with a solution of methanol.

The HPLC purified synthetic oligonucleotide 5′-d-(TAATTAAGCATAATAT)-3′ was purchased from Microsynth (Balgach, Switzerland). For ESI-MS analysis of the oligonucleotide, samples contained a platinum complex:oligonucleotide ratio of 3:1 (75:25 μM) in 10 mM ammonium acetate (pH 7.4) and were incubated for 24 h at 37 °C. ESI-MS spectra were recorded on an Ultima II q-TOF mass spectrometer (Waters, Manchester, U.K.) fitted with a standard Z-spray ion source operating in negative ion mode. Experimental parameters were set as follows: capillary voltage, 3 kV; sample cone, 50 V; source temperature, 80 °C; desolvation temperature, 200 °C; acquisition window, m/z 500−1500 in 1 s. A volume of 5 μL of sample (20 μM) was introduced into the mass spectrometer by infusion at a flow rate of 20 μL/min with a solution of ACN/H_2O 50:50 (v:v). External calibration was carried out with a solution of phosphoric acid at 0.01% in 50% acetonitrile. Data acquisition and analysis were carried out using the MassLynx 4.1 software bundle (Waters).

ESI-MS spectra of 9-EtG samples were recorded by direct introduction (5 μL/min) into an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA) equipped with a conventional ESI source. The working conditions were as follows: spray voltage 5 kV, capillary voltage 35 V, and capillary temperature 275 °C. Sheath gas was set at 8 au. For spectra acquisition, a nominal resolution (at m/z 400) of 100 000 and Xcalibur 2.0 software (Thermo) were used.

Horse heart cytochrome c was purchased from Sigma (Code C7752). Metal complexes/cyt c adducts were prepared in tetramethyl ammonium acetate buffer (TMAA) (pH 7.4) with a protein concentration of 10^{-4} M and platinum to protein ratio of 3:1. The reaction mixtures were incubated at 37 °C for different time intervals over 48 h. Samples were extensively ultrafiltered using Centricon YM-3 (Amicon Bioseparations, Millipore Corporation) in order to remove the unbound platinum complex. After a 20 fold dilution with HCOOH 0.1%, ESI-MS spectra were recorded by an Orbitrap high-resolution mass spectrometer as detailed above. The working conditions were as follows: spray voltage 3.1 kV, capillary voltage 45 V, and capillary

Table 1. Antiproliferative Activities (TGI) of 1−6 in Comparison to Cisplatin in Human Solid Tumor Cells[a]

compound	TGI (μM)				
	HBL-100	HeLa	SW1573	T-47D	WiDr
1	20 ± 5.6	58 ± 16	29 ± 12	46 ± 9.3	48 ± 12
2	41 ± 7.2	27 ± 8.2	61 ± 6.0	>100	>100
3	45 ± 2.1	33 ± 4.2	>100	>100	>100
4	3.3 ± 0.6	5.8 ± 2.1	4.3 ± 1.5	8.5 ± 2.1	8.0 ± 2.5
5	4.5 ± 0.1	2.5 ± 0.7	8.3 ± 0.9	54 ± 3.2	64 ± 7.3
6	48 ± 6.3	33 ± 7.2	>100	>100	>100
cisplatin	14 ± 2.1	15 ± 6.1	12 ± 2.6	>100	>100

[a]Values are given in mean ± standard deviation and are means of three to eight experiments.

temperature 220 °C. Sheath gas was set at 17 au, and auxiliary gas was kept at 1 au. For acquisition, Xcalibur 2.0 software (Thermo) was used, and monoisotopic and average deconvoluted masses were obtained by using integrated Xtract tool. For spectra acquisition, a nominal resolution (at m/z 400) of 100 000 was used.

NMR Spectroscopy. For characterization of the Pt adducts, ^{1}H, ^{13}C, 2D [^{1}H, ^{13}C] HSQC, and ^{195}Pt NMR spectra were recorded on either Bruker AMX-300 or DRX-500 spectrometers. ^{195}Pt NMR chemical shifts were externally referenced to Na_2PtCl_6 (0 ppm).

DNA Electrophoresis. Samples with pBR322 plasmid DNA were prepared in 50 mM phosphate buffer (pH 7.4). The concentration of plasmid in the reaction mixture was 0.5 μg/μL, and the concentration of the complexes was varied to give different metal-to-base pair stoichiometries (r = 0.1, 0.05 and 0.01). The mobility of the metal complex-treated pBR322 samples was analyzed by gel electrophoresis on a 0.8% (w/v) agarose gel (Boehringer-Mannheim, Mannheim, Germany) at 90 V/cm at 25 °C in tris-acetate/EDTA buffer. The gel was stained for 30 min in 0.5 g/mL (w/v) ethidium bromide, and the bands were analyzed with a UVP gel scanner.

