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The Formation of DNA Interstrand Cross-Links by a Novel Bis-[Pt_2Cl_4(diminazene aceturate)$_2$]Cl_4·$4H_2O$ Complex Inhibits the B to Z Transition

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ABSTRACT

We present data demonstrating that the cytotoxic compound [Pt_2Cl_4(diminazene aceturate)$_2$]Cl_4·$4H_2O$ (Pt-berenil) circumvents cisplatin resistance in ovarian carcinoma cells. The analysis of the interaction of Pt-berenil with linear and supercoiled DNA indicates that this compound induces the formation of a large number of covalent interstrand cross-links on DNA and that this number is significantly higher than that produced by *cis*-diamminedichloroplatinum(II) (*cis*-DDP). Renaturation experiments, interstrand cross-link assays, and electron microscopy indicate that the kinetics of DNA interstrand cross-link formation caused by Pt-berenil binding is faster than that caused by *cis*-DDP at similar levels of platinum bound to DNA. Furthermore, the number of DNA interstrand cross-links in Pt-berenil-DNA complexes is influenced by supercoiling. Circular dichroism experiments show that Pt-berenil strongly inhibits the B-DNA-to-Z-DNA transition of poly(dG-m^5 dC)·poly(dG-m^5dC) at salt concentrations (3 mM $MgCl_2$) at which the native methylated polynucleotide readily adopts the Z-DNA conformation, which suggests that the induction of interstrand cross-links by Pt-berenil inhibits the Z-DNA transition. On the basis of these results, we propose that bis(platinum) compounds with structure similar to Pt-berenil may act as blockers of DNA conformational changes and may also display activity in cisplatin-resistant cells.

It has been postulated that the DNA interstrand cross-link adducts formed by platinum drugs may contribute to the cytotoxicity of these compounds (Leng and Brabec, 1994). In fact, the interstrand cross-links formed in DNA by *cis*-diamminedichloroplatinum(II) (*cis*-DDP) between guanine residues in opposite strands preferentially inhibit DNA or RNA polymerases in vitro (Lemaire et al., 1991; Vrána et al., 1996). Moreover, the binding of platinum drugs on DNA may induce conformational changes of the double helix structure that may be responsible for the drug activity (Johnson et al., 1989; Leng and Brabec, 1994; Pil and Lippard, 1997). The secondary structure most commonly adopted by DNA in vivo is the B-form, which is right-handed; however, DNA may also adopt other conformations (Cantor, 1981). Thus, a new conformational state of the DNA, namely Z-DNA, characterized by a left-handed conformation of the helix, has been described (Pohl and Jovin, 1972; Herbert and Rich, 1996). It is now well established that DNA sequences that are in Z conformation or having potential to adopt the Z-DNA form can be found in biological systems (Nordheim et al., 1981; Nordheim and Rich, 1983; Lancilloti et al., 1987; Casasnovas et al., 1989; Wittig et al., 1991). Moreover, a possible role for Z-DNA in gene regulation has also been proposed (Jiménez-Ruíz et al., 1991).

Pohl and Jovin showed that at high salt concentration, poly(dG-dC)·poly(dG-dC) undergoes a cooperative, conformational transition from the B-DNA form toward the Z-DNA form (Pohl and Jovin, 1972). Later on, Behe and Felsenfeld noticed that the methylated polymer poly(dG-m^5 dC)·poly(dG-m^5dC) also undergoes a transition from the B form to the Z form, but it does so at lower salt concentrations (Behe and Felsenfeld, 1981). Malinge and Leng (1984) found that binding of the antitumor drug *cis*-DDP to poly(dG-m^5dC)·poly(dG-m^5dC) in B conformation induces a conformational change toward a distorted Z-like form and that binding of *cis*-DDP to the polynucleotide in Z conformation stabilizes its Z-DNA structure. Moreover, it has been also observed that a family of *trans*-bis(platinum) complexes provokes the B-to-Z transition in poly(dG-m^5dC)·poly(dG-m^5dC) (Johnson et

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ABBREVIATIONS: *cis*-DDP, *cis*-diamminedichloroplatinum(II); berenil, diminazene aceturate; CD, circular dichroism; Pt-berenil, [Pt_2Cl_4(diminazene aceturate)$_2$]Cl_4·$4H_2O$; r_i, input molar ratio of platinum to nucleotides; r_b, molar ratio of platinum bound to nucleotides; TXRF, total reflection X-ray fluorescence; DMEM, Dulbecco's modified Eagle's medium; kb, kilobase pair(s).

al., 1992). In contrast, the clinically ineffective *trans*-isomer of *cis*-DDP inhibits B-to-Z transition in DNA (Peticolas and Thomas, 1985; Rahmouni et al., 1985; Zaludová et al., 1997).

