Comparison of Chemical Reactivity, Cytotoxicity, Interstrand Cross-Linking and DNA Sequence Specificity of Bis(platinum) Complexes Containing Monodentate or Bidentate Coordination Spheres with Their Monomeric Analogues†

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ABSTRACT: The properties of a new bis(platinum) complex containing two monodentate coordination spheres, [{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N(CH_2)_4NH_2$]$Cl_2$ (1,1/t,t), are reported. Comparison is made with respect to chemical reactivity, in vitro biological activity in murine and tumor cells, DNA conformational changes, cross-linking efficiency, and sequence specificity between this complex and the previously reported complex containing two bidentate platinum atoms, [{Pt(mal)(NH_3)}$_2$$H_2N(CH_2)_4NH_2$] (2,2/c,c), as well as with their respective monomeric analogues, [PtCl(dien)]Cl and *cis*-[$PtCl_2(NH_3)_2$](*cis*-DDP). While both bis(platinum) complexes are active against cis-DDP-resistant cells, the monodenate bis(platinum) complex (1,1/t,t) has a lower resistance factor than the complex with bidentate coordination spheres (2,2/c,c). More importantly, this property is repeated in a human ovarian carcinoma cell line. DNA-binding studies show that DNA interstrand cross-linking is more efficient for the 1,1/t,t complex. DNA sequencing studies employing the exonuclease activity of T4-polymerase demonstrate that there are a variety of binding sites; some are common to all complexes and some common to both bis(platinum) complexes, while the monodentate 1,1/t,t species also reacts at unique sites, not attacked by any of the other complexes studied. The circular dichroism of CT DNA modified by the 1,1/t,t complex is also unique and is not seen for any of the other agents.

Bis(platinum) complexes containing two $Pt(amine)_2$ units linked by a variable-length diamine chain, [{*cis*-$PtCl_2$-(NH_3)}$_2$$H_2N(CH_2)_nNH_2$], are of chemical (Farrell et al., 1988; Farrell & Qu, 1989) and biological (Roberts et al., 1989; Farrell et al., 1990) interest because they show high activity in vitro and in vivo against tumor cell lines resistant to *cis*-[$PtCl_2(NH_3)_2$] (cisplatin, *cis*-DDP)[1] (Kraker et al., 1989). The problem of both acquired and inherent resistance of tumor cells presents a principal limitation to the more widespread clinical use of cisplatin. Platinum compounds are believed to mediate their cytotoxic effects through their interaction with DNA. The principal lesion of *cis*-DDP on DNA is the intrastrand link between two adjacent guanines or adjacent adenine/guanine bases (Farrell, 1989). These adducts block both replication and transcription (Sherman & Lippard, 1987; Reedijk et al., 1987). Some monomeric analogues of *cis*-DDP containing structurally different amines, and in particular those containing 1,2-diaminocyclohexane, are non-cross-resistant and have been extensively studied as potential "second-generation" clinical candidates (Burchenal & Kalaher, 1978). Although differential DNA repair is implicated in their mechanism of non-cross-resistance (Eastman & Schulte, 1988), these analogues show essentially the same pattern of adduct formation as *cis*-DDP (Page et al., 1990; Jennerwein et al., 1989).

Recently, DNA-binding studies have shown that bis(platinum) complexes form unique interstrand cross-links by binding of one Pt atom to each strand of DNA (Roberts et al., 1989). These complexes are of special interest therefore because they are capable of molecular interactions not accessible to monomeric complexes. By virtue of their unique DNA-binding properties, bis(platinum) compounds may not only be non-cross-resistant with cisplatin but may also display a broader spectrum of clinical activity. It is important therefore to delineate the minimal structural features of bis(platinum) complexes required for cytotoxicity, especially in *cis*-DDP-resistant cells. The tetrafunctional nature of the bis(platinum) complexes allows for a complex array of inter- and intrastrand cross-links upon reaction with DNA. To correlate the biological effects with different DNA lesions, it is necessary to determine the contribution from an interstrand cross-link, independent of any contribution from the *cis*-DDP-like intrastrand cross-link. We have now prepared a bis(platinum) complex with monodentate coordination spheres, [{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N(CH_2)_nNH_2$]$Cl_2$. This paper reports on its synthesis, characterization, in vitro cytotoxicity, DNA binding, and cross-linking efficiency. Comparison is made with the bis(platinum) complexes containing bidentate platinum and their respective monomeric analogues.

Experimental Procedures

Starting Materials and Physical Methods. The complex *trans*-[$PtCl_2(NH_3)_2$] was prepared by the published method (Kaufman & Cowan, 1963). 1,4-Diaminobutane was purchased from Aldrich and used without further purification.

