DNA Binding Mode of the Cis and Trans Geometries of New Antitumor Nonclassical Platinum Complexes Containing Piperidine, Piperazine, or 4-Picoline Ligand in Cell-Free Media. Relations to Their Activity in Cancer Cell Lines†

Jana Kasparkova,*,‡ Victoria Marini,‡ Yousef Najajreh,§ Dan Gibson,§,|| and Viktor Brabec‡

Institute of Biophysics, Academy of Sciences of the Czech Republic, CZ-61265 Brno, Czech Republic, and Department of Medicinal Chemistry and Natural Products, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem 91120, Israel

Received February 10, 2003; Revised Manuscript Received March 20, 2003

ABSTRACT: The global modification of mammalian and plasmid DNAs by novel platinum compounds, *cis*- or *trans*-[$PtCl_2(NH_3)(Am)$], where Am = NH_3, nonplanar heterocycle piperidine, piperazine, or aromatic planar heterocycle 4-picoline, was investigated in cell-free media using various biochemical and biophysical methods. These modifications have been compared with the activity of these new compounds in several tumor cell lines including those resistant to antitumor *cis*-diamminedichloroplatinum(II) (cisplatin). The results show that the replacement of the NH_3 group in cisplatin by the heterocyclic ligands does not considerably affect the DNA binding mode of this drug. Cytotoxicity studies have revealed that the replacement lowers the activity of the platinum compound in both sensitive and resistant cell lines. It has been suggested that the reduced activity of these analogues of cisplatin is associated with some features of the damaged DNA and/or its cellular processing. Alternatively, the reduced activity of the analogues of cisplatin might also be due to the factors that do not operate directly at the level of the target DNA, such as intracellular platinum uptake. In contrast to the analogues of cisplatin, the replacement of one ammine group by the heterocyclic ligand in its clinically ineffective trans isomer (transplatin) results in a radical enhancement of its activity in tumor cell lines. Importantly, this replacement also markedly alters the DNA binding mode of transplatin. The results support the view that one strategy of how to activate the trans geometry in bifunctional platinum(II) compounds including circumvention of resistance to cisplatin may consist of a chemical modification of the ineffective transplatin that results in an increased stability of its intrastrand cross-links in double-helical DNA and/or in an increased efficiency to form interstrand cross-links.

Platinum anticancer agents are in widespread clinical use especially against testicular, ovarian, and head and neck carcinomas (*1*−*3*). They are believed to owe their anticancer activity to their ability to bind covalently to cellular DNA and to alter its structure, thereby affecting subsequent cellular processes that eventually result in the death of the cancer cell (*4*−*6*). The first platinum antitumor drug introduced in the clinic was *cis*-diamminedichloroplatinum(II)[1] (cisplatin, Figure 1a). It binds preferentially to two adjacent N7 atoms in the major groove of B-DNA, forming an intrastrand cross-link (CL) that kinks the double helix toward the major groove (*7*). The interstrand CLs, the intrastrand CLs between nonadjacent bases, and the monofunctional adducts represent only minor lesions of cisplatin. 1,2-Intrastrand CLs of

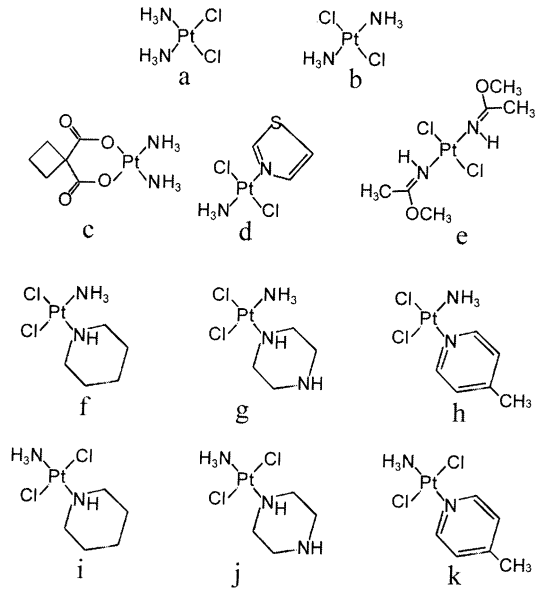

FIGURE 1: Structures of the platinum complexes.

† Supported by the Grant Agency of the Academy of Sciences of the Czech Republic (Grants A5004101, KJB5004301) and the Grant Agency of the Czech Republic (Grant 305/01/418). J.K. is the international research scholar of the Howard Hughes Medical Institute. The research of J.K. and V.B. was also supported in part by the Wellcome Trust (U.K.). V.M. is supported by a doctoral fellowship from the Faculty of Sciences, Masaryk University, Brno. Y.N. thanks the David R. Bloom Center for Drug Design and for partial support. D.G. acknowledges partial support from the Alex Grass Center for Drug Design.

* To whom correspondence should be addressed. Phone: 420-541517174. Fax: 420-541240499. E-mail: jana@ibp.cz.

‡ Academy of Sciences of the Czech Republic.

§ Hebrew University of Jerusalem.

|| Affiliated with the David R. Bloom, Center for Pharmacy at The Hebrew University of Jerusalem, Israel.

10.1021/bi0342315 CCC: $25.00
Published on Web 04/30/2003

cisplatin can terminate DNA and RNA synthesis and are recognized by some cellular proteins that bind to it, and it is this protein binding that may mediate the drug's cytotoxicity (*5*, *8*−*10*).

The clinically ineffective trans isomer of cisplatin (transplatin, Figure 1b) also covalently binds to DNA bases. The lesions formed by transplatin in double-helical DNA are monofunctional adducts and interstrand and intrastrand CLs that are recognized by cellular proteins distinctly less often than the CLs of cisplatin. Unlike cisplatin, because of steric reasons, transplatin cannot form intrastrand CLs in double-helical DNA between adjacent base residues. It can cross-link two bases on the same strand only if they are separated by at least one intervening base, forming mostly 1,3-GNG CLs (where N = adenine, cytosine, or thymine). Importantly, these CLs are stable in single-stranded DNA, while in double-stranded DNA some of these CLs readily isomerize to the interstrand CLs (*11*). The clinical ineffectiveness of transplatin might be associated with its reduced capability to form bifunctional adducts in double-helical DNA (*12*). It has even been suggested (*12*, *13*) that in cells transplatin forms only a small amount of interstrand CLs, since the slow transformation of the monofunctional adducts to interstrand CLs allows sufficient time for inactivation of the monofunctional adducts by intracellular sulfur-containing nucleophiles. Hence, one strategy to activate the trans geometry might consist of chemical modification of transplatin that enhances the stability of the intrastrand CLs formed by the *trans*-platinum compounds in double-helical DNA and/or increases the rate of the formation of their interstrand CLs.

Following the discovery of the anticancer activity of cisplatin and the clinical inactivity of transplatin, most of the initial efforts of the medicinal chemists to develop analogues with superior therapeutic properties focused on the preparation of neutral, cis-oriented complexes with two inert amine ligands and one or two semilabile ligand(s) in the other two coordination positions [see, for example, *cis*-diamminecyclobutanedicarboxylatoplatinum(II) (carboplatin, Figure 1c)]. Since the early days, many examples of "nonclassical" platinum compounds possessing antitumor activity have been described. Several of these complexes are already in advanced clinical trials (for reviews, see refs *10*, *14*−*18*).

The trans geometry in dichloroplatinum(II) compounds has already been activated by various ways. The replacement of at least one ammine ligand in trasplatin by a planar amine ligand [such as quinoline, thiazole (Figure 1d), or pyridine] (*15*, *19*) or *trans*-[$PtCl_2$(*E*-iminoether)$_2$] (Figure 1e) (*16*) represents examples of such an activation. While cisplatin interacts with double-helical DNA, forming preferentially 1,2-GG (or AG) intrastrand CLs, the "nonclassical" trans disposed bidentate complexes cannot form in double-helical DNA intrastrand CLs between adjacent purines (*20*), and consequently, they exhibit different DNA binding modes (*10*, *18*).

