0026-895X/03/6304-933–944$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:933–944, 2003

Vol. 63, No. 4
2180/1055024
Printed in U.S.A.

Antitumor and Cellular Pharmacological Properties of a Novel Platinum(IV) Complex: *trans*-[$PtCl_2(OH)_2$(Dimethylamine)(Isopropylamine)]

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Received September 30, 2002; accepted January 14, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The antitumor and cellular pharmacological properties of the *trans*-Pt(IV) complex, *trans*-[$PtCl_2(OH)_2$(dimethylamine)(isopropylamine)] (compound **2**) has been evaluated in comparison with its corresponding *trans*-Pt(II) counterpart, *trans*-[$PtCl_2$(dimethylamine)(isopropylamine)] (compound **1**). The results reported here indicate that compound **2** markedly circumvents cisplatin resistance in 41McisR and CH1cisR ovarian tumor cell lines endowed with different mechanisms of resistance (decreased platinum accumulation and enhanced DNA repair/tolerance, respectively). However, compound **1** is able to circumvent cisplatin resistance only in CH1cisR cells. Interestingly, at equitoxic concentrations, compounds **1** and **2** induce a higher amount of apoptotic cells than cisplatin in CH1cisR cells. Moreover, the number of apoptotic cells induced by compounds **1** and **2** correlates with their ability to form DNA interstrand cross-links in CH1cisR cells. Although compounds **1** and **2** showed remarkable cytotoxic activity, only compound **2** was able to inhibit the growth of CH1 human ovarian carcinoma xenografts in mice. Binding studies with serum albumin indicate that compound **1** possesses a much higher reactivity against albumin than compound **2**. Moreover, the level of binding of compound **1** to plasma proteins during the period 15 min to 1 h after administration to mice (15 mg/kg, i.p.) is 2.5-fold higher than that of compound **2.** Therefore, the lack of in vivo antitumor activity shown by compound **1** might be related to its extracellular inactivation before reaching the tumor site because of its high rate of binding to plasma proteins.

The use of *cis*-diamminedichloroplatinum(II), known as cisplatin or *cis*-DDP (Fig. 1), in cancer chemotherapy has a made a major impact on the observed response rates of some tumor types, such as testicular or ovarian carcinoma (Wong and Giandomenico, 1999). However, cisplatin has two major drawbacks: 1) severe toxicity that includes nephrotoxicity, neurotoxicity and ototoxicity and 2) the acquisition or presence of resistance to the drug (Cohen and Lippard, 2001). Because tumor resistance to cisplatin limits its efficacy, there is urgent need to discover new platinum complexes capable of overcoming cisplatin resistance.

Because enhanced removal of cisplatin-DNA adducts has been reported as one of the main causes of cell resistance to cisplatin, there is general consensus that this particular resistance mechanism may be circumvented by platinum complexes that bind differently to DNA than cisplatin does (Farrell, 1993; Zdraveski et al., 2002). One platinum compound that possesses DNA-binding properties distinct from those of cisplatin is its *trans* isomer transplatin, *trans*-diamminedichloroplatinum(II), or *trans*-DDP (Fig. 1). In fact, it has been found than *cis*-DDP mainly forms 1,2 intrastrand cross-links on DNA, whereas the main DNA adducts of *trans*-DDP are 1,3 intrastrand and interstrand cross-links (Cohen and Lippard, 2001). Unfortunately, early structure-activity relationship studies showed that *trans*-DDP and other *trans*-Pt complexes were inactive as antitumor drugs (Connors et al., 1979). However, in 1989, Farrell et al. (1989) reported the first cytostatic *trans*-Pt(II) complexes. In recent years, several classes of biologically active *trans*-platinum complexes have been reported (Natile and Coluccia, 2001). Among these

This work was supported by Spanish Comisión Interministerial de Ciencia y Tecnologica grant SAF00-0029. Support and sponsorship by Cost Actions D20/0001/00 and D20/0003/00 is kindly acknowledged. An institutional grant from Fundación Ramón Areces is also acknowledged.

ABBREVIATIONS: *cis*-DDP, cisplatin, *cis*-diamminedichloroplatinum(II); *trans*-DDP, transplatin, *trans*-diamminedichloroplatinum(II); DMEM, Dulbecco's modified Eagle's medium; PI, propidium iodide; TXRF, total reflection X-ray fluorescence; PBS, phosphate-buffered saline; ICL, interstrand cross-link; MTD, maximum tolerated dose; HSA, human serum albumin; RF, resistance factor; r_i, input molar ratio of platinum to nucleotide; dsDNA, double strand DNA.

unusual classes of platinum drugs, it has been recently found that *trans*-Pt(II)Cl_2 complexed with an asymmetric set of aliphatic amines is able to circumvent cisplatin resistance. It is known that resistance to cisplatin is multifactorial and includes three main mechanisms: decreased cellular accumulation of cisplatin, increased cytoplasmic detoxification (through increased levels of glutathione and metallothioneins), and increased DNA repair/tolerance of platinum-DNA adducts (Pérez, 1998). It has also been postulated that alterations in the apoptotic cell death pathway may constitute a fourth mechanism of cisplatin resistance (González et al., 2001).

We have recently reported that the *trans*-platinum(II) complex with mixed aliphatic amines, *trans*-[$PtCl_2$(dimethylamine)(isopropylamine)] (compound **1**; Fig. 1) circumvents cisplatin resistance in cell lines that overexpress *ras* oncogenes such as HL60 human leukemic cells and Pam 212-*ras* murine keratinocytes (Montero et al., 1999). In addition, compound **1** is also able to circumvent resistance to cisplatin in A2780cisR ovarian tumor cells, which exhibit resistance through a combination of the three main mechanisms mentioned above (Pérez et al., 2001). Moreover, we observed that circumvention of cisplatin resistance by compound **1** is associated with a higher level of apoptosis induction relative to *cis*-DDP (Pérez et al., 1999; Montero et al., 2002). Of interest also was the observation that in A2780cisR cells, there is a correlation between the DNA interstrand cross-linking efficiency of compound **1** and its ability to induce apoptosis (Montero et al., 2002).

The desires to develop an orally active platinum drug, to improve quality of life of patients, and to expand platinum chemotherapy to outpatient treatment have stimulated the research on Pt(IV) compounds (Fuertes et al., 2002). In fact, the compound [bis-acetato amminedichloro(cyclohexylamine) platinum(IV)], also called JM216 (or satraplatin) has shown activity against some cisplatin-resistant human ovarian carcinoma cell lines. In addition, phase I to III clinical trials have shown that JM216 is an orally active platinum drug (Kelland et al., 1993: Judson et al., 1997). On the other hand, JM335 [*trans*-ammine(cyclohexylamine-dichlorodihydroxo) platinum(IV)] has been reported as the first *trans*-platinum(IV) complex endowed with antitumor activity against several human ovarian carcinoma xenografts (Kelland et al., 1994). On these grounds, we report here the cytotoxic activity of the *trans*-platinum(IV) complex, *trans*-[$PtCl_2(OH)_2$(dimethylamine)(isopropylamine)] (compound **2**; Fig. 1) in pairs of cisplatin-sensitive and -resistant human ovarian tumor cell lines. The resistant cell lines show acquired resistance to cisplatin and were selected with regard to the three major mechanisms of resistance to the drug. In addition, these cell lines have been used previously to identify novel platinum complexes capable of circumventing cisplatin resistance (Kelland et al., 1992, 1994, 1995). Because cellular and molecular pharmacology studies are essential to understand the relationships between structure and anticancer properties, we have compared the cellular accumulation, DNA binding, interstrand cross-linking efficiency, apoptosis induction, and binding to serum albumin and plasma proteins of the *trans*-Pt(IV) complex (compound **2**) with that of its corresponding *trans*-Pt(II) analog (compound **1**). Finally, the in vivo antitumor activity of compounds **1** and **2** has been evaluated in mice bearing tumor xenografts.

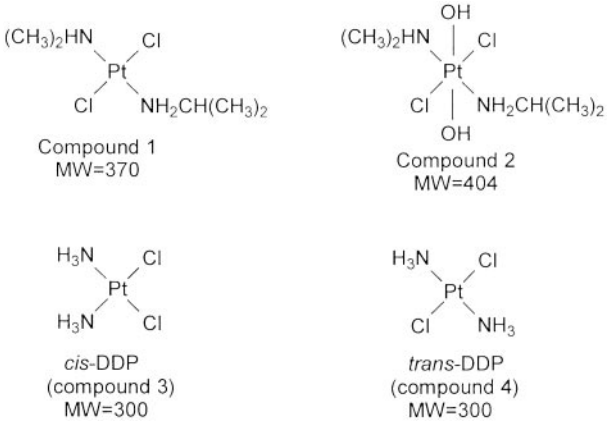

Fig. 1. Structures and molecular weights of the platinum complexes used in this work.

