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Intrinsically Fluorescent Cytotoxic Cisplatin Analogues as DNA Marker Molecules

Julio J. Criado,*,† Emilio R. Fernández,† Juan L. Manzano,† Angel Alonso,‖ Susana Barrena,‡ Manuel Medarde,§ Rafael Pelaez,§ M. Dolores Tabernero,⊥ and Alberto Orfao‡

Departamentos de Química Inorgánica and Química Farmacéutica, Facultad de Farmacia, and Centro de Investigación del Cáncer (CIC), Campus "Miguel de Unamuno", Universidad de Salamanca, 37007-Salamanca (Spain), Departamento de Química Analítica, Facultad de C. Químicas, Plz. de la Merced s/n, Universidad de Salamanca, 37008-Salamanca, (Spain), and Unidad de Investigación, Hospital Universitario de Salamanca, 37007-Salamanca (Spain). Received September 3, 2004;
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Two square planar derivatives of $Pt(en)Cl_2$ with intrinsic fluorescence in aqueous solution at room temperature, with quantum yields (Φ) 0.11 and 0.10, respectively, have been synthesized and characterized as [Pt(en)(CG)Cl] (Complex **1**) and [$Pt(en)(CG)_2$] (Complex **2**) (en = ethylenediamine, CG = cholylglycinate). Complexes **1** and **2** exchange just one ligand (chloride or cholylglycinate, respectively) when reacted with water or 5′-GMP to give the same chemical species. After reaction with DNA oligonucleotides or DNA plasmids, they show enhanced emission in the visible region, which lasts for long periods of time and makes them potentially useful DNA marker molecules. Incubation with nucleated blood cells followed by microscopic analyses revealed that they enter the cells within minutes of exposure, selectively stain the DNA, and persist after more than 48 h of exposure. Complexes **1** and **2** display cell cycle phase-independent cytotoxic activity against cisplatin-resistant CHO (Chinese hamster ovarian) tumor cells, with an early onset of their effects. Their slightly different biological effects, as compared to cisplatin, are considered to be linked to the bile acids and their vector properties and to the preferential formation of monoadducts.

INTRODUCTION

Cisplatin has become one of the most widely used anticancer agents, being especially effective in the treatment of patients with testicular and ovarian cancers as well as of hematologic malignancies, such as Hodgkins disease and childhood leukemias (*1*). Its severe toxicity and the development of resistance to the drug has led to the synthesis and evaluation of thousands of cisplatin analogues (*2*). In such complexes, leaving groups appear to influence solubility, toxicity, and both the tissue and intracellular distribution of the drug, while stable (carrier) am(m)ine ligands determine the structure of DNA adducts and cellular responses (*1*). The only analogues approved worldwide for patient treatment, the dicarboxylates carboplatin and oxaliplatin, appear to follow these rules (*2*, *3*).

Many and diverse biochemical and biophysical methods have been applied to elucidate critical aspects of the mechanisms of action of cytotoxic platinum complexes, in particular those of cisplatin (*4*). Platinum(II) complexes are considered to be nonfluorescent in solution at room temperature (except those carrying additional fluorophores), and, to the best of our knowledge, no intrinsically fluorescent cisplatin analogue with cytotoxic activity has been described so far. Recently reported approaches using platinum complexes with appended fluorophores have been only partially successful since, once inside the cell, fluorescence was not long-lasting (*5*). To achieve dynamic pictures of the biological effects of cisplatin analogues, it would be very interesting to have intrinsically fluorescent compounds with cytotoxic activity, especially against drug-resistant cell lines (*6*, *7*).

In recent years, we and others have explored the potential usefulness of bile acids as exchangeable platinum(II) ligands (*8*), which might reduce both toxicity and resistance owing to their lower lability (carboxylate vs chloride ligands), their amphipathic character, and their vectoriality (drug targeting) (*9*−*11*). In the present paper we describe for the first time two new intrinsically fluorescent square-planar platinum(II) complexes carrying one or two cholylglycinate ligands (**1** and **2**, Figure 1) that emit at room-temperature both once free in solution and after reaction with DNA. They were synthesized from $Pt(en)Cl_2$ using previously described protocols, and their reactions with different potential cellular ligands, such as water, GMP and DNAs of different sizes and sources, together with their fluorescence and cytotoxic properties have been studied. The implications on the cellular and biological effects of these compounds are also discussed.

EXPERIMENTAL SECTION

Chemicals. Dichloro(ethylenediamine)platinum(II), $Pt(en)Cl_2$, was purchased from Aldrich (St Louis, MO). Sodium cholylglycinate (NaCG), guanosine 5′-monophos-

* To whom correspondence should be addressed. Fax: +34-923 29 45 15. E-mail: jjct@usal.es.
† Departamento de Química Inorgánica, Universidad de Salamanca.
§ Departamento de Química Farmacéutica, Universidad de Salamanca.
‡ Centro de Investigación del Cancer (CIC), Universidad de Salamanca.
‖ Departamento de Química Analítica, Universidad de Salamanca.
⊥ Hospital Universitario de Salamanca.