We have recently described a new series of antitumor "nonclassical" *cis*- and *trans*-Pt(II) complexes having nonplanar heterocyclic amine ligands, such as piperidine and piperazine (Figures 1f,g,i,j) (*21*, *22*). Some of these compounds, especially the trans analogues, have the ability to overcome multifactorial cisplatin resistance in human ovarian cell lines. The ability to circumvent cisplatin resistance is also thought to be related to the DNA binding properties of the platinum complexes and particularly to their ability to form lesions in DNA that are different from those formed by cisplatin (*10*, *23*, *24*). Thus, to attain the ability to rationally design novel antitumor platinum drugs capable of circumventing inherent or acquired resistance to cisplatin, it is necessary to understand how the DNA binding properties of antitumor platinum complexes that are not cross-resistant with cisplatin differ from the DNA binding properties of cisplatin and transplatin. In this work, we describe the DNA binding properties of *cis*-[$PtCl_2(NH_3)$(Am)], where Am = NH_3, nonplanar heterocycle piperidine (Figure 1f), piperazine (Figure 1g) or aromatic planar heterocycle 4-picoline (Figure 1h), and their trans analogues (Figures 1i−k) and compare these binding properties with the activity of the new compounds in several tumor cell lines including those resistant to cisplatin.

MATERIAL AND METHODS

Starting Materials. cis- and *trans*-[$PtCl_2(NH_3)$(Am)], where Am = piperidine, piperazine, or 4-picoline (Figures 1f−k), were prepared by the methods described in detail previously (*21*). Cisplatin and transplatin (Figure 1a,b) were obtained from Sigma (Prague, Czech Republic). The stock solutions of the platinum complexes at 5×10^{-4} M in 10 mM $NaClO_4$ were prepared in the dark at 25 °C. Calf thymus (CT) DNA (42% G + C, mean molecular mass of ca. 20 000 kDa) was prepared and characterized as described previously (*25*, *26*). Plasmids pSP73KB [2455 base pairs (bp)] and pUC19 (2686 bp) were isolated according to standard procedures. The synthetic oligodeoxyribonucleotides (Figure 2) were synthesized and purified as described previously (*27*). Restriction endonucleases were purchased from New England Biolabs. Ethidium bromide and agarose were from Merck KgaA. Klenow fragment of DNA polymerase I was from Boehringer-Mannheim Biochemica (Mannheim, Germany). T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). Acrylamide, bis(acrylamide), urea, and NaCN were from Merck KgaA (Darmstadt, Germany). Dimethyl sulfate (DMS) was from Sigma (Prague, Czech Republic). The radioactive products were from Amersham.

Platination Reactions. CT or plasmid DNAs were incubated with the platinum complex in 10 mM $NaClO_4$ at 37 °C in the dark. After 48 h, the samples of plasmid DNA were precipitated by ethanol and redissolved in the medium required for subsequent biochemical or biophysical analysis whereas the samples of CT DNA were exhaustively dialyzed against such a medium. An aliquot of these samples was used to determine the value of r_b (r_b is defined as the number

[1] Abbreviations: bp, base pair; cisplatin, *cis*-diamminedichloroplatinum(II); CD, circular dichroism; CL, cross-link; CT, calf thymus; DMS, dimethyl sulfate; DPP, differential pulse polarography; FAAS, flameless atomic absorption spectrophotometry; FPLC, fast protein liquid chromatography; IC_{50}, the concentration of the compound that afforded 50% cell killing; PAA, polyacrylamide; 4-pic, 4-picoline; pip, piperidine; pz, piperazine; r_b, the number of molecules of the platinum compound bound per nucleotide residue; r_i, the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA; t_m, melting temperature; Δt_m, the difference between the t_m values of platinated and nonmodified DNAs; transplatin, *trans*-diamminedichloroplatinum(II).

Abbreviation	Sequence
CGC	5'-TCTCTCCTC**G**CTCTTCCTCT AGAGAGGAGCGAGAAGGAGA-5'
CGA	5'-TCTCTCCTC**G**ATCTTCCTCT AGAGAGGAGCTAGAAGGAGA-5'
TGC	5'-TCTCTCCTT**G**CTCTTCCTCT AGAGAGGAACGAGAAGGAGA-5'
TGT	5'-TCTCTCCTT**G**TTCTTCCTCT AGAGAGGAGCGAGAAGGAGA-5'
CGCGC	5'-CCTCTCTCTC**G**C**G**CTCTTCT GGAGAGAGAG**C**GCGAGAAGA-5'
TGCGT	5'-CCTCTCTCTT**G**C**G**TTCTTCT GGAGAGAGAA**C**GCAAGAAGA-5'
TGTGT	5'-CCTCTCTCTT**G**T**G**TTCTTCT GGAGAGAGAA**C**ACAAGAAGA-5'

FIGURE 2: Sequences of the synthetic oligodeoxyribonucleotides used in the present study with their abbreviations. The top and bottom strands of each pair are designated top and bottom, respectively, in the text. The bold letter in the top strands of the duplexes CGC, CGA, TGC, and TGT indicates the location of the monofunctional adduct of the *trans*-platinum compound formed before interstrand cross-linking reaction in the way described in Materials and Methods. The bold letters in the top strands of the duplexes CGCGC, TGCGT, and TGTGT indicate the location of the 1,3-intrastrand CL after modification of the oligonucleotides by the *trans*-platinum compound in the way described in Materials and Methods. The bold letters in the bottom strands of these duplexes indicate the location of the sites that were involved in the interstrand CLs after linkage isomerization reaction was completed (see the text).

of molecules of the platinum compound bound per nucleotide residue) by flameless atomic absorption spectrophotometry (FAAS) or differential pulse polarography (DPP) (*28*).

The single-stranded oligonucleotides [the top strands of the duplexes TGT, CGC, TGC, CGA, TGTGT, TGCGT, and CGCGC (for their sequences, see Figure 2)] were reacted in stoichiometric amounts with transplatin and its analogues tested in the present work. The platinated oligonucleotides were repurified by ion-exchange fast protein liquid chromatography (FPLC). It was verified by platinum FAAS and by the measurements of the optical density that the modified oligonucleotides contained one platinum atom. It was also verified using DMS footprinting of platinum on DNA (*29*−*31*) that one platinum molecule was coordinated to a single guanine at its N7 position in the top strands of the duplexes CGA, CGC, TGC, and TGT or to two guanines at their N7 position in the top strands of the duplexes CGCGC, TGCGT, and TGTGT. The platinated top strands were allowed to anneal with unplatinated complementary strands (the bottom strands in Figure 2) in 0.1 M $NaClO_4$. Other details are in the text (vide infra) or have been described previously (*27*, *29*, *32*).

Interstrand Cross-Link Assay. Platinum complexes at varying concentrations were incubated for 24 h with 500 ng of pSP73KB DNA after it had been linearized by EcoRI and 3′-end-labeled by means of the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. The platinated samples were precipitated by ethanol, the pellets were dissolved in 18 μL of 30 mM NaOH with 1 mM EDTA, 6.6% sucrose, and 0.04% bromophenol blue, and samples were immediately analyzed for platinum content by FAAS and for DNA interstrand CLs in the same way as described in recent papers (*29*, *33*, *34*). Briefly, the amount of interstrand CLs was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%). After the electrophoresis was completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified. The frequency of interstrand CLs, % ICL/Pt (the number of interstrand CLs per adduct), was calculated as % ICL/Pt = XL/(4910r_b) (pSP73KB plasmid contained 4910 nucleotide residues). XL is the number of interstrand CLs per one molecule of the linearized DNA duplex, which was calculated assuming Poisson distribution of the interstrand CLs as XL = $-\ln A$, where A is the fraction of molecules running as a band corresponding to the non-cross-linked DNA.

Unwinding of Negatively Supercoiled DNA. The unwinding of closed circular supercoiled pUC19 plasmid DNA was measured by an agarose gel mobility shift assay (*35*). The unwinding angle Φ induced per Pt-DNA adduct was calculated upon the determination of the r_b value at which the complete transformation of the supercoiled to relaxed form of the plasmid was attained. Samples of pUC19 plasmid were incubated with platinum compounds for 48 h, precipitated by ethanol, and redissolved in TAE buffer (0.04 M Tris-acetate + 1 mM EDTA, pH 7.0). An aliquot of the precipitated sample was subjected to electrophoresis on 1% agarose gels running at 25 °C in the dark with TAE buffer with a voltage set at 30 V. The gels were then stained with ethidium bromide, followed by photography on Polaroid 667 film with a transilluminator. The other aliquot was used for the determination of r_b values by FAAS.

DNA Melting. The melting curves of CT DNAs were recorded by measuring the absorbance at 260 nm. The melting curves were recorded in a medium containing 10 mM or 0.2 M $NaClO_4$ with 1 mM Tris-HCl/0.1 mM EDTA, pH 7.4. The value of the melting temperature (t_m) was determined as the temperature corresponding to a maximum in the first-derivative profile of the melting curves. The t_m values could be thus determined with an accuracy of ±0.5 °C.