Preparation of [{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N(CH_2)_4NH_2$]$Cl_2$. To *trans*-[$PtCl_2(NH_3)_2$] (1.5 g) suspended in H_2O (150 mL) was added 1,4-butanediamine in H_2O (0.25 mL in 5 mL). The

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[1] Abbreviations: dien, diethylenetriamine; [PtCl(dien)], Pt(dien), chloro(diethylenetriamine)platinum(II) chloride; *cis*-DDP, cisplatin, *cis*-[$PtCl_2(NH_3)_2$], *cis*-diamminedichloroplatinum(II); dach, 1,2-diaminocyclohexane; [Pt(*R*,*R*-dach)SO_4], (sulfato)[(*R*,*R*)-1,2-diaminocyclohexane]platinum(II); 1,1/t,t, [{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N$-$(CH_2)_4NH_2$]$Cl_2$, ($\mu$-1,4-diaminobutane)bis{*trans*-chlorodiammineplatinum(II)} dichloride; mal, malonato; 2,2/c,c, [{Pt(mal)(NH_3)}$_2$$H_2N$-$(CH_2)_4NH_2$], ($\mu$-1,4-diaminobutane)bis{ammine(malonato)platinum(II)}; CT, calf thymus; FAAS, flameless atomic absorption spectroscopy.

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suspension was stirred for 4 h at 50–60 °C to give a clear solution. The solution was left stirring overnight at room temperature. Upon evaporation to small volume (approximately 5 mL) a white solid product was filtered and washed thoroughly with cold water. The solid was recrystallized (3×) from EtOH to give the final product in 46% yield. The complex was characterized by elemental analysis, ^{1}H NMR (relative to TMS) at 2.74 and 1.79 ppm, and ^{195}Pt NMR (relative to $PtCl_6^{2-}$) at −2436.7 ppm. The IR spectrum shows bands typical of bridging diamine and ν(Pt–Cl) = 330 cm^{-1}.

Spectroscopic Measurements. IR spectra were obtained as KBr disks on Nicolet FT6000 series and Perkin-Elmer 1430 spectrophotometers. NMR spectra were run on Bruker 250- and 270-MHz spectrometers. ^{195}Pt NMR spectra (250 MHz) were run in D_2O with reference to a Na_2PtCl_6 solution in D_2O as external reference. Samples were run at a pulse width of 10 μs with a relaxation delay of 0.5 s. Usually a sweep width of 30 kHz was used and 5000–10 000 scans were adequate. All shifts are positive to lower shielding. UV/visible spectra and T_m measurements were performed on a Perkin-Elmer Lambda 4B instrument. Circular dichroism spectra were obtained in a 3-cm circular quartz cell on a Jobin-Yvon Autodichrograph Mark V instrument.

Platinum–DNA Binding

Treatment of Calf Thymus (CT) DNA by the Platinum Compounds. CT DNA was treated with the platinum compounds and was used for r_b determination as previously described (Roberts et al., 1989). Briefly, 10 mL of CT DNA (4 μg/mL) was treated with various concentrations of platinum compounds in a buffer containing Tris-HCl pH 7.5 (10 mM), EDTA (1 mM), and NaCl (10 mM) (TEN Buffer) at 37 °C for 1 h. The reactions were terminated by raising the salt concentration to 100 mM. The samples were extensively dialyzed against TEN buffer. The treated CT DNA was then used in melting temperature determinations, CD measurements, or flameless atomic absorption spectroscopy (FAAS) for the determination of r_b.

Interstrand Cross-Link Assay. The (*Dde*I–*Eco*RI)$_{65bp}$ fragment of pBR322 was isolated and labeled as described previously (Roberts et al., 1989). This DNA fragment was treated in the presence of CT DNA (4 μg/mL) with the platinum compounds in a 30-μL reaction containing TEN buffer for 1 h at 37 °C. The incubations were terminated by the addition of NaCl to a final concentration of 100 mM and microdialyzed (Pierce) against TEN buffer. A portion of the reactions (10 μL) was mixed with an equal volume of formamide dyes and the amount of cross-links was analyzed by electrophoresis on 8% polyacrylamide sequencing gels under denaturing conditions.

Cross-link experiments with the self-complementary 5′-end labeled 12-mer TTTTGGCCAAAA were performed essentially as described above. The reaction products were analyzed by electrophoresis on 20% polyacrylamide sequencing gels under denaturing conditions.

Quantitation of DNA Interstrand Cross-Links. The radioactivity in the bands corresponding to the single-strand DNA and cross-linked duplex were quantified by using a Betascope 603 blot analyzer (Betagen, Waltham, MA). The percentage of DNA that was cross-linked was calculated as the fraction of radioactivity associated with the slowly migrating broad band, as compared to the total radioactivity in both the single-strand and cross-linked bands. Autoradiography was conducted by placing the dried gel onto Kodak XAR5 X-ray film for 15–30 h, in the presence of intensifying screens at −70 °C.