Materials and Methods

Materials. *cis*- and *trans*-DDP and also pUC8 plasmid DNA were purchased from Sigma (Alcobendas, Spain). *trans*-[$PtCl_2$(dimethylamine)(isopropylamine)] was synthesized following a well-established procedure, which exploits the difference in the *trans* effect of halide and amine ligands in platinum(II) complexes to achieve selective substitution and therefore control of stereochemistry (Montero et al., 1999).

Synthesis of *trans*-[$PtCl_2(OH)_2$(Dimethylamine) (Isopropylamine)]. The oxidation of platinum(II) complexes with hydrogen peroxide yields platinum(IV) complexes in which the stereochemistry of the platinum(II) complex is retained and *trans*-hydroxo ligands are added; 0.27 mmol of *trans*-[$PtCl_2$(dimethylamine)(isopropylamine)] was suspended in 2 ml of water, and hydrogen peroxide (1.62 mmol) was added. The mixture was stirred and heated at 60°C in the darkness for 24 h. Afterward, stirring was maintained at room temperature in the presence of light to complete the decomposition of the unreacted H_2O_2 molecules. The product was collected by filtration as a pale yellow solid, washed with ether, and dried under vacuum. Yield, 89%. Melting point, 173.2 to 174.1°C. IR (ν/cm): 3534 (O-H), 557 (Pt-OH), 346 (Pt-Cl). ^{1}H-NMR (δ ppm) 3.28 (H1), 1.30 (H2), 2.47 (H1′). ^{13}C-NMR (δ ppm) 47.3 (C1), 21.6 (C2), 41.2 (C1′). ^{195}Pt-NMR (δ ppm) 786.5. Anal. ($C_5H_{18}N_2Cl_2Pt$) C, H, N, Cl.

Cell Culture and Platinum Drugs Cytotoxicity. The pairs of cisplatin-sensitive and -resistant human ovarian tumor cell lines A2780/A2780cisR, CH1/CH1cisR, and 41M/41McisR (Hills et al., 1989; Kelland et al., 1992) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum together with 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37°C in an atmosphere of 95% air/5% CO_2. Cell death was evaluated by using a system based on the tetrazolium compound 3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, which is reduced by living cells to yield a soluble formazan product that can be assayed colorimetrically (Alley et al., 1988). Exponentially growing cells were plated at a density of 10^4 cells per well in 96-well sterile plates in 100 μl of DMEM, and were incubated for 3 to 4 h. Stock solutions of the platinum compounds (1 mg/ml) in DMEM were added to the wells at final concentrations ranging from 0 to 350 μM, in a volume of 100 μl per well. After 24 or 72 h of incubation with the drugs, 50 μl of a freshly diluted 3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (1:5 in culture medium) was added to a final concentration of 1 mg/ml into each well, and the plate was further incubated for 5 h. Cell survival was evaluated by measuring the absorbance at 520 nm, using a Microplate reader 2001 (Cambrex Bio Science Walkersville, Inc., Walkersville, MD). IC_{50} values were calculated from curves constructed by

plotting cell survival (percentage) versus compound concentration (micromolar). IC_{50} is defined as the drug concentration that produces 50% of cell killing. The mean IC_{50} values were obtained from four independent experiments carried out with quadruplicate cultures. Cell killing was also evaluated using the sulforhodamine B as described previously (Kelland et al., 1994)

Quantification of Apoptosis by Annexin V Binding and Flow Cytometry. Exponentially growing CH1cisR cells were exposed to concentrations equal to the IC_{50} of the platinum drugs for 24 h. Subsequently, attached and detached cells were recovered, mixed, and resuspended in annexin V binding buffer (BD Biosciences PharMingen, San Diego, CA). Propidium iodide (PI, 2.5 μl; Sigma) and 1 μg/ml of annexin V-fluorescein isothiocyanate (BD Biosciences PharMingen) were added, and the cells were left at room temperature before flow cytometric analysis in a FACScalibur apparatus (BD Biosciences, San Jose, CA). The percentage of apoptotic cells induced by each platinum drug (percentage of annexin V-positive/PI negative cells) was calculated from the annexinV/PI scattergrams (Pestell et al., 2000). The data were obtained from four independent experiments with duplicates.

Total Reflection X-Ray Fluorescence Measurements. The analysis by total reflection X-ray fluorescence (TXRF) of the platinum content in biological samples was performed using a Extra-II spectrometer (Seifert, Ahrensburg, Germany). TXRF determinations were carried out according to a procedure previously reported (Fernández-Ruíz et al., 1999). Briefly, a 100-μl aliquot of a sample was introduced in a test tube of 2 ml. This solution was standardized with 100 ng/ml of Vanadium [Merck (Darmstadt, Germany) ICP Vanadium standard solution]. Afterward, the sample was introduced into a high-purity nitrogen flow concentrator at a temperature of 70°C until the volume was reduced five times. An aliquot of 5 μl was then taken, deposited on a previously clean quartz-made reflector, and dried on a ceramic plate at a temperature of 50°C. The entire process was done in a laminate flow chamber (Model A-100). The samples were analyzed following the X-ray molybdenum line under working conditions of 50 kV and 20 mA with a live-time of 1000 s and a dead time of 35%. Spectra were recorded between 0 and 20 keV. Fifteen elements (P, S, K, Ca, V, Fe, Cu, Zn, As, Br, Rb, Sr, Ni, Mn, and Pt) were simultaneously analyzed to obtain a correct deconvolution of profiles associated with the general spectrum. The Pt line was used for Pt quantification. The analytical sensitivity of the TXRF measurements was 0.3 to 22.4 ng of Pt in a solution volume of 100 μl, with repeatability between 2 and 8% (n = 8, four independent experiments with duplicate cultures).

Measurements of Platinum Accumulation in Culture Cells. Cultures plates containing exponentially growing 41M or 41McisR cells in 10 ml of DMEM (cell density = 2×10^5 cells/ml) were exposed to either 10 μM or equitoxic concentrations (IC_{50}) of the platinum drugs dissolved in DMEM for 1, 5, 12, and 24 h. Cells were washed with ice-cold phosphate-buffered saline (PBS), scraped, and resuspended in 700 μl of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.4% Triton X-100, incubated at 4°C for 15 min and centrifuged at 12,000 rpm for 15 min in a centrifuge. Afterward, supernatants were treated for 3 h at 37°C with 20 μg/ml of proteinase K (Roche Diagnostics, Indianapolis, IN). The platinum content in the samples was determined by TXRF as described above. Experiments were carried out as four independent experiments with duplicate cultures.

Determination of Platinum Binding to DNA in Culture Cells. Culture plates containing exponentially growing CH1or CH1cisR cells in 10 ml of DMEM (cell density, 2×10^5 cells/ml) were exposed to either 10 μM or equitoxic concentrations (IC_{50}) of the platinum drugs dissolved in DMEM. The plates were incubated for 1, 5, 12, and 24 h under the conditions described above. After drug incubation, culture medium was removed from the plates and the cell plates were washed with PBS. Subsequently, the cells were lysed with 700 μl of a buffer solution containing 150 mM Tris-HCl, pH 8.0, 100 mM EDTA, and 100 mM NaCl, incubated for 15 min at 4°C and centrifuged at 12,000 rpm for 15 min in a Microfuge (Beckman Coulter, Fullerton, CA). Supernatants were treated for 3 h at 37°C with 20 μg/ml of proteinase K (Roche Diagnostics). Afterward, supernatants were incubated for 16 h at 37°C with 4 μl of RNase A of 100 μg/ml (Roche Diagnostics). Finally, DNA was extracted with a volume of phenol/chloroform/isoamyl alcohol (50:49:1), precipitated with 2.5 volumes of cold ethanol, and 0.1 volumes of 3 M sodium acetate, washed with 75% of ethanol, dried, and resuspended in 1 ml of water. The DNA content in each sample was measured by UV spectrophotometry at 260 nm in a Shimadzu UV-240 spectrophotometer, and platinum bound to DNA was determined by TXRF. The data were obtained from four independent experiments with duplicate cultures.