Rearrangement of Intrastrand Cross-Links of Trans Compounds. The platinated oligodeoxyribonucleotides (top strands of the duplexes CGCGC, TGCGT, and TGTGT; for their sequences, see Figure 2) at 20 μM were annealed with the unplatinated complementary strands in 0.2 M $NaClO_4$/5 mM Tris-HCl, pH 7.5/0.1 mM EDTA at 20 °C for 30 min and then for 2 h at 4 °C. The resulting duplexes at 2 μM (or higher) were subsequently incubated at 37 °C. At various time intervals, aliquots were withdrawn and analyzed by electrophoresis in denaturing 12% polyacrylamide (PAA)/8 M urea gel. The bases involved in the interstrand CLs were determined by Maxam−Gilbert footprinting (*29*, *36*).

Other Physical Methods. Absorption spectra were measured with a Beckmann DU-7400 spectrophotometer. FAAS measurements were carried out on a Unicam 939 AA spectrometer with a graphite furnace. For FAAS analysis, DNA was precipitated with ethanol and dissolved in 0.1 M HCl. DPP curves were recorded with the aid of an EG&G PARC electrochemical analyzer, model 384B. FPLC purification was carried out on a Pharmacia Biotech FPLC system with MonoQ HR 5/5 column. Circular dichroism

(CD) spectra were recorded at 25 °C using a JASCO spectropolarimeter, model J720. The gels were dried and visualized by using a molecular dynamics phosphor imager (Storm 860 system), and the radioactivities associated with bands were quantitated with the ImageQuant software.

Cytotoxicity. Cultures of pairs of cisplatin sensitive and resistant ovarian cancer cell lines (A2780/A2780cisR, 41M/41McisR, and CH1/CH1cisR) were described elsewhere (*37*). Cell survival in the cultures treated with the platinum complexes was evaluated by the microculture tetrazolium assay (*38*) as described in our recent paper (*21*). Briefly, the platinum compounds were added to 96-microwell plates containing the cell cultures at final concentrations in the range 0−0.2 mM. After 24 h, cell survival was evaluated by measuring absorbance at 520 nm. IC_{50} values (the concentration of the compound that afforded 50% cell killing) were calculated from the plots of cell survival vs compound concentration. Experiments were performed in quadruplicate. Other details can be found in the previously published papers (*21*, *37*, *38*).

RESULTS

DNA Binding. Solutions of double-helical CT DNA at a concentration of 0.032 mg/mL were incubated with the platinum complexes tested in the present work at an r_i of 0.05 in 10 mM $NaClO_4$ at 37 °C (r_i is defined as the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA). At various time intervals, an aliquot of the reaction mixture was withdrawn and assayed by DPP for platinum not bound to DNA. The amount of platinum bound to DNA (r_b) was calculated by subtracting the amount of free (unbound) platinum from the total amount of platinum present in the reaction. No changes in the pH of the reaction mixture containing DNA and platinum compounds were measured within 48 h after mixing DNA with the platinum complex. The amount of the platinum compounds bound to DNA increased with time. In these binding reactions, the times at which the binding reached 50% ($t_{50\%}$) were in the range of 20−113 min, and they are summarized for all compounds in Tables 2 and 3. The value of $t_{50\%}$ for the reaction of cisplatin or transplatin with DNA under comparable conditions was ∼120 min. These results indicate that the rates of binding of *cis*- and *trans*-$[PtCl_2(NH_3)(pip)]$ to natural double-helical DNA are comparable to those of cisplatin or transplatin, whereas the rates of binding of *cis*- and *trans*-$[PtCl_2(NH_3)(pz)]$ or *cis*- and *trans*-$[PtCl_2(NH_3)$-(4-pic)] are considerably higher.

In further experiments, CT DNA was incubated with the new analogues of cisplatin and transplatin at $r_i = 0.2$, and essentially the same rates of the binding were observed as at $r_i = 0.05$. The binding of these new platinum compounds to CT DNA was also quantified in the other way. Aliquots of the reaction withdrawn at various time intervals were quickly cooled on an ice bath and then exhaustively dialyzed against 10 mM $NaClO_4$ at 4 °C to remove the free (unbound) platinum compound. The content of platinum in these DNA samples was determined by FAAS. Results identical to those obtained using the DPP assay were obtained. The binding experiments of the present work indicate that the modification reactions resulted in the irreversible coordination of the new analogues of cisplatin and transplatin to polymeric double-helical DNA, which also facilitates sample analysis. Hence, it is possible to prepare easily and precisely the samples of DNA modified by the platinum complex at a preselected value of r_b. The samples of DNA modified by new platinum compounds and analyzed further by biophysical or biochemical methods were prepared in 10 mM $NaClO_4$ at 37 °C. If not stated otherwise, after 24 h of the reaction of DNA with the complex, the samples were precipitated in ethanol and dissolved in the medium necessary for a particular analysis and the r_b value in an aliquot of this sample was checked by FAAS. In this way, the analyses described in the present paper were performed in the absence of unbound (free) platinum complex.

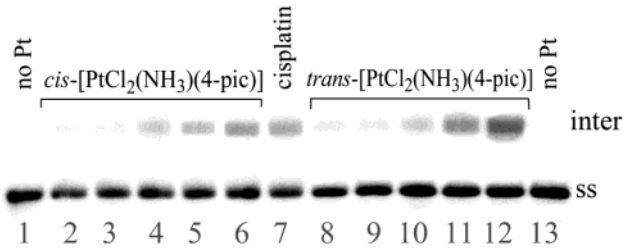

FIGURE 3: Formation of the interstrand CLs by platinum complexes in linearized pSP73KB plasmid (2455bp). Autoradiograms are shown of denaturing 1% agarose gels of linearized DNA, which was 3′-end-labeled. The interstrand cross-linked DNA appears as the top bands migrating on the gel more slowly than the single-stranded DNA (contained in the bottom bands). Plasmid linearized by EcoRI was nonplatinated (control) (lanes 1 and 13) or incubated for 24 h with cisplatin (lane 7), *cis*-$[PtCl_2(NH_3)(4\text{-pic})]$ (lanes 2−6), or *trans*-$[PtCl_2(NH_3)(4\text{-pic})]$ (lanes 8−14). r_b values are the following: 0.00001 (lane 8), 0.00005 (lanes 2 and 9), 0.0001 (lanes 3 and 10), 0.0005 (lanes 4 and 11), 0.001 (lanes 5, 7, and 12), 0.002 (lane 6).

Interstrand Cross-Linking. Bifunctional platinum compounds that covalently bind to DNA form various types of interstrand and intrastrand CLs. Considerable evidence suggests that the antitumor efficacy of bifunctional platinum compounds is the result of the formation of these lesions, but their relative efficacy remains unknown. Therefore, we have decided to quantitate the interstrand cross-linking efficiency of all the analogues of cisplatin and transplatin tested in the present work in linearized pSP73KB plasmid (2455 bp). This plasmid DNA was linearized by EcoRI (EcoRI cuts only once within the pSP73KB plasmid) and modified by the platinum complexes. The samples were analyzed for the interstrand CLs by agarose gel electrophoresis under denaturing conditions (*29*). Upon electrophoresis, the 3′-end-labeled strands of linearized pSP73KB plasmid containing no interstrand CLs migrate as a 2455-base single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species (shown in Figure 3 for *cis*- and *trans*-$[PtCl_2(NH_3)$-(4-pic)]).