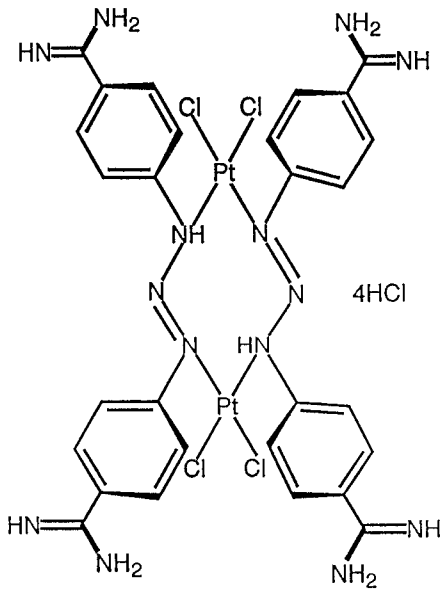
We have previously reported that the cytotoxic bis(platinum) complex $[Pt_2Cl_4(\text{diminazene aceturate})_2]Cl_4{\cdot}4H_2O$ (Pt-berenil) (Fig. 1) induces drastic changes on DNA secondary structure, leading to a compaction of the double helix, and that the diminazene aceturate (berenil) ligand directs the DNA binding of the Pt-berenil drug (González et al., 1996, 1997). We show herein that the interaction between the Pt-berenil compound and DNA results in the formation of a large number of covalent interstrand cross-links and that this number is significantly higher than that caused by *cis*-DDP-DNA interaction. Our data indicate, moreover, that the kinetics of DNA interstrand cross-link formation by Pt-berenil binding is faster than that of *cis*-DDP-DNA interaction and that the number of DNA interstrand cross-links in Pt-berenil-DNA complexes is influenced by supercoiling. To determine whether the large number of DNA interstrand cross-links formed by the drug affect the conformational changes of the double helix, we have analyzed the effect of Pt-berenil binding on the $MgCl_2$-induced B-DNA-to-Z-DNA transition of poly(dG-m^{5}dC)·poly(dG-m^{5}dC). We have chosen this methylated polymer because the dinucleotide sequence "m^{5}dC-dG" appears so frequently in eukaryotic DNA [it may account for more than half of all "d(CpG)" sequences (Razin and Riggs, 1980)]. Moreover, we have chosen $MgCl_2$ as the Z-DNA inducer because it allows work at Cl^- concentrations close to those found intracellularly (Rich et al., 1984). The results show that Pt-berenil strongly inhibits the salt-induced B-DNA-to-Z-DNA transition of poly(dG-m^5 dC)·poly(dG-m^{5}dC). We think that these results are interesting in view of the fact that the currently known platinum drugs, in contrast with Pt-berenil, induce the B-DNA-to-Z-DNA transition (Malinge and Leng, 1984; Johnson et al., 1992; Zaludová et al., 1997). Because we also present data here demonstrating that Pt-berenil is active in *cis*-DDP-resistant cells, we propose that other bis(platinum) compounds with structures similar to Pt-berenil might be designed in the search for new cytotoxic platinum compounds capable of circumventing cisplatin resistance.

Experimental Procedures

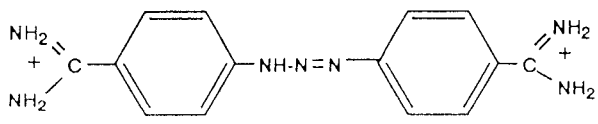
Materials. The stock solutions of the *cis*-DDP, berenil, and Pt-berenil compounds were prepared to a final concentration of 1 mg/ml. Pt-berenil was synthesized as described previously (González et al., 1996). Cis-DDP was kindly supplied by Bristol-Myers S.A. (Madrid, Spain). The Pt-berenil and *cis*-DDP compounds were dissolved in 10 mM $NaClO_4$. The drug solutions were freshly prepared before use.

Calf thymus DNA was purchased from Sigma Chemical Co. (St. Louis, MO). The pF18 plasmid DNA (2927 base pairs in length) containing a Z-DNA forming sequence (Jiménez-Ruíz et al., 1989) was isolated from the *Escherichia coli* JM83 strain by a modification of the alkaline lysis method (Maniatis et al., 1989). The *Bam*HI restriction enzyme and the Klenow fragment of *E coli* DNA polymerase I were obtained from Boehringer Mannheim (Madrid, Spain). [α-^{32}P]dCTP (10 mCi/ml) was purchased from Amersham International (Madrid, Spain).

Cytotoxicity Assays. The ovarian carcinoma cell lines A2780, A2780cisR, CH1, CH1cisR, SKOV-3 and SKOV-3cisR were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of newborn 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. A2780cisR, CH1cisR, and SKOV-3cisR are a sublines of their respective parent lines that have acquired cisplatin resistance (Kelland et al., 1993). Cell survival in compound-treated cultures was analyzed by using a system based on the tetrazolium compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, which is reduced by living cells to yield a soluble formazan product that can be assayed colorimetrically (Alley et al., 1988). Cells were plated in 96-well sterile plates, at a density of 10^4 cells/well in 100 μl of medium and were incubated for 3–4 h. Compounds dissolved in DMEM were added to final concentrations from 0.1 to 100 μM, in a volume of 100 μl/well. Ninety-six hours later, 50 μl of a freshly diluted 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (1/5 in culture medium) was



Platinum Berenil complex



Berenil

Fig. 1. Chemical structure of Pt-berenil (A) and berenil (B).

added at a concentration of 1 mg/ml into each well and the plates were incubated for 5 h at 37°C in a humidified 5% CO_2 atmosphere. Cell survival was evaluated by measuring the absorbance at 520 nm, using a Whittaker Microplate Reader 2001. All experiments were made in quadruplicate.

Platination Reactions. Calf thymus DNA, pF18 DNA, and poly(dG-m^5 dC)·poly(dG-m^5dC) at concentrations of 200 μg/ml were incubated with the platinum drugs at $r_i = 0.1$ in 10 mM $NaClO_4$ at 37°C. At various time periods (10 min, 30 min, 1 h, 2 h, 3 h, 18 h, and 24 h), aliquots of the reaction mixture were withdrawn and assayed by total X-ray fluorescence (TXRF) for platinum not bound to DNA (Niemann et al., 1990; Wobrauschek, 1994; González et al., 1996). The molar ratio of platinum bound to nucleotides (r_b) was calculated by subtracting the amount of free (unbound) platinum present in the reaction mixture. In agreement with previously reported data (González et al., 1996), it was observed that the kinetics of DNA platination by both Pt-berenil and *cis*-DDP were similar and that after 24 h of incubation, DNA platination reached a plateau because > 95% of the *cis*-Pt(II) centers from both Pt-berenil and *cis*-DDP were bound to DNA.