Kinetics of DNA Interstrand Cross-Links (ICLs) Formation in Culture Cells. DNA ICLs were determined by alkaline filter elution using CH1 and CH1cisR cell lines as described previously (Alvarez-Valdés et al., 2002). The DNA of the cells was labeled by seeding 10^6 cells in P100 tissue culture plates and growing for 24 h in the presence of 0.03 μCi/ml [^{14}C]thymidine (specific activity, 51 mCi/mmol; Amersham Biosciences). A plate of cells to be used as an internal standard in the assay was labeled overnight with 0.17 μCi of [methyl-^{3}H]thymidine (specific activity, 5 mCi/mmol) plus 10^{-5} M unlabeled thymidine. CH1 or CH1cisR cells (^{14}C-labeled) were treated with equitoxic doses (IC_{50} for 24 h of drug treatment) of compound **1**, compound **2**, and *cis*-DDP for 1, 5, 12, and 24 h. In addition, an untreated control plate was included in all experiments. Immediately after drug treatment, the drug was washed off using ice-cold PBS. Test (^{14}C-labeled) cells and internal standard (^{3}H-labeled) cells were then irradiated on ice with 5 and 1 Gy, respectively, of ^{60}Co γ-rays from a 2000-Ci source (dose rate, 2/Gy). Approximately 10^6 cells of a 1:1 mix of test and internal standard cells were then added to duplicate 2-μm pore size 25-mm polycarbonate filters (Millipore Co., Bedford, MA) in 5 ml of ice-cold PBS. Cells were then lysed by two additions of 10 ml of lysis buffer (2% SDS, in 0.1 M glycine and 0.02 M EDTA, pH 10). In the first 10 ml, proteinase K (0.5 mg/ml; Sigma, Alcobendas, Spain) was added immediately before use. DNA was then eluted at pH 12 using 10 ml of 0.1 M tetrapropylammonium hydroxide, containing 0.1% SDS and 0.02 M EDTA. The elution rate was 0.010 ml/min (using a Pharmacia Biotech peristaltic pump), and fractions were collected at 90-min intervals over 24 h. The ^{14}C and ^{3}H DNA radioactivity was then determined in each fraction and from the filters by liquid scintillation counting (1209 Rackbeta; PerkinElmer Wallac). Results are expressed as fraction ^{14}C retained versus fraction ^{3}H (internal standard). DNA interstrand cross-link (ICL) units/dalton $\times 10^9$ were calculated using the expression:

$$ICL_{\text{index}} = \left[\left(\frac{1 - r_0}{1 - r}\right)^{1/2} - 1\right]P_b$$

where r and r_o are the fractions of ^{14}C-labeled DNA for treated versus control cells remaining on the filter when 60% of ^{3}H-labeled DNA is retained on the filter, and P_b is the radiation-induced break probability per dalton. Control experiments were carried out to test for the presence of cisplatin-induced single-strand breaks. The data were obtained from four independent experiments with duplicate cultures.

DNA Interstrand Cross-Linking Efficiency in Linear pUC8 DNA. DNA interstrand cross-links formation was evaluated as described previously (González et al., 1999). To linearize pUC8 plasmid (Maniatis et al., 1989), its DNA was digested in 150 mM NaCl with 10 units/mg of DNA of *Bam*HI (unique restriction site in pUC8 DNA) at 37°C for 4 h. The linear double-stranded plasmid DNA was 3′-end labeled by incubation with 2.5 mCi/mg DNA of [α-^{32}P]dCTP and 1.25 units/mg DNA of the Klenow fragment of *E. coli* DNA polymerase I for 30 min at room temperature. The reaction was stopped by heating

at 70°C for 5 min. The unincorporated radioactivity was removed by passing the labeling reaction through a Sephadex G-50 column. The labeled DNA was precipitated with 0.1 volumes of sodium acetate and 2 volumes of cold ethanol. Sonicated CT DNA was added to the eluted solution of the labeled pUC8 DNA to a final DNA concentration of 180 μg/ml. Afterward, the DNA, at a concentration of 90 ng/ml, was incubated in 10 mM $NaClO_4$ with the platinum drugs at $r_i = 0.05$ (molar ratio of Pt to nucleotides) for several periods of time. Then, aliquots of 10 μl were removed, and the reactions were ended by addition of an equal volume of the loading dye (90% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue). The DNA was melted for 10 min at 90°C and chilled on ice; 1.5% agarose gel electrophoresis in denaturing conditions was carried out at 20 V for 16 h. The gels were dried and autoradiographed. Band quantification was made using a model 300A densitometer (Amersham Biosciences).

***In Vivo* Antitumor Efficacy in CH1 Tumor Xenografts.** A protocol previously reported was followed with some minor modifications (Gowan et al., 2002). First of all, the maximum-tolerated doses of compounds **1** and **2** were determined in NCr nude mice after single intraperitoneal injection of the compounds in 10% DMSO/90% H_2O and in H_2O, respectively. Approximately 2-mm^2 fragments of CH1 human ovarian carcinoma xenografts were implanted into adult female Ncr nude mice, by trocar, subcutaneously in the flank under halothane anesthesia. Once palpable (approximately 6–8 mm diameter) mice were randomized (six animals) into control or treatment groups and therapy started (day 0). Platinum drugs were given by intraperitoneal injection on days 0 and 7 for compound **1** in 10% DMSO/90% H_2O and on days 0, 7, and 14 in H_2O for compound **2** at their predetermined maximum tolerated doses (MTD, 30 and 15 mg/kg, respectively). Animals were weighted and tumor volumes were determined by caliper measurements twice weekly from day 0. Tumor volumes were determined using the formula volume = $a \times b^2 \times \pi/6$, where a and b are orthogonal tumor diameters. Results were expressed as relative tumor volumes. Drug efficacy was determined in terms of a treated/control volume ratio at particular days after the start of treatment. There were no drug-induced deaths with either drug. Compound **1** gave no body weight loss. There was transient body weight loss with compound **2** at day 3 that recovered by day 7. All animal procedures were carried out according the guidelines set out by the Institute of Cancer Research Animal Ethics Committee and the United Kingdom Coordinating Committee on Cancer Research Committee on the Welfare of Animals in experimental Neoplasia (Workman et al., 1999).

Binding of Platinum Compounds to Human Serum Albumin. Briefly, human serum albumin (HSA) in (0.05 μM) in PBS, pH 7.0, was incubated with 0.25 μM of the platinum drugs for several periods of time (1, 3, 7, and 14 days). After incubation, samples were dialyzed overnight against PBS, pH 7.0, to remove unbound drug. Aliquots of 100 μl of control HSA and of HSA incubated with the drugs were subjected to TXRF to determine Pt content. The amount of Pt present in the control samples was subtracted from the amount of Pt present in the dialysis membranes containing HSA. The dialysis was performed in plastic containers. Contaminating metals were removed from plastic ware and buffers by routine treatment with Chelex-100 (Bio-Rad, Hercules, CA) as described previously (Fuertes et al., 2001). PBS contains a concentration of NaCl of 137 mM, which is similar to that present in serum. The data were obtained from four independent experiments with duplicate samples.

Binding of Platinum Compounds to Plasma Proteins in Mice. Total plasma platinum and ultrafilterable platinum in the plasma were determined according to a previously reported procedure (Zhang et al., 2002). Freshly prepared solutions of compounds **1** and **2** were administered by single intraperitoneal injection to Balb C mice (six animals per drug) at a dose of 15 mg/kg. Blood samples (0.1–0.2 ml) were collected from the femoral artery at 0.25, 1, 2, and 3 h into heparinized microtubes, followed by centrifugation at 12,000 rpm for 15 min to isolate the plasma. Afterward, platinum content in aliquots of plasma was determined by TXRF. To determine free and plasma protein-bound platinum fractions, aliquots of plasma were deproteinized by passage through 25 kDa cut-off filters. Platinum content in aliquots of ultrafiltrates was measured by TXRF. All animal procedures were carried out according to the guidelines of Animal Ethics Committee on the Welfare of Animals (Workman et al., 1999).

Statistical Analysis. Where appropriate, statistical significance was tested using a Student's test (two-tailed, unpaired). A P value of <0.05 was considered significant.