The experiments were carried out with DNA samples that were modified by the platinum complexes for 24 h at various r_b values. The bands corresponding to the more slowly migrating interstrand-cross-linked fragments were seen for r_b values as low as 5×10^{-5} (Figure 3, lanes 2 and 8). The intensity of the more slowly migrating band increased with the growing level of the modification. The radioactivity associated with the individual bands in each lane was measured to obtain estimates of the fraction of non-cross-linked or cross-linked DNA under each condition. The

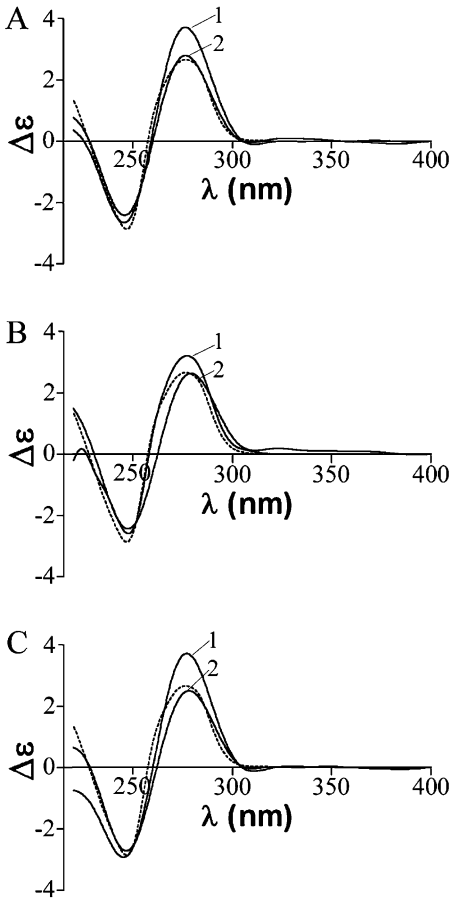

FIGURE 4: CD spectroscopy of calf thymus DNA modified by cisplatin and transplatin analogues at $r_b = 0.05$. CD spectra were recorded for DNA in 10 mM $NaClO_4$. CD spectra of DNA modified by *cis*- and *trans*-[$PtCl_2(NH_3)$(pip)] (A), *cis*- and *trans*-[$PtCl_2(NH_3)$(pz)] (B), and *cis*- and *trans*-[$PtCl_2(NH_3)$(4-pic)] (C). Curves: (dashed lines) control (nonmodified) DNA; (1) analogues of cisplatin; (2) analogues of transplatin.

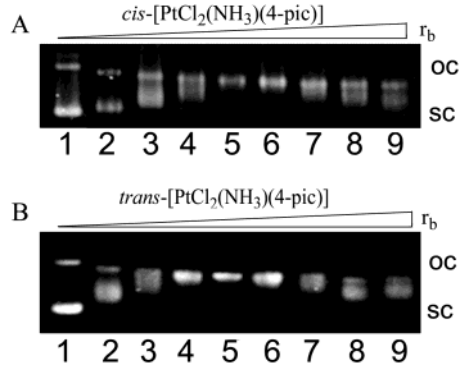

FIGURE 5: Unwinding of supercoiled pUC19 plasmid DNA modified by *cis*-[$PtCl_2(NH_3)$(4-pic)] (A) and *trans*-[$PtCl_2(NH_3)$(4-pic)] (B). Lanes in part A: (1) control, nonmodified DNA; (2−9) $r_b = 0.050$, 0.062, 0.074, 0.083, 0.089, 0.10, 0.11, 0.121, respectively. Lanes in part B: (1) control, nonmodified DNA; (2−9) $r_b = 0.020$, 0.032, 0.036, 0.043, 0.049, 0.055, 0.062, 0.065, respectively. The top bands correspond to a form of nicked plasmid (oc), and the bottom bands correspond to a closed negatively supercoiled plasmid (sc).

frequency of interstrand CLs (% ICL/Pt) was calculated using the Poisson distribution from the fraction of non-cross-linked DNA in combination with the r_b values and the fragment size. The DNA interstrand cross-linking efficiency of all new analogues of cisplatin or transplatin tested in the present work was almost independent of r_b and was in the range of 4−40% (Tables 2 and 3). Interstrand cross-linking efficiency of all new analogues of cisplatin (4−6%) was very similar to that of the parent cisplatin [6% (*29*, *39*)]. In contrast, the interstrand cross-linking efficiency found for the new analogues of transplatin (18−40%) was considerably higher than that found for transplatin [12% (*29*)].

The samples of linearized DNA modified by the compounds tested in the present work at $r_b = 0.001$ and 0.01 were also analyzed in 1% nondenaturing agarose gel (not shown). No new, more slowly migrating bands were observed, which indicates that no CLs between DNA strands belonging to different duplexes are formed.

Circular Dichroism Spectroscopy. CD spectral characteristics were compared for CT DNA in the absence and in the presence of the new analogues of cisplatin or transplatin tested in the present work at $r_b = 0.05$ (Figure 4). Upon the binding of these compounds to CT DNA, the conservative CD spectrum normally found for DNA in the canonical B-conformation is considerably transformed at wavelengths below 300 nm. There was a significant increase in the intensity of the positive band around 280 nm if DNA was modified by the analogues of cisplatin. This increase was similar to that observed if DNA was under identical conditions modified by cisplatin (*40*). On the other hand, if DNA was modified by the analogues of transplatin, this increase, although considerably lower, was only observed when DNA was modified by *trans*-[$PtCl_2(NH_3)$(pip)] (Figure 4A). No such increase in the intensity of the positive band around 280 nm was seen if DNA was modified by *trans*-[$PtCl_2(NH_3)$(pz)], and even a small decrease of this band was noted if DNA was modified by *trans*-[$PtCl_2(NH_3)$(4-pic)]. On the basis of the analogy with the changes in the CD spectra of DNA modified by cisplatin and clinically ineffective transplatin (*40*), it might be suggested that the binding of all new cisplatin analogues results in the conformational alterations in double-helical DNA of nondenaturational character similar to those induced in DNA by the parent cisplatin (*40*). The CD results (Figure 4) also suggest that the binding of trans analogues affects DNA conformation differently from cis compounds and that the character of the conformational changes induced in DNA by *trans*-platinum compounds corresponds to denaturational alterations (*40*).

DNA Unwinding. Electrophoresis in native agarose gel is used to determine the unwinding induced in negatively supercoiled pUC19 plasmid by monitoring the degree of supercoiling (*35*) (Figure 5). A compound that unwinds the DNA duplex reduces the number of supercoils in closed circular DNA so that their number decreases. This decrease upon binding of unwinding agents causes a decrease in the rate of migration through agarose gel, which makes it possible to observe and quantify the mean value of unwinding per adduct.

Figure 5 shows electrophoresis gels from experiments in which variable amounts of *cis*- and *trans*-[$PtCl_2(NH_3)$(4-pic)] (panels A and B, respectively) have been bound to a mixture of relaxed and negatively supercoiled pUC19 DNA as examples. Interestingly, all new analogues of cisplatin and transplatin accelerated the mobility of the relaxed form similarly as does cisplatin, whose bifunctional binding to DNA shortens and condenses the DNA helix (*41*, *42*). The

mean unwinding angle is given by $\Phi = 18\ \sigma/r_b(c)$, where σ is the superhelical density and $r_b(c)$ is the value of r_b at which the supercoiled and nicked forms comigrate (*35*). Under the present experimental conditions, σ was calculated to be -0.055 on the basis of the data of cisplatin for which the $r_b(c)$ was determined in this study and $\Phi = 13°$ was assumed. By use of this approach, the DNA unwinding angles in the range $(12-13) \pm 1°$ were determined for all new analogues of cisplatin, which are thus similar to those found for cisplatin 13° (*35*). On the other hand, the unwinding angles determined for all new analogues of transplatin were markedly higher $(17-30) \pm 3°$ than those determined for transplatin (9°) and cisplatin (*35*).

DNA Melting. CT DNA was modified by the new analogues of cisplatin and transplatin tested in the present work to $r_b = 0.05$ in 10 mM $NaClO_4$ at 37 °C for 24 h. The samples were divided into two parts, and in one part the salt concentration was further adjusted by addition of $NaClO_4$ to 0.2 M. Hence, the melting curves for DNA modified by the platinum compounds to the same level were measured in the two different media, at low and high salt concentrations (Tables 2 and 3). The effect on t_m is dependent on the salt concentration. At high salt concentrations (0.2 M), a decrease of t_m is observed for the modifications by all new analogues of cisplatin, which was similar to that observed for the modification by the parent cisplatin. On the other hand, modification of DNA by transplatin analogues affected t_m measured at high salt concentration only very slightly, similar to the case of the modification by transplatin. If the concentration of salt in the medium in which the melting curves were measured was low (0.01 M), the modification of DNA by cisplatin analogues still resulted in a decrease of t_m, which was, however, less than that observed when the melting was analyzed at high salt concentration and was again similar to that observed for DNA modified by cisplatin. The only exception was the t_m of DNA modified by positively charged *cis*-$[PtCl_2(NH_3)(pz)]^+$, which was noticeably increased because of the platination. The modification by transplatin analogues resulted in a more pronounced increase of t_m, although similar to that due to the modification by the parent transplatin.

