Effects of Geometric Isomerism and Ligand Substitution in Bifunctional Dinuclear Platinum Complexes on Binding Properties and Conformational Changes in DNA†

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ABSTRACT: The DNA binding profile of a series of dinuclear platinum complexes [{*trans*-PtCl(L)$_2$}$_2$H$_2$N(CH$_2$)$_n$NH$_2$]$^{2+}$ (L = NH$_3$ or py; 1,1/t,t/NH$_3$ and 1,1/t,t/py, respectively) and [{*cis*-PtCl(NH$_3$)$_2$}$_2$H$_2$N(CH$_2$)$_n$NH$_2$]$^{2+}$ (1,1/c,c/NH$_3$) was examined to compare the effects of geometrical isomerism and the presence of ligands other than NH$_3$ in the coordination sphere. Steric effects, because of the geometry of the leaving groups *cis* to the diamine bridge or the presence of planar pyridine ligands, result in diminished binding to calf thymus DNA for these isomers. In contrast, the pyridine derivative shows a distinct binding preference for poly(dG-dC)·poly(dG-dC) in comparison to both NH$_3$ isomers. Both NH$_3$ complexes induce the B → Z transition in poly(dG-dC)·poly(dG-dC), but the presence of a pyridine ligand stabilizes the B conformation. The bifunctional binding of the NH$_3$ isomers results in unwinding of supercoiled pUC19 plasmid DNA equivalent to *cis*-DDP, while the unwinding of the pyridine derivative is approximately twice that of the mononuclear *trans*-[PtCl$_2$(py)$_2$]. DNA-DNA interstrand cross-linking is very efficient for all three agents, but sequencing studies indicated that only the 1,1/t,t/NH$_3$ derivative is capable of forming a (Pt,Pt) intrastrand cross-link to the adjacent guanines of a d(GpG) sequence. The effects on DNA caused by bifunctional binding of dinuclear complexes are compared with those from the mononuclear [PtCl$_2$(NH$_3$)$_2$] isomers. The results are discussed with respect to the antitumor activity of the dinuclear series.

Dinuclear platinum complexes are a class of compounds of considerable interest for their antitumor and DNA-binding properties (Farrell, 1993, and references therein, 1995a). The most general formula for the dinuclear complexes we have studied is [{PtCl$_m$(NH$_3$)$_{3-m}$}μ-H$_2$N-R-NH$_2${PtCl$_n$(NH$_3$)$_{3-n}$}]$^{[(2-m)+(2-n)]+}$ (m or n = 0−3 and R is a linear or substituted aliphatic linker). When m = 1, each platinum has one leaving group and the DNA binding is bifunctional. These compounds are of special interest because they do *not* contain a *cis*-[PtCl$_2$(amine)$_2$] unit yet are cytotoxic and antitumor active at doses (1−3 mg/kg) similar to that of *cis*-DDP[1] (Manzotti et al., 1994; Farrell et al., 1994). The properties of monofunctional Pt coordination spheres and an overall 2+ charge violate all previous structure−activity relationships for platinum complexes.

To date, we have reported on the properties of [{*trans*-PtCl(NH$_3$)$_2$}$_2$H$_2$N(CH$_2$)$_n$NH$_2$]$^{2+}$ (1,1/t,t/NH$_3$) which has been shown to be particularly effective against *cis*-DDP-resistant cells both *in vitro* and *in vivo* (Manzotti et al., 1994). The complexes cause significant DNA (Pt,Pt) interstrand (Zou et al., 1994; Farrell et al., 1990a) as well as (Pt,Pt) intrastrand cross-links (Zou et al., 1994; Bloemink et al., 1992). Binding to DNA not only occurs at sequences preferred by *cis*-DDP, but other sequences, especially alternating purine-pyrimidine GCGC sequences, are attacked (Zou et al., 1994; Wu et al., 1994; Farrell et al., 1990a). The 1,1/t,t/NH$_3$ complexes were also found to promote the B → Z conformational change of poly(dG-dC)·poly(dG-dC) at very low binding as evident from circular dichroism studies (Johnson et al., 1992).

The presence of a formally bifunctional unit allows us to compare how the conformational changes induced by the dinuclear complexes compare with those induced by the bifunctional binding of *cis*-DDP and *trans*-DDP. In principle, the bifunctional DNA binding modes of dinuclear platinum complexes may be manipulated by the geometry of the complex, especially with respect to the relationship of leaving groups to diamine bridge, and the nature of the diamine bridge itself as well as the other ligands in the Pt coordination spheres. Geometric isomerism exists in this case because the Cl leaving groups are either *cis* or *trans* to the diamine bridge (Figure 1). We have now succeeded in preparing the [{*cis*-PtCl(NH$_3$)$_2$}$_2$H$_2$N(CH$_2$)$_n$NH$_2$]$^{2+}$ (1,1/c,c/NH$_3$) isomer (Qu et al., 1995).