Mapping Sites of Platinum–DNA Adducts by the 3′ → 5′ Exonuclease Activity of T4-Polymerase. The 3′ → 5′ exonuclease activity of T4-polymerase was used to monitor the sites of DNA lesions in a 49-bp DNA fragment (Royer-Pokora et al., 1981; Fuchs et al., 1983). The sequence is the following:

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5'-GACTACTTGGTACACTGACGCGAGCTCGCGGAAGCTCATTCCAGTGCGC-3'

3'-CTGATGAACCATGTGACTGCGCTCGAGCGCCTTCGAGTAAGGACACGCG-5'
  1.....5.....10.....15.....20.....25.....30.....35.....40.....45.....
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The duplex was synthesized as two separate oligonucleotides on a Du Pont automatic DNA synthesizer (generator DNA synthesizer). The oligonucleotides were gel purified by electrophoresis on 12% polyacrylamide gels under denaturing conditions (8 M urea). The "top-strand" oligomer was terminally labeled on the 5′-end by standard techniques and annealed to its complement. The fully duplex DNA was purified on a 12% polyacrylamide native gel.

The 49-bp duplex was treated with the platinum compounds as described above. The treated DNA fragments (2–5 ng) were digested with the 3′ → 5′ exonuclease activity of T4-polymerase (10 units) in a reaction containing Tris-HCl (50 mM), KCl (50 mM), and $MgCl_2$ (10 mM) for 10 min at 37 °C. The reactions were terminated by the addition of EDTA to 25 mM.

Reversal of Platinum–DNA Adducts. Since cross-linked DNA and platinum–DNA adducts would affect the migration properties of the DNA, it was necessary to remove all the Pt–DNA adducts prior to the analysis of the digestion products on DNA sequencing gels. Removal of the platinum adduct as $[Pt(CN)_4]^{2-}$ was accomplished by incubating the treated DNA with NaCN (0.3 M, pH 8.0) for 3 h at 37 °C and then dialyzing it against TEN buffer for 1 h. The DNA samples were lyophilized to dryness and resuspended in a formamide/dye solution. The reaction products were analyzed by electrophoresis on 12% polyacrylamide sequencing gels under denaturing conditions. Dried gels were subjected to autoradiography using Kodak XAR-5 film with intensifying screens for 18–36 h at −70 °C.

DNA Sequencing. Sequencing of the uniquely 5′ or 3′-terminally labeled 49-bp duplex was performed by using standard Maxam–Gilbert procedures (Maxam & Gilbert, 1980).

Cytotoxicity Assays. The cytotoxicity assays were carried out by standard procedures. For the L1210 leukemia cell line, the assays were performed by previously published procedures (Farrell et al., 1990). The 1,1/t,t and 2,2/c,c complexes, along with *cis*-DDP and Pt(dien), were assayed in the same set of experiments for stricter comparison. The result on the 2,2/c,c complex II agree with those found earlier (Farrell et al., 1990). The ovarian carcinoma lines have been previously described and the cytotoxicity in ovarian carcinoma cells was determined by a published method (Hills et al., 1989).

Plasmid Incision Assay

Enzymes. The UvrA, UvrB, and UvrC subunits were purified from *Escherichia coli* overproducer strain CH296 containing plasmids pUNC45, pUNC211, and pDR3274, respectively (obtained from A. Sancar, University of North Carolina). The purification procedure was as described previously (Sancar et al., 1987).

UvrABC Nuclease Incision Reaction. UvrABC digestions were performed by incubating 1.1 pmol of UvrA, 2.1 pmol of UvrB, 5.5 pmol of UvrC, and 1.0 μg of supercoiled PM2 DNA containing 10–15 platinum–DNA lesions/molecule, in a 50-μL reaction containing 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5),

Table I: In Vitro Cytotoxicity of Bis(platinum) Complexes in Murine L1210 Leukemias and Human Tumor Ovarian Carcinomas Sensitive and Resistant to Cisplatin

	Part A		
	L1210 leukemia ID_{50} (μM)[a]		
	L1210/0	L1210/DDP[b]	L1210/dach
[{*cis*-$PtCl_2(NH_3)$}$_2$$H_2N(CH_2)_4NH_2$][c]	0.28	1.88 (6.5)	0.41 (1.4)
[{Pt(mal)(NH_3)}$_2$$H_2N(CH_2)_4NH_2$]	1.69	6.61 (3.91)	2.82 (1.67)
[{*cis*-$PtCl_2(NH_3)$}$_2$$H_2N(CH_2)_5NH_2$][c]	0.37	1.0 (2.7)	0.45 (1.22)
[{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N(CH_2)_4NH_2$]$Cl_2$	3.5	0.87 (0.25)	1.23 (0.29)
[PtCl(dien)]Cl	>10	>10	>10
cis-[$PtCl_2(NH_3)_2$]	0.22	9.89 (45)	0.91 (4.14)
[Pt(*R*,*R*-dach)SO_4]	0.22	1.7 (7.7)	5.36 (24.4)
	Part B		
	ovarian carcinoma IC_{50} (μM)[d]		
	CH1	41M	41m/cisR[b]
[{*cis*-$PtCl_2(NH_3)$}$_2$$H_2N(CH_2)_5NH_2$]	0.05	0.31	0.75 (2.4)
[{Pt(mal)(NH_3)}$_2$$H_2N(CH_2)_4NH_2$]	0.39	2.3	7.0 (3)
[{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N(CH_2)_4NH_2$]$Cl_2$	1.2	8.0	5.2 (0.65)
cis-[$PtCl_2(NH_3)_2$]	0.11	0.25	1.64 (6.5)

[a] All values are the average of three experiments. L1210/0 sensitive to *cis*-DDP; L1210/DDP resistant to *cis*-DDP; L1210/dach resistant to [Pt(*R*,*R*-dach)SO_4]. [b] Resistance factor in parentheses and defined as ID_{50} (or IC_{50}) resistant ÷ ID_{50} (IC_{50}) sensitive. [c] From Farrell et al. (1990). [d] See Hills et al. (1989).