Characterization of DNA Adducts of Transplatin Analogues by Thiourea. Cisplatin, transplatin, and analogous bifunctional platinum compounds coordinate to DNA in a two-step process, forming first the monofunctional adducts preferentially at guanine residues, which subsequently close to bifunctional lesions (*4*, *40*, *43*−*45*). Thiourea is used to labilize monofunctionally bound transplatin from DNA (*46*). The displacement of transplatin is initiated by coordination of thiourea trans to the nucleobase. Because of the strong trans effect of sulfur, the nucleobase nitrogen−platinum bond is weakened and thus becomes susceptible to further substitution reactions. Consequently, transplatin in monofunctional DNA adducts is effectively removed, whereas bifunctional adducts of transplatin are resistant to thiourea treatment (*46*).

The initial experiments, aimed at the characterization of DNA adducts of transplatin and its analogues, were conducted employing thiourea as a probe for DNA monofunctional adducts formed by *trans*-platinum compounds (*46*). Double-stranded DNA was incubated with transplatin or its analogues at a drug-to-nucleotide ratio of $r_i = 0.05$ in 10 mM $NaClO_4$ at 37 °C. At various times the reaction was stopped by adjusting the NaCl concentration to 0.2 M and by immediate cooling to −20 °C. In parallel experiments, the reactions were stopped by addition of 10 mM thiourea solutions. These samples were incubated for 10 min at 37 °C and then quickly cooled to −20 °C. The samples were then exhaustively dialyzed against 0.2 M NaCl and subsequently against H_2O at 4 °C, and the platinum content was determined by FAAS (Figure 6).

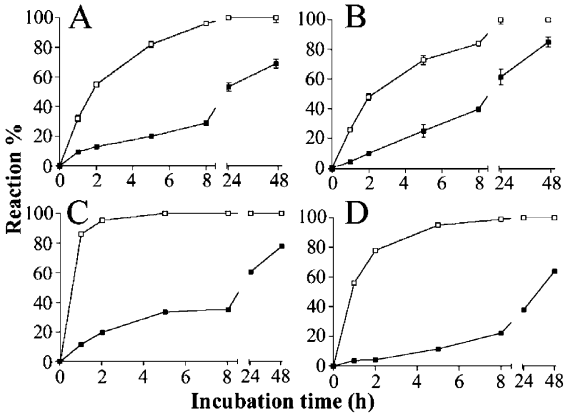

FIGURE 6: Kinetics of reaction of transplatin (A), *trans*-$[PtCl_2(NH_3)(pip)]$ (B), *trans*-$[PtCl_2(NH_3)(pz)]^+$ (C), and *trans*-$[PtCl_2(NH_3)(4-pic)]$ (D) with double-helical DNA at $r_i = 0.05$ in 10 mM $NaClO_4$ at 37 °C. DNA concentration was 0.15 mg/mL. Reactions were stopped with (■) or without (□) 10 mM thiourea (10 min), and platinum associated with DNA was assessed by FAAS. Data points measured in triplicate varied ±2% from their mean.

The reactions of DNA with transplatin and its analogues were complete after 24 h (Figure 6), consistent with the results of DNA binding experiments performed using DPP. Thiourea displaced ca. 80−90% *trans*-platinum compounds from DNA at early time intervals (1−5 h, Figure 6). At longer incubation times (24−48 h), thiourea was less efficient in removing transplatin from DNA. It can be concluded that at these time intervals a considerable fraction of monofunctional adducts of transplatin had closed to a bifunctional lesion (*46*, *47*). Thus, after a reaction period of 48 h, only ca. 15% *trans*-$[PtCl_2(NH_3)(pip)]$, 22% *trans*-$[PtCl_2(NH_3)(pz)]$, 34% *trans*-$[PtCl_2(NH_3)(4\text{-pic})]$, or 35% transplatin was displaced from double-stranded DNA, which implies that ca. 85%, 78%, 66%, or 65% monofunctional adducts, respectively, had evolved to bifunctional lesions. The same order of the efficiency of monofunctional adducts of *trans*-platinum compounds to evolve to bifunctional lesions was found at any time of the platination reaction; i.e., monofunctional adducts of *trans*-$[PtCl_2(NH_3)(pip)]$ and *trans*-$[PtCl_2(NH_3)(pz)]^+$ close to become bifunctional lesions with a considerably higher rate than those of transplatin, while DNA monofunctional adducts of *trans*-$[PtCl_2(NH_3)(4\text{-pic})]$ are transformed to bifunctional CLs with an overall rate similar to that of transplatin. It was verified that 5−60 min incubations with 10 mM thiourea gave the same results as those shown in Figure 6.

We have also verified that the different amount of DNA adducts of transplatin and its analogues removed from DNA by thiourea is not due to a different efficiency of thiourea to displace the monofunctional adducts of these different trans compounds from DNA. We prepared short 20-mer oligodeoxyribonucleotides (the top strands of the duplexes CGA,

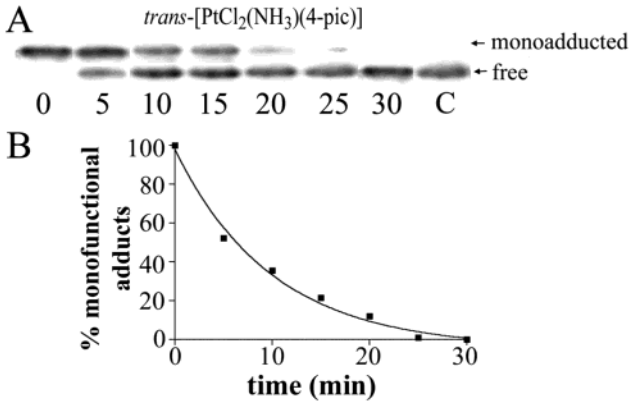

FIGURE 7: Reaction of 10 mM thiourea with the 20 μM duplex TGT containing the monofunctional adduct of *trans*-[$PtCl_2(NH_3)$-(4-pic)] at 37 °C. At various time intervals, the aliquots were withdrawn and analyzed by electrophoresis in 24% PAA/8 M urea gel. (A) Autoradiogram: the numbers below each lane indicate the time of the incubation with thiourea in minutes; (lane C) unplatinated duplex. (B) Dependence on time of the percentage of monofunctional adducts of *trans*-[$PtCl_2(NH_3)$(4-pic)] that remained on the duplex after the treatment with thiourea. This percentage was calculated from the ratio of the radioactivity in each lane in part A associated with the band corresponding to the platinated strand (top bands in part A) to the sum of the radioactivities associated with both platinated and unplatinated strands (multiplied by 100). For other details, see the text.

CGC, TGC, and TGT; for their sequence, see the Figure 2) containing a single, site-specific monofunctional adduct formed at the central G residue by either *trans*-platinum compound tested in the present work. These top strands were radioactively labeled at their 5′-ends, hybridized with their complementary strands in 0.1 M $NaClO_4$ at 0 °C for 2 h, and immediately treated with 10 mM thiourea under the same conditions as were those used in the experiments employing calf thymus DNA (vide supra). It was also verified by analyzing these duplexes by electrophoresis in denaturing 24% PAA/8 M urea gel that under these conditions the monofunctional adducts did not close to interstrand CLs. The aliquots of these reaction mixtures were withdrawn at various time intervals, the reaction was terminated by immediate cooling of these samples on dry ice, and the samples were analyzed on denaturing 24% PAA/8 M urea gel (Figure 7A). As shown in Figure 7A for the duplex TGT containing the monofunctional adduct of *trans*-[$PtCl_2(NH_3)$(4-pic)], the formation of this adduct resulted in a slower migration of the oligonucleotide (cf. lanes 1 and 8 in Figure 7A). The amount of radioactivity associated with the band corresponding to this more slowly migrating species decreased with the time of the incubation of the platinated duplexes with thiourea with a concomitant increase in the amount of radioactivity associated with the band corresponding to the unplatinated oligonucleotide (Figure 7B). The half-time of this reaction was 8 min for the monofunctional adduct of all *trans*-platinum compounds in all duplexes tested, which indicates that the monofunctional adducts of all *trans*-platinum compounds were removed from DNA by thiourea with the same efficiency and independently of the nucleotide sequence context.