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[1] Abbreviations: *cis*-DDP, *cis*-[PtCl$_2$(NH$_3$)$_2$], cisplatin, *cis*-diamminedichloroplatinum; *trans*-DDP, *trans*-[PtCl$_2$(NH$_3$)$_2$]; dien, diethylenetriamine; py, pyridine; 1,1/t,t/NH$_3$, [{*trans*-PtCl(NH$_3$)$_2$}$_2$H$_2$N(CH$_2$)$_4$NH$_2$]$^{2+}$; 1,1/t,t/py, [{*trans*-PtCl(py)$_2$}$_2$H$_2$N(CH$_2$)$_4$NH$_2$]$^{2+}$; 1,1/c,c/NH$_3$, [{*cis*-PtCl(NH$_3$)$_2$}$_2$H$_2$N(CH$_2$)$_4$NH$_2$]$^{2+}$; CD, circular dichroism; FAAS, flameless atomic absorption spectroscopy; r_b, the number of bound platinum complexes per nucleotide; CT, calf thymus; SC, supercoiled; OC, open circle; r_b(c), value at which the SC and OC forms of DNA comigrate; A/F, number of adducts per fragment; XL, fraction of counts running as interstrand cross-linked band; XL/F, ratio of interstrand cross-links to total platinum bound; XL/A, ratio of interstrand cross-links per adduct; HMG, high-mobility group; ID$_{50}$, inhibitory dose at which cell growth is inhibited by 50%; bp, base pair.

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A further modification to allow for systematic variation of DNA binding properties is in the nature of the amine coordinated to the Pt centers. Especially, we have shown that the DNA binding properties of *cis*- and *trans*-$[PtCl_2(amine)_2]$ differ dramatically between amine = NH_3 and amine = pyridine (Zou et al., 1993). We therefore decided to examine the effects on DNA binding of incorporation of planar pyridine ligands into the 1,1/t,t structure. To this end, the complex $[\{trans\text{-}PtCl(py)_2\}_2H_2N(CH_2)_nNH_2]^{2+}$ (1,1/t,t/py) was synthesized. This paper reports a comparison of the DNA binding and protein recognition properties of the three 1,4-butanediamine-linked dinuclear Pt complexes differing in geometry and the nature of coordinated ligands.

MATERIALS AND METHODS

Synthesis of Complexes. The complex $[\{trans\text{-}PtCl(py)_2\}_2H_2N(CH_2)_4NH_2]Cl_2$ was synthesized using *trans*-$[PtCl_2(py)_2]$ and the published general procedure for the 1,1/t,t/NH_3 complexes (Qu & Farrell, 1990, 1992). The complex $[\{cis\text{-}PtCl(NH_3)_2\}_2H_2N(CH_2)_4NH_2](NO_3)_2$ (1,1/c,c/NH_3) was prepared from *cis*-$[PtCl_2(NH_3)_2]$ and $AgNO_3$ using procedures similar to those for the 1,1/t,t/NH_3 complexes (Qu et al., 1995). All complexes were characterized by elemental analysis, ^1H and ^{195}Pt NMR spectroscopy, and mass spectrometry. Characterization data were in complete agreement with the proposed structures.

Spectroscopic Measurements. Circular dichroism spectra were recorded on a Jobin-Yvon Autodichrograph Mark V spectrophotometer using a quartz cell with a 1 cm path length. Flameless atomic absorption spectroscopy (FAAS) measurements were carried out on a Perkin-Elmer 560 instrument with a graphite furnace.

Quantitation of Pt−DNA Binding and Conformational Changes. Platination of DNA and calculation of r_b were carried out as previously described (Zou et al., 1994; Farrell et al., 1990a). CT DNA (0.02 or 0.05 mg/mL) or poly(dG-dC)·poly(dG-dC) (0.016 mg/mL) was incubated with varying concentrations (5−200 μM) of platinum complexes in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) for 24 h at 37 °C, followed by addition of 200 mM NaCl to terminate the reactions. Unbound platinum complexes were removed by extensive dialysis against TE buffer at 4 °C. The Pt content of the samples was determined by FAAS by suspending the solutions in 2% nitric acid and heating at 50 °C for 24 h or 70 °C for 1 h to hydrolyze the DNA. The r_b values were also determined and were used for calculation of the cross-link per adduct ratio in the interstrand cross-link assay. Samples for circular dichroism spectra and the circular dichroism studies were performed as described previously (Johnson et al., 1992; Wu et al., 1994).

Unwinding of Negatively Supercoiled DNA. Unwinding of closed circular supercoiled pUC19 plasmid DNA was monitored by an agarose gel mobility shift assay (Keck & Lippard, 1992). The unwinding angle, ϕ, induced per platinum−DNA adduct was calculated upon the determination of the r_b value corresponding to the coalescence point where the two forms of DNA (supercoiled and relaxed) comigrate. Samples of dinuclear platinum complexes were incubated at 37 °C with 3 μg of pUC19 plasmid for 24 h. All samples were then extensively dialyzed against TE buffer using a BRL 1202 MD microdialysis system at 4 °C in the dark. An aliquot of the dialyzed solutions was subjected to electrophoresis on 1% agarose gels running at 25 °C in the dark with TAE buffer (Tris-acetate/EDTA) with voltages set between 30 and 40 V. The gels were then stained with ethidium bromide, followed by photography on Polaroid 667 film with UV transillumination. The other aliquot was used for the determination of r_b values, calculated on the basis of DNA concentration (ϵ_{260} = 6600 M^{-1} cm^{-1}) and Pt bound measured by FAAS.