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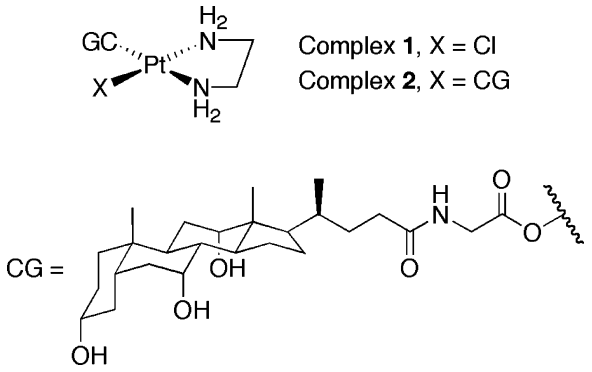

Figure 1. Structures of the platinum complexes **1** and **2**.

phate (sodium salt; 5′-GMP), penicillin/streptomycin, sucrose, spermine tetrahydrochloride, trypsin, trypsin inhibitor, RNAse, Nonidet Np40, and propidium iodide were obtained from Sigma (St Louis, MO). HBSS (Hank's balanced salt solution), DMEM cell culture medium, L-glutamine, fetal calf serum, and sodium citrate were all purchased from Gibco BRL (Carlsbad, CA). Trisodium citrate and dimethyl sulfoxide were obtained from Merck (Darmstadt, Germany). All other reagents were of high purity and were used as purchased without any further purification. Oligonucleotides **I** (self-complementary) 5′-3′d(TATGATCATA), **II** (self-complementary) 5′-3′d(TGATCATGATCA), and **III** (single strand, non self-complementary) 5′-3′d(TTAGGGTTA) were purchased from Sigma Genosys (Cambridge, UK), with purification in reverse phase. Purified *Trichoderma harzianum* HH3-DNA (linear, 563 bp) was kindly provided by the Department of Microbiology (University of Salamanca, Salamanca, Spain), and the CH1cisR ovarian cancer cell line was kindly provided by Dr. J. M. Pérez (Madrid, Spain).

Analytical Methods. Chemical analyses for C, H, and N were performed on a Perkin-Elmer 2400 elemental analyzer (Applied Biosystems, Foster City, CA). Platinum was determined by atomic absorption on a Hitachi Z-8100 flameless graphite furnace spectrophotometer (Hitachi, Tokio, Japan) set at a wavelength of 265.9 nm, using the following temperature program: 90 °C (20 s), 100 °C (20 s), 800 °C (20 s), 1600 °C (30 s), 2800 °C (5 s), and 3000 °C (4 s). Infrared (IR) spectra were recorded in the 4000−200 cm^{-1} range on a Perkin-Elmer FT-IR 1730 instrument coupled to a Perkin-Elmer Data Station (Applied Biosystems). KBr pellets and spectrophotometric grade Nujol (Fluka, St. Louis, MO) or polyethylene disks (Aldrich) were used to record the spectra above and below 400 cm^{-1}, respectively. Electrospray ionization mass spectra (ESI-MS) were recorded on a Waters ZQ 4000 (Waters S.A., Milford, MA); positive and negative ion mass spectra were obtained by signal averaging of 25 consecutive shots; MALDI-TOF spectra were recorded on a Bruker, Reflex III (delayed extraction) instrument (Bruker, Karlsruhe, Germany) equipped with a nitrogen laser (λ = 337 nm, 3 ns pulse width). Electrical conductivity in solution was measured using a CDM2e conductimetry radiometer with a CDC-104 immersion cell (Radiometer, Copenhagen, Denmark). Temperature was controlled in a Unitherm water bath (Selecta, Barcelona, Spain) with a precision of ±0.01 °C. ^{1}H (400 MHz), ^{13}C (102.6 MHz), and ^{195}Pt (64.5 MHz) NMR spectra were obtained in D_2O, methanol-d_4 or DMSO-d_6 solutions on a Bruker DX400 instrument (Bruker, Germany). Carbon resonances were distinguished by DEPT-90 and DEPT-135 experiments. TMS was used as internal standard for ^{1}H and ^{13}C spectra. Using K_2PtCl_6 (δ = 0 ppm) as external standard, 300.000 scans, 2 s relaxation delay, and a 48 h acquisition time were used to obtain the ^{195}Pt spectra. Fluorescence measurements were performed in water at room temperature in a Shimadzu RF 5000 spectrofluorometer system (Shimadzu, Tokyo, Japan) equipped with a 150 W xenon lamp and a sensitivity unit. The optimal excitation wavelength was obtained from a fluorescence excitation spectra acquired at the optimal emission wavelength. Excitation and emission peaks were as follows: (1) free [Pt(en)(CG)Cl] and free [Pt(en)$(CG)_2$] in solution (301 nm, 384 nm) and (330 nm, 436 nm), respectively, (2) platinum complexes bound to oligonucleotides **I**, **II**, and **III** (352 nm, 437 nm), and (3) platinum complexes bound to HH3-DNA (350 nm, 437 nm).

Synthesis and Characterization of the Platinum Compounds. *Ethylenediaminechlorocholylglycinateplatinum(II), Pt(en)(CG)Cl*. Complex **1** was obtained by slowly adding a 1 mM NaCG solution to a 1 mM Pt(en)Cl_2 solution at 50 °C, with subsequent stirring for 24 h in the darkness (final pH = 5.6−5.7) (*10*, *12*). Liquid−liquid extraction on C18 cartridges yielded (after final methanol extraction) the newly formed platinum complexes (60% from the starting NaCG) free of excess unreacted Pt(en)Cl_2. Preparative TLC chromatography using butyl acetate/methanol 65/35 (v/v) gave complex **1** (R_f = 0.30, final yield 29%) as a yellow solid after evaporation to dryness in P_4O_{10}.