DNA Renaturation Experiments. Sonicated calf thymus DNA (average length of 500 base pairs) was dissolved in 0.02 × standard saline citrate (150 mM NaCl, 15 mM sodium citrate, pH 7.0) at a concentration of 20 μg/ml. Aliquots of 1 ml of the calf thymus DNA solution were modified by the drugs at r_b of 0.05. The DNA was melted at 45°C to 95°C. After 2 min at 95°C, control DNA was allowed to reanneal by decreasing the temperature from 95°C to 12°C above the melting temperature of native DNA (57 ± 0.5°C) at a 1°C/min rate. DNA modified by Pt-berenil or *cis*-DDP were exposed to comparable renaturation conditions. Thus, Pt-berenil-modified DNA or *cis*-DDP-modified DNA were allowed to reanneal by decreasing the temperature at a 1°C/min rate from 95°C to 12°C above the melting temperature of Pt-berenil-DNA (62 ±0.7°C) or *cis*-DDP-DNA complexes (55 ± 0.3), respectively. The decrease in chromicity upon renaturation was measured in a Beckman Acta CIII spectrophotometer attached to a temperature programmer. The percentage of DNA renaturation was calculated from the hypochromicity value at 95°C.

Interstrand Cross-Link Assay. To linearize the pF18 plasmid, the DNA was digested in 150 mM NaCl with 10 units/μg DNA of *Bam*HI (unique restriction site in pF18) at 37°C for 4 h. The plasmid DNA was 3′-end labeled by incubation with 2.5 mCi/mg DNA of [α-^{32}P]dCTP and 1.25 units/μg 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 DNA was added to the eluted solution of the labeled pF18 DNA to a final DNA concentration of 180 μg/ml. Afterwards, the DNA, at a concentration of 90 ng/ml, was incubated with the Pt-berenil and *cis*-DDP drugs in 10 mM $NaClO_4$ at r_i of 0.1 for different periods of incubation. Then, 10-μl aliquots were removed and the reaction was 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). At each period of incubation, the Pt bound to DNA (r_b) was monitored by TXRF. The DNA was melted for 10 min at 90°C and chilled on ice. Agarose gel (1.5%) electrophoresis in denaturing conditions was carried out at 20 V for 16 h (Maniatis et al., 1989). Interstrand cross-link formation was also detected in covalently closed circular pF18 plasmid DNA (density of supercoiling, $\sigma = -0.067$). Supercoiled pF18 plasmid was incubated with Pt-berenil or *cis*-DDP under the conditions indicated above and subsequently linearized with *Bam*HI endonuclease, 3′-end labeled with [α-^{32}P]dCTP, denatured by heat, and subjected to agarose gel electrophoresis in denaturing conditions. The gels were dried and autoradiographed. Band quantification was made using a Molecular Dynamics model 300A densitometer (Sunnyvale, CA).

Determination of the Cross-Linking Rate. The number of cross-links was estimated assuming a Poisson distribution [$(XL/fg = -\ln(1 - F_{DS})$] (Vos and Hanawalt, 1987). *XL/fg* represents the number of cross-links per fragment. F_{DS} results from dividing the peak of the integrated area of the double-stranded band by the sum of the integrated areas of the double-stranded and single-stranded bands. The (*XL/fg*)/fragment size ratio gives the number of cross-links per kilobase pair (kb).

Electron Microscopy of Pt-berenil-pF18 DNA Complexes. Supercoiled ($\sigma = -0.067$) or linear pF18 plasmid DNA (2 μg) were incubated at 37°C in 10 mM $NaClO_4$ with Pt-berenil at $r_i = 0.1$ until an $r_b = 0.05$ was achieved. Then, the DNA was precipitated with 2.5 volumes of cold ethanol and 0.1 volume of 3 M sodium acetate, pH 4.8. Afterward, the precipitate was washed with 100 μl of 75% ethanol. Sample aliquots of the Pt-berenil-pF18 DNA complexes (DNA concentration = 0.1 μg/ml) were denatured and extended as a monolayer on an aqueous hypophase according to the method of Sogo and Thoma (1989). The DNA interstrand cross-links were visualized in electron micrographs of the denatured supercoiled and linear DNA samples.

Determination of Platinum Bound to DNA In Vivo. Culture plates containing 10 ml of HeLa cells (human cervix carcinoma line) in DMEM (cell density, 2×10^5 cells/ml) were preincubated at 37°C for 24 h in an atmosphere of 5% CO_2. Then, 500 μl of culture medium containing the desired amount of drug to reach a final drug concentration of 35 μM (the IC_{50} value of Pt-berenil in HeLa cells) was added to the plates. Subsequently, the plates were incubated for 48 h at 37°C and 5% CO_2. Afterward, the cells were centrifuged at 1500 rpm for 5 min, washed with phosphate-buffered saline, resuspended in 400 μl of 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 during 15 min in a Microfuge (Hettich Microfuge, Madrid, Spain). The supernatants were then treated for 3 h at 37°C with 20 mg/ml of proteinase K in a buffer containing 150 mM NaCl, 40 mM Tris·HCl, pH 8.0, 40 mM EDTA, and 1% SDS. Finally, the DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 2.5 volumes of cold ethanol and 0.1 volumes of 3 M sodium acetate, washed with 75% ethanol, dried, and resuspended in 1 ml of water. The DNA content in each sample was measured by UV spectrophotometry at 260 nm. The platinum bound to DNA was determined by TXRF (Niemann et al., 1990; Wobrauschek, 1994). Experiments were carried out in triplicate.