Interstrand Cross-Link Assay and Sequence Specificity of Platinum−DNA Adducts. The experiments were conducted in the same way as described previously (Zou et al., 1994; Farrell et al., 1990a). The 5′-terminally [^{32}P]-labeled 49 bp oligodeoxynucleotides of sequence

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5′-GACTACTTGGTACACTGACGCGAGCTCGCGGAAGCTCATTCCAGTGCGC-3′ (top)
3′-CTGATGAACCATGTGACTGCGCTCGAGCGCCTTCGAGTAAGGTCACGCG-5′(bottom)
   1   5   10   15   20   25   30   35   40   45
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were prepared on a Du Pont automatic DNA synthesizer and purified by electrophoresis on 12% denaturing polyacrylamide gels. The protocols followed that of previous reports (Zou et al., 1994). Briefly, the DNA fragments were identified by comparison with the Maxam−Gilbert products produced under the same conditions (Maxam & Gilbert, 1980). For interstrand cross-linking, a mixture (20 μL) of the 5′-terminally labeled 49-mer oligonucleotides in the presence of CT DNA (0.023 mg/mL) and dinuclear platinum agents with several different concentrations was incubated in TE buffer for 24 h at 37 °C. The reactions were terminated by addition of 100 mM NaCl. After heating at 95 °C for 3 min, a mixture of the samples and formamide dyes with equal volume was loaded on 8% polyacrylamide denaturing sequencing gels for electrophoresis. The dried gels were then autoradiographed with Kodak XAR-5 films in the presence of intensifying screens at −70 °C. To quantify the interstrand cross-linked DNA which appeared as the bands migrating more slowly than the singly stranded DNA on the gel, the radioactivity of all bands was measured by a Betascope 603 blot analyzer (Betagen, Waltham, MA). Mapping of DNA lesions induced by platinum complexes in the 49 bp oligonucleotides prepared above was conducted as previously described (Zou et al., 1993; Farrell et al., 1990a). Briefly, the duplex (5−10 ng/μL, radiolabeled on either strand) was incubated with platinum complexes (0.5 μM) in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) at 37 °C. Total incubation time was 3 h for the bis(platinum) NH_3 agents and 12 h for the pyridine derivative. The treated DNA fragment was then digested by the 3′ → 5′ exonuclease activity of T4 DNA polymerase of 10 units in a total volume of 40 μL containing 50 mM Tris-HCl, 50 mM KCl, and 10mM $MgCl_2$ for 90 min at 37 °C. The reactions were terminated by addition of EDTA to 25 mM, followed by denaturing the enzyme by bringing the sample to 90 °C for 5 min. The bound platinum was removed as $[Pt(CN)_4]^{2-}$ in a reaction with 0.3 M NaCN, pH 8.0, for 3 h at 37 °C or overnight at room temperature. The reaction products were diluted by about 5 times with a formamide/dyes sample buffer and directly loaded on gels without dialysis. The resulting DNA fragments were identified by comparison with the Maxam−Gilbert products (Maxam & Gilbert, 1980) of the same 5′-end-labeled 49 bp DNA by prolonged electrophoresis on 12% polyacrylamide sequencing gels under denaturing conditions. The Maxam−Gilbert

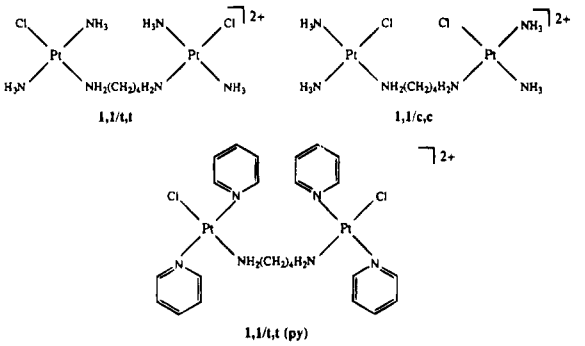

FIGURE 1: Structures of dinuclear platinum complexes used in this study. Counteranions are omitted for clarity. We have adopted a system where the numbers refer to the number of chlorides on each platinum atom and the lettering refers to the geometry of the leaving group with respect to the nitrogen of the bridging diamine. The differences in coordination sphere for complexes derived from $[PtCl_2(NH_3)_2]$ or $[PtCl_2(py)_2]$ are further delineated by the suffix /NH_3 or /py as appropriate.

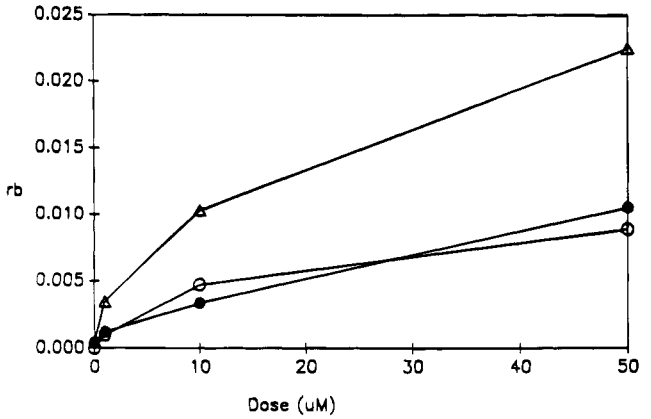

FIGURE 2: Kinetics of binding of dinuclear platinum complexes to calf thymus DNA at 37 °C for 24 h (see Materials and Methods for full details). [{*trans*-$PtCl(NH_3)_2$}$_2H_2N(CH_2)_4NH_2$]$^{2+}$ (1,1/t,t) is △. 1,1/c,c/NH_3 = [{*cis*-$PtCl(NH_3)_2$}$_2H_2N(CH_2)_nNH_2$]$^{2+}$ (1,1/c,c) is ●. [{*trans*-$PtCl(py)_2$}$_2H_2N(CH_2)_4NH_2$]$^{2+}$ (1,1/t,t/py) is ○.

sequencing was carried out by using a NEN Du Pont sequencing kit.

RESULTS

The structures of the complexes studied are given in Figure 1. The characterization data (^1H and ^{195}Pt NMR, IR, and C, H, N analysis) for all new complexes were in agreement with the proposed structures.

Initial attempts to prepare the *cis* isomer of the pyridine complex did not give pure material and were not pursued further. DNA binding was assayed with respect to quantitation of the r_b values, conformational changes, interstrand cross-linking, and sequence specificity.