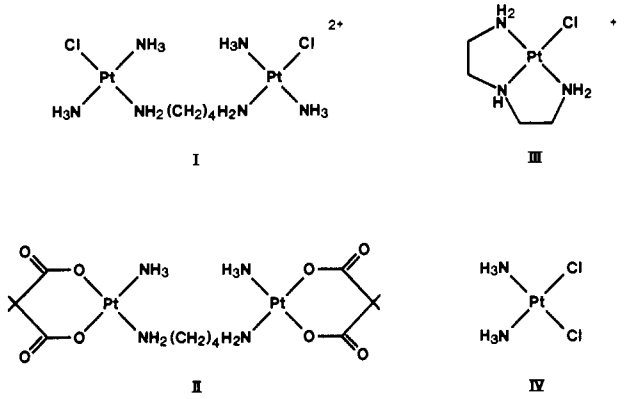


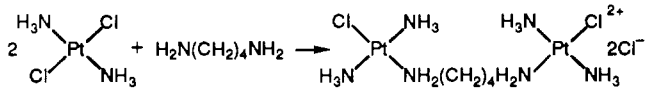
FIGURE 1: The structures of bis(platinum) complexes with monodentate and bidentate coordination spheres and their monomeric analogues. I, [{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N(CH_2)_4NH_2$]$Cl_2$, 1,1/t,t; II [{Pt(mal)$(NH_3)$}$_2$$H_2N(CH_2)_4NH_2$], 2,2/c,c; III, [PtCl(dien)]Cl, Pt(dien); IV, *cis*-[$PtCl_2(NH_3)_2$], *cis*-DDP. The abbreviations are used throughout this text and in all figure legends.

50 mM KCl, 2 mM ATP, 5 mM DTT (ABC buffer), and 100 μg/mL bovine serum albumin for 30 min at 37 °C. The DNA in buffer was prewarmed for 5 min at 37 °C prior to the addition of the UvrA, UvrB, and UvrC subunits.

Results

The complexes studied are shown in Figure 1. Consistent with our previous scheme (Farrell et al., 1990), complex I is abbreviated as 1,1/t,t, signifying monodentate platinum coordination spheres with the sole chloride trans to the diamine bridge. Comparison was made with [{Pt(mal)(NH_3)}$_2$$H_2N(CH_2)_4NH_2$] (II), 2,2/c,c, and their monomeric analogues [PtCl(dien)]Cl (III), Pt(dien), and *cis*-[$PtCl_2(NH_3)_2$] (IV), *cis*-DDP. The choice of the malonate derivative II rather than the strictly analogous chloride complex was influenced by its enhanced water solubility and our use of this derivative in our previous DNA-binding studies.

The hitherto unreported 1,1/t,t complex was prepared by reaction of 1 equiv of 1,4-diaminobutane with 2 equiv of *trans*-[$PtCl_2(NH_3)_2$]:



The value of the ^{195}Pt NMR chemical shift is intermediate between the values found for [$PtCl_2$(amine)$_2$] and [Pt(amine)$_4$] coordination spheres in bis(platinum) complexes and confirms the proposed [PtCl(amine)$_3$] coordination. The white air-stable solid is readily soluble in warm water.

Cytotoxicity Studies. Cytotoxicity data in L1210 cell lines sensitive and resistant to cisplatin and the 1,2-diaminocyclohexane analogue [Pt(*R*,*R*-dach)SO_4] are collected in Table IA. The results for the 2,2/c,c complex II are also consistent with those previously obtained. The cytotoxicity of the 1,1/t,t complex is somewhat reduced in comparison to the 2,2 chloride analogues in *cis*-DDP-sensitive cells. However, its resistance factor, defined as ID_{50}(resistant)/ID_{50}(sensitive), is substantially reduced in comparison to that of the 2,2c,c complexes. Further, the monodentate [PtCl(dien)]Cl is, as expected, inactive in all cell lines at concentrations up to 10 μM. The 1,1/t,t complex represents a unique example of a platinum species that is at the least equally as active in cell lines sensitive and resistant to *cis*-DDP or Pt(dach).

To confirm that these observations apply to human tumors the compounds were assayed for cytotoxicity in the panel of ovarian carcinoma cell lines developed by the group of Harrap (Hills et al., 1989). Selected results are shown in Table IB. There is in fact a remarkable similarity in the behavior pattern of the complexes toward these clinically relevant human cell lines and the murine L1210 leukemia results. Thus, both 2,2/c,c complexes (n = 4, 5) have a lowered resistance factor in comparison to *cis*-DDP and equivalent sensitivity in the sensitive cell line. The 1,1/t,t complex I again displays enhanced cytotoxicity in the resistant line and thus a resistance factor of less than 1.