In Vivo Detection of DNA Modifications Induced by Binding of Pt-Berenil. The HeLa cells cultivated and treated with 35 μM concentrations of the Pt-berenil drug for 24 h were harvested with trypsin/EDTA and centrifuged. The pellets were treated as described in the previous section to isolate the DNA. Aliquots (250 μl) of genomic DNA (60 ng/ml of DNA) were digested to completion with *Eco*RI. Subsequently, 20-μl aliquots were subjected to 1% agarose gel electrophoresis during 16 h at 1.5 V/cm and transferred to a nylon membrane (Amersham). Southern blot detection of genomic DNA was performed using a [^{32}P]-labeled 18 S ribosomal DNA probe (10 mCi/ml).

CD Spectroscopy. CD spectra were recorded in a JASCO J-600 spectropolarimeter interfaced to a 486 PC. Measurements were performed at 37°C using 1-cm path-length cells. Each spectrum represents the mean of three scans. Aliquots containing 15 μg/ml of poly(dG-m^5 dC)·poly(dG-m^5dC) were prepared from the stock solution. CD spectra were run in a range of wavelength from 220 to 320 nm and at a speed of 50 nm/min. Scans were recorded at 0.4 nm intervals.

Measurements of the salt-induced B-to-Z transition of aliquots of native poly(dG-m^5 dC)·poly(dG-m^5dC) dissolved in 10 mM $NaClO_4$ were carried out by adding the desired amount of concentrated $MgCl_2$ (1 M) to the DNA solution to reach a final $MgCl_2$ concentration of 1.3 mM or 3 mM. The CD spectrum of the polynucleotide was recorded at several incubation times. The midpoint times of the salt induced B-DNA-to-Z DNA transition of the polynucleotide were cal-

culated by plotting the ellipticity values at 250 nm and 290 nm versus time for each $MgCl_2$ concentration. The midpoint of the B-to-Z DNA transition is obtained when the ellipticities at 250 nm and 290 nm reach the same value (Cheng et al., 1983).

To evaluate the effect of the drugs on the $MgCl_2$-induced B-to-Z transition of poly(dG-m^5 dC)·poly(dG-m^5dC), aliquots of the polynucleotide were preincubated in 10 mM $NaClO_4$ with Pt-berenil or berenil for 3 h at 37°C to achieve an $r_b = 0.05$. Subsequently, $MgCl_2$ was added to reach a concentration of 1.3 mM or 3 mM. The CD spectra were recorded at the same temperature for different time intervals. All the experiments were done in triplicate.

Results

Cytotoxicity of Pt-Berenil in Cisplatin-Resistant Cells. We reported previously that Pt-berenil has a cytotoxic activity in a micromolar range similar to that of cisplatin against HL-60 and U-937 leukemic cells (González et al., 1996). In view of these encouraging results, we have tested the cytotoxicity of Pt-berenil against an ovarian carcinoma panel, including cells sensitive and resistant to *cis*-DDP. Table 1 shows the IC_{50} values of Pt-berenil and *cis*-DDP against A2780, A2780cisR, CH1, CH1cisR, SKOV, and SKOVcisR tumor cells (Kelland et al., 1993). It may be observed that Pt-berenil has a cytotoxic activity similar to that of *cis*-DDP in the A2780, CH1, and SOV cisplatin-sensitive cells (IC_{50} values for Pt-berenil of 0.20 μM, 0.70 μM, and 0.30 μM, respectively, versus IC_{50} values for *cis*-DDP of 0.30 μM, 3.30 μM, and 0.10 μM, respectively). Interestingly, however, in A2780cisR, CH1cisR, and SKOVcisR cells, Pt-berenil circumvents the acquired cisplatin resistance because this compound exhibits IC_{50} values of 0.70 μM, 0.36 μM, and 10.40 μM, respectively, versus IC_{50} values for *cis*-DDP of 3.30 μM, 0.65 μM, and 39.60 μM, respectively. Thus, the data suggest that the Pt-berenil complex may be considered to be a potential antitumor agent, because it has resistance factor indexes (see Table 1) that are significantly lower than cisplatin against the panel of human ovarian carcinoma cells tested.

DNA Interstrand Cross-Link Formation by Pt-Berenil. Previously reported data indicated that the binding of Pt-berenil to DNA significantly increases the melting temperature of the double helix above that produced by the ligand berenil and that the increase is incubation time-dependent (González et al., 1996). DNA renaturation experiments of Pt-berenil-DNA and berenil-DNA complexes suggested that Pt-berenil might induce covalent DNA interstrand cross-links because the percentage of DNA renaturation in Pt-berenil-DNA was much higher than that of berenil-DNA. The rationale of the experiments was that linked homologous sequences should have, in solution, renaturation kinetics faster than the same nonlinked sequences because of a "zippering" effect provided by a nucleation site for helix formation at the cross-link site. Figure 2 shows that the percentage and kinetics of reassociation of the DNA of the Pt-berenil-DNA complexes is significantly higher than that of the control DNA and that of the berenil-DNA complexes. Because after 25 min of reassociation, the chromicity of the Pt-berenil-DNA complexes was the same as that of the native Pt-berenil-DNA complexes, it is likely that most of the DNA of the complex had renatured and that interstrand nucleation points had been formed along the DNA. That the nucleation sites of the DNA from the Pt-berenil-DNA complexes might be caused by stable interstrand cross-links may be deduced from the fact that the bridging of berenil to DNA after denaturation of the helix does not affect the reassociation kinetics. The incubation time of the DNA with the Pt-berenil drug influences the number of the nucleation points, because the percentage of DNA reassociation is proportional to the period of complex formation (data not shown). A similar assay was performed with the drug *cis*-DDP, which has been shown to form interstrand cross-links at a low ratio (Lemaire et al., 1991). The results obtained confirmed previous reported data indicating that the number of interstrand cross-links (potential nucleation sites) caused by *cis*-DDP-DNA interaction should be low, because the percentage and kinetics of DNA reassociation relative to native DNA was not significantly altered.