Quantitation of DNA Binding. Quantitation of DNA binding shows some interesting differences for the NH_3 isomers (Figure 2). More rapid binding of the 1,1/t,t/NH_3 isomer to CT DNA occurs, and the binding of the *cis* isomer is approximately half that of the *trans* isomer for any given dose (Figure 2). This is an interesting steric effect of the leaving group (Cl or H_2O from the aquated species) *cis* to the diamine bridge. Also, little precipitation of DNA is noted at higher concentrations of the 1,1/c,c used, whereas at doses of the 1,1/t,t isomer >100 μM, our experience is that substantial precipitation of DNA occurs (see also Figure 5).

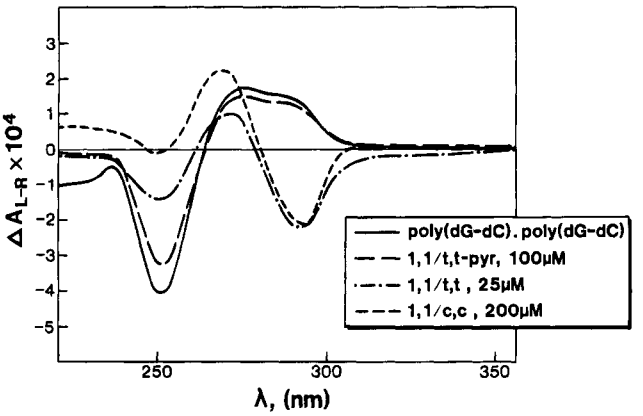

FIGURE 3: Circular dichroism spectra of poly(dG-dC)•poly(dG-dC) adducted by dinuclear platinum complexes. The doses are adjusted to give approximately equivalent r_b values, calculated as outlined in Materials and Methods. The doses correspond to r_b = 0.12 (1,1/t,t), 0.15 (1,1/t,t/py), and 0.135 (1,1/c,c).

The binding of the pyridine complex is similar to that of the 1,1/c,c/NH_3 species, again presumably due to steric effects. Thus, both coordination geometry and the nature of the ligands attached to Pt affect the rate of DNA binding. When poly(dG-dC)•poly(dG-dC) is the substrate, a similar difference is observed between the two NH_3 isomers, but eventually, in both cases, the binding reaches a plateau (Wu et al., 1994). This plateauing is not observed for binding of 1,1/t,t/py to poly(dG-dC)•poly(dG-dC) (data not shown) and may reflect binding to Z-form DNA of the NH_3 complexes (see below).

Conformational Changes Induced by Dinuclear Platinum Complexes. Previous studies had indicated that an interesting difference between mononuclear and dinuclear platinum complexes was that the Z form of poly(dG-dC)•poly(dG-dC) was induced very efficiently upon binding of the 1,1/t,t/NH_3 complex (Johnson et al., 1992) but not by *cis*-DDP (Malfoy et al., 1981; Ushay et al., 1982). The generality of this feature was examined with the complexes studied here (Figure 3). The 1,1/c,c/NH_3 complex induces the B → Z transition of poly(dG•dC)-poly(dG•dC) at equivalent r_b to the *trans* isomer. Note that the CD spectra of the 1,1/t,t- and 1,1/c,c-adducted poly(dG-dC)•poly(dG-dC) are not identical, implying that the adducts formed are also structurally distinct, albeit similar. In contrast, upon replacement of NH_3 by pyridine in the 1,1/t,t structure, the [{*trans*-$PtCl(py)_2$}$_2H_2N(CH_2)_4NH_2$]$^{2+}$ cation stabilizes the B form, an interesting effect of the planar ligand. Planar intercalating ligands such as ethidium bromide also stabilize B-form DNA and can reverse the Z form induced by small cations (Na^+) and hydrophobic species (EtOH) (Lamos et al., 1986; Walker et al., 1985). It is possible that the orientation of the planar ligands relative to the purine and pyrimidine bases helps to stabilize the B form in a manner similar to that of the intercalating agents.

DNA Unwinding. To examine further the nature of conformational changes, we measured the unwinding angles for all three compounds (Table 1 and Figure 4). The degree of topological unwinding of supercoiled plasmid DNA by platinum complexes is dependent on the geometry of the complex and the structure of the specific adduct (Keck & Lippard, 1992). The unwinding angles induced by the pair of [{$PtCl(NH_3)$}$_2H_2N(CH_2)_4NH_2$]$^{2+}$ isomers are similar to that found for *cis*-DDP and are typical of bifunctional binding

Table 1: Unwinding of Supercoiled DNA by Platinum Complexes[a]

complex	$r_b(c)$	unwinding angle (deg)
$[PtCl(NH_3)_3]^+$	0.176	6
trans-DDP	0.053	9−10
cis-DDP	0.043	13
trans-$[PtCl_2(py)_2]$	0.033	17
$[\{trans\text{-}PtCl(NH_3)_2\}_2H_2N(CH_2)_4NH_2]^{2+}$	0.057	10
$[\{cis\text{-}PtCl(NH_3)_2\}_2H_2N(CH_2)_4NH_2]^{2+}$	0.045	12
$[\{trans\text{-}PtCl(py)_2\}_2H_2N(CH_2)_4NH_2]^{2+}$	0.013	42

[a] The unwinding angle is given by $\Phi = 18\sigma/r_b(c)$, where $r_b(c)$ is the value at which the two forms of DNA (supercoiled and relaxed) comigrate. The analysis follows that of Keck and Lippard (1992), with an unwinding angle for *cis*-DDP of 13° used to calculate σ, giving a superhelical density of −0.308 under the present conditions. See Materials and Methods for details. The values for the DDP isomers and $[PtCl(NH_3)_3]^+$ are from Keck and Lippard (1992), while that of *trans*-$[PtCl_2(py)_2]$ is from Zou et al. (1993).