Taken together, the data demonstrate that the structural feature of a bis(platinum) complex with monodentate coordination spheres is sufficient, of and by itself, to produce cytotoxicity, *especially in cis-DDP-resistant cells*. The demonstration of this property in the human tumors is of considerable clinical importance.

DNA Binding. Given the cytotoxicity results, it is of particular interest to elucidate the similarities and differences in the behavior of both types of bis(platinum) complexes toward DNA and to relate these properties to their biological effects. The DNA binding and interstrand cross-linking were examined by a combination of physical and molecular techniques in-

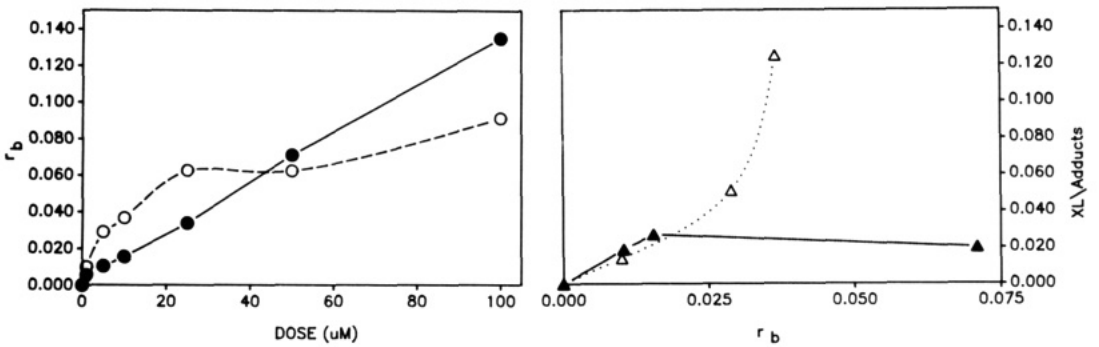


FIGURE 2: Interaction of bis(platinum) complexes with DNA. (A, Left) CT DNA (4 μg/mL) was treated with various concentrations of either complex II, 2,2/c,c (closed symbols), or complex I, 1,1/t,t (open symbols), for 1 h at 37 °C. Following extensive dialysis, the DNA was hydrolyzed by overnight treatment with nitric acid (2%). The bound Pt was determined by FAAS. (B, Right) Labeled 65 base pair DNA fragment was treated with the bis(platinum) complexes in the presence of CT DNA (4 μg/mL). From the r_b data the average number of adducts was calculated on a per fragment basis. The number of cross-links/fragment was determined by using denaturing gel electrophoresis as described in the Materials and Methods and Figure 3. Closed symbols = II, 2,2/c,c, and open symbols = I, 1,1/t,t. Error estimates given in Table II.

cluding CD, NMR, T_m measurements, and polyacrylamide gel electrophoresis.

The reaction of both the 1,1/t,t complex (I) and the 2,2/c,c complex (II) with stoichiometric amounts of 5′-GMP was initially studied by ^{195}Pt NMR spectroscopy. In both cases the final products obtained corresponded to complete chloride substitution by the purine mononucleotide and clean transformations from one species to the other occurred. The ^{195}Pt NMR chemical shifts of the products, (−2568 ppm for $[\{Pt(NH_3)_2(5'\text{-GMP})\}_2NH_2(CH_2)_4NH_2]^{2+}$ and −2473 ppm for $[\{Pt(NH_3)(5'\text{-GMP})_2\}_2NH_2(CH_2)_4NH_2]^{4+}$) are consistent with those obtained for the monomeric analogues $[Pt(dien)(5'\text{-GMP})]^+$ (−2873 ppm) (Y. Qu and N. Farrell, unpublished results) and *cis*-$[Pt(NH_3)_2(5'\text{-GMP})_2]^{2+}$ (−2455 ppm) (Miller & Marzilli, 1985). Overall, the results indicate that any differences in DNA binding between the bis(platinum) complexes are unlikely to be due to any intrinsic differences in DNA reactivity or affinity (i.e., irrespective of any particular mode of binding). The reaction of the 2,2/c,c complex is slower in comparison to that of the 1,1/t,t species but this is related to the use of the relatively inert leaving group malonate. The kinetics of carboxylate displacement by DNA (Knox et al., 1986), however, are much faster than for small ligands (Canovese et al., 1988).

The reactivity of the bis(platinum) complexes toward DNA was further examined by determination of Pt-bound DNA (r_b). Figure 2A shows a plot of r_b versus concentration (1–100 μM) upon incubation with both bis(platinum) complexes 1,1/t,t and 2,2/c,c followed by dialysis to remove unbound Pt. The relative binding is similar in both cases. The 1,1/t,t complex displays a plateauing effect at concentrations of approximately 30 μM, and this effect is not observed for the 2,2/c,c complex. The relative charges and the number of H-bonding amine groups on the two bis(platinum) compounds may result in electrostatic interactions with the phosphodiester backbone of DNA, which may further contribute to the observed differences.