*Complex **1**, Pt(en)(CG)Cl*. Yellowish solid. Soluble in H_2O, MeOH, DMSO. Mp (with dec) = 173 ± 2 °C. EA Calcd. for $C_{28}H_{50}ClN_3O_6Pt$: % C 44.53; % H 6.67; % N 5.56; % Pt 25.83. Found: % C 45.16; % H 6.81; % N 5.52; % Pt 26.11. IR (ν, cm^{-1}): 3392, 1601, 1409, 542, 349. ESI-MS (*m/z*): 754.7 $[M]^+$, 741.4 $[M - Cl - H + Na]^+$, 718.6 $[M - Cl - H]^+$, 510.4 $[CG + 2Na]^+$, 488.4 $[CG + H + Na]^+$. ^{1}H NMR (CD_3OD) δ = 0.71 (3H, s; Me-18); 0.90 (3H, s; Me-19); 1.03 (3H, d, J = 6.5 Hz; Me-21); 2.53 (4H, bs; en); 3.37 (1H, m; H-3); 3.80 (1H, bs; H-7); 3.96 (1H, bs; H-12); 4.02 (2H, s; H-25). ^{13}C NMR (CD_3OD) δ = 188.6 (C- 26); 179.6 (C-24); 72.7 (C-12); 71.4 (C-3); 67.8 (C-7); 53.5 (C-25); 48.2 (en); 46.5 (C-17); 46.0 (C-13); 45.4 (en); 41.6 (C-5); 41.6 (C-14); 39.4 (C-8); 38.8 (C-4); 35.8 (C-20); 35.0 (C-1); 34.4 (C-6); 34.3 (C-10); 31.9 (C-22); 30.5 (C-23); 29.4 (C-2); 29.2 (C-11); 27.9 (C-16); 26.3 (C-9); 22.7 (C-15); 21.6 (C-19); 16.5 (C-21); 11.5 (C-18). ^{195}Pt NMR (D_2O) δ = −1970 ppm.

Ethylenediaminebischolylglycinateplatinum(II), Pt(en)$(CG)_2$. Complex **2** was obtained using two different approaches: The first was identical to that described above, except that 2 equiv of NaCG was used, giving complex **2** (R_f = 0.24, 12% final yield). The second method was used in order to remove traces of complex **1**: a 1 mM solution of Pt(en)Cl_2 was reacted with an excess of $AgNO_3$ for 24 h; the precipitate formed was filtered out, and 2 equiv of NaCG was slowly added as a 1 mM solution. The subsequent procedures were identical to those described above for complex **1**. At the end, complex **2** gave a single TLC band (R_f = 0.24, 35% final yield).

*Complex **2**, Pt(en)$(CG)_2$*. Yellow solid. Soluble in H_2O, MeOH, DMSO. Mp (with dec) = 184 ± 2 °C. EA Calcd. for $C_{54}H_{92}N_4O_{12}Pt$: % C 54.76; % H 7.83; % N 4.73; % Pt 16.47. Found: % C 54.93; % H 7.78; % N 4.52; % Pt 17.01. IR (ν, cm^{-1}): 3429, 1617, 1384, 536. MALDI-TOF-MS (*m/z*): 1228.8 $[M + 2Na - 1]^+$, 1207.9 $[M + Na + 1]^+$, 1184.6 $[M + 1]^+$, 997.8 $[(NaCG)_2 + Na]^+$, 719.5 $[M - CG]^+$, 510.4 $[CG + 2Na]^+$, 488.4 $[CG + Na]^+$. ^{1}H NMR (CD_3OD) δ = 0.70 (3H, s; Me-18); 0.90 (3H, s; Me-19); 1.02 (3H, d, J = 6.5 Hz; Me-21); 2.48 (2H, bs; en); 3.35 (1H, m; H-3); 3.73 (2H, bs; H-25); 3.78 (1H, bs; H-7); 3.95 (1H, bs; H-12). ^{13}C NMR (CD_3OD) δ = 176.5 (C-26); 176.3 (C-24); 74.0 (C-12); 72.9 (C-3); 69.0 (C-7); 48.1 (C-17); 47.5

(en); 47.5 (C-13); 44.6 (C-25); 43.2 (C-14); 42.9 (C-5); 41.0 (C-8); 40.4 (C-4); 37.0 (C-20); 36.5 (C-1); 35.9 (C-6); 35.8 (C-10); 34.3 (C-22); 33.3 (C-23); 31.3 (C-2); 29.5 (C-11); 28.7 (C-16); 27.8 (C-9); 24.3 (C-15); 23.2 (C-19); 17.7 (C-21); 13.0 (C-18). ^{195}Pt NMR (D_2O) $\delta = -1420$ ppm.

NMR Reaction of Complexes 1 and 2 with 5′-GMP. One milligram of complex **1** (or complex **2**) was added as a solid to 0.5 mg (or 0.3 mg) of 5′-GMP sodium salt dissolved in 400 μL of 10 mM phosphate buffer in D_2O (pH = 7.2). Complex **2** immediately gave a clear solution, but complex **1** only dissolved after 24 h at 50 °C. ^{1}H NMR spectra at different time-points were recorded at 25 °C using a presaturation pulse to remove the residual solvent peak (spectral width = 8000 Hz). ROESY and DQF−COSY spectra of the final 1:1 complexes were also acquired at 25 °C.

Microscopic Analyses. Peripheral blood (PB) samples were collected from three healthy volunteers. Three 100 μL aliquots from each PB sample were incubated with 2 mL of an hypotonic solution (Quicklysis, Cytognos, Salamanca, Spain) for 10 min at room temperature to lyse nonnucleated red cells. After this incubation period, nucleated cells were centrifuged (5 min at 540*g*) and washed once in a phosphate-buffered saline (PBS) solution (pH = 7.6). Then, the cell pellet was resuspended in 50 μL of PBS and placed on a slide to which 50 μL of a solution of either complex **1** (5 μM) or complex **2** (5 μM) in water or just 50 μL of water was added. Fluorescence measurements were performed using an Olympus BX40 fluorescence microscope (Olympus, Hamburg, Germany) equipped with a mercury arc lamp using a 350 ± 25 nm band-pass excitation filter and a 400 ± 10 nm long pass emission filter. Fluorescence emission was captured through a CCD camera and printed in a 512 × 512 pixel picture.