(Table 1). The unwinding is in fact almost twice that of the monofunctional $[PtCl(NH_3)_3]^+$ previously studied (Keck & Lippard, 1992). However, we do not take this result to mean that the conformational changes induced by the dinuclear complexes are simply the sum of two "monomeric" or monofunctional lesions, as has been suggested (Keck & Lippard, 1992). The total unwinding caused by localized binding of the two monofunctional centers, separated by 4−6 bp, is likely to be transmitted over a longer distance and to be reflected as the result of the overall bifunctional binding at the site of platination (see Discussion below).

Substitution of a planar ligand such as pyridine for NH_3 in the monomeric *trans*-$[PtCl_2(amine)_2]$ dramatically increases the unwinding efficiency (Zou et al., 1993). As with monomeric complexes, the substitution of py for NH_3 greatly enhances the unwinding of supercoiled DNA, which is essentially double that of the mononuclear complex. The value obtained represents one of the largest unwinding angles reported for complex (drug) modification of DNA. Note further that the phenomenon of reappearance of supercoiled DNA above the coalescence point, observed previously for *trans*-$[PtCl_2(py)_2]$ (Zou et al., 1993), is also seen in the present case, further indicating how the presence of a planar ligand affects the details of Pt−DNA binding.

DNA−DNA Interstrand Cross-Linking. DNA−DNA interstrand cross-linking is very efficient for all complexes studied (Figure 5). Electrophoresis under denaturing conditions of the end-labeled 49 bp oligonucleotide modified by the platinum complexes allows a quantitative assessment of the number of DNA interstrand cross-links formed per DNA adduct (Roberts et al., 1989; Zou et al., 1993). The single-stranded DNA fragment was identified as running fastest on the gel, and the interstrand cross-linked fragment as the double-stranded DNA appeared as the bands migrating more slowly on the gel. Quantitation showed that geometry and nature of coordinated ligand affected the efficiency of interstrand cross-linking, being approximately 80% of all adducts for the 1,1/c,c/NH_3 case compared to 52% and 41% for the 1,1/t,t/NH_3 and 1,1/t,t/py isomers, respectively (Table 2).

Adduct Formation and Sequence Specificity for Dinuclear Platinum Complexes. The inhibition of the 3′ → 5′ exonuclease activity of T4 polymerase (Malinge et al., 1987) was used to monitor the sites of DNA adducts and DNA−DNA interstrand cross-links on a 49 bp oligonucleotide of defined

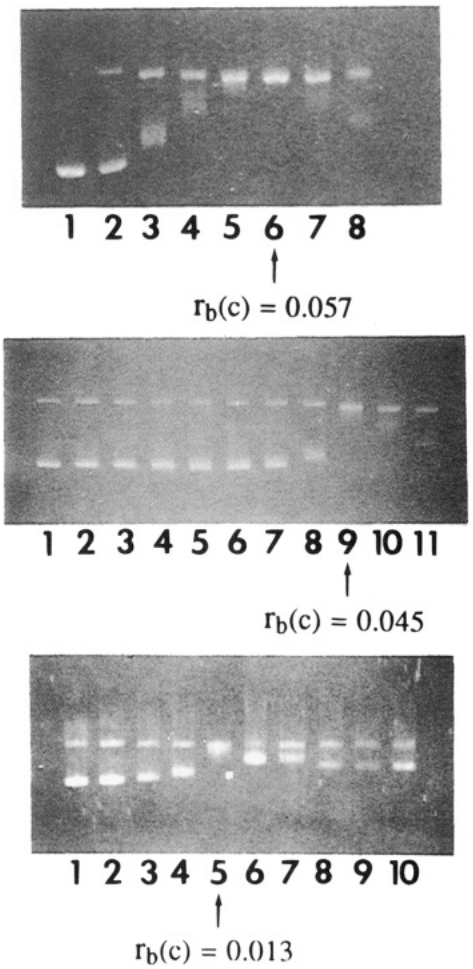

FIGURE 4: Unwinding of supercoiled pUC19 plasmid DNA by dinuclear platinum complexes. See also Table 1. The coalescence point where the two forms of DNA (supercoiled and relaxed) comigrate corresponds to $r_b(c)$, and values were calculated for $[\{trans\text{-}PtCl(NH_3)_2\}_2H_2N(CH_2)_4NH_2]^{2+}$ (top), $[\{cis\text{-}PtCl(NH_3)_2\}_2H_2N(CH_2)_4NH_2]^{2+}$ (middle), and $[\{trans\text{-}PtCl(py)_2\}_2H_2N(CH_2)_4NH_2]^{2+}$ (bottom) as described previously (Zou et al., 1993). In the top panel, the plasmid was incubated with 1,1/t,t/NH_3 to give r_b values of 0, 0.010, 0.023, 0.037, 0.051, 0.057, 0.069, and 0.143 corresponding to lanes 1−8, respectively. In the middle gel, the r_b values were 0, 0.004, 0.008, 0.011, 0.011, 0.016, 0.016, 0.027, 0.045, 0.078, and 0.0126 in lanes 1−11, respectively. For the bottom gel, the r_b values were 0, 0.001, 0.005, 0.009, 0.013, 0.015, 0.053, 0.063, 0.065, and 0.064 in lanes 1−10, respectively. The coalescence point where the two forms of DNA (supercoiled and relaxed) comigrate corresponds to $r_b(c)$ = 0.057, 0.045, and 0.013 as indicated.

sequence (Zou et al., 1994). Interstrand cross-linking sites for all three complexes are apparent because of the presence of polymerase inhibition sites at the alternating purine-pyrimidine $d(C_{19}G_{20}C_{21}G_{22})$ and $d(C_{27}G_{28}C_{29}G_{30}G_{31})$ sequences, and for both NH_3 complexes, the full digestion pattern is essentially identical to that described previously (Zou et al., 1994), with the major exception of the $T_8G_9G_{10}T_{11}$ region discussed in detail below.