Results

Cytotoxic Activity. The cytotoxicity of the platinum complexes in the pairs of cisplatin-sensitive and -resistant human ovarian tumor cell lines, 41M/41McisR, CH1/CH1cisR, and A2780/A2780cisR is shown in Table 1. These cell lines were selected because they cover all of the known major mechanisms of resistance to cisplatin. 41McisR cells are resistant primarily through reduced drug accumulation, CH1cisR cells through enhanced DNA repair/tolerance and A2780cisR cells through a combination of decreased accumulation, enhanced DNA repair/tolerance, and elevated glutathione levels (Hills et al., 1989; Kelland et al., 1992). The cytotoxic activity after 24 h of incubation of the tumor cell lines with compounds **1**, **2**, cisplatin, and transplatin was expressed as IC_{50} (drug concentration reducing the number of living cells by 50%). As expected, the results of Table 1 show that transplatin was devoid of activity ($IC_{50} > 200$ μM). However, the *trans*-platinum complexes **1** and **2** showed a cytotoxic potency comparable with cisplatin, with IC_{50} values in the low micromolar range (1.3 to 57 μM). The ability of compounds **1** and **2** to circumvent cisplatin-acquired resistance was determined from the resistance factor, RF, defined as the ratio of IC_{50} resistant line to IC_{50} parent line. An RF of <2 was considered to denote non–cross-resistance (Kelland et al., 1994); compound **1** displayed greater cross-resistance to cisplatin compared with compound **2**. In fact,

TABLE 1

Cytotoxic effect of the *trans*-platinum complexes in pairs of cisplatin-sensitive and -resistant ovarian tumor cell lines

RF is the ratio of (IC_{50} resistant line)/(IC_{50} sensitive line). Values are means ± S.D. of four independent experiments in quadruplicate cultures.

	IC_{50}								
Compound	A2780	A2780cisR	RF	CH1	CH1cisR	RF	41M	41McisR	RF
					μM				
1	9.0 ± 1.0	57.0 ± 5.2	(6.3)	19.0 ± 1.2	15.0 ± 1.5	(0.8)	13.0 ± 0.9	54.0 ± 5.3	(4.1)
2	5.7 ± 1.3	8.4 ± 0.8	(1.5)	7.0 ± 1.7	5.7 ± 1.0	(0.8)	23.5 ± 2.0	1.3 ± 0.5	(0.05)
cis-DDP	4.0 ± 0.4	58.0 ± 4.0	(14.5)	13.0 ± 1.3	50.0 ± 3.1	(3.8)	56.0 ± 3.2	128.0 ± 4.3	(2.3)
trans-DDP	>200	>200		>200	>200		>200	>200	

compound **1** exhibited non–cross-resistance only in the pair of cell lines CH1/CH1cisR (RF = 0.8). In contrast, compound **2** was able to circumvent cisplatin resistance in the three pairs of cell lines A2780/A2780cisR, CH1/CH1cisR, and 41M/41McisR (RF = 1.5, 0.8, and 0.05, respectively). Similar results were obtained for the RF values when the period of incubation with the platinum drugs was extended to 72 h; however, these values were slightly increased over those obtained at 24 h of incubation (data not shown).

Platinum Accumulation in 41McisR Cells. It is known that 41McisR cells are primarily resistant to cisplatin through decreased platinum accumulation (Kelland et al., 1992). Because the cytotoxicity data indicate that compound **2** circumvents cisplatin resistance in this cell line, we have measured by TXRF the intracellular platinum levels after exposure of 41McisR and 41M cells to 10 μM and equitoxic concentrations (IC_{50}) of compounds **1**, **2**, and cisplatin for several periods of time. Figure 2 shows that the intracellular accumulation of compounds **1**, **2**, and cisplatin increased as a function of the period of drug treatment in 41McisR cells. Thus, the intracellular levels of compounds **1**, **2**, and cisplatin were, respectively, 0.15, 0.30, and 0.05 μmol/2 $\times$ 10^6 cells after 1 h of incubation and progressively increased to reach, respectively, 0.42, 0.60, and 0.30 μmol/2 $\times$ 10^6 cells after 5 h of incubation, 0.55, 0.70 and 0.45 μmol/2 $\times$ 10^6 cells after 12 h of incubation, and 0.60, 0.75, and 0.47 μmol/2 $\times$ 10^6 cells after 24 h of incubation. Across the four times of incubation tested, platinum accumulation levels for compound **1** and cisplatin were an average of 72 and 48% in the 41McisR line compared with the parent 41M line, respectively. However, there was no significant difference ($P < 0.05$) in platinum accumulation between 41M and 41McisR lines, at any period of treatment with compound **2** (data not shown). In addition, platinum accumulation levels at concentrations equal to the IC_{50} values of compound **1** and cisplatin were even lower than at 10 μM in 41McisR cells compared with 41M cells. For instance, after 24 h of incubation with the 41McisR cell line at concentrations equal to their corresponding IC_{50} values, intracellular platinum levels for compound **1** and cisplatin were 1.20 μmol/2 $\times$ 10^6 cells and 6.40 μmol/2 $\times$ 10^6 cells, respectively. In contrast, intracellular platinum levels in the 41M cell line for compound **1** and cisplatin were 2.80 μmol/2 $\times$ 10^6 cells and 32.00 μmol/2 $\times$ 10^6 cells, respectively. No significant difference ($P < 0.05$) in platinum accumulation between 41M and 41McisR lines were detected at the IC_{50} of compound **2** for any of the periods of incubation tested. These data indicate that compound **2** is more efficiently accumulated in 41McisR cells than both compound **1** and cisplatin. So, the results suggest that circumvention of cisplatin resistance in 41McisR cells by compound **2** is related to its higher efficiency of intracellular accumulation relative to the parent drug cisplatin.

Platinum Binding to DNA in CH1cisR Cells. The CH1cisR line may be considered a tumor cell model in which cisplatin resistance may be studied at the level of drug-DNA adducts. In fact, enhanced removal of or increased tolerance to platinum-DNA adducts mainly contributes to the resistance of CH1cisR cells to cisplatin (Kelland et al., 1994). Because compounds **1** and **2** are both able to circumvent cisplatin resistance in CH1cisR cells, we quantified by TXRF platinum-DNA binding levels in CH1cisR cells and CH1 cells incubated with 10 μM or the concentrations equal to the IC_{50} values of compounds **1**, **2**, and cisplatin for several periods of time. Figure 3 shows that the binding of compounds **1**, **2**, and cisplatin began to be quantifiable only after 1 h of incubation (60, 25, and 40 nmol Pt/g DNA, respectively). Platinum binding to DNA for compounds **1**, **2**, and cisplatin progressively increased to reach, respectively, 375, 300, and 450 nmol Pt/g DNA after 5 h of incubation and 520, 450, and 580 nmol Pt/g DNA after 12 h of incubation. After 24 h of incubation with compounds **1**, **2** and cisplatin, platinum binding to DNA of CH1cisR cells was 600, 580, and 640 nmol Pt/g DNA, respectively. Therefore, Fig. 3 shows that in CH1cisR cells, the levels of DNA binding of compounds **1** and **2** are slightly

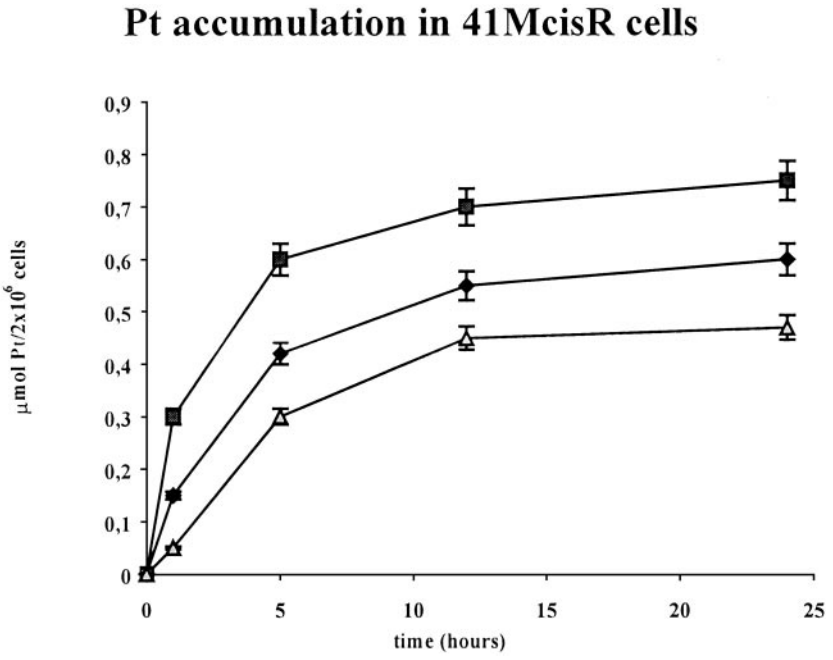

Fig. 2. Kinetics of platinum accumulation of 10 μM of compounds **1** (♦), **2** (■), and *cis*-DDP (△) in 41McisR cells as measured by TXRF. The results are expressed as means ± S.D. (n = 8, four independent experiments with duplicate cultures).

lower than those of cisplatin. Across the four times of incubation tested, no significant difference in the numbers of platinum DNA adducts were observed between CH1cisR and CH1 cell lines for compounds **1** and **2** and cisplatin (data not shown). On the other hand, after 24 h of incubation at equitoxic concentrations (IC_{50}), platinum-DNA binding levels in CH1cisR cells were 3-fold higher for cisplatin than for compounds **1** and **2** (2560, 840, and 820 nmol of Pt/g of DNA, respectively). Therefore, the data indicate that because the DNA binding of compounds **1** and **2** is lower than that of cisplatin, the overall levels of DNA platination do not seem to be responsible for the circumvention of cisplatin resistance displayed by compounds **1** and **2** in CH1cisR cells.