Cytotoxicity Assays. The cytotoxic activity of the two newly synthesized platinum compounds **1** and **2** was evaluated in comparison with that of the most widely used platinum compound, cisplatin. To this end, CH1cisR ovarian cancer cells were cultured in parallel in the presence of different concentrations (0, 1, 2.5, 5, and 10 μM) of complex **1**, complex **2**, or cisplatin in DMEM medium supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin. For the analysis of the cytotoxic activity, cells were harvested either prior to adding the platinum compounds or at 24 and 48 h after they were placed in culture. To harvest the cells they were incubated (5 min at 37 °C) with a solution containing 0.25% (w/v) of trypsin in Hank's balanced salt solution.

Evaluation of the cytotoxic activity was performed using conventional flow cytometry (FACScalibur, Becton/Dickinson Biosciences, San Jose, CA) techniques that allow the simultaneous measurement of cell death and cell proliferation. Briefly, harvested cells were centrifuged (5 min at 540*g*) and resuspended at a concentration of 10^4 cells/μL of 70% ethanol. After an incubation period of at least 30 min at 4 °C, cells were washed once in sodium citrate buffer−sucrose 250 mM, trisodium citrate 40 mM, 5% (v/v) dimethyl sulfoxide, 0.1% (v/v) Nonidet P40, 0.5 mM trishydroxymethylaminomethane, and 1.5 mM spermine tetrahydrochloride and resuspended in the same citrate buffer at a concentration of 10^4 cells/μL. To 10^6 cells in 100 μL, 1.5 mL of a solution containing 0.5 g/L of RNAse and 0.1 g/L of trypsin inhibitor in citrate buffer was added; after gently mixing, cells were incubated for another 10 min at room temperature. Once this incubation period had ended, 1.5 mL of a solution containing 0.42 g/L of propidium iodide in sodium citrate buffer was added, and another incubation for 15 min (room temperature) was performed in the darkness. Measurements of cell DNA contents were performed for at least 10^4 cells for each experimental condition.

Statistical Analysis. The equations for kinetic studies were adjusted to the experimental data by means of a nonlinear regression program available in the SIMFIT in the statistics package (Bardsley 1992 SIMFIT package 3.2. Department of Obstetrics and Gynecology, Manchester, UK).

For the calculation of the ratio of dead/apoptotic cells and the distribution of surviving cells in the different cell cycle phases, the ModFIT software program was used (Verity Software, Topsham, MA). The Mann−Whitney *U* test was used to estimate the statistical significance of the differences observed between groups. *P* values <0.05 were considered to be associated with statistical significance.

RESULTS

Structure of the Platinum Complexes 1 and 2. Both platinum complexes were characterized by a combination of spectroscopic techniques, since no crystals suitable for X-ray diffraction studies were available. In the MS, the peaks corresponding to platinated species were easily recognized due to six different isotopic masses for the metal (190, 192, 194, 195, 196, and 198). The observed molecular ions (754.7 for complex **1** and 1184.6 for complex **2**) are consistent with the molecular formulas $C_{28}H_{50}ClN_3O_6Pt$ (calcd. mass: 754.3) and $C_{54}H_{92}N_4O_{12}Pt$ (calcd. mass: 1183.64). Remarkable fragmentation peaks corresponded to the loss of a chloride from complex **1** or a cholylglycinate from complex **2** ($[M\mathbf{1} - Cl - H]^+ = 718.6$ and $[M\mathbf{2} - CG]^+ = 719.5$).

The ^{195}Pt NMR resonance for complex **1** is centered at −1970 ppm indicating a square planar complex with a PtN_2OCl environment, while the ^{195}Pt NMR signal for complex **2** is centered at −1470 ppm indicating a PtN_2O_2 square planar complex (*13*). The carboxylate ν_{as} and ν_s absorption bands in the IR spectra of complex **1** (1601 and 1409 cm^{-1}) and complex **2** (1637 and 1384 cm^{-1}) also point to monodentate binding of the cholylglycinate ligands (*10*). For complex **1**, a typical stretching vibrational mode of Pt−Cl at 349 cm^{-1} was observed (*10*, *14*).

The ^{1}H NMR and ^{13}C NMR spectra for complex **1** showed peaks corresponding to one cholylglycinate and to the ethylenediamine. The ^{1}H NMR and ^{13}C NMR spectra for complex **2** displayed peaks corresponding to two cholylglycinates and to the ethylenediamine. There is a single set of resonances for the two cholylglycinate ligands in complex **2** and the two ethylenediamine carbons of complex **2** are isochronous.

Both complexes are soluble in methanol and DMSO and dissolve over time in water, complex **2** being more water soluble than complex **1** (*14*). Aqueous solutions of both compounds showed a concentration-dependent, broad, and unstructured (*15*−*17*) fluorescence emission band (Figure 3), which persisted for weeks. The absorption spectra of these platinum compounds were typically formed by very intense charge-transference bands in the UV zone (Figure 2). These bands hide weaker d−d transitions located to greater wavelengths which did not show up at the concentrations used (10^{-4} M) in the registered absorption spectra.

The quantum yields (Φ) in water were calculated as being of 0.11 for complex **1** and of 0.10 for complex **2** (relative to quinine sulfate) (*18*).

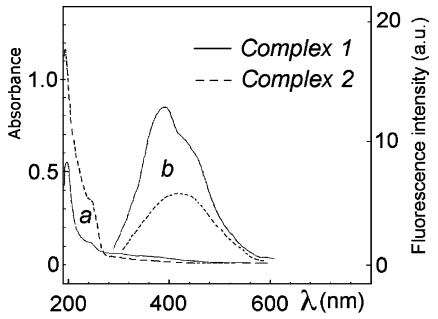

Figure 2

Figure 2. Electronic absorption (a) and emission (b) spectra at room temperature of platinum complexes in water solution ($c = 1 \times 10^{-4}$ M). Excitation wavelength: $\lambda_{ex} = 301$ nm (Complex **1**); $\lambda_{ex} = 330$ nm, (Complex **2**); fluorescence intensity in arbitrary units.