Interstrand Cross-Link Formation. The presence of monodentate or bidentate coordination spheres in bis(platinum) complexes may result in altered patterns of interstrand cross-linking. These differences could be reflected in the efficiency of cross-link formation, its sequence specificity, or the conformational distortion induced on the helix by the total spectrum of adducts formed in each case.

The interstrand cross-linking, Figure 3, was first assayed by using the (*Dde*I–*Eco*RI)$_{65}$ fragment of pBR322 as described previously (Roberts et al., 1989). The complexes were incubated with this fragment in the presence of CT DNA (4 μg/mL) at varying concentrations for 1 h. Upon electrophoresis under denaturing conditions, non-cross-linked 5′-labeled fragments migrate as 65-base single strands where cross-linked fragments migrate as a higher molecular weight species. It is apparent that cross-linking occurs at concentrations as low as 0.5 μM for the 1,1/t,t complex (lane 4). At intermediate concentrations up to 50 μM distinct bands are seen in the gel (lanes 5 and 6). These bands probably correspond to structurally different cross-linked species and may indicate that there is some sequence specificity to cross-link formation. In agreement with our previous results, the 2,2/c,c complex also caused cross-links, but at higher concentrations than 1,1/t,t. No cross-linking was observed for either Pt(dien) or *cis*-DDP at 100 μM concentration. Quantitation of cross-links per adducts formed was carried out in essentially the same manner as previously described (Roberts et al., 1989). Briefly, the dried gel was scanned for radioactivity by using a Betascope 603 blot analyzer to obtain estimates of the fraction of non-cross-linked fragment under each condition. The frequency of interstrand cross-links was calculated from the fraction of non-cross-linked fragment, r_b values, and the fragment size by using the Poisson distribution. The relative cross-linking efficiencies are summarized in Table II and Figure 2B. The purpose of these experiments was to provide a comparison of the interstrand cross-linking of structurally different bis(platinum) complexes. At the concentrations

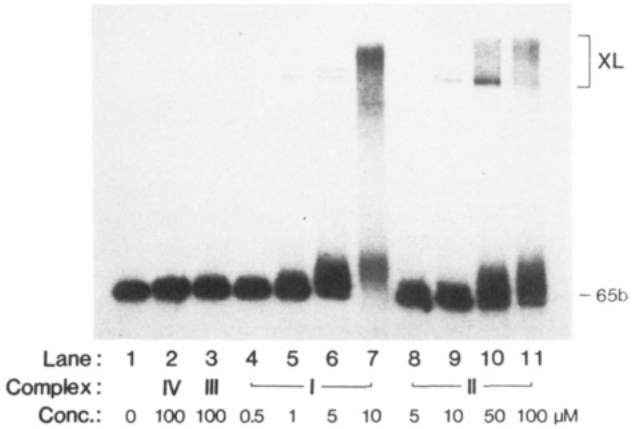


FIGURE 3: Interstrand cross-link formation by bis(platinum) complexes. Uniquely 5′-labeled 65-bp *Dde*I–*Eco*R1 fragment of pBR322 was treated with the indicated concentrations of either 1,1/t,t (I), 2,2/c,c (II), Pt(dien) (III), or *cis*-DDP (IV). The frequency of interstrand cross-link formation was quantitated by analysis on denaturing 8% polyacrylamide gels (Materials and Methods). The cross-linked DNA runs as smear and is indicated as XL.

Table II: Frequency of Interstrand Cross-Link Formation of Bis(platinum) Complexes[a]

complex, type[b]	concn (μM)	r_b[c]	A/F	1 − XL[d]	XL/F[e]	XL/A
I, 1,1/t,t	1	0.010 ± 0.004	1.3	0.982	0.018	0.014
	5	0.029 ± 0.014	3.64	0.832	0.184	0.051
	10	0.037 ± 0.018	4.81	0.55	0.60	0.125
	50	0.0622 ± 0.006	8.09	*f*	*f*	*f*
II, 2,2/c,c	5	0.010 ± 0.001	1.3	0.976	0.025	0.019
	10	0.0155 ± 0.002	2.02	0.947	0.055	0.027
	50	0.017 ± 0.012	9.23	0.835	0.18	0.020
	100	0.135 ± 0.035	17.55	0.755	0.28	0.016

[a] A/F, adducts per fragment; XL, interstrand cross-links; XL/F interstrand cross-links per fragment; XL/A interstrand cross-links per adduct. [b] Complex I, [{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N(CH_2)_4NH_2$]$Cl_2$; complex II, [{$Pt(mal)(NH_3)$}$_2$$H_2N(CH_2)_4NH_2$]. [c] Total Pt bound as measured by FAAS. Mean of three experiments ±SD. [d] Mean of two experiments, where XL = the fraction of counts running as the cross-link band. [e] Assuming a Poisson distribution of cross-links, the XL/F was calculated by the amount of non-cross-linked DNA, where XL/F = −ln (1 − XL). [f] Cross-link frequency was impossible to determine due to aggregation of the sample in the well.