Stability of the 1,3-GNG Intrastrand Cross-Links of Transplatin Analogues. The 1,3-GNG intrastrand CL of transplatin (N = any nucleotide) is stable within single-stranded DNA under physiological conditions. Within double-

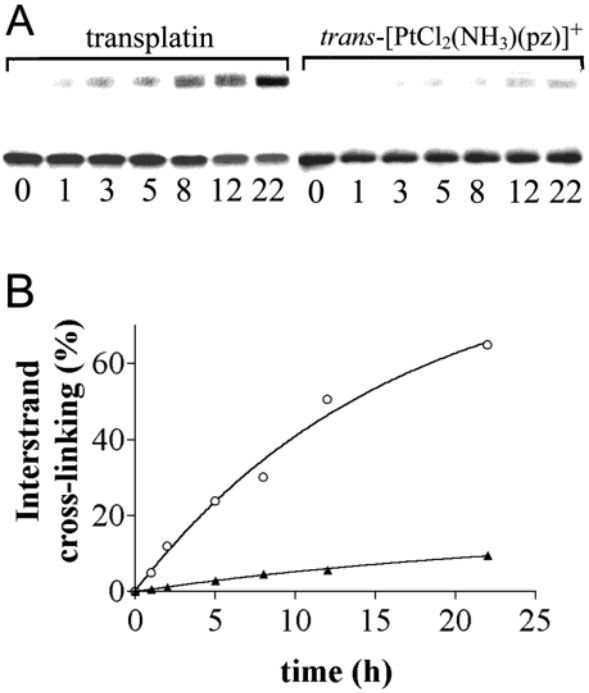

FIGURE 8: Rearrangement of the 1,3-intrastrand CLs formed by transplatin or *trans*-$[PtCl_2(NH_3)(pz)]^+$ in the duplex TGTGT. The samples of the 2 μM duplexes were incubated at 37 °C in 0.2 M $NaClO_4$, 5 mM Tris-HCl buffer (pH 7.5), and 0.1 mM EDTA. At various time intervals, the aliquots were withdrawn and analyzed by electrophoresis in 12% PAA/8 M urea gel. (A) Autoradiograms of the gels of the duplex modified by transplatin (left) or *trans*-$[PtCl_2(NH_3)(pz)]^+$ (right) radioactively labeled at the 5′-end of its top strand. Incubation times in minutes are indicated under each lane. Lane 0 refers to the 5′-end-labeled single-stranded top (platinated) strand. (B) Plot of the percentages of 1,3-interstrand CL of transplatin (○) or *trans*-$[PtCl_2(NH_3)(pz)]^+$ (▲) vs time. These percentages were calculated from the ratio of the radioactivity in each lane in part A associated with the band corresponding to the top bands in part A to the sum of the radioactivities associated with both bands (multiplied by 100). For other details, see the text.

helical DNA, its stability is markedly reduced and is even notably smaller than that of the interstrand CLs (preferentially formed by this platinum compound between guanine and complementary cytosine residues). Consequently, the pairing of single-stranded DNA containing 1,3-GNG intrastrand CL of transplatin with their complementary DNA sequences results in a rearrangement of these intrastrand adducts into interstrand CLs (*11*).

The stability of 1,3-GNG intrastrand CLs of transplatin and its analogues containing heterocyclic ligands was investigated using 20-mer oligodeoxyribonucleotides (the top strands of the duplexes CGCGC, TGCGT, and TGTGT shown in Figure 2) that were radioactively labeled at their 5′-ends and platinated so that they contained single and central, site-specific 1,3-GCG or GTG intrastrand CL. The single-stranded oligonucleotides containing this CL or the corresponding duplexes were incubated in 0.2 M $NaClO_4$ at 37 °C. At various time intervals, aliquots were withdrawn and analyzed by gel electrophoresis under denaturing conditions (Figure 8A).

The 1,3-GNG intrastrand adducts of all *trans*-platinum compounds within the single-stranded oligonucleotide were inert over a long period of time (>5 days) (not shown). It was verified by DMS footprinting that no rearrangement of

Table 1: IC_{50} Mean Values (±SD) Obtained for the Platinum Compounds Tested in the Present Work

	IC_{50} (μM)					
	A2780	A2780cisR	CH1	CH1cisR	41M	41McisR
cisplatin	2.2 ± 0.6	38 ± 3	6 ± 1	23 ± 3	26 ± 2	107 ± 8
cis-[$PtCl_2(NH_3)$(pip)]	26 ± 3	234 ± 17	36 ± 4	263 ± 17	64 ± 5	315 ± 24
cis-[$PtCl_2(NH_3)$(pz)]$^+$	10 ± 1	25 ± 2	28 ± 2	56 ± 3	46 ± 3	112 ± 12
cis-[$PtCl_2(NH_3)$(4-pic)]	23 ± 4	198 ± 9	24 ± 2	274 ± 15	52 ± 4	312 ± 28
transplatin	>200	>200	>200	>200	>200	>200
trans-[$PtCl_2(NH_3)$(pip)]	5 ± 0.7	20 ± 2	15 ± 2	94 ± 6	27 ± 2	150 ± 8
trans-[$PtCl_2(NH_3)$(pz)]$^+$	5 ± 1	44 ± 4	12 ± 3	34 ± 4	52 ± 5	155 ± 12
trans-[$PtCl_2(NH_3)$(4-pic)]	7 ± 1	80 ± 5	22 ± 3	154 ± 8	32 ± 3	187 ± 15

the 1,3-intrastrand CL occurred within this period. In contrast, this adduct formed by transplatin after pairing the platinated single-stranded oligonucleotides with their complementary strands was labile, consistent with the previous results (*11*). As a function of time, the radioactivity associated with the band corresponding to the 1,3-intrastrand CL decreased with a concomitant increase of the radioactivity associated with the new, more slowly migrating species that migrated at the same rate as the 20-bp duplex containing a single, interstrand CL of transplatin (shown for the duplex TGTGT containing the 1,3-GTG intrastrand CLs of transplatin or *trans*-[$PtCl_2(NH_3)$(pz)]$^+$ in Figure 8A). This result was interpreted to mean that the 1,3-intrastrand CL was transformed into an interstrand CL (*11*). For instance, after 24 h of incubation of the duplex TGTGT containing the 1,3-intrastrand CL of transplatin, 70% of the 1,3-intrastrand CLs were transformed into the interstrand CLs. On the other hand, the yields of these rearrangement reactions involving the 1,3-intrastrand CLs of transplatin analogues containing piperidine, piperazine, or 4-picoline ligand were radically lower (shown for the 1,3-intrastrand CL formed by *trans*-[$PtCl_2(NH_3)$(pz)]$^+$ in the duplex TGTGT in Figure 8B). After 24 h of incubation of the duplexes TGTGT containing the 1,3-intrastrand CL of *trans*-[$PtCl_2(NH_3)$(pip)], *trans*-[$PtCl_2(NH_3)$(pz)], or *trans*-[$PtCl_2(NH_3)$(4-pic)], only 4−19% of the 1,3-intrastrand CLs were transformed into the interstrand CLs. Importantly, similar slow rates were observed for the rearrangement into the interstrand CLs of the 1,3-intrastrand CLs of all three *trans*-platinum compounds tested in the present work in the other two duplexes CGCGC and TGCGT (not shown).

The bases in the interstrand CLs resulting from the rearrangement of the 1,3-intrastrand CLs were identified from Maxam−Gilbert footprinting in the same way as in the previous papers (*11*) (not shown). The results indicated that the interstrand CLs of all *trans*-platinum compounds in all three duplexes were always formed between the platinated 5′ G of the 1,3-GNG intrastrand CL and the complementary C.

Cytotoxicity. The cytotoxic activity of the *cis*- and *trans*-platinum compounds tested in the present work was determined against three pairs of cisplatin sensitive and resistant cancer cell lines (see Table 1) in the same way as in our recent work (*21*). These pairs of cell lines were selected on the basis of encompassing the known major mechanisms underlying resistance to cisplatin: A2780cisR being resistant through a combination of decreased uptake, enhanced DNA repair/tolerance and elevated reduced glutathione levels, 41McisR primarily through reduced drug transport, and CH1cisR through enhanced DNA repair/tolerance. The compounds were incubated for 24 h with the tumor cell lines, and the cell survival in the culture treated with the platinum compounds was evaluated as described previously (*21*, *38*).