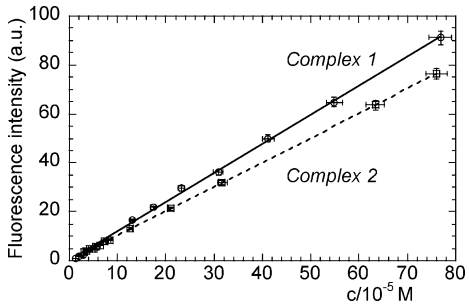

Figure 3. Concentration dependency of the fluorescence intensity of the platinum complexes. Concentrations are expressed in 10^{-5} M and the fluorescence intensity in arbitrary units (a.u.). Emission wavelengths for each complex were selected from the emission spectra (Figure 2) to achieve the maximal emission intensity: Complex **1**, $\lambda_{ex} = 301$ nm, $\lambda_{em} = 384$ nm; Complex **2**: $\lambda_{ex} = 330$ nm, $\lambda_{em} = 436$ nm. For each concentration series, small aliquots of a recently prepared aqueous solution of the corresponding complex were sequentially added to the cuvette, equilibrated for 10 min and measured at room temperature.

Kinetic Studies. Molar conductivities at 37 °C of complexes **1** or **2** (10^{-3} M solutions) were of 144 and 150 S cm^2 mol^{-1}, respectively, in agreement with a 1:1 electrolyte ratio. The hydrolysis kinetics at 37 °C in water and in solutions of increasing NaCl concentrations (4, 150, and 500 mM) were followed by the overtime variation in absorbance at 245 nm. The decay curve was adjusted to a pseudo first-order rate equation, $A = A_0e^{-kt} + C$, where A is absorbance at time t; A_0 is absorbance at time zero; k_{obs} is the rate constant (h^{-1}), and C is a constant term introduced to take into account the baseline variation and the absorption of other nonreacting species (Figure 4). The calculated values for the rate constants (k_{obs}) and the half-lives are summarized in Table 1.

Reactivity of Complexes 1 and 2 with 5-GMP. The reaction between complex **1** or complex **2** and 5′-GMP at 37 °C in a 1:1 molar ratio (10^{-4} M each) was followed by mass spectrometry (Figure 5). Briefly, unreacted 5′-GMP was quantified by peak integration (35 spectra) relative to an internal standard and comparison with a calibration curve (obtained by fitting different 5′-GMP standards to a straight line). Total 5′-GMP was estimated as the sums of the $m/z = 362$ [(5′-GMP) − H]$^+$ and $m/z = 384$ [5′-GMP) − H + Na]$^+$ ions (calibration curve: $0.999 > r^2 < 0.999$, $n = 4$, RSD = 0.3% − 0.03%). Both complexes reacted slowly since 5′-GMP consumptions greater than 95% took 187 and 124 h, respectively.

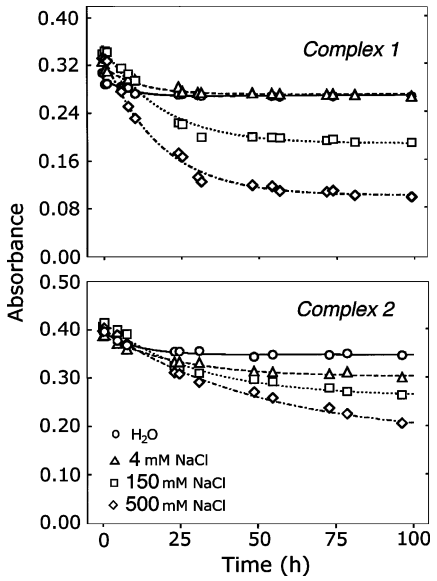

Figure 4. Absorbance vs time curves for complexes **1** and **2**. The hydrolysis reactions for complexes **1** and **2** were followed by monitoring the decay of the 245 nm absorption band at 37 °C. Increasing concentrations of sodium chloride (4, 150, and 500 mM) were selected to mimic the effect of different environments on the hydrolysis kinetics. The traces connecting the experimental data-points were drawn by SIMFIT and correspond to first order decays.

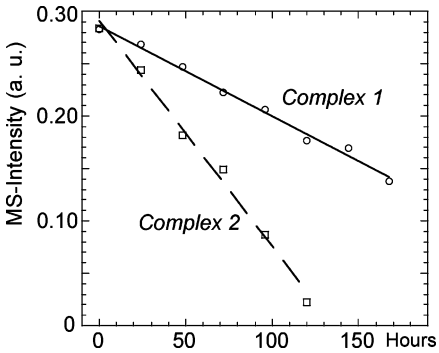

Figure 5. Effect of complex **1** and complex **2** on free 5′-GMP concentration according to time curves in the presence of complexes **1** and complex **2**. Free 5-GMP concentration was monitored by the MS-intensity measured at different time points (see text for details).

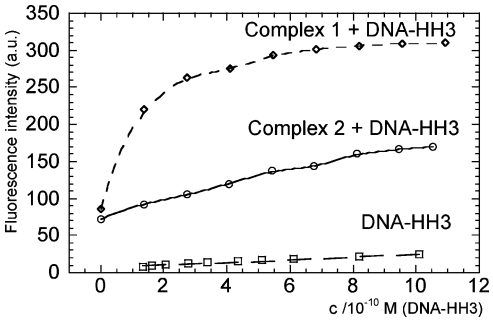

Figure 6. Fluorescence of the platinum complex **1** and complex **2** after incubation with HH3-DNA. Fluorescence intensity of increasing concentrations of HH3-DNA incubated for 24 h. with a 7.2×10^{-4} M solution of complex **1** in water and a 1.3×10^{-4} M solution of complex **2**. ($\lambda_{ex} = 350$ nm, $\lambda_{em} = 437$ nm.) The solutions remained fluorescent for periods of time longer than two months.