To have a more direct evidence of the capacity of the Pt-berenil drug to induce DNA interstrand cross-linking, we compared the intensity of double-stranded DNA forms versus single-stranded DNA forms after melting and subsequent electrophoresis in denaturing conditions of Pt-berenil-DNA complexes and *cis*-DDP-DNA complexes formed after various incubation times with the DNA. Figure 3A shows that, as expected, a unique band on the agarose gel corresponding to the single-stranded DNA form was obtained when the control pF18 DNA was melted (Fig. 3, lane 2). Because the DNA of the melted berenil-pF18 DNA complexes migrated also as a single-stranded DNA band (Fig. 3, lane 13) the berenil ligand must not induce heat stable DNA interstrand cross-links. The berenil-DNA electrostatic interactions were most likely destroyed by melting. On the other hand, DNA bands migrating as double-stranded DNA forms were detected in the melted Pt-berenil-DNA and *cis*-DDP-DNA complexes (Fig. 3, lanes 6 to 12). The ratio of the intensity of the double-stranded DNA band versus the intensity of the band migrating as single-stranded DNA increased as the period of incubation of the DNA with the drug also increased. We interpreted this result in terms of an increase in the number of plasmid DNA molecules having Pt-DNA interstrand cross-links. Because we observed that the kinetics of pF18 DNA platination was similar for both drugs (see legend of Fig. 3A), we made a comparison of the number of DNA interstrand cross-links formed by Pt-berenil and *cis*-DDP. It was observed (Fig. 3B) that the slope of the regression line obtained varied significantly. Our calculations indicated that, on the

TABLE 1
IC_{50} values obtained for compounds Pt-berenil and cis-DDP against several ovarian carcinoma cell lines

	Ovarian carcinoma cell line panel (IC_{50} ± S.D.)					
	A2780	A2780cisR	CH1	CH1cisR	SKOV-3	SKOV-3cisR
Pt-berenil	0.20 ± 0.01	0.70 ± 0.04 (3.5)	0.30 ± 0.03	0.36 ± 0.02 (1.2)	5.20 ± 0.03	10.40 ± 0.6 (2)
cis-DDP	0.30 ± 0.02	3.30 ± 0.05 (11)	0.10 ± 0.01	0.65 ± 0.05 (6.5)	4.40 ± 0.03	39.60 ± 0.8 (9)
Berenil	>10	>10	>10	>10	>100	>100

Numbers in parentheses, resistance factors: IC_{50} resistant line/IC_{50} parent line.

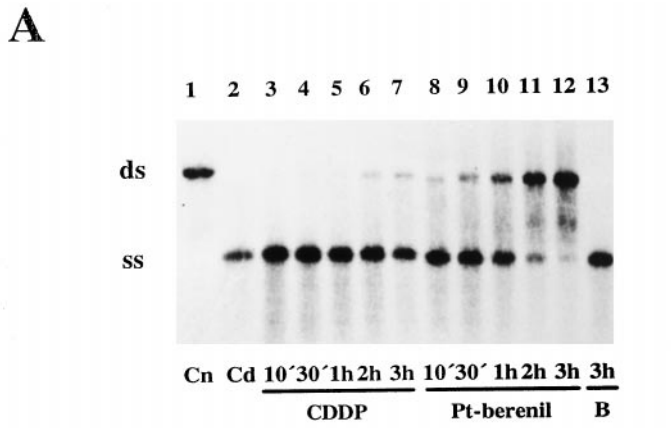
average, there must be 1.98 interstrand cross-links per plasmid molecule in the Pt-berenil complexes formed after 3 h of incubation (r_b = 0.048) in contrast to 0.18 cross-links per plasmid in the *cis*-DDP-DNA complexes formed also in 3 h (r_b = 0.047).

To analyze whether the Pt-berenil-induced DNA interstrand cross-link formation is affected by DNA supercoiling, we also performed agarose gel electrophoresis in denaturing conditions of covalently close circular pF18 plasmid DNA (σ = −0.067), incubated with the compounds and subsequently linearized and melted (Fig. 4). We observed that the level of DNA platination and the number of DNA interstrand cross-links increased slightly for both drugs, because after 3 h of incubation, the number of interstrand cross-links per plasmid molecule was 3.75 in Pt-berenil-DNA complexes (Fig. 4, lane 7; r_b = 0.055) and 0.20 in *cis*-DDP-DNA complexes (Fig. 4, lane 12; r_b = 0.052).

The Pt-berenil-DNA cross-links were visualized by electron microscopy (Sogo and Thoma, 1989). Although on the average a single cross-link was observed in most of the Pt-berenil-treated linear pF18 plasmids, in agreement with the calculated presence of 1.98 cross-links per plasmid as indicated above (Fig 5C), some of the linear fragments presented two cross-links. Multiple bubbles, most likely corresponding to the presence of various interstrand cross-links, were observed when the supercoiled DNA was treated with the drug (Fig 5D).

Binding of Pt-Berenil to DNA In Vivo. The binding of Pt-berenil and *cis*-DDP molecules to the DNA in whole cells was determined by quantification of Pt atoms bound to DNA using TXRF (Niemann et al., 1990; Wobrauschek, 1994). The data indicate that under the conditions used, the number of Pt-berenil molecules bound to genomic DNA in Pt-berenil treated HeLa cells was 3-fold higher than the number of molecules bound to the genomic DNA in the *cis*-DDP treated cells. Thus, at a concentration of 35 μM, the drug, 50 μg of DNA, and 0.016% Pt-berenil was bound to DNA, compared with 0.005% of *cis*-DDP. We observed, moreover, that the lower DNA binding rate of *cis*-DDP was associated with its slower cell uptake relative to Pt-berenil since after 48 h of incubation of the Hela cells with 35 μM, the drugs (equivalent to 2.1 × 10^{17} drug molecules), 93% of the input Pt-berenil molecules and 84% of the input *cis*-DDP molecules, were transported into the cells (data not shown).