DNA ICL Formation in CH1cisR Cells. It has been reported that resistance of tumor cells to cisplatin may be associated with increased repair efficiency of DNA interstrand cross-links (Petersen et al., 1996). So, we have analyzed in CH1cisR cells whether the ability of compounds **1** and **2** to form DNA ICLs may account for their circumvention of cisplatin resistance. CH1cisR cells were exposed to equitoxic doses (IC_{50}) of compounds **1**, **2,** and cisplatin and the ICL indexes (see *Materials and Methods*) induced by these platinum complexes were determined after several periods of incubation. Figure 4 shows that compounds **1**, **2**, and cisplatin produced increasing levels of ICLs when the period of drug treatment increased. Interestingly, compounds **1** and **2** induced a higher amount of ICLs than cisplatin at all the periods of incubation tested. Moreover, the quantity of DNA ICLs formed by compound **1** is slightly higher than that of compound **2**. For instance, after 5 h of drug treatment, the ICL index of compounds **1** and **2** was 4.4- and 2.8-fold higher, respectively, than that of cisplatin. After 24 h of drug treatment, the ICL index of compounds **1** and **2** was 3.0- and 2.6-fold higher, respectively, than that of cisplatin. No significant difference ($P < 0.05$) in ICL indexes was observed for compounds **1** and **2** in the CH1cisR line compared with the parental CH1 cell line. Interestingly, however, after 24 h of incubation at the IC_{50} of cisplatin, there was an average of only 48% ICL formed by the drug in the CH1cisR line versus the CH1 line. In fact, ICL units/Dalton $\times 10^9$ were 0.45 in the CH1cisR line and 0.94 in the CH1parent line. These data indicate that the higher efficiency of DNA ICLs formation of compounds **1** and **2** relative to cisplatin may be at least in part responsible for the circumvention of cisplatin resistance shown by both *trans*-platinum compounds in CH1cisR cells.

Apoptosis Induction in CH1cisR Cells. As indicated above, our data suggest that the efficiency of DNA ICL formation of compounds **1** and **2** may be related with the circumvention of cisplatin resistance shown by both *trans*-platinum compounds in CH1cisR cells. We have also analyzed by annexin V-PI flow cytometry whether compounds **1** and **2** are able to induce apoptosis in CH1cisR cells after 24 h of incubation at equitoxic concentrations (IC_{50} values). Annexin V binds phosphatidyl serine residues, which are asymmetrically distributed toward the inner plasma membrane but migrate to the outer plasma membrane during apoptosis (Fuertes et al., 2003). Figure 5 shows that treatment of CH1cisR cells with compounds **1** and **2** induced a greater increase in the Annexin V-positive/PI-negative cell population (right bottom quadrant) than treatment with *cis*-DDP did (Fig. 5, B–D, respectively). The annexin V-positive/PI-negative cell population constitutes the fraction of apoptotic cells, and the percentage of cells undergoing apoptosis may be calculated from the dots of the right bottom quadrants of the scattergrams of Fig. 6 (Van Engeland et al., 1998). The data show that at concentrations equal to the IC_{50} values, compounds **1** and **2** induced apoptosis in 29 and 25% of CH1cisR cells, respectively. The percentage of cells undergoing necrosis was calculated as the sum of the dots of the right upper plus left upper quadrants in the scattergrams (Montero et al., 2002). Therefore, at concentrations equal to their IC_{50} values, compounds **1** and **2** also induced necrotic cell death in 21 and 25% of the CH1cisR cells, respectively. In addition, the percentage of apoptotic cells induced by compounds **1** and **2** was approximately 2-fold higher than that induced by *cis*-DDP (13%). Moreover, cisplatin induced cell death by necrosis in 37% of the population of CH1cisR cells.

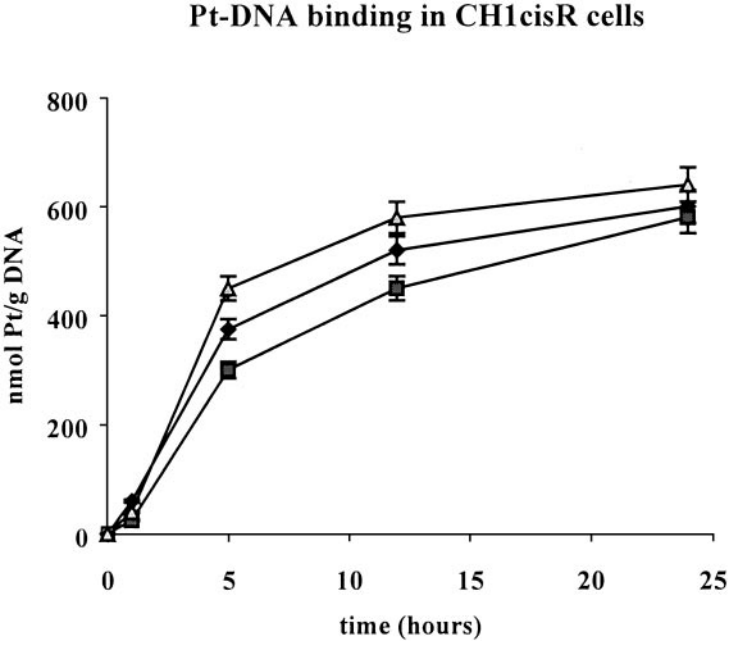

Fig. 3. Kinetics of platinum binding to DNA of 10 μM of compounds **1** (♦), **2** (■), and *cis*-DDP (△) in CH1cisR cells as measured by TXRF. The results are expressed as means ± S.D. (n = 8, four independent experiments with duplicate cultures).

These data indicate that circumvention of cisplatin resistance by compounds **1** and **2** may also be related to their ability to induce apoptosis.

DNA ICL Formation in Linear pUC8 Plasmid DNA. We had previously reported that compound **1** induces a greater number of ICLs in linear pBR322 plasmid DNA as well as in oligonucleotide duplexes relative to *cis*-DDP (Pérez et al., 2000a; Montero et al., 2002). We have also compared the efficiency of ICL formation of compounds **1** and **2** in linear pUC8 DNA. Figure 6 shows the kinetics of ICL formation in linear pUC8 of both *trans*-platinum compounds after several incubation times at a molar ratio of Pt to nucleotide (r_i) of 0.05. It may bee seen that after 5 h of incubation with compounds **1** and **2** there is not evidence of ICLs formation because their corresponding DNA bands migrate with an electrophoretic mobility similar to that of single-stranded DNA of control denatured pUC8 (lanes 3, 4, and 2, respectively). In addition, both *cis*- and *trans*-DDP are also unable to form ICLs in linear pUC8 DNA after 5 h of incubation at $r_i = 0.05$ (lanes 11 and 12, respectively). However, after 10 h of incubation, compound **1** forms some ICLs because its DNA band migrates with a electrophoretic mobility similar to that of double-stranded DNA (dsDNA) of control native linear pUC8 (lanes 6 and 1, respectively). In contrast, compound **2** is still unable to form ICLs; its DNA band migrates as single-stranded DNA (lane 5). After 24 and 48 h of incubation, both compounds **1** and **2** forms ICLs in linear pUC8 DNA because their DNA bands migrate as dsDNA (lanes 7, 8, 9, and 10, respectively). Interestingly, the electrophoretic mobility decreases and the intensity increases for the bands of dsDNA of compounds **1** and **2** (compare lanes 8 and 7 with lanes 9 and 10, respectively) when the period of incubation increases as an indication that the number of DNA interstrand adducts increases. These data indicate that the kinetics of ICL formation in linear pUC8 DNA of compound **1** is faster than that of compound **2**.