When dinuclear complexes with monofunctional coordination spheres bind to DNA through the first Pt atom, the second step may result in either a (Pt,Pt) intrastrand or a (Pt,Pt) interstrand cross-link (Figure 6). The 1,1/t,t complex shows an intense stop site comigrating with $d(T_{11})$ which corresponds to inhibition at the $d(G_9G_{10})$ sequence (Figure 7). This is compatible with formation of a (Pt,Pt)−d(GpG) intrastrand adduct, and sequence specificity studies confirmed

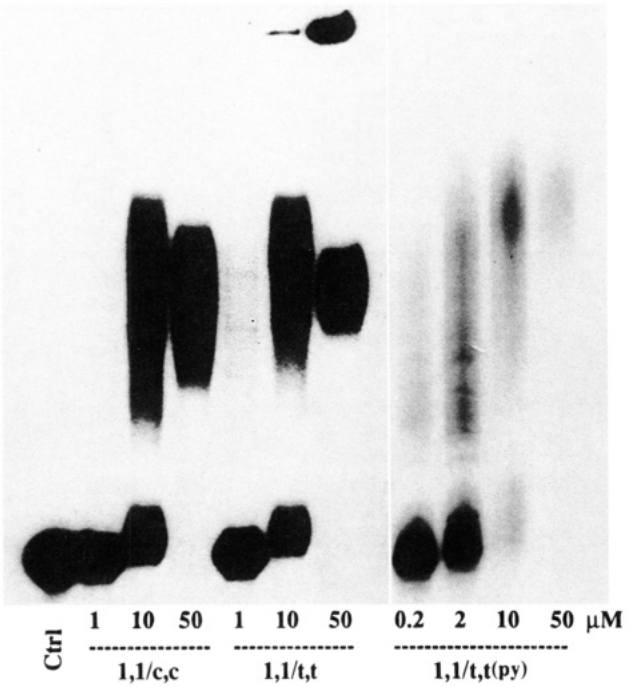

FIGURE 5: DNA−DNA interstrand cross-link formation induced by platinum complexes. See also Table 2. The interstrand cross-linked DNA appears as the bands migrating more slowly than the singly stranded DNA on the gel. The three lanes for each complex correspond to the doses in micromolar incubated with the 49 bp oligonucleotide. See Materials and Methods for details.

Table 2: Frequency of Interstrand Cross-link Formation by Dinuclear Platinum Complexes[a]

complex	r_b[b]	A/F[c]	XL[d]	XL/F[e]	XL/A[f]
1,1/c,c/NH_3	0.001	0.10	0.08	0.08	0.8
1,1/t,t/NH_3	0.003	0.29	0.14	0.15	0.52
1,1/t,t/py	0.001	0.10	0.04	0.041	0.41

[a] See Materials and Methods for details and Figure 1 for abbreviations. [b] The r_b values were measured for the interaction of the complexes (1 μM concentration) with CT DNA (0.023 mg/mL). Note the diminished binding of the 1,1/c,c/NH_3 isomer due to steric effects. [c] A/F = number of adducts per fragment. [d] XL = the fraction of counts running as the cross-linked band. [e] XL/F = Ratio of interstrand cross-links to total Pt bound [see Roberts et al. (1989) and Farrell et al. (1990)]. [f] XL/A = number of interstrand cross-links per adduct.

this assignment (Zou et al., 1994). Model complexes for this adduct have been prepared by binding of the Pt centers to the two G bases of d(GpG) (Bloemink et al., 1992). This (Pt,Pt) intrastrand adduct is thus the direct analog of the major adduct of *cis*-DDP (Sherman et al., 1985; den Hartog et al., 1982). Of great interest is the fact that this band is unique to the 1,1/t,t/NH_3 complex and is missing from the digestion pattern of both the 1,1/c,c/NH_3 and the pyridine complex (Figure 7).

The steric effects of two *trans*-[$PtCl(py)_2$] moities may be easily visualized to result in severe steric crowding. For the NH_3 complexes, space-filling models indicated that only the 1,1/t,t isomer was capable of forming the (Pt,Pt)−d(GpG) intrastrand cross-link. In principle, both the 1,1/c,c and 1,1/t,t isomers are capable of bridging the expected 4 Å distance between adjacent guanine N7 atoms in a B-form GGCC sequence. However, only the diamine chain in the 1,1/t,t case can be rotated around the two Pt square planes to allow simultaneous Pt binding at both guanine N7 positions. The greater steric demands of the two leaving groups *cis* to the

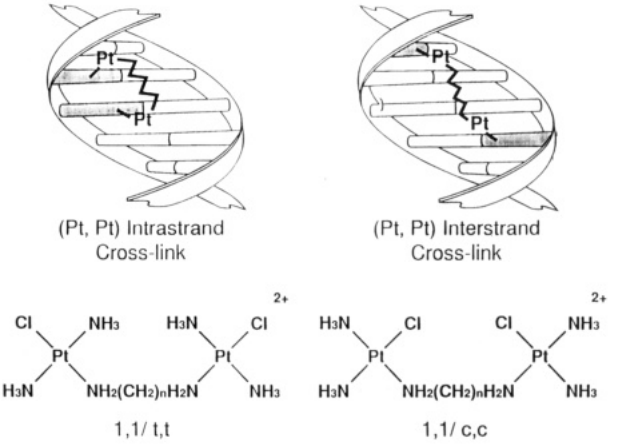

FIGURE 6: Schematic representation of the limiting modes of bifunctional DNA binding of the *cis* and *trans* isomers of the dinuclear platinum complexes [{$PtCl(NH_3)_2$}$_2\mu$-$H_2N(CH_2)_4NH_2$]$^{2+}$.