To demonstrate that the Pt-berenil drug binds to DNA in vivo, we carried out Southern blot experiments in which



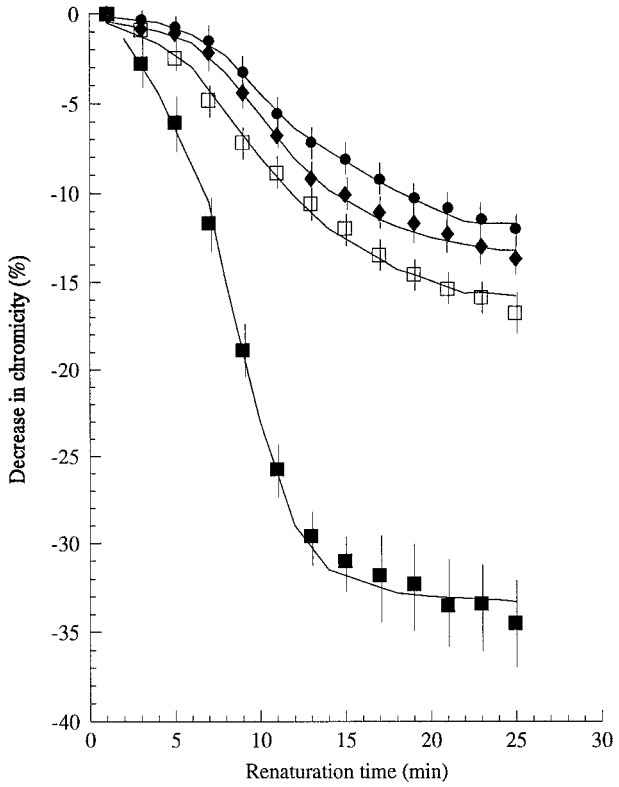


Fig. 2. Kinetics of DNA renaturation of control DNA (●), Pt-berenil-DNA (■), *cis*-DDP-DNA (□), and berenil-DNA (♦) complexes formed at r_b = 0.05.

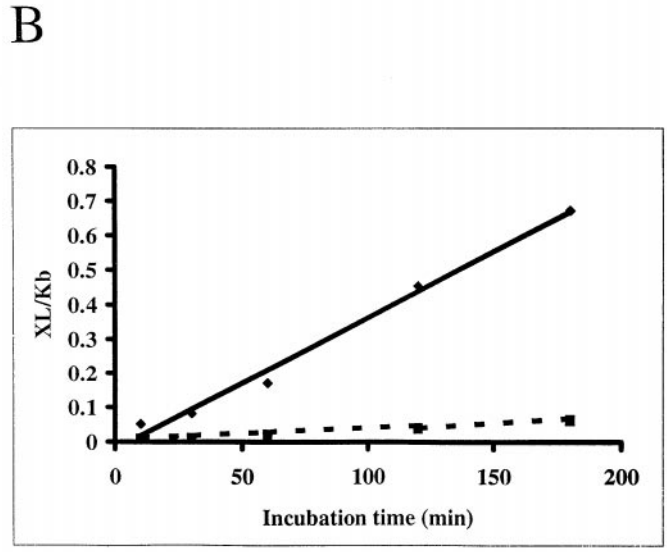


Fig. 3. A, pattern of single and double stranded DNA bands after melting of the *cis*-DDP-pF18 DNA, Pt-berenil-pF18 DNA and berenil-pF18 DNA complexes. The complexes were formed by incubation of the drugs with the DNA at an r_i value of 0.1 for 10 min, 30 min, 1 h, 2 h, and 3 h. (ds, double stranded form; ss, single stranded forms; Cn, native DNA; Cd, denaturated DNA; B, berenil). B, cross-linking rates for *cis*-DDP-DNA (■) and Pt-berenil-DNA (♦). The mean r_b values obtained for Pt-berenil at the incubation times indicated above were 0.005, 0.010, 0.015, 0.030, and 0.048, respectively, and for *cis*-DDP were 0.002, 0.010, 0.017, 0.036, and 0.047, respectively. The standard deviation in all cases was <5%.

genomic *Eco*RI digested DNA extracted from drug-treated and untreated HeLa cells was hybridized with an 18 S ribosomal DNA probe (Vos and Hanawalt, 1987). The results of the experiments are shown in Fig. 6. As expected, two DNA bands of 4- and 9.5-kb length were detected when the native genomic DNA from drug-untreated cells was hybridized to the probe (Fig. 6, lane 1). Also two bands corresponding to a theoretical length of 3 kb and 6.6 kb hybridizing with the probe were observed in the DNA from the cells treated with the Pt-berenil drug. The increase in electrophoretic mobility of the DNA bands from Pt-berenil-treated cells relative to control DNA (Fig. 6) suggests that in vivo, Pt-berenil induces DNA compaction, probably through interstrand cross-link formation.

Effect of Pt-Berenil on the $MgCl_2$-Induced B-DNA to Z-DNA Transition of Poly(dG-m^5 dC)·Poly(dG-m^{5}dC). Table 2 shows the $MgCl_2$ concentrations and the times needed to reach the B-to-Z transition midpoint ($t_{1/2}$) of control polynucleotide and of the polynucleotide modified by Pt-berenil and berenil at $r_b = 0.05$. It may be observed that at an $MgCl_2$ concentration of 1.3 mM, the B-DNA-to-Z-DNA transition of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) has a midpoint-time of 65 min. Similar results were obtained when the polynucleotide was modified by berenil, although this drug provoked a delay of the B-to-Z transition, because the midpoint-time was obtained after 87 min of incubation with the 1.3 mM $MgCl_2$. Surprisingly, however, when the polynucleotide was modified by Pt-berenil, the B-to-Z transition midpoint was not reached even after 120 min at the same $MgCl_2$ concentration. Moreover, it is interesting to note that after 5 min of incubation with 3 mM $MgCl_2$, the shape of the CD spectrum of the polynucleotide modified by Pt-berenil is characteristic of the B conformation. In contrast, an inversion of the CD spectrum characteristic of the Z conformation is observed in the polynucleotide modified by berenil and in control polynucleotide after 5 min of incubation with 3 mM $MgCl_2$ (Fig. 7).