Table 2: Summary of DNA Binding Characteristics of *cis*-[$PtCl_2(NH_3)$(pip)] (cis-pip), *cis*-[$PtCl_2(NH_3)$(pz)] (cis-pz), *cis*-[$PtCl_2(NH_3)$(4-pic)] (cis-pic), and Cisplatin

	cis-pip	cis-pz	cis-pic	cisplatin
DNA binding ($t_{50\%}$),[a] min	113	35	21	120[b]
% interstrand CLs/adduct[c]	5	6	4	6[d]
CD band at 278 nm[c]	increase	increase	increase	increase[e]
unwinding angle/adduct,[c] deg	13	13	12	13 [f]
melting temperature (Δt_m)[c,g]				
low ionic strength, °C	−2.5	3.5	−2.5	−2.5 [h]
high ionic strength, °C	−6.0	−4.5	−4.8	−4.1 [h]

[a] The time at which the binding reached 50%. [b] Bancroft et al. (*45*). [c] DNA modified for 48 h. [d] Brabec and Leng (*29*). [e] Brabec et al. (*40*). [f] Keck and Lippard (*35*). [g] Δt_m is defined as the difference between the t_m values of platinated and nonmodified DNAs. [h] Zaludova et al. (*48*).

The IC_{50} values are shown in Table 1. The analogues of cisplatin in which one ammine ligand was replaced by the heterocyclic ligand were somewhat less active than the parent cisplatin in both sensitive and resistant cell lines with only one exception; *cis*-[$PtCl_2(NH_3)$(pz)] was slightly more potent in the resistant line A2780cisR. On the other hand, the analogues of transplatin in which one ammine ligand was replaced by the heterocyclic ligand were radically more potent than the parent transplatin in the lines both sensitive and resistant to cisplatin. Importantly, the analogues of transplatin were in most cases considerably more potent than their cis congeners. The only exception was *trans*-[$PtCl_2(NH_3)$(pz)], which exhibited in three lines (A2780cisR, 41M, and 41McisR) activity that was comparable to that of its cis congener or that was slightly lower. Thus, the replacement of one ammine ligand by the heterocyclic ligand markedly enhanced the activity of transplatin in cancer cell lines, whereas a consequence of this replacement in cisplatin lowered its cytotoxicity.

DISCUSSION

Cis Geometry. Our cytotoxicity studies (Table 1) show that in most systems tested in the present work the replacement of one NH_3 group by the heterocyclic ligand in the molecule of cisplatin lowers the activity of this drug in both sensitive and resistant cell lines. However, this replacement does not considerably affect the DNA binding mode of cisplatin in a cell-free medium studied by some biochemical and biophysical methods (Table 2).

The only dissimilarities in the DNA binding mode of cisplatin and its analogues observed in the present work consist of the enhanced rate of binding exhibited by *cis*-

Table 3: Summary of DNA Binding Characteristics of *trans*-[$PtCl_2(NH_3)$(pip)] (trans-pip), *trans*-[$PtCl_2(NH_3)$(pz)] (trans-pz), *trans*-[$PtCl_2(NH_3)$(4-pic)] (trans-pic), and Transplatin

	trans-pip	trans-pz	trans-pic	transplatin
DNA binding ($t_{50\%}$),[a] min	113	20	50	120 [b]
% interstrand CLs/adduct[c]	26	18	40	12[d]
% monofunctional lesions/adduct[c]	15	22	34	35
CD band at 278 nm[c]	small increase	no change	decrease	decrease[e]
unwinding angle/adduct,[c] deg	30	17	26	9 [f]
melting temperature (Δt_m)[c,g]				
low ionic strength, °C	7.6	6.3	7.2	9.2 [h]
high ionic strength, °C	−1.9	−1.9	0.6	0.6 [h]
stability of intrastrand CLs	high	high	high	low

[a] The time at which the binding reached 50%. [b] Bancroft et al. (*45*). [c] DNA modified for 48 h. [d] Brabec and Leng (*29*). [e] Brabec et al. (*40*). [f] Keck and Lippard (*35*). [g] Δt_m is defined as the difference between the t_m values of platinated and nonmodified DNAs. [h] Zaludova et al. (*48*).

$[PtCl_2(NH_3)(pz)]^+$ or *cis*-$[PtCl_2(NH_3)(4\text{-pic})]$ and the melting temperature of DNA modified by *cis*-$[PtCl_2(NH_3)(pz)]^+$ measured at low salt concentration (Table 2). While the reasons for the faster binding of *cis*-$[PtCl_2(NH_3)(4\text{-pic})]$ are unknown, the positive charge on cationic *cis*-$[PtCl_2(NH_3)(pz)]^+$ may facilitate its rapid binding to DNA, perhaps by some sort of rapid electrostatic preassociation. However, the cytotoxicity data (Table 1) suggest that the enhanced rate of binding of *cis*-$[PtCl_2(NH_3)(pz)]^+$ and *cis*-$[PtCl_2(NH_3)(4\text{-pic})]$ to DNA observed in cell-free media does not affect in a fundamental way the activity of these compounds in tumor cell lines tested in the present work.

Similarly, an extra positive charge on *cis*-$[PtCl_2(NH_3)(pz)]^+$ could be responsible for an increase of the melting temperature of DNA modified by this compound and measured at low salt concentration. Previously, three important factors have been invoked to account for the thermal stability of DNA modified by platinum complexes: stabilizing effects of the positive charge on the metal moiety and of DNA interstrand CLs and a destabilizing effect of conformational distortions such as intrastrand CLs induced in DNA by platinum coordination (*48*). The dependence of transition melting temperature on ionic strength was explained by competing electrostatic effects as salt concentration was varied. Under the incubation conditions, we expect all new platinum analogues to have produced a range of adducts. The observed change in melting temperature will reflect the relative proportion and contribution of the two limiting binding modes. Inherently, we predict conformational distortions due to the adduct formation to destabilize the helix, as has been consistently observed in studies with cisplatin or other antitumor platinum compounds. In contrast, interstrand cross-linking is predicted to stabilize the helix by preventing strand dissociation. At low ionic strength, it is reasonable to conclude that the increases in t_m are caused by interstrand CLs formed by the platinum compounds and by positive charges on these platinum compounds. On the other hand, at high salt concentration the stabilizing effects are reduced, since electrostatic effects of the platinum compounds are apparently lowered with increasing concentration of Na^+ counterions. Hence, it is possible that the decrease in t_m observed at high and low ionic strength is a consequence of conformational changes induced by the adducts of new analogues of cisplatin that then dominate the combination of "stabilizing" effects. At high salt concentration, the stabilizing effects are reduced, since electrostatic effects of the platinum compounds are apparently lowered with increasing concentration of Na^+ counterions. Importantly, cisplatin and all its analogues tested in the present work affect DNA properties (conformational alterations such as those revealed by CD spectroscopy and DNA unwinding, Table 2) in a similar way. The only major difference between *cis*-$[PtCl_2(NH_3)(pz)]^+$ and cisplatin or its other analogues that could affect stability of platinated DNA appears to be a higher positive charge on *cis*-$[PtCl_2(NH_3)(pz)]^+$ that can substantially affect DNA stability at low but not at high salt concentration. Thus, the increase of the melting temperature of DNA modified by *cis*-$[PtCl_2(NH_3)(pz)]^+$ observed at low and not at high salt concentration is apparently associated with its higher positive charge that can contribute to a higher "electrostatic" stabilizing effect in comparison with cisplatin and its other analogues only at low ionic strength.

A careful examination of the cytotoxicity data in Table 1 reveals that *cis*-$[PtCl_2(NH_3)(pz)]^+$ is consistently more potent in cisplatin resistant cell lines than other analogues of cisplatin tested in the present work. Thus, the results of the present work suggest that a higher charge on cisplatin analogues containing aliphatic heterocyclic ligands may be a factor promoting circumvention of cisplatin resistance.

The replacement of the NH_3 group in cisplatin by the heterocyclic ligands has not changed its DNA binding mode evaluated in the present work by some biochemical and biophysical methods after global modification of DNA. It is, therefore, reasonable to suggest that the reduced activity of these analogues of cisplatin may be associated with some features of the damaged DNA and/or its cellular processing not perceptible by the methods used in the present work. Alternatively, the reduced activity of the analogues of cisplatin (Table 1) might also be due to the factors involved in the mechanism underlying antitumor effects of cisplatin that do not operate directly at the level of the target DNA. Among these factors might be, for instance, those that affect the amount of platinum complex that can reach target DNA in cancer cells by changing the cell accumulation of the complexes. To understand the reduced activity of the analogues of cisplatin containing the nonleaving heterocyclic ligand in tumor cell lines, however, further structural and biochemical studies are required.

Trans Geometry. In contrast to analogues of cisplatin, the replacement of one ammine group by a heterocyclic ligand such as piperidine, piperazine, and 4-picoline in ineffective transplatin results in a radical enhancement of its activity in tumor cell lines both sensitive and resistant to cisplatin (Table 1). Concomitantly, this replacement markedly alters the DNA binding mode of transplatin (Table 3). Hence, the results of

the present work further sustain the hypothesis that platinum drugs that bind to DNA in a fundamentally different manner from that of cisplatin may have altered pharmacological properties (*10*, *16*, *19*).