RESULTS AND DISCUSSION

Synthesis and Characterization of Iodido−Platinum(II) Complexes. Complexes **1**−**6** (Chart 1) were prepared according to established synthetic procedures as reported in the Experimental Section, and the resulting products were extensively characterized by standard methods (Experimental Section).

Remarkably, all compounds display a good solubility in aqueous media in the presence of 1% DMSO. Freshly prepared solutions show relatively intense absorption bands around 300 nm that are interpreted in terms of iodide to platinum LMCT (ligand to metal charge transfer) transitions. The absorption bands manifest no appreciable changes over 24 h observation, implying that these compounds are relatively stable in aqueous solution. Representative spectral profiles for **3** are shown in Figure S1 of the Supporting Information.

Antiproliferative Properties and Flow Cytometry Studies. The cytotoxic activity of the six platinum complexes was evaluated against a panel of human tumor cell lines with diverse drug sensitivity profiles, including HBL-100 (breast), HeLa (cervix), SW1573 (nonsmall cell lung), T-47D (breast), and WiDr (colon), using the SRB assay.[21] The experimental TGI (Total Growth Inhibition)[22] values after 48 h treatment at 37 °C are summarized in Table 1 and compared to those of cisplatin (see Experimental Section for details). Notably, for all selected cell lines, the *cis* series **1**−**3** resulted to be moderately active in comparison to cisplatin, with **1** being in general the most effective out of the *cis* complexes. Conversely, the *trans* complexes **4**−**6** showed a pronounced variability in their cytotoxic effects, with **4** being the most effective of the series (TGI values in the low μM range in all cases) followed by **5** and **6**, respectively. Notably, **4** was about 3 fold more active than cisplatin in HBL-100, HeLa, and SW1573 cell lines, and even 10 fold more effective in the cisplatin resistant cancer cell lines T-47D and WiDr. These results suggest that the occurrence must be due to relevant differences in the respective mechanisms of action.

To gain a deeper insight into the effects that the two series of platinum(II) complexes produce on tumor cells and to highlight possible differences in their respective modes of action, cell cycle analysis by flow cytometry was performed on the five tested cancer cell lines after 24 h exposure to selected iodido−platinum compounds (Figure 1). The study was restricted to the most active compounds of the two series, i.e. **1** (*cis*) and **4** (*trans*), and results were compared to those of the standard drug cisplatin. Cells were exposed to each agent at two different drug concentrations, which were chosen based on two parameters:[23] the TGI values of the selected compounds and the sensitivity of the cell line to drug treatment, since at too high drug doses extensive cell death prevents examination of the cell cycle phase distribution. The obtained results show that the new compounds induce cell death (>20% and without any evident cell cycle arrest) in all cell lines, which mainly occurs at G_1 or S phase (Figure 1A). At variance, cisplatin produces a clear S-phase arrest and the amount of cell death is lower (10−20%), even at the highest applied dose. Moreover, the possibility of G_2/M intervention of the new drugs can be discarded based on inspection of the obtained data.

Combining annexin V-FITC, a fluorescent marker for the detection of phosphatidyl chlorine residues in cell membranes by flow cytometry, with propidium iodide (PI), it is possible to discriminate between viable cells, cells in early apoptosis, cells in late apoptosis, and necrotic cells. Exposure of the five cell lines to each compound for 24 h revealed that **4** induced apoptosis to a larger extent than cisplatin. Figure 1B reports representative results obtained for HeLa cells. In summary, the cell cycle analysis points out that when compared to cisplatin, the new Pt(II) complexes produced greater cell death without cell cycle arrest. The results suggest that the mechanism of action of these new compounds may differ profoundly from that of the reference drug cisplatin.