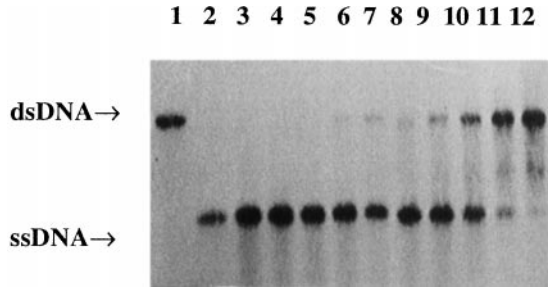


Fig. 4. A, pattern of single- and double-stranded DNA bands after *Bam*HI digestion and subsequent melting of the *cis*-DDP-pF18 DNA and Pt-berenil-pF18 DNA formed in covalently closed circular pF18 DNA ($\sigma = -0.067$) at r_i values of 0.1 for 10 min, 30 min, 1 h, 2 h, and 3 h. Lane 1, control unmelted pF18 DNA; lane 2, control melted pF18 DNA; lanes 3 through 7, *cis*-DDP-pF18 DNA complexes formed after 10 min, 30 min, 1 h, 2 h, and 3 h, respectively; lanes 8 through 12, Pt-berenil-pF18 DNA complexes formed after 10 min, 30 min, 1 h, 2 h, and 3 h, respectively. (dsDNA, double-stranded pF18 DNA; ss, single-stranded pF18 DNA). The r_b mean values obtained in supercoiled pF18 DNA for Pt-berenil at the incubation times indicated above were 0.007, 0.014, 0.020, 0.040, and 0.055, respectively, and for *cis*-DDP were 0.005, 0.016, 0.021, 0.038, and 0.052, respectively. The standard deviation in all cases was <5%.

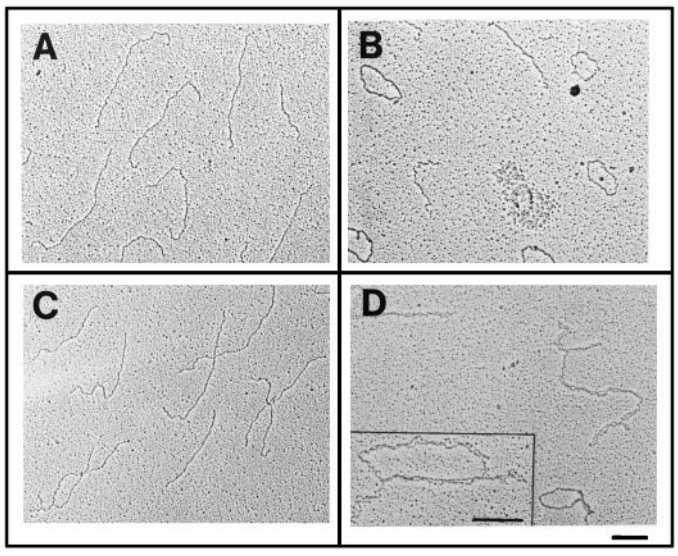


Fig. 5. Electron micrographs of linear pF18 DNA (A), supercoiled pF18 DNA (B) and linear (C) and supercoiled (D) pF18 DNA incubated with Pt-berenil at $r_b = 0.05$. The supercoiling density, σ, of covalently closed circular pF18 DNA was −0.067.

Discussion

We have presented in this article evidence showing the ability of the cytotoxic compound Pt-berenil to form drug-DNA interstrand cross-links. The kinetics and percentage of reassociation of the DNA from the Pt-berenil-DNA complexes suggested that the renaturation of the helix is caused by the existence of a "zippering" effect. Most probably, Pt-DNA interstrand cross-link adducts were formed at the "zippering site." This interpretation is consistent with other data show-

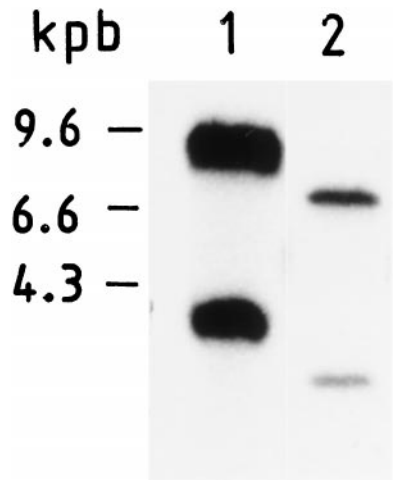


Fig. 6. Hybridization of a 18 S ribosomal DNA probe to genomic DNA from HeLa cells digested with *Eco*RI endonuclease. Lane 1, DNA from nontreated HeLa cells. Lane 2, DNA from HeLa cells treated with 35 μM Pt-berenil.

TABLE 2

$MgCl_2$ concentrations and the times needed to reach the B-to-Z transition midpoint ($T_{1/2}$) of control polynucleotide and that of the polynucleotide modified by Pt-berenil and berenil at $r_b = 0.05$ after addition of growing concentrations of $MgCl_2$. The experiments were done in triplicate.