NMR monitoring of the reaction of complexes **1** and **2** with 5′-GMP revealed an over time decrease in the signal corresponding to the guanosine H8 proton (8.1 ppm),

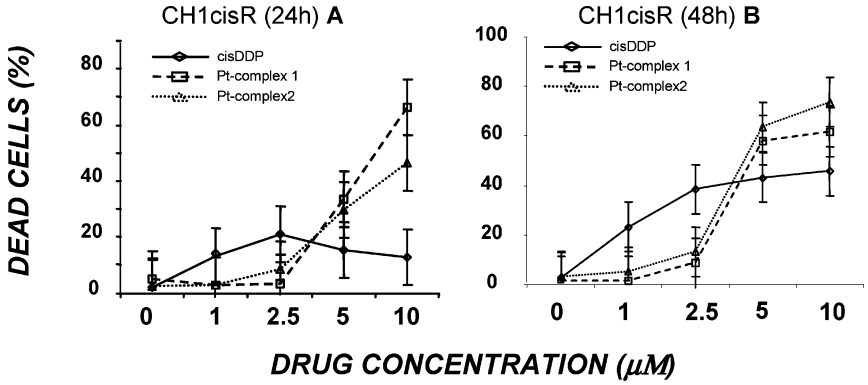

Figure 7. Cytotoxic activity of different doses of Pt complex **1** and complex **2** as compared to cisplatin against CH1cisR ovarian cancer cells after 24 h (panel A) and 48h (panel B) of culture. Results expressed as percentage of dead CH1cisR cells. Statistically significant differences ($p < 0.04$) were observed for the percentage of dead cells at 24 h for cisplatin as compared to both complex **1** and complex **2** at doses of 10 μM.

Table 1. Kinetic Parameters for Hydrolysis Reactions [$C = 10^{-4}$ M, 37 °C, final pH = 4.5 for Complex 1 and pH 6.0 for Complex 2]

	H_2O	4 mM NaCl	150 mM NaCl	500 mM NaCl
		Complex **1**		
k_{obs} (h^{-1})	$1.22 \pm 0.02 \times 10^{-1}$	$8.4 \pm 0.02 \times 10^{-2}$	$5.38 \pm 0.01 \times 10^{-2}$	$5.24 \pm 0.01 \times 10^{-2}$
$t_{1/2}$ (h)	5.68	8.23	12.88	13.22
		Complex **2**		
k_{obs} (h^{-1})	$0.93 \pm 0.01 \times 10^{-1}$	$0.39 \pm 0.02 \times 10^{-1}$	$0.32 \pm 0.02 \times 10^{-1}$	$0.19 \pm 0.01 \times 10^{-1}$
$t_{1/2}$ (h)	7.45	17.77	21.66	36.48

together with the appearance of a new resonance at 8.7 ppm (identical for both platinum compounds). The chemical shift changes for the sugar and the steroid moieties were less pronounced.

Fluorescence Properties of the Platinum Complexes 1 and 2 after Reaction with DNAs. All measurements were performed under the conditions employed for the microscopic experiments ($\lambda_{ex} = 350$ nm and $\lambda_{em} = 437$ nm) described below, to render the comparisons meaningful. The complexes were reacted separately with 5′-GMP, oligonucleotides **I**, **II**, and **III** (see Experimental Section) and HH3-DNA.

Incubation of 10^{-4} M [Pt(en)(CG)Cl] (**1**) and [Pt(en)-(CG)$_2$] (**2**) solutions with a 1:1 molar ratio of 5′-GMP led to fluorescence "quenching" . On the other hand, addition of increasing amounts of either oligonucleotides **I**, **II**, or **III** or HH3-DNA led, after 5 min, to an increase in fluorescence intensity higher than the sum of the fluorescence intensities of the individual components (*19*). The solutions remained fluorescent for periods longer than 2 months. No major differences in fluorescence emission were observed with increasing salt concentration (4, 150, and 500 mM of sodium chloride) when compared with aqueous solution (Figure 6).

Cytotoxic Activity of Platinum Complexes 1 and 2 and Microscopic Analyses. As shown in Figure 7, platinum complexes **1** and **2** displayed an important cytotoxic activity against the CH1cisR ovarian cancer cell line. This cytotoxic activity was already evident after 24 h of culture, and its maximum was reached for the highest doses assayed: 5 and 10 μM. Interestingly, after 24 h no significant differences were observed between the proportion of dead cells obtained with both complexes ($p > 0.05$). In contrast, the cytotoxic activity of these two complexes after 24 h of culture was significantly ($p < 0.04$) higher than that of cisplatin, this latter compound requiring longer periods of time (48 h) to reach identical cytotoxic effects, see Figure 7.

Analysis of the relative distribution of surviving cells in culture along the G_0/G_1, S and G_2/mitosis cell cycle