Reactivity with Biomolecules. To investigate the mode of action of these promising diiodido−Pt(II) complexes at a molecular level and to highlight preferential affinity for proteins or nucleic acids, we studied their reactivity with a variety of model biomolecules, including modified amino acids, cytochrome c, 9-ethylguanine, and a small oligonucleotide (Chart 2). The selected biomolecules are widely used as models in metallodrugs interaction studies.[24−28]

Reactivity with N-AcMet and N-AcCys. Initially, the interaction of the *cis* complexes **2** and **3** with N-AcMet was

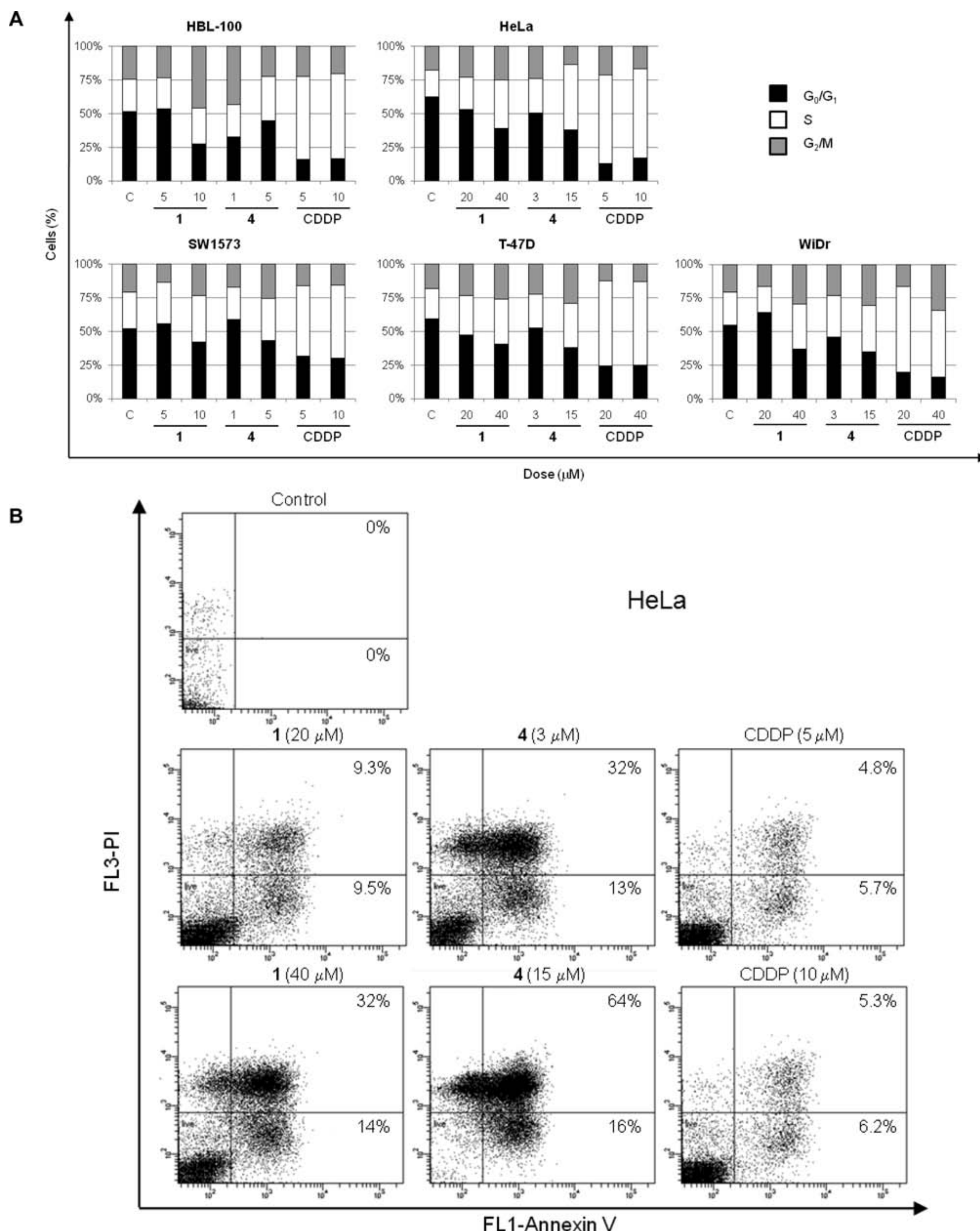
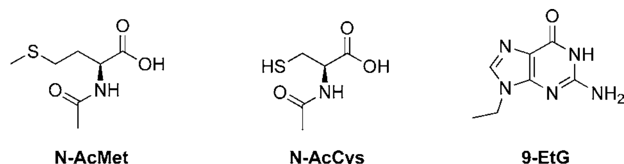