In Vivo Antitumor Efficacy in CH1 Xenografts. In view of the interesting cytotoxic properties of compounds **1** and **2**, the in vivo antitumor activity of both *trans*-Pt compounds was also determined against CH1 human ovarian tumor xenograft implanted in mice. Approximately 2-mm^2 fragments of CH1 xenografts were implanted into adult female nude mice. Once the tumors had grown enough (6 to 8 mm of diameter) mice were randomized into control or treatment groups and therapy started (day 0). The drugs were administered by single intraperitoneal injection at their predetermined MTDs. The MTDs were 30 and 15 mg/kg for compounds **1** and **2**, respectively. Compound **1** was administered on days 0 and 7 and compound **2** on days 0, 7, and 14. The results of Fig. 7 indicate that the mice treated with the MTD of compound **2** had a significant delay in CH1 tumor progression relative to control untreated mice. In fact, the data of Fig. 7 show that at day 10, the tumor volumes of control mice were about 3-fold higher than those of mice treated with compound **2** ($P < 0.05$). Moreover, the tumor volume reached by control mice at day 10 was similar to that reached at day 15 by mice treated with compound **2**. Interestingly, however, compound **1** did not show any statistically significant effect in CH1 tumor progression relative to control mice. These results indicate that compound **2** shows a promising level of in vivo antitumor activity against a human tumor xenograft in comparison to both the Pt(II) counterpart (compound **1**) and transplatin (Kelland et al., 1995).

In Vitro Binding of Compounds 1 and 2 to Serum Albumin. Albumin plays a central role in the molecular pharmacology of drugs used in cancer chemotherapy. In fact, HSA interferes with certain anticancer agents, changing their biological effectiveness (Trynda-Lemiesz et al., 1999). Figure 8 shows the binding of compounds **1**, **2**, *cis*-DDP, and *trans*-DDP to HSA as a function of incubation time over a 14-day period. It may be observed that binding of compound **1** to HSA was the most effective case. When the platinum complexes were incubated at 5-fold excess to HSA, the amounts of platinum bound to HSA after 1 day of incubation were similar for all the compounds. However, after 3 days of incubation, 1.94 mol of compound **1** was bound per mol of HSA, whereas only 0.77

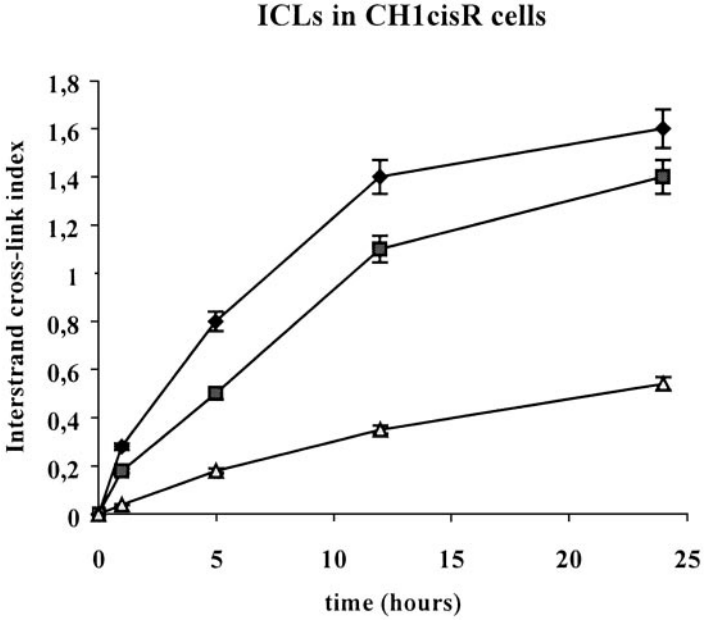

Fig. 4. Alkaline elution plots showing the time course for formation of DNA ICLs in CH1cisR cells at equitoxic doses (IC_{50}) of compounds **1** (♦), **2** (■), and *cis*-DDP (△). The results are expresses as means ± S.D. ($n = 8$, four independent experiments with duplicate cultures).

mol of compound **2** and 0.74 mol of *cis*-DDP were bound, respectively. However, binding of *trans*-DDP to HSA was similar to that of compound **1**: 1.65 mol of *trans*-DDP per mol of protein. The differences in binding to HSA increased with the period of incubation of the platinum drugs with the protein. So, after 7 days, 3.77 mol of compound **1** was bound per mol of HSA, whereas only 1.06 mol of compound **2** and 0.86 mol of *cis*-DDP were bound. Binding of *trans*-DDP to HSA was 2.22 mol of *trans*-DDP per mol of protein. After 14 days, 4 mol of compound **1** was bound per mol of HSA, whereas only 1.36 mol of compound **2** and 1.86 mol of *cis*-DDP were bound. Binding of *trans*-DDP to HSA was 2.79 mol of *trans*-DDP per mol of protein. These data indicate that the binding ability of compound **1** toward HSA is higher not only than that of compound **2** but also than those of *cis*- and *trans*-DDP.

In Vivo Binding of Compounds 1 and 2 to Whole Plasma Proteins. We also analyzed the in vivo binding of compounds **1** and **2** to whole plasma proteins in Balb C mice. Therefore, we measured, by TXRF, total platinum concentrations in plasma as well as ultrafiltrable platinum concentrations. Pharmacokinetic studies after i.p. administration of compounds **1** and **2** to mice at a dose of 15 mg/kg revealed a biexponential decay in plasma. In fact, ultrafiltrable platinum in plasma after i.p administration of a dose of 15 mg/kg of compounds **1** and **2** declines rapidly ($t_{1/2\alpha}$ of 32 and 34 min, respectively), followed by a slow elimination ($t_{1/2\beta}$ of 42 and 44 h, respectively). Table 2 shows the concentration of ultrafiltrable platinum as well as the percentage of total plasma platinum bound to plasma proteins for compounds **1** and **2** during the first 3 h after administration of the drugs to mice. It may be observed that 15 min after dosing with compounds **1** and **2**, the

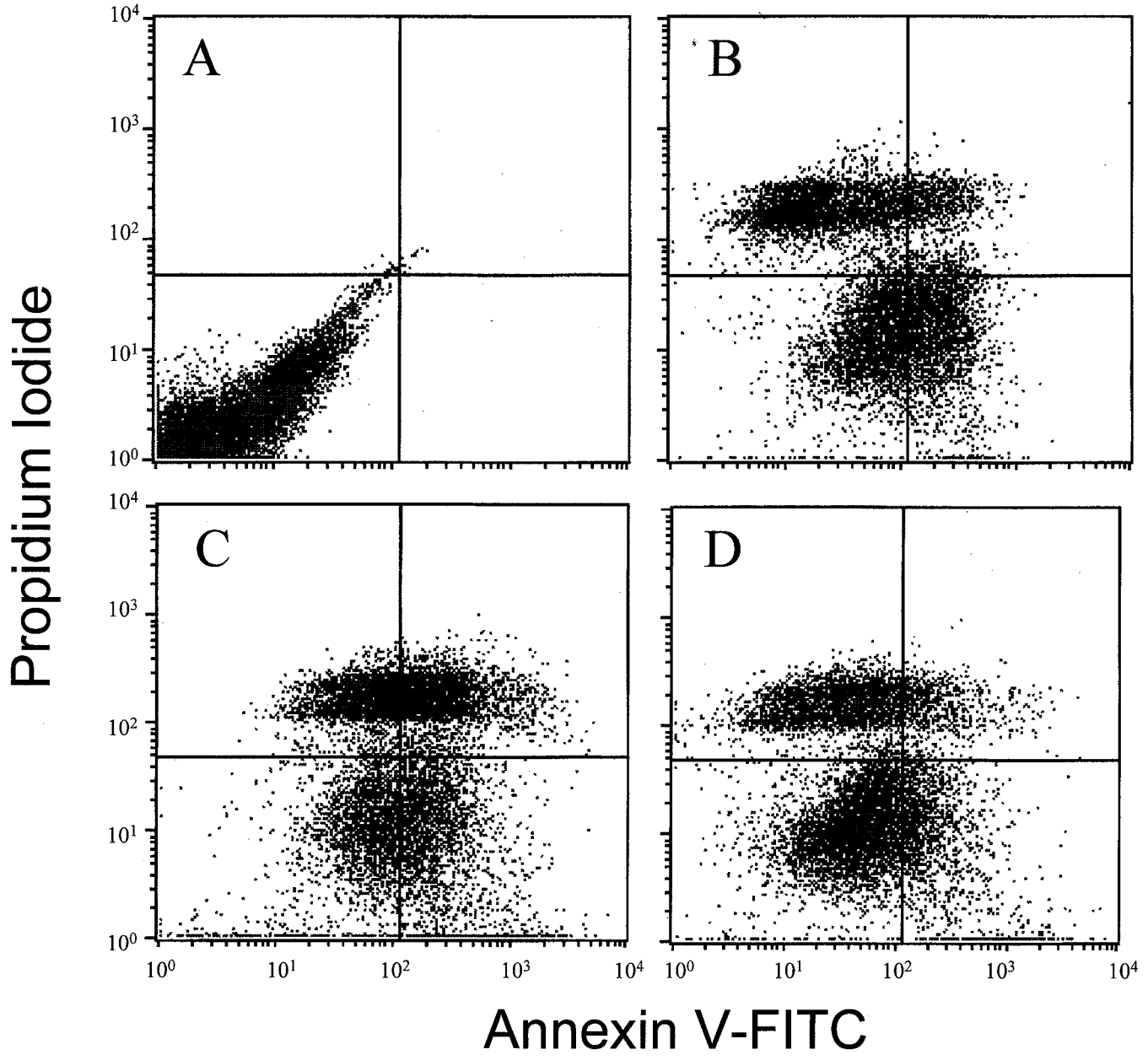

Fig. 5. Quantification of apoptosis after 24-h exposure to concentrations equal to the IC_{50} values of the platinum drugs. Representative annexin V/PI fluorescence scattergrams showing CH1cisR cells. Control (A), compound **1** treatment (B), compound **2** treatment (C), and *cis*-DDP treatment (D).