Table III: Effect of Bis(platinum) Complexes on Melting Behavior of Calf Thymus DNA

complex	concn (μM)	r_b[b]	T_m (°C)[c]	ΔT_m (°C)	λ_{max} (nm)
			74.5		257.0
I, 1,1/t,t	25	0.0161	83.8	9.3	261.0
I	50	0.0255	83.8	9.3	263.4
I	100	0.0463	85.0	10.5	266.0
II, 2,2/c,c	100	0.0183	78.8	4.3	267.3

[a] Complex I, [{*trans*-$PtCl(NH_3)_2$}$_2$$H_2N(CH_2)_4NH_2$]$Cl_2$; complex II, [{$Pt(mal)(NH_3)$}$_2$$H_2N(CH_2)_4NH_2$]. [b] Total Pt bound as measured by FAAS. [c] Mean of two experiments.

employed, cross-linking was equivalent for both the 1,1/t,t and 2,2/c,c compounds at low r_b values (<0.025). At $r_b > 0.025$, the 1,1/t,t compound (I) produced significantly more cross-links than the 2,2/c,c compound (II) when compared on a per adduct basis. This comparison is in interesting contrast to the overall binding discussed above; the r_b for the 2,2/c,c increases with greater Pt concentration whereas the r_b for 1,1/t,t rises to a maximum and then levels off. The lower estimate of cross-linking for 2,2/c,c in comparison to previous calculations (Roberts et al., 1989) can be ascribed to the use of fresh solutions in the present study. In addition, to help approximate in vivo conditions, experiments used buffer solutions containing NaCl, which will tend to slow the reaction by either inhibiting Pt attack on DNA or slowing down conversion of monodentate adducts to cross-links.

Conformational Alteration of DNA by Bis(platinum) Complexes. The total spectrum of lesions produced by any 2,2/c,c complex at any given time will probably contain a number of cis-DDP-like adducts. A pertinent question, then, is to what extent any conformational changes on DNA are dictated by the interstrand cross-link per se and whether the changes are the same or different between 1,1 and 2,2 complexes and whether these alterations are similar for identical sequences of DNA. In order to investigate these questions, physical and DNA sequencing studies were initiated.

Physical Studies. Physical studies of the melting behavior, UV/visible spectra, and circular dichroism spectra of platinated DNA give information on the stability and conformational changes induced upon complex binding. The melting behavior of platinated DNA is dependent on the r_b of the sample (Johnson et al., 1989). For both Pt(dien) and cis-DDP at $r_b < 0.05$ there is a slight increase but at higher r_b the T_m decreases. Our experiments (not shown here) confirmed this behavior. Both bis(platinum) complexes, on the other hand, showed significant increases in T_m, Table III. Equivalent (equimolar in complex concentration) binding of monomeric species would not produce such increases. The results are consistent with interstrand cross-linking by the bis(platinum) complexes with resultant stabilization of the double-stranded structure. An accurate measure of T_m was difficult to obtain because the curves only began to display a plateau above 90

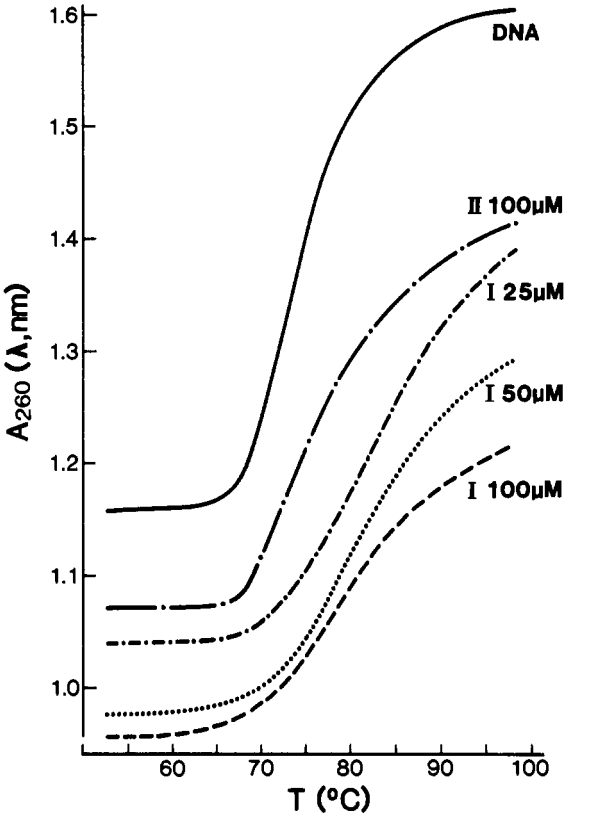


FIGURE 4: Influence of bis(platinum) complexes on the melting behavior of Calf Thymus DNA. I, 1,1/t,t; II, 2,2/c,c. Error estimates, where appropriate, given in Table III.