Figure 1. (A) Cell cycle phase distribution of untreated cells (C) and cells treated with compounds **1**, **4**, and cisplatin (CDDP) for 24 h at two drug doses. (B) Annexin V and PI staining of untreated HeLa cells (Control) and HeLa cells treated for 24 h with compounds **1**, **4**, and cisplatin (CDDP) at two drug doses. Viable cells are Annexin- and PI-. Early apoptotic cells are Annexin+ and PI-. Late apoptotic cells are Annexin+ and PI+. Quantification of apoptosis showed the percentage of cells that were apoptotic.

analyzed by MS following established procedures.[11] Figure S2A of the Supporting Information shows the representative spectrum obtained in the case of **2** in which the formation of a number of metallodrug-amino acid adducts was observed containing Pt^{2+} or $[PtI]^+$ and $[PtI_2(\text{dimethylamine})]$ fragments

Chart 2. Structures of the Modified Amino Acids and DNA Model Compound Used in This Study



(Table 2). This phenomenon was previously observed for the first compound of the *cis* series: compound **1**.[11]

Table 2. Main Peaks Present in the ESI Mass Spectra of 2 Treated with N-AcMet. Recorded after 24 h. Incubation at 37 °C

m/z experimental	*m/z* theoretical	N-AcMet complex adduct
703.98	703.99	(N-AcMet)2-PtI
830.89	830.90	(N-AcMet)2-PtI2
875.95	875.95	(N-AcMet)2-Pt(dma)I2

The reaction of the *trans* complex **4** with N-AcMet was also studied by MS and ^{1}H NMR. However, no adduct formation occurred over 4 days, and the starting materials could be recovered practically intact from the solution. When a similar study was repeated allowing N-AcCys to react with **4**, the mass spectrum obtained after 4 h incubation at 37 °C (Figure S2 of the Supporting Information, spectrum B) showed a main peak at 602.04 *m/z*, which is assigned to a *trans*-[PtI(ipa)$_2$(N-AcCys)] adduct. Remarkably, the resulting metallodrug-amino acid adduct contains the [PtI(ipa)$_2$]$^{2+}$ fragment implying that loss of one iodide ligand has occurred. However, this reactivity of the compound is very low. In fact, it is only possible to detect adduct formation by ^{195}Pt NMR after overnight incubation of the sample, as shown in Figure S3A of the Supporting Information, where the starting material **4** produces the signal at −3333.66 ppm and a new signal arises at −3238.12 ppm upon adduct formation. To prove the nature of this adduct, the reaction of **4** with N-AcCys was forced using $AgNO_3$ to afford the metathesis of the iodide ligands and to achieve immediate formation of the aqua complex facilitating the reaction with N-AcCys. As a result of these conditions, the major adduct in the ^{195}Pt NMR spectrum (Figure S3B of the Supporting Information) now corresponds to the expected signal at −3235.06 ppm. Moreover, mass spectrometry analysis of the latter sample showed the presence of the species at 602.04 *m/z* corresponding to the *trans*-[PtI(ipa)$_2$(N-AcCys)] adduct (Figure S4 of the Supporting Information).

Reactivity with Cytochrome c. Subsequently, both *cis*- and *trans*-iodido platinum(II) complexes were reacted with the model protein cytochrome c (cyt c), and their adducts were studied by electrospray ionization (ESI) MS as it has been previously reported for compound **1**.[11] Thus, **2** and **3** from the *cis* series were reacted with cyt c under physiological-like conditions (25 mM TMAA buffer pH 7.4 at 37 °C) at a metal:protein molar ratio of 3:1. As shown in Figure 2, for the *cis* complex **2**, extensive binding to cyt c is observed after 24 h incubation. Deconvolution of the spectra shows intense peaks corresponding to platinated species. The resulting metal−protein species contain Pt^{2+}, $[PtI]^+$, PtI_2, $2[PtI]^+$, $2PtI_2$, and $3[PtI]^+$ fragments, while complete loss of the amine ligands has occurred. The results are in excellent accordance with the reactivity reported for **1**.[11] Interestingly, the presence of cyt c