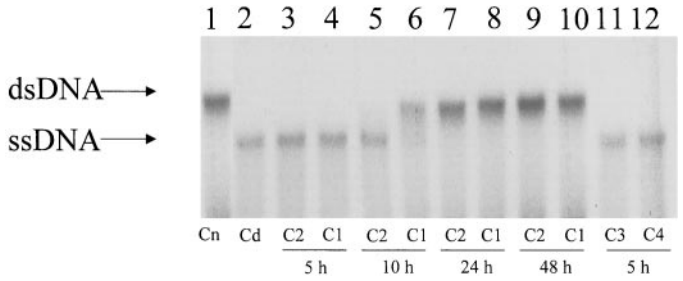

Fig. 6. Kinetics of DNA ICLs formation in linear pUC8 DNA by compound **2** (lanes 3, 5, 7, and 9) and compound 1 (lanes 4, 6, 8, and 10) at r_i = 0.05. Cn, control native linear pUC8 DNA (ds DNA, lane 1); Cd, control denatured native linear pUC8 DNA (ss DNA, lane 2). C3 and C4, linear pUC8 DNA incubated with *cis*-DDP and *trans*-DDP at r_i = 0.05, respectively (lanes 11 and 12). r_i, input molar ratio of Pt to nucleotide.

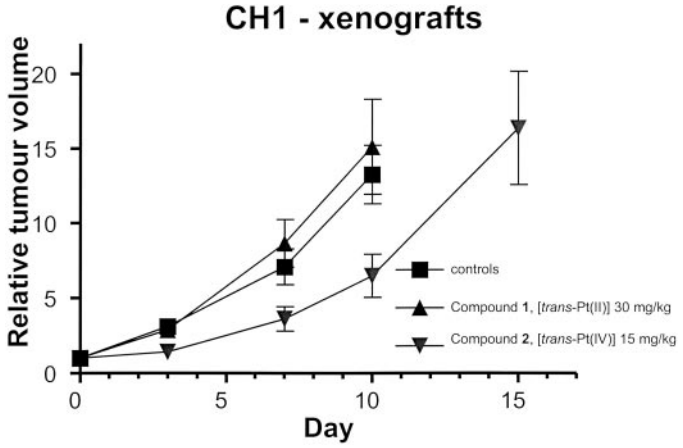

Fig. 7. Tumor growth curves (expressed a mean relative tumor volume) for mice bearing CH1 human ovarian carcinoma s.c. xenografts treated when tumor reached 6–8 mm of diameter. ■, compound **1** (30 mg/kg i.p. single injection on days 0 and 7); ▼, compound 2 (15 mg/kg i.p. single injection on days 0, 7, and 14); ▲, control untreated tumor-bearing mice. Values represent mean ± S.D.

plasma concentration of free platinum is 2.5-fold higher for compound **2** (11.20 ± 3.20 μg/ml) than for compound **1** (4.58 ± 1.20 μg/ml). Moreover, the percentage of total plasma platinum bound to plasma proteins is 2-fold lower for compound **2** (35.51 ± 4.52%) than for compound **1** (70.21 ± 2.20%). Similarly, 1 h after i.p. administration of compounds **1** and **2,** the plasma concentration of free platinum was 2.5-fold higher for compound **2** than for compound **1**. The percentage of plasma platinum bound to plasma proteins was 1.7-fold lower for compound **2** than for compound **1**. Two and 3 h after i.p. administration of compounds **1** and **2** to mice, the plasma concentration of free platinum markedly decreased for both *trans*-platinum compounds, but the percentage of plasma platinum bound to plasma proteins was still lower for compound **2** relative to compound **1**. In summary, the data in Table 2 indicate that during the first hour after i.p. administration of compounds **1** and **2** to mice, binding of compound **1** to plasma proteins was significantly higher than that of compound **2**.

Discussion

The original standard structure-activity relationships for platinum drugs established that antitumor activity was found only in *cis*-Pt complexes, not in their corresponding *trans* isomers [e.g., *cis*-DDP versus *trans*-DDP (Connors et al., 1979)]. However, since the first report of cytotoxic *trans*-platinum complexes (Farrell et al., 1989), several research groups have pursued the concept of activating the *trans* geometry. Therefore, substitution of the NH_3 inert ligands in transplatin has led to an increasing number of biologically active *trans*-platinum complexes, especially in terms of in vitro growth inhibition of tumor cells (Pérez et al., 2000b). The main purpose of this work was to compare the cellular and molecular pharmacological properties of the new *trans*-Pt(IV) complex, *trans*-[$PtCl_2(OH)_2$ (dimethylamine)(isopropylamine)] (compound **2**), with those of its corresponding *trans*-Pt(II) complex, *trans*-[$PtCl_2$(dimethylamine)(isopropylamine)], (compound **1**). The results reported here indicate that compound **2** exhibits a greater spectrum of cytotoxicity than compound **1**. In fact, compound **2** is able to strongly circumvent cisplatin resistance in two ovarian

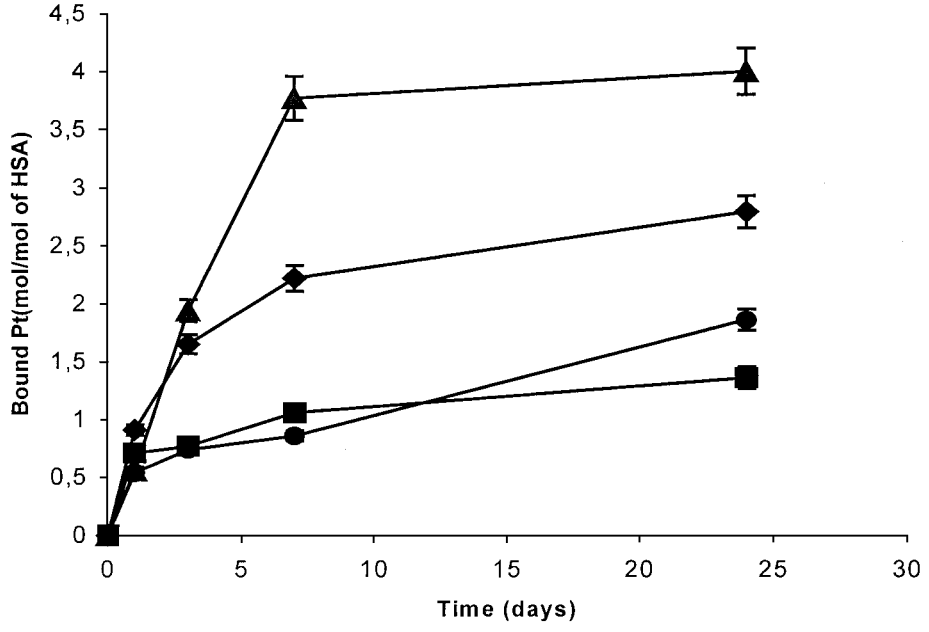

Fig. 8. Binding of platinum complexes to HSA as a function of incubation time over a 14-day period. Pt content was determined by TXRF. Compound **1** (▲), compound **2** (●), *cis*-DDP (■), and *trans*-DDP (♦). Concentration of HSA, 0.05 μM; concentration of platinum complexes, 0.25 μM; molar ratio of Pt/HSA, 5. The results are expressed as means ± S.D. (n = 8, four independent experiments with duplicate samples).

TABLE 2
Free and bound platinum
Time-concentration courses of ultrafiltrable free platinum in plasma and of platinum bound to plasma proteins as measured by TXRF after i.p. administration of compounds **1** and **2** to Balb C mice at a dose of 15 mg/kg. Values are means ± S.D. (n = 6 animals).