Similarly as in the case of cisplatin analogues containing piperazine and 4-picoline ligand, these analogues of transplatin bind to DNA considerably more quickly than the parent transplatin and its analogue containing piperidine ligand (Table 3). Nevertheless, it seems less likely that this faster binding to DNA is associated in a fundamental way with the activity of these transplatin analogues in tumor cell lines tested in the present work.

An increase of t_m observed at low salt concentration (Table 3) can be interpreted to mean that under these conditions "stabilizing" effects of interstrand CLs and positive charges on platinum moieties dominate over the "destabilizing" effects of conformational alterations induced by the adducts of *trans*-platinum compounds. That the adducts of new analogues of transplatin change the conformation of DNA is further supported by the other data on DNA melting (a decrease of t_m due to the modification of DNA by *trans*-$[PtCl_2(NH_3)(pip)]$ and *trans*-$[PtCl_2(NH_3)(pz)]^+$, Table 3). An interesting and as yet unresolved question is why high salt concentration appears to result in an overall destabilization due to the binding of *trans*-$[PtCl_2(NH_3)(pip)]$ and *trans*-$[PtCl_2(NH_3)(pz)]^+$ and not due to the binding of *trans*-$[PtCl_2(NH_3)(4\text{-pic})]$. At high salt concentration, the stabilizing effects are reduced, since electrostatic effects of the platinum compounds are apparently lowered with increasing concentration of Na^+ counterions so that the major stabilizing factors are interstrand CLs (vide supra). It is possible that the observed decrease in t_m at high ionic strength is a consequence of the domination of "destabilizing" effects of conformational changes induced by the adducts of *trans*-$[PtCl_2(NH_3)(pip)]$ and *trans*-$[PtCl_2(NH_3)(pz)]^+$ over the "stabilizing" effects of their interstrand CLs. On the other hand, the observed increase in t_m of DNA modified by *trans*-$[PtCl_2(NH_3)(4\text{-pic})]$ at high ionic strength is a consequence of the domination of "stabilizing" effects of interstrand CLs formed by this compound over the "destabilizing" effects of conformational changes induced by its adducts. Consistent with this interpretation is the finding that *trans*-$[PtCl_2(NH_3)(4\text{-pic})]$ forms in DNA pronouncedly more interstrand CLs than *trans*-$[PtCl_2(NH_3)(pip)]$ and *trans*-$[PtCl_2(NH_3)(pz)]^+$. The melting of DNA modified by platinum compounds is a complex process that may involve other factors not considered in the present work. Thus, a more definitive evaluation of all effects of the replacement of ammine groups in cisplatin or transplatin by the heterocyclic ligands on DNA melting remains to be determined.

The values of unwinding angles are affected by the nature of the ligands in the coordination sphere of platinum and the stereochemistry at the platinum center. A previous systematic work revealed that the platinum(II) compounds fall into different classes according to their DNA binding modes (*35*). It has been shown that platinum(II) compounds with the smallest unwinding angles (6°) are those that can bind DNA only monofunctionally {[PtCl(dien)]Cl or $[PtCl(NH_3)_3]Cl$}. Another group of platinum compounds is composed of those that bind to DNA in a bifunctional manner. The compounds that belong to this category unwind DNA by 10−13° and include cisplatin, transplatin, and antitumor bifunctional dinuclear complexes such as $[\{trans\text{-}PtCl(NH_3)_2\}_2(\mu\text{-}H_2N(CH_2)_nNH_2)]Cl_2$, $n = 2-6$. The observation that all new analogues of cisplatin or transplatin tested in the present work cannot be grouped with monofunctional platinum(II) compounds is readily understood in terms of adduct structures in which the complexes are preferentially coordinated to DNA in a bifunctional manner.

Interestingly, the unwinding angles produced by the analogues of transplatin (Table 3) are considerably higher than those produced by cisplatin or transplatin (*35*). One plausible explanation for this observation might be consistent with a possibility that the additional contribution to unwinding is associated with the interaction of the piperidine, piperazine, or 4-picoline group with the duplex upon covalent binding of platinum. In this way, the piperidine, piperazine, or 4-picoline moiety in DNA adducts of transplatin analogues are geometrically well positioned to interact with the double helix. Thus, the results of unwinding experiments represent good support for the view that piperidine, piperazine, or 4-picoline ligands in transplatin analogues tested in the present work interact substantially with the double helix upon covalent binding of the platinum complex. Importantly, this specific DNA binding mode is only observed for the analogues of transplatin and not for their cis isomers. This is consistent with the idea that the formation of the major adducts of the cis isomers, presumably 1,2-intrastrand CLs, does not allow positioning of the piperidine, piperazine, or 4-picoline moiety in these adducts that would be favorable for their interaction with the double helix.

The analogues of transplatin show a noticeably higher interstrand cross-linking efficiency than cisplatin or transplatin (Figure 3 and Table 3). The CLs are formed in DNA by bifunctional platinum compounds in a two-step process. In the first step, the monofunctional adducts are formed preferentially at guanine residues that subsequently close to the CLs. The unexpected high rate of interstrand CL formation by transplatin analogues containing a heterocyclic ligand relative to that determined for "classical" transplatin may imply that conformational changes in double-stranded DNA, induced by the heterocyclic ligand in a monofunctional adduct, modulate the second binding step and facilitate formation of an interstrand CL, in contrast to the situation for classical transplatin. Further studies are required aimed at determining the sites involved in the interstrand CLs of these analogues and the conformational alterations induced in DNA by all types of adducts to identify more factors responsible for high interstrand cross-linking efficiency of this class of platinum compounds. Nevertheless, one of the striking consequences of the replacement of one ammine ligand in "classical" transplatin by the piperidine, piperazine, or 4-picoline ligand is a markedly higher rate of DNA interstrand cross-linking by these compounds. The clinical ineffectiveness of transplatin has been proposed to be also associated with its reduced capability to form bifunctional adducts in double-helical DNA (*12*, *13*). A considerably higher interstrand cross-linking efficiency of transplatin analogues containing the piperidine, piperazine, or 4-picoline ligand in comparison with transplatin (Table 3) remains an important candidate for the factor responsible for their markedly higher activity in tumor cells.

It has been also demonstrated (*11*) that transplatin does not form stable intrastrand CLs in double-helical DNA, and

this property of transplatin was also related to its clinical inefficiency (*13*, *49*). We have demonstrated in the present work (Figure 8) that the replacement of one ammine ligand in "classical" transplatin by the piperidine, piperazine, or 4-picoline ligand results in a distinctively enhanced stability of the 1,3-GNG intrastrand CLs formed by these compounds in double-helical DNA. Hence, the markedly higher activity of transplatin analogues containing the piperidine, piperazine, or 4-picoline ligand in tumor cell lines also correlates with the higher stability of their intrastrand CLs.

In conclusion, the mechanism underlying antitumor activity of platinum compounds is a complex process involving a number of factors. Among these factors are also those that do not operate directly at the level of the DNA adducts, such as those affecting the amount of platinum complex that can reach DNA [by influencing either the cell accumulation of the complexes or the levels of intracellular platinophiles that act as detoxification agents (metallothioneine and glutathione)]. Nevertheless, it is generally accepted that DNA is an important and major pharmacological target of platinum compounds (*4*, *6*, *10*). The present work correlates the DNA binding mode in a cell-free medium of the bifunctional analogues of cisplatin and transplatin containing one piperidine, piperazine, or 4-picoline ligand with their activity in several tumor cell lines. The results offer strong experimental support for the view that one strategy of how to activate trans geometry in bifunctional platinum(II) compounds and to circumvent resistance to cisplatin consists of a chemical modification of the "classical" transplatin that would result in an increased stability of the intrastrand CLs of these *trans*-platinum compounds in double-helical DNA and/or in their increased efficiency to form interstrand CLs. The present work also suggests that such a modification may be accomplished by the replacement of one ammine group by a nonplanar heterocyclic ligand, such as piperidine and piperazine, or by an aromatic planar heterocyclic ligand, such as 4-picoline.

ACKNOWLEDGMENT

The authors acknowledge that their participation in the EC COST Chemistry Action D20 enabled them to exchange regularly the most recent ideas in the field of platinum anticancer drugs with several European colleagues.

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BI0342315