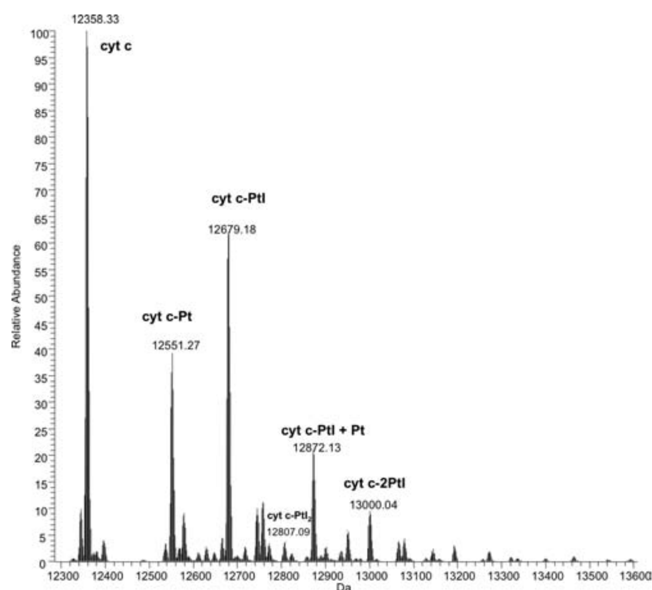


Figure 2. Deconvoluted ESI mass spectrum of cyt c treated with *cis*-[PtI$_2$(dma)$_2$] (**2**) and incubated for 24 h in buffer TMAA buffer (pH 7.4).

adducts with Pt(amine) species is not observed as previously described for cisplatin and other *cis* or *trans* analogues with chloride ligands.[24,25,29] Poly platinum cyt c adducts were also detected (a mixture of PtI and/or PtI$_2$ fragments), indicating the presence of several available Pt binding sites. In contrast, the reactivity of the corresponding *trans*-iodido platinum(II) complexes with cyt c resulted to be less pronounced than that for *cis* analogues. For example, although caution has to be used in the quantification of formed adducts, only compound **6** (*trans*-[PtI$_2$(ma)$_2$]) from the *trans* series showed appreciable adduct formation after 24 h incubation (Figure 3). A possible

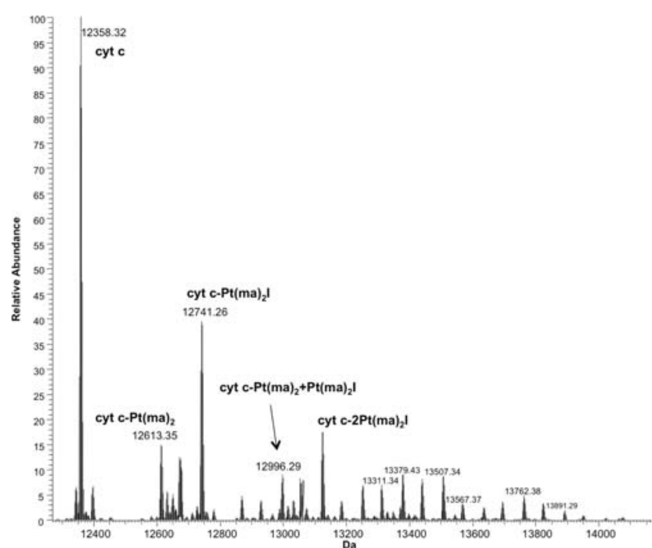


Figure 3. Deconvoluted ESI mass spectrum of cyt c treated with *trans*-[PtI$_2$(ma)$_2$] (**6**) and incubated for 24 h in buffer TMAA buffer (pH 7.4).

explanation for the observed effects might be the steric hindrance of bulkier carrier ligands in **4** and **5**. The smaller size of the methylamine will justify the formation of the observed platinum-cyt c adducts containing fragments with the

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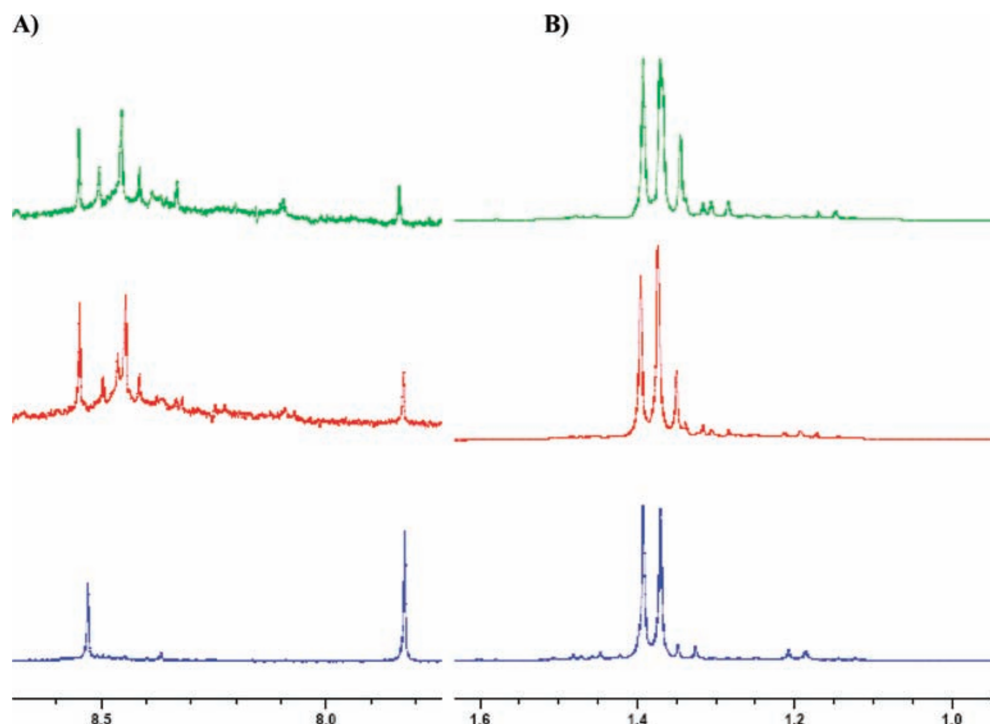

Figure 4. Progress of the reaction of 9-EtG treated with **1** in acetone:D_2O at 37 °C (metal:9-EtG ratio = 4:1), followed by 1H NMR. Details of the spectra in A (aromatic area) and B (aliphatic area) before and after addition at 2, 6, 24, and 48 h (from the ppm scale to the top).

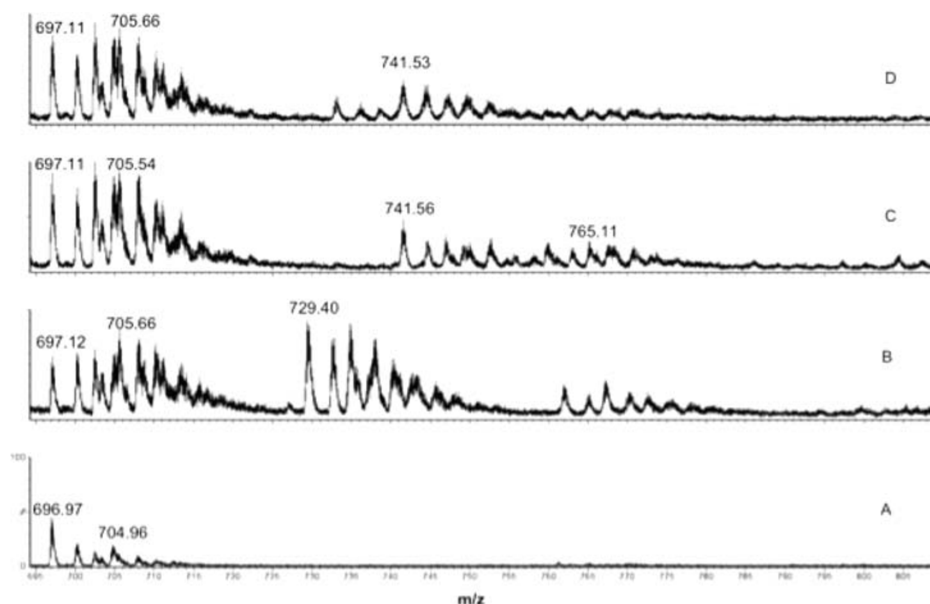

Figure 5. Negative ion ESI-MS spectra of the oligonucleotide SS focusing on the 7^- charge state (A) and SS incubated with cisplatin (B), **1** (C), and **4** (D) (metal complex:SS ratio = 1:1) for 24 h at 37 °C.

presence of both amines ligands, with the main adducts in the spectrum corresponding to cyt c-$[Pt(ma)_2]^{2+}$ or cyt c-$[PtI(ma)_2]^+$ species at about 12613 and 12741 Da, respectively.

Reactivity with 9-EtG. Afterward, the reactivity of one compound representative of each series, *cis* (**1**) and *trans* (**4**), with 9-EtG, used here to model the reactions with DNA purine nucleobases,[30] was monitored by NMR spectroscopy as described in the Experimental Section.

The ^1H NMR spectra of **1** with 9-EtG recorded at different times over 72 h incubation at 37 °C are shown in Figure 4. When following the changes in the H8 peak of 9-EtG, after 24 h, a low-intensity signal arises at about 8.50 ppm corresponding to the H8 resonance of a new adduct (Figure 4A). A pronounced speciation is clearly detected after 72 h in the same aromatic area. Extensive adducts formation is also detected in the aliphatic area (Figure 4B), though the signals of the metal complex overlap with those of complex **1** making the complete assignment of each adduct very difficult. Most importantly, from the obtained spectra, no isopropylamine free ligand is detected at 0.9 ppm (methyl group from free ipa).

When similar experiments were repeated with the *trans* isomer **4**, some differences in the reactivity were highlighted

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compared to **1** (Figure S5 of the Supporting Information). In fact, the reaction of **4** with 9-EtG is faster, as adduct formation can be observed by ^{1}H NMR at 8.60 ppm after 10 min. This trend of reactivity is consistent with previous observations for the aquation of *trans*-platinum complexes with aliphatic amines, where the faster rate of hydrolysis of the *trans* isomer has been attributed to the different *trans*-labilizing abilities of the chlorides.[31−33] Remarkably, as described for **1**, when forcing the release of the iodide ligands using $AgNO_3$ and about two equivalents of 9-EtG (Experimental Section), we could isolate the bis-adduct *trans*-$[Pt(ipa)_2(9\text{-EtG})_2](SbF_6)_2$ (Figure S6 of the Supporting Information).

Reactivity with Single-Stranded Oligonucleotide. The reactivity of the complexes toward the single-stranded oligonucleotide 5′-d(TAATTAAGCATAATAT)-3′ (SS) was also studied by MS and compared with cisplatin. In a typical experiment, each compound was added to an aqueous solution of this oligonucleotide (at 1:1 molar ratio) and allowed to incubate for 24 h at 37 °C. Afterward, samples were analyzed by ESI-MS in negative mode according to established procedures.[11] The full scan mass spectra of SS incubated with **1**, **4**, and cisplatin showed the formation of comparable adducts corresponding to $[SS+Pt(amine)_2]$ moieties (Figure 5). For example, the SS-**1** monoadduct corresponding to $[SS+Pt(ipa)_2]^{7-}$ species was detected at about 741 m/z (Figure 5C), while the $[SS+Pt(NH_3)_2]^{7-}$ adduct at about 729 m/z was observed for cisplatin (Figure 5B). In general, the reactivity of the *trans* compounds was lower (Figure 5D) than that of cisplatin, the latter being able to platinate SS very efficiently after 24 h. The results are in good accordance with previously reported data on similar Pt(II) compounds with chloride ligands.[11]

Afterward, the reactivity of both *cis* and *trans* complexes with pBR322 plasmid DNA was analyzed by gel-electrophoresis according to established procedures, using cisplatin as a reference.[11] None of the complexes of the series from **2** to **6** altered the DNA electrophoretic mobility, as was previously observed for complex **1**.[11] Representative results for complex **4** are reported in Figure S7 of the Supporting Information.

■ CONCLUSIONS

Following recent encouraging results,[11] we have expanded here the exploration of "*rule-breaker*" *cis*-diiodido diamine platinum(II) complexes and extended the study to include their *trans*-diiodido diamine platinum(II) analogues. Six platinum(II) complexes featuring two iodide ligands, either in *cis* or *trans* configuration, were prepared, characterized, and investigated. The antiproliferative effects of these compounds were evaluated in a panel of representative human cancer cell lines. All compounds manifested relevant antiproliferative properties in vitro, with *trans* complexes being, in general, more active than their *cis* counterparts. In particular, the two *trans* complexes **4** and **5** showed a far greater cytotoxic potency than the corresponding *cis* complexes **1** and **2** and the standard anticancer agent cisplatin. Notably, *trans* complex **6** turned out to be far less effective, showing a cytotoxic potency similar to its *cis* analogue **3**. Flow cytometry studies allowed us to shed some light on the mode of action of these novel compounds in comparison to cisplatin. In particular, our flow cytometry results suggest the occurrence of a largely different mechanism for these iodido−platinum complexes compared to cisplatin, which is known to arrest cells at the G_1/S transition and early S phase of the cell cycle. Further mechanistic insight was obtained from Annexin V binding studies; remarkably, evident dose−dependent apoptosis was observed in cancer cell lines treated with the new compounds. In agreement with the results of the antiproliferative tests, the *trans* complex **4** exhibited a better performance than the *cis* complex **1**. Surprisingly, for cisplatin-treated cells, the amount of apoptotic cells was found to be quite low. In this particular context, it has been proposed that cisplatin-induced cell death does not always arise from "classic" apoptosis.[34]

It is reasonable to ascribe differences in the biological profiles of these novel iodido−platinum complexes to specific differences in their reactivity with biomolecules that might modify their ultimate targets. Thus, the reactivity of these Pt(II) compounds with model biomolecules was extensively explored at a molecular level, mainly combining MS and NMR methods. In a previous work, the unexpected reactivity of *cis*-$[PtI_2(\text{isopropylamine})_2]$ (**1**) against cyt c, as well as against N-AcMet, suggests a methionine residue (Met-65) as the likely Pt binding site on cyt c. Here, we have extended our investigation to the newly synthesized *cis/trans*-$[PtI_2(\text{amine})_2]$ complexes and screened their reactivity with sulfur donors of thioether or thiol type, such as N-AcMet and N-AcCys, respectively, and with cyt c. Interestingly, these studies revealed a very similar reactivity profile for the three *cis*-type platinum complexes featuring the release of the amine ligand with partial or complete retention of the iodide ligands upon binding to model amino acids or peptides. In contrast, *trans*-type complexes manifested a lower propensity to form adducts with amino acids or with cyt c, and a more classical reactivity consisting of the preferential release of the iodide ligands upon protein binding was revealed. Moreover, it is remarkable that the *trans*-iodido complexes are poorly reactive in the presence of thioether-type sulfur donors (N-AcMet and Met-65 in cyt c), while they react better with thiols (N-AcCys). The reactivity of these two classes of Pt(II) complexes with nucleic acids was also investigated using 9-EtG and the single strand oligonucleotide SS as models. The obtained results revealed, for both *trans*- and *cis*-type complexes, that adduct formation occurs with retention of the amine spectator ligands upon nucleobase binding. Yet, *trans* complexes resulted to be more reactive than the *cis* analogues. It must be noted that the reactivity of the reported platinum(II) complexes, both *cis* and *trans*-configured, is lower compared to cisplatin, and a higher compound concentration is accordingly required to detect reactions with DNA bases and to isolate possible adducts. Such less pronounced reactivity might also explain why these complexes leave electrophoretic mobility of plasmid DNA essentially unaltered at concentrations that typically induce remarkable mobility shifts in the case of cisplatin. These results are also consistent with the observed effects on the cell cycle.

In terms of possible molecular mechanisms of action for diiodido−diamine Pt(II) complexes the reported results indicate the following:

- The *trans* complexes are markedly less reactive than their *cis* isomers with model amino acids and peptides, especially when Met donors are present. In addition, the *cis* compounds manifest a peculiar reactivity, which involves the release of the amine ligands and retention of the iodides upon biomolecule binding.
- In the presence of DNA model compounds, the reactivity of the *trans* complexes is more pronounced than that of

the *cis* analogues, and in both cases it follows pathways typical of classical Pt(II) compounds.

- Both *cis* and *trans* complexes are in general less reactive with biomolecules (either peptides or nucleic acids) than cisplatin.

These findings might partially explain the observed antiproliferative effects of the diiodido−diamine Pt(II) complexes as compared to cisplatin. Interestingly, the highly cytotoxic *trans*-Pt(II) complexes **4** and **5** are less reactive with model peptides but more efficient in binding to nucleic acids with respect to the *cis* analogues. In addition, the most reactive compound with cyt c in the *trans* series (**6**) is also the least cytotoxic. If DNA is the pharmacological target for the *trans* compounds, certainly their "unease" of binding to peptides might favor their uptake in the cell nuclei and subsequent efficient DNA damage. This could also explain their potency of inhibition of cell growth even in cisplatin resistant cell lines, being more selective than cisplatin in binding to biomolecules (i.e., preferring nucleic acids to proteins).

Certainly, more challenging is the interpretation of the mechanisms of action of the *cis* diiodido−diamine Pt(II) compounds, presenting not only a marked preference for amino acids/peptides binding over DNA but also a peculiar reactivity involving the release of the amine ligands. Other studies are in progress to identify potential protein targets for this promising class of cytotoxic agents (e.g., zinc finger proteins).

In conclusion, the herewith reported results highlight the occurrence of different interaction modes for diiodido−diammine platinum(II) complexes toward representative biomolecules that may ultimately explain the observed conspicuous differences in their respective biological actions. Beyond the geometrical configuration, even the nature of the amine ligand seems to play a crucial role in determining the overall biological profile.

■ ASSOCIATED CONTENT

Supporting Information

UV spectra over 24 h of complex **3** (10^{-4} M) in Milli-Q water, mass spectra of N-AcMEt and N-AcCys with **2** and **4**, respectively, ^{195}Pt NMR spectra of **4** and N-AcCys, ^{1}H NMR progress spectra from 9-EtG treated with **4** and **1**, and gel electrophoresis of pBR322 plasmid DNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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