°C, Figure 4. For comparative purposes the T_m for each concentration of 1,1/t,t and 2,2/c,c was therefore calculated at an absorbance corresponding to a 19% increase—half of the 38% increase in the control DNA. No linear increase in T_m is observed for 1,1/t,t upon increased r_b, nor is there a significant difference between the bis(platinum) complexes in the stabilization curve. Similarly, the T_m's of samples treated for 16 h showed no significant increases over those of samples treated for only 1 h. The results imply therefore that once a certain minimum number of interstrand cross-links are

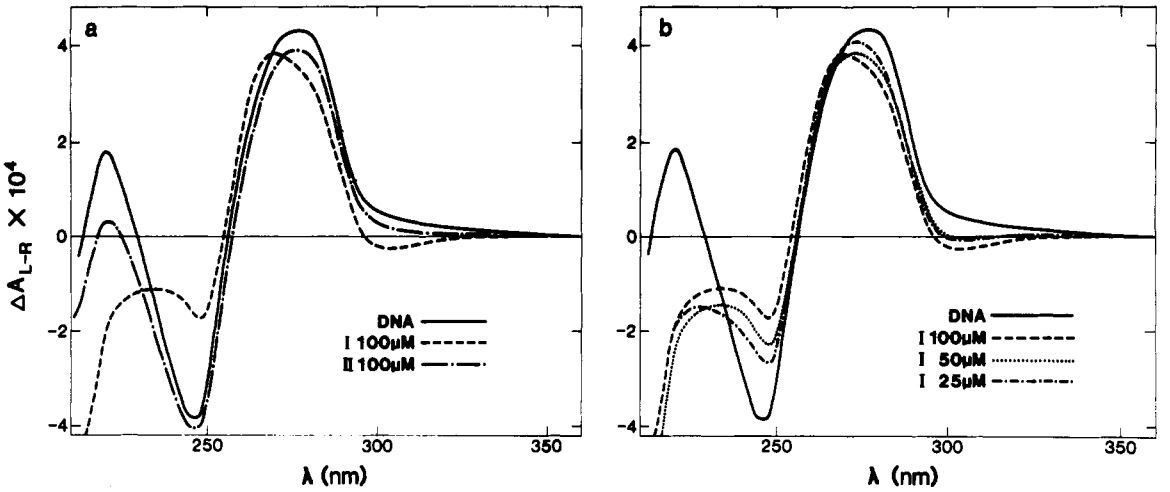


FIGURE 5: Circular dichroism spectra of calf thymus DNA modified by bis(platinum) complexes. I, 1,1/t,t; II, 2,2/c,c. The DNA was treated as under Materials and Methods with the indicated concentration of complex.

formed the resultant thermal stabilization is not enhanced by increased binding, at least up to a measurable temperature of 90–95 °C.

The UV spectrum of *cis*-DDP-modified DNA is characterized by a hyperchromic effect and a slight red shift in the absorption maximum (Horacek & Drobnik, 1971). The UV spectrum of DNA modified by both bis(platinum) complexes also shows a slight red shift, which is greater for the 2,2/c,c complex. In contrast to monomeric platinum complexes, the 1,1/t,t complex shows an apparent hypochromic effect in the UV spectrum. The nature of the hypochromicity is not known. It is conceivable that formation of interhelical cross-links may lead to large aggregates of DNA precipitating from solution (Roberts et al., 1989). The subsequent decrease in DNA concentration would thus result in a lower absorbance and an apparent hypochromic shift.

The circular dichroism spectra of DNA upon reaction with bis(platinum) complexes are shown in Figure 5. Circular dichroism gives information on the overall conformational changes induced by platination. The major change for *cis*-DDP at low r_b is a slight increase in ellipticity of the positive band and the binding of Pt(dien) also shows little perturbation of the structure, in agreement with the extensive studies already reported for these two complexes in the literature (Johnson et al., 1989). The spectrum of DNA modified by the 1,1/t,t complex, especially, shows significant differences in comparison to those of all the other complexes (at the same r_b). Particularly notable is the decrease in intensity of the negative band centered at 246 nm with increasing r_b, Figure 5b. The decrease in intensity is accompanied by a slight red shift of the maximum to 248 nm. The positive band centered at 275 nm does not undergo any major decrease in absorbance but the maximum is again shifted slightly to 270 nm. It is further notable that the CD spectrum of DNA modified by the 2,2/c,c complex is very similar to that of *cis*-DDP-modified DNA, indicating a relatively minor modification of the overall structure. This result also implies that intrastrand cross-links from the individual platinum atoms of a 2,2/c,c bis(platinum) complex form a significant part of the total adducts formed in this case.

Recognition of Platinum-Damaged DNA by UvrABC Nuclease. The bis(platinum) complexes appear to produce novel DNA lesions, and it was of interest to determine whether these DNA adducts were recognized by a DNA repair enzyme. The bacterial repair enzyme from *E. coli*, UvrABC nuclease, recognizes diverse types of DNA damage (Van Houten, 1990). The enzyme appears to recognize lesion-induced helical distortions in the DNA rather than specific modified nucleotides. This enzyme is therefore a sensitive probe of sequence specificity and conformational changes of DNA adducts. The ability of the UvrABC nuclease complex to recognize and incise DNA containing platinum