Time after Injection	Ultrafiltrable Platinum in Plasma		Total Plasma Platinum Bound to Plasma Proteins	
	1	**2**	**1**	**2**
	$\mu g/ml$		%	
0.25 h	4.58 ± 1.20	11.24 ± 3.20	70.21 ± 2.20	35.51 ± 4.52
1 h	0.62 ± 0.12	1.55 ± 0.22	88.04 ± 2.61	51.44 ± 5.22
2 h	0.13 ± 0.03	0.40 ± 0.12	96.41 ± 1.13	84.65 ± 2.13
3 h	0.05 ± 0.01	0.15 ± 0.02	99.24 ± 0.24	92.31 ± 0.62

tumor cell lines endowed with different mechanisms of resistance (41McisR and CH1cisR). However, compound **1** is able to circumvent cisplatin resistance only in CH1cisR cells. Our cytotoxicity data suggest that compound **2** circumvents cisplatin resistance in 41McisR cells mainly through reduced drug accumulation (RF = 0.05). In fact, intracellular platinum levels measured in 41McisR after several periods of treatment with 10 μM or concentrations equal to the IC_{50} values of the platinum complexes indicate that intracellular accumulation of compound **2** is 1.5- to 2-fold higher than that of *cis*-DDP. Intracellular accumulation of compound **2** is also 1.25- to 1.5-fold higher than that of compound **1**. Moreover, there was no significant difference ($P < 0.05$) in platinum accumulation between 41M and 41McisR lines at any period of treatment with compound **2.** On the other hand, compounds **1** and **2** exert a remarkable cytotoxic effect against CH1cisR cells, which show acquired cisplatin resistance through enhanced DNA repair/enhanced tolerance (RF = 0.8 for both *trans*-Pt compounds). Of interest is the observation that the *cis*-Pt(II) analog of compound **1** is devoid of cytotoxic activity against CH1cisR cells (Pantoja et al., 2002). TXRF measurements show that the levels of platinum binding to DNA for compounds **1** and **2** are slightly lower than those of *cis*-DDP in CH1cisR cells incubated with 10 μM (a drug concentration located between the IC_{50} values of compounds **1** and **2**) or with concentrations equal to the IC_{50} values of the Pt complexes. Similar results were obtained in the parental CH1 cell line. Therefore, the amount of DNA platination does not seem to be involved in the circumvention of cisplatin resistance exhibited by compounds **1** and **2** in CH1cisR cells. However, there are strong differences in the number of DNA ICLs formed by both compounds **1** and **2** in comparison with *cis*-DDP in both CH1cisR and CH1 cells as measured by alkaline filter elution. After 24 h of incubation at equitoxic doses (IC_{50} values), the ICL indexes of compounds **1** and **2** in CH1cisR cells are 3- and 2.6-fold higher than that of *cis*-DDP, respectively. Similar results were obtained for both *trans*-platinum compounds in the parental CH1 cell line. In contrast, after 24 h of incubation at the IC_{50} of cisplatin, there was an average of only 48% ICL formed by the drug in the CH1cisR line versus the 41M line. So, it is likely that the ICLs formed by compounds **1** and **2** may be less efficiently repaired in CH1cisR cells than those of cisplatin because of the different nature of the DNA ICL lesion. In fact, we have previously reported that compound **1** forms DNA ICLs between complementary guanine and cytosine residues, whereas the ICLs of cisplatin are formed between two guanines (Montero et al., 2002). On the other hand, our assays in linear pUC8 plasmid DNA indicate that the kinetics of DNA ICL formation of compound **1** is faster than that of compound **2**, which may be related to the fact that Pt(IV) complexes must be reduced to Pt(II) complexes before binding to DNA. In fact, it is currently thought that Pt(IV) compounds are prodrugs that become active after in vivo reduction to Pt(II) compounds (Fuertes et al., 2002). Because DNA ICLs have often been involved in the cytotoxicity of Pt(II) and Pt(IV) complexes, it is likely that the circumvention of cisplatin resistance shown by compounds **1** and **2** in CH1cisR cells is related to their ability to form this particular type of DNA adduct (Kelland et al., 1994; Montero et al., 2002). Increasing evidence indicates that in some tumor cell lines, an important cause of cisplatin resistance may be associated with the inability of this drug to induce apoptosis (González et al., 2001). We show in this article that at equitoxic doses (IC_{50}), compounds **1** and **2** induce a higher amount of apoptotic cells and therefore a lower amount of necrotic cells than *cis*-DDP in the CH1cisR line. Moreover, the number of apoptotic cells induced by compounds **1** and **2** correlates with the levels of DNA ICLs formed by both drugs in CH1cisR cells. It is accepted that DNA damage and subsequent induction of apoptosis may be the main mechanism of cytotoxic activity of platinum complexes (González et al., 2001). The results reported here indicate that in CH1cisR cells, the higher ability to induce apoptosis of compounds **1** and **2** in comparison with *cis*-DDP may be associated with their higher efficacy of DNA ICL formation. On the other hand, the annexin V/PI fluorescence data suggest that there is a relationship between the cytotoxic potency of the platinum drugs and their ability to induce apoptosis in cisplatin-resistant cells. So, as previously reported in A2780cisR cells, we have also found in CH1cisR cells that the lower the dose of drug needed to kill cisplatin-resistant cells, the higher the percentage of apoptosis induction (Montero et al., 2002).

Although compounds **1** and **2** show remarkable activity against human ovarian carcinoma lines in vitro, only compound **2** is able to inhibit the growth of CH1 xenografts in mice. So, our data support previous findings that indicated that in pairs of *trans*-Pt(II) and *trans*-Pt(IV) complexes showing in vitro activity, only the *trans*-Pt(IV) counterparts were endowed with in vivo antitumor activity. Moreover, the most active platinum complexes were the dihydroxo-Pt(IV) complexes and not the tetrachloro-Pt(IV) analogs. (Kelland et al., 1994, 1995). It has been reported that the lack of antitumor activity of most *trans*-Pt(II) complexes including *trans*-DDP might be related to their chemical instability in biological media as well as to their high rates of binding to serum proteins and their high rates of biotransformation in the liver, which would preclude the delivery of enough amount of active species to the tumor site (Kelland et al., 1995; Pérez et al., 2000b). However, it should be pointed out that there are relevant examples of *trans*-Pt(II) complexes endowed with important in vivo antitumor activity as is the case of *trans*-$[PtCl_2(iminoether)_2]$ (Leng et al., 2000). On the other hand, the interaction of anticancer drugs with blood constituents, particularly with serum albumin, may have a major impact on drug pharmacology and efficacy. In fact, the differences in efficacy, activity, and toxicity between the most widely used

platinum antitumor drugs cisplatin and carboplatin may be discussed in relation to the differences of plasma protein binding to both compounds (O'Dwyer et al., 2000). The data presented here clearly indicate that binding of compound **2** to HSA is much lower than that of compound **1**. Moreover, our data also show that *trans*-DDP exhibits higher binding to HSA than compound **2** and *cis*-DDP. So, it is likely that as previously reported for transplatin, inactivation of compound **1** through binding to serum albumin may also play an important role in the lack of in vivo antitumor activity shown by this drug (Trynda-Lemiesz et al., 1999). On the other hand, our in vivo studies on the extent of binding of compounds **1** and **2** to plasma proteins after i.p. administration to mice show that there are remarkable pharmacokinetic differences between both drugs. Thus, the percentage of binding of compound **1** to plasma proteins during the period 15 min to 1 h after drug treatment is 2.5-fold higher than that of compound **2**. Because albumin is the major component of plasma proteins (Trynda-Lemiesz et al., 1999), these data support the lower in *vitro* binding to HSA of the *trans*-Pt(IV) compound **2** relative to the *trans*-Pt(II) compound **1**. Therefore, these results suggest that the lack of in vivo antitumor activity of compound **1** may be related to its high rate of binding to plasma proteins (mainly albumin), which may strongly decrease the effective concentration of drug that reaches the tumor cell.

In summary, the results reported here show that the *trans*-Pt(IV) compound **2**, *trans*-[$PtCl_2(OH)_2$(dimethylamine)(isopropylamine], exhibits in vitro cytotoxicity as well and in vivo antitumor activity. However, the data show that its *trans*-Pt(II) analog, compound **1**, lacks in vivo antitumor activity, although it possesses cytotoxic activity. In addition, the results indicate that the reactivity of compound **2** is lower than that of compound **1**, because it is much more inert to ligand substitution. Altogether, our results support the hypothesis that because of their decreased reactivity, Pt(IV) complexes may act as prodrugs, which reach efficiently the tumor site to be transformed into active Pt(II) species within the cell.

Acknowledgments

We thank Johnson-Matthey Chem-Ltd (Reading, UK) for their generous gift of K2PtCl4.

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