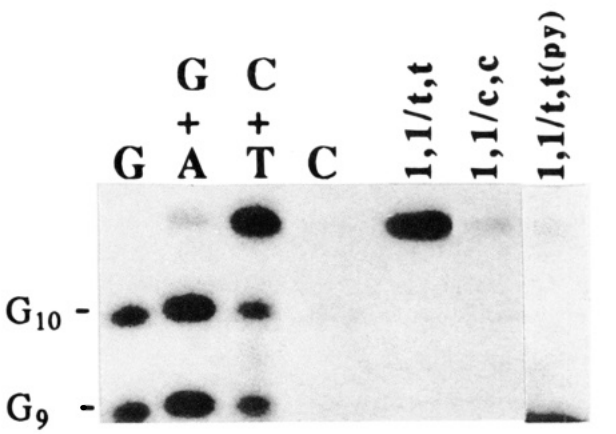

FIGURE 7: Sequence specificity of platinum complex binding to DNA. See Materials and Methods for details. The gel is obtained upon prolonged electrophoresis of a typical sequencing gel using T4 DNA polymerase (Zou et al., 1994). Assignment of the binding sites is based on the fact that the degraded fragments migrate 0.5−1.5 nucleotides more slowly than the Maxam−Gilbert fragments which identify the modified sites (Malinge et al., 1987; Zou et al., 1993). Lanes G, G+A, C+T, and C were loaded with Maxam−Gilbert sequencing products of G, A+G, T+C, and C reactions, respectively.

diamine bridge prevent a favorable orientation of the linker chain to permit simultaneous binding at both guanines. The diminished interstrand cross-linking of the 1,1/t,t isomer to that of the 1,1/c,c complex (see Table 2) may now be explained by the fact that only in the former case will there be a contribution to adduct formation from the (Pt,Pt) intrastrand cross-link, thus competing with interstrand cross-linking. Further, the 1,1/c,c compound may thus be considered a "pure" interstrand cross-linker. The generality of this finding for different chain lengths is currently under investigation.

DISCUSSION

This study forms part of our efforts to explore the relationship between DNA binding and cytotoxicity for mononuclear and dinuclear platinum complexes. A full analysis of factors affecting cytotoxicity should also take into account the relative uptake of the various platinum complexes. This is especially important in comparing activity in cisplatin-resistant cells where accumulation deficit is likely to be a significant component of the developed resistance

Table 3: Comparison of Effects of Bifunctional DNA Binding and Biological Activity of Mononuclear and Dinuclear Platinum Complexes

complex	unwinding angle[a]	interstrand cross-linking	intrastrand cross-linking	B → Z induction	HMG protein recognition[b]	ID_{50} (μM)[c]
cis-DDP	11°	low (<5% total)	d(GG), d(AG), d(GNG) minor	no	1.0	0.42 (28.6)
trans-DDP	9°	low (<5% total)	d(GNG) major	no	not recognized	–
trans-$[PtCl_2(py)_2]$[d]	17°	25%	not seen	no	ND	1.2 (0.92)
1,1/t,t	10°	52%	d(GG)	yes	0.3	3.4 (0.5)
1,1/c,c	12°	80%	not seen	yes	0.15	0.25 (1.44)
1,1/t,t/py	42°	41%	not seen	no	ND	>10

[a] See Table 1 for details. [b] Relative recognition of Pt-damaged DNA by HMG protein extracts adapted from Skov et al. (1993) and Farrell (1995c). The extent of protein recognition was performed using a damaged DNA affinity precipitation assay as described in Hughes et al. (1992) and Marples et al. (1994). Briefly, DNA bound to cellulose was modified by binding of Pt complexes. Protein extract (confirmed to contain HMG proteins by comparison with authentic samples) from CHO cells was mixed with the platinated (amount modified to give equivalent r_b for all samples) or undamaged DNA cellulose suspension. Unbound protein was washed away and the bound protein subsequently removed, separated from DNA−cellulose, and visualized by staining and one-dimensional SDS (sodium dodecyl sulfate) gel electrophoresis. See Marples et al. (1994) for details. An arbitrary value of 1.0 was assigned to the *cis*-DDP intensity, and the relative intensities from treatment with 1,1/t,t/NH_3 and 1,1/c,c/NH_3 were calculated. ND is not determined. [c] Data from Farrell et al. (1994). Data were obtained in L1210 cells sensitive and resistant to *cis*-DDP and the ID_{50} values calculated as per Farrell et al. (1990b). Resistance factors [ID_{50}(resistant)/ID_{50}(sensitive)] in parentheses. Thus, the 1,1/t,t/NH_3 complex with a resistance factor <1 is more capable of overcoming resistance in L1210 cells than the 1,1/c,c isomer (RF > 1). [d] Data from Zou et al. (1993) and Farrell (1995b).