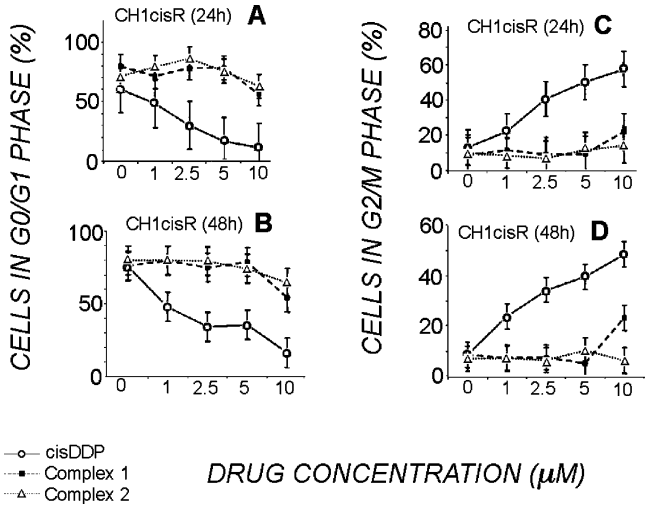

Figure 8. Cell cycle distribution of live CH1cisR (panels A to D) cells, after being cultured for 24 h (panels A, C), and 48 h (panels B, D), in the presence of Pt complex **1**, complex **2**, and cisplatin. Percentage value of G_0/G_1 cells is shown in panels A and B, and the proportion to G_2/M cells is displayed in the other panels (C and D). Statistically significant differences ($p < 0.03$) were found in the percentage of both G_2/M- and G_0/G_1-cell cycle phase cells cultured in the absence versus the presence of cisplatin at doses >2.5 μM for 24 and 48 h, except for the comparisons of the percentage of G_2/M cells treated with 2.5 μM cisplatin for 24 h.

phases showed that neither of the two platinum complexes synthesized induced a significant accumulation of cells in any specific cell cycle phase ($p > 0.05$). By contrast, cisplatin induced a dose-dependent accumulation of CH1cisR cells in the G_2/M cell cycle phases ($p < 0.03$), in line with previous findings (*20*). At identical doses, cisplatin requires that cells must have reached G_2, to enter apoptosis and finally die (*20*, *21*), see Figure 8.

PB nucleated cells incubated with either platinum complexes **1** or **2** became fluorescent after 10 min, mainly within the cells' nuclei, under microscopic analysis

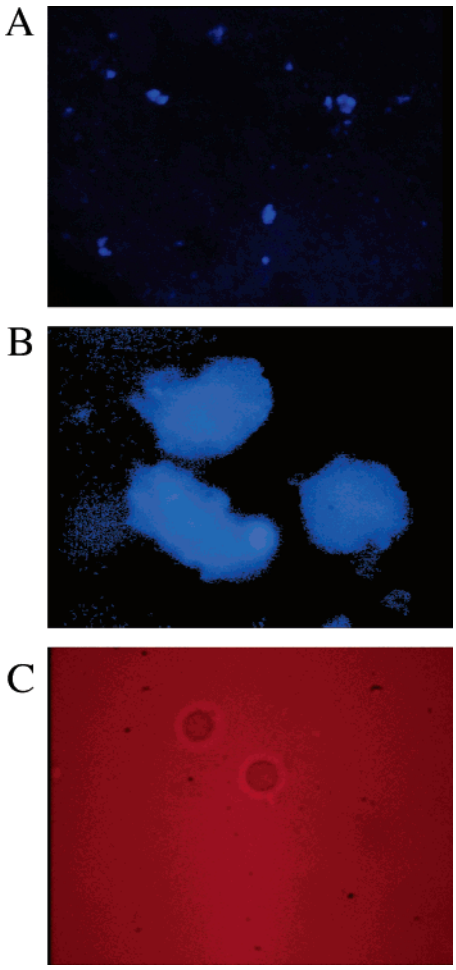

Figure 9. Illustrative photographs of the fluorescence emission observed after incubating peripheral blood nucleated cells with complex **1** (complex **2** leads to very similar photographs). Panel A shows a 20× magnification and panel B a 500× magnification. Panel C shows that these cells remained alive and did not incorporate propidium iodide.

(Figure 9). Cells treated with both platinum complexes remained fluorescent for more than five weeks.

DISCUSSION

The spectroscopic data for complex **1** and complex **2** agree with the expected [Pt(en)(CG)Cl] and [Pt(en)$(CG)_2$] square planar complexes, very similar to the closely related [Pt$(NH_3)_2$(CG)Cl] and [Pt$(NH_3)_2(CG)_2$] compounds we have recently described (*10*). Both complexes carry two putative leaving ligands, one chloride and one cholylglycinate for complex **1** and two cholylglycinates for complex **2**. The cholylglycinate ligands in both complexes are spectroscopically very similar, suggesting identical binding modes. Minor differences are seen for the atoms next to the Pt atom, the C-26 carboxylates and the ethylenediamine carbons and protons, which are isochronous in the NMR spectra of complex **2** but not in the asymmetric complex **1** (with different trans ligands: chloride and cholylglycinate). In complex **2**, the two cholylglycinates give rise to only one set of resonances, indicating a rapid rotation on the NMR time scale of the bile acids ring system, as would be expected for a freely rotating linear chain linking the Pt atom and the steroid skeleton.

Aqueous solutions of either complex show a persistent, concentration-dependent, broad, and unstructured fluorescence emission band. To the best of our knowledge, there are no previous reports of fluorescent platinum complexes in water at room temperature (other than those with attached fluorescent ligands) (*5*). The half-lives for the complexes measured in the kinetic studies (see Table 1) imply that both complexes are almost completely hydrolyzed after 48 h in pure water, but their solutions remain fluorescent.

Substitution of the chloride ion by water in cisplatin is the first kinetic step in its mechanism of action (*22*−*25*), prior to reaction with its final targets. Complexes **1** and **2** show higher aqueous solubility but slower kinetic profiles than cisplatin. The higher stability in water of complex **2** as compared to complex **1** relates to the substitution of the chloride ligand by the second carboxylate group (a worse leaving group). Such a substitution in cisplatin analogues (e.g. carboplatin and oxaliplatin) is considered to be responsible for their increased water solubility, as well as their reduced kinetic reactivity and toxicity (*1*). For complexes **1** and **2**, the higher the NaCl concentration, the longer their half-lives, as observed upon increasing NaCl concentrations from those observed in the cytoplasmic compartment (4 mM) to the plasma ones (150 mM). This behavior parallels that of cisplatin and accounts for its higher intracellular than extracellular reactivity (*1*).