Drug	$MgCl_2$ concentrations	$T_{1/2} \pm$ S.D.
	mM	*min*
Control	1.3	65 ± 1.0
Berenil	1.3	87 ± 2.5
Pt-berenil	1.3	>120

ing that several platinum compounds are also able to form DNA interstrand adducts (Lemaire et al., 1991). When we compared the percentage of renaturation of the DNA from the Pt-berenil-DNA complexes with that of the DNA from *cis*-DDP-DNA complexes we observed that the renaturation rate was higher in the Pt-berenil treated DNA than in the *cis*-DDP-treated DNA, as an indication that the number of "zippering" points related to interstrand cross-links should be high in the Pt-berenil-treated DNA.

The existence of interstrand cross-links in Pt-berenil-treated DNA was shown by the presence of double-stranded DNA forms in plasmids treated with the drug after denaturation. The data revealed that at r_b around 0.05, there is at least one interstrand cross-link per linear pF18 plasmid DNA molecule and that, on the average, the number of DNA interstrand cross-links produced by Pt-berenil is 11 times higher than that of *cis*-DDP. Moreover, the data indicate that under our experimental conditions, the number of interstrand cross-links per plasmid molecule produced by Pt-berenil in supercoiled pF18 DNA is about 2-fold higher than that produced by the drug in linear DNA (3.75 versus 1.98). On the other hand, *cis*-DDP also increases the number of interstrand cross-links in supercoiled pF18 DNA relative to linear pF18 DNA, but to a much lower extent than Pt-berenil. These results are in agreement with previously reported data indicating that supercoiling affects the formation of interstrand adducts by *cis*-DDP (Vrána et al., 1996). A similar phenomenon has been also observed in the binding to DNA of several other drugs, such as psoralen or ethidium bromide (Cook et al., 1989).

Electron micrographs taken from preparations of denatured Pt-berenil-pF18 DNA complexes confirmed that the intercatenary interaction of the Pt-berenil drug with the DNA through the *cis*-Pt(II) centers is favoured in supercoiled plasmids, whereas in linear pF18, only one site of cross-link was present in most of the DNA molecules several sites of cross-linking were visualized in the supercoiled DNA forms. Thus, because Pt-berenil unwinds the double helix in supercoiled plasmids (González et al., 1996), it is expected to bind more readily to negatively supercoiled DNA.

The CD data show that binding of Pt-berenil to poly(dG-m^5dC)·poly(dG-m^5dC) produces a strong inhibition of the salt-induced B-DNA-to-Z-DNA transition. In fact, when the polynucleotide is modified by the drug at $r_b = 0.05$, the B-to-Z equilibrium is not reached under salt conditions (3 mM $MgCl_2$) at which the native polynucleotide readily adopts the Z-DNA form (Fig. 7; Behe and Felsenfeld, 1981; Gonzalez et al., 1998). Interestingly, the CD data indicate that the polynucleotide modified by Pt-berenil is in B form even after 5 min of incubation with this salt concentration. On the other hand, our CD data indicate that the inhibition of the B-DNA-to-Z-DNA transition of poly(dG-^{5}dC)·poly(dG-m^5dC) produced by berenil is much lower than that of Pt-berenil. Because Pt-berenil forms a high number of DNA interstrand cross-links through the *cis*-Pt(II) centers]in contrast with berenil, which binds electrostatically to DNA (Brown et al., 1990)[, it is most likely that such a high number of interstrand cross-links may block the uncoiling of the DNA double helix required to undergo the B-DNA-to-Z-DNA transition.

The cytotoxicity data reported in this article indicate that Pt-berenil circumvents resistance to cisplatin in several ovarian tumor cell lines. It has been postulated that the DNA interstrand cross-links formed by platinum drugs may contribute to the antitumor activity of these compounds (Leng and Brabec, 1994). Therefore, it is tempting to speculate about the possibility that the ability of Pt-berenil to form DNA interstrand cross-links may be in part responsible for its cytotoxic activity in *cis*-DDP-resistant cells.

In summary, the results presented here support the hypothesis that the synthesis of platinum compounds having biologically relevant molecules as ligands might lead to the development of cytotoxic agents active in cisplatin resistant cells and with potential to form specific types of DNA adducts.

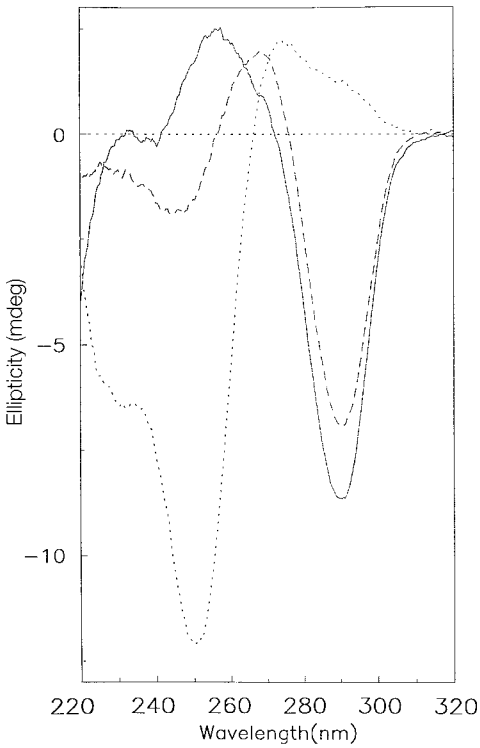


Fig. 7. CD spectra of poly(dG-m^5dC)·poly(dG-m^5dC) (—), poly(dG-m^5dC)·poly(dG-m^5dC)-Pt-berenil complexes formed at $r_b = 0.05$ (·····) and poly(dG-m^5dC)·poly(dG-m^5dC)-berenil complexes (– – –) formed at $r_b = 0.05$ after 5 min of incubation with 3 mM $MgCl_2$ at 37°C. Ellipticity units are given in mdeg. (·····) Ellipticity baseline (at 0).

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