(Farrell, 1993, and references therein). In our experience, measurement of uptake of charged complexes has been technically difficult due to immediate ($t = 0$) nonspecific association of the complex with cellular membranes. To avoid such problems, a host-cell reactivation (HCR) assay using transfection of a platinated plasmid was employed (Johnson et al., 1995). The global array of DNA adducts derived from the 1,1/t,t/NH_3 and 1,1/c,c/NH_3 complexes was at least as efficient as those derived from *cis*-DDP in inhibiting DNA replication and transcription. As such, it is instructive to examine the DNA binding profile of the dinuclear complexes, summarized in Table 3. What are the structural features of the (Pt,Pt) interstrand and intrastrand cross-links that may contribute to the observed cytotoxicity?

(Pt,Pt) Interstrand Cross-Links. All complexes studied are very efficient cross-linking agents. The pyridine analog is significantly less cytotoxic than the NH_3 counterparts (see Table 3). Likewise, the dinuclear compound [*trans*-{PtCl-(NH_3)(quin)}$_2$$NH_2(CH_2)_6NH_2]^{2+}$, where one NH_3 group is displaced by the planar quinoline ligand, is an efficient cross-linking agent but not a potent cytotoxic agent (Kharatishvili et al., 1995). This latter compound is derived from *trans*-$[PtCl_2(NH_3)$(quinoline)], which shows cytotoxicity and DNA binding profiles similar to those of *trans*-$[PtCl_2$(pyridine)$_2]$ (van Beusichem & Farrell, 1992; Skov et al., 1993; Farrell, 1995b). Therefore, interstrand cross-linking is not by itself a sufficient requirement for cytotoxicity. Neither dinuclear complex containing planar ligand is capable of inducing the B → Z conformational change, and the results imply that this switch may be an essential part of the mechanism of action of the NH_3 compounds. Other unusual structures such as hairpin-like formation recently observed for the 1,1/t,t/NH_3 case may be also be sterically inaccessible to complexes with planar ligands (Yang et al., 1995).

(Pt,Pt) Intrastrand Cross-Links. An interesting observation from these results is that the relative formation of bifunctional (Pt,Pt) intrastrand and (Pt,Pt) interstrand adducts from bis(platinum) complexes may be controlled by geometric isomerism. The structural feature of the 1,1/t,t geometry allows manipulation of both the relative amounts of (Pt,Pt) intrastrand cross-link and the conformational change induced by such binding. This point is reinforced by results showing that the bending of a site-specific intrastrand d(GpG) adduct is flexible and significantly less than that of *cis*-DDP (V. Brabec and N. Farrell, unpublished results).

Contributions of DNA Conformational Changes to Cytotoxicity. In contrast to the situation between *cis*- and *trans*-DDP, both dinuclear geometries are antitumor active but are distinguished in their ability to overcome acquired *cis*-DDP resistance [see Table 3 and Manzotti et al. (1994)]. The different biological properties of the pair of dinuclear complexes in comparison to the $[PtCl_2(NH_3)_2]$ pair may reflect "downstream" effects upon repair and protein recognition of the different DNA adducts, which may be inherently more cytotoxic or more difficult to repair than those of *cis*-DDP.

The global conformational changes of importance to protein recognition of DNA are unwinding and bending. At equivalent r_b, *trans*-DDP is less effective at unwinding than *cis*-DDP (Cohen et al., 1979; Scovell & Collart, 1985). For bifunctional binding of dinuclear platinum complexes, unwinding equivalent to *cis*-DDP is achievable but without the necessity of the *cis*-DDP structure. If unwinding is responsible for recognition of *cis*-DDP-damaged DNA (Bellon et al., 1991; van Houten, 1990), then similar effects should be observed from the dinuclear complexes. Unwinding is thus unlikely to be the cause of any differential effects in cells caused by dinuclear platinum complexes.

The bending of DNA upon *cis*-DDP binding is likely to be responsible for the recognition of Pt-damaged DNA by damage recognition proteins containing the HMG structural motif (Lilley, 1992; Pil & Lippard, 1992; Kaszpárová & Brabec, 1995). The role of such proteins in the mechanism of *cis*-DDP cytotoxicity and their relevance to cellular resistance to *cis*-DDP and repair of *cis*-DDP-DNA adducts are currently areas of active research (Billings et al., 1992; Brown et al., 1993; Bissett et al., 1993; Chu & Chang, 1990). We have shown (Farrell, 1995a,c; Skov et al., 1993) that HMG proteins recognize DNA damaged by both dinuclear compounds but not as efficiently as for *cis*-DDP-damaged DNA (see Table 3). At equivalent r_b, recognition is 30 and 15% for the 1,1/t,t and 1,1/c,c complexes, respectively, relative to that for *cis*-DDP. The interaction of true damage recognition proteins with platinated DNA could initiate the repair process, or alternatively, access to the platinated site

by repair proteins could be inhibited (Lippard, 1993; Chu, 1994). The dinuclear complexes are the first examples of highly cytotoxic agents which are *not* recognized by HMG proteins, suggesting that the nature of bis(platinum)−DNA adducts allows for a systematic design of a "HMG bypass" mechanism. Whether the structural differences in the intrastrand cross-links formed by *cis*-DDP and the 1,1/t,t complexes may explain the differences in HMG protein recognition is under investigation.

The results presented here confirm the utility of the dinuclear motif in modulating and directing biochemical interactions and support the viability of a molecular mechanism for systematically altering the antitumor activity of *cis*-DDP and its structural analogs. Thus, the use of bis(platinum) complexes as clinical agents is highly promising. They will further serve as very useful probes for interpreting the functions of proteins which bind specifically to *cis*-DDP-damaged DNA.

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