The hydrolysis reactions of the two complexes follow single decay kinetics of increasing rates with decreasing NaCl concentrations (see Table 1). The two complexes initially hydrolyze to the same putative aquo-complex (*26*), $[Pt(en)(CG)H_2O]^+$, in agreement with the equivalent fragmentation peaks of the MS ($[M\mathbf{1} - Cl - H]^+ = 718.6$ and $[M\mathbf{2} - CG]^+ = 719.5$):

$$[Pt(en)(CG)Cl] + H_2O \rightarrow [Pt(en)(CG)H_2O]^+ + Cl^-$$

$$[Pt(en)(CG)_2] + H_2O \rightarrow [Pt(en)(CG)H_2O]^+ + CG^-$$

A second decay curve is not observed for either complex, even for very long reaction times. This observation suggests that there is only one replaceable ligand in these complexes, as opposed to cisplatin and its analogues, where two substitutions are seen from the kinetics of hydrolysis reactions. These double substitutions allow the formation of DNA bis-adducts, which are deemed important for the cytotoxic activity of cisplatin analogues (probably due to intra- and interstrand DNA cross-links) (*2*−*4*).

As a model system, the reaction of the complexes with 5′-GMP (cisplatin reacts with 5′-GMP to give mixtures of the mono- and the bisadducts) were studied by MS and NMR. The final time-points showed the [Pt(en)(CG)(5′-GMP)]$^+$ monoadduct with $m/z = 1082.5$, together with the residual unreacted complex. No platinated peak which could be ascribed to the generation of a [Pt(en)-$(5′\text{-GMP})_2]^+$ bis-adduct was detected, in agreement with the previously described kinetic studies. The ^{1}H NMR chemical shift changes are in agreement with the N7 platination of guanine. Guanine N7 is readily accessible in the DNA major groove and, owing to its high basicity among the four nucleotide bases, it is the major binding target on DNA for antitumor platinum complexes (*27*−*29*). Summarizing, complex **1** and **2** seem to have a single reactive ligand, the second one being inert or reacting much slower under the experimental conditions used, and they both lead to the same chemical species. The reaction of the complexes with 5′-GMP led to a substantial decrease in their fluorescence intensities. The endpoint

solutions gave, after correcting for the concentration differences, fluorescent intensities very similar to each other, as expected if they contain the same final products.

To determine the nature of the fluorescent species observed in the microscopic images described below, we further studied the reactivity of the complexes against different oligonucleotides and DNA structures by following changes in the emission spectra. A substantial increase in fluorescence intensity is observed, ascribed to the formation of monoadducts of the complexes with the guanine bases of the DNA. There was no fluorescence decay observed even for periods longer than 2 months, suggesting that the monoadducts are stable in vitro and do not evolve toward bis-adducts, even for oligonucleotide **III**, which bears three Gs in a row. The decreased fluorescence intensity of the 5′-GMP adducts and the increased emission attained with oligonucleotides and other DNAs is very fortunate, since it reduces the background fluorescence in microscopic images.

The microscopic studies confirmed that complexes **1** and **2** in individual cells mainly target the cells' nuclei, supporting their preferential binding to DNA. Interestingly, the increase in cell fluorescence was rapidly observed after 10 min, in keeping with the results described using oligonucleotides **I**, **II**, and **III**, and HH3-DNA. In addition, cells treated with both platinum complexes remained fluorescent for more than five weeks. Accordingly, both complexes emit enough fluorescence to allow unequivocal identification of cells containing the platinum complexes bound to their DNA and they remain stable. Such features, in a heterogeneous sample composed of a mixture of different cell subsets, would facilitate the visualization of individual cells containing platinum complexes inside by conventional microscopic techniques. This also indicates that even if cells containing platinum remained alive for relatively long periods of time they would not be able to rapidly eliminate such platinum complexes from the nuclei into the cytoplasmic compartment or the extracellular media. This would further facilitate a close analysis of the activity of these compounds through the possibility of identifying the cells targeted, provided that both platinum complexes do in fact retain cytotoxic activity.

The two synthesized platinum complexes showed a dose-dependent cytotoxic activity. Interestingly, once compared to cisplatin, both complex **1** and complex **2** showed significantly a higher cytotoxic activity at early time periods after they have been added to the cell culture, even in the absence of a high number of DNA bis-adducts. Since the formation of bis-adducts appears to be directly linked to the cytotoxic activity of cisplatin, our results suggest that the two platinum complex assayed might have formed other different type of adducts (*19*, *30*−*33*) which would also be capable of inducing cytotoxicity. The linkage of platinum compounds to bile acids would also help to explain the different pharmacokinetics observed for the two new complexes as compared to cisplatin. In addition, they exert their cytotoxic effects in the absence of significant accumulation of cells at a specific cell cycle phase.

Analysis of the relative distribution of surviving cells in culture along the G_0/G_1, S, and G_2/mitosis cell cycle phases showed that neither of the two platinum complexes synthesized induced a significant accumulation of cells in any specific cell cycle phase ($p > 0.05$). By contrast, cisplatin induced a dose-dependent accumulation of CH1cisR cells in the G_2/M cell cycle phases ($p < 0.03$), in line with previous findings (*20*). At identical doses, cisplatin requires that cells must have reached G_2, to enter apoptosis and finally die (*20*, *21*).

Such cell-cycle associated differences could be explained, at least to a certain extent, in terms of the formation of different adducts with the platinum complexes **1** and **2** as compared to cisplatin, which could lead to the occurrence of different interactions of the platinum compound once inside the cell. A potential advantage of the newly synthesized platinum complexes would rely on their potential cytotoxic effects on proliferating and resting tumor cells, provided the fact that this is not associated with an increase in normal tissue toxicity. Further studies are necessary to explore these potential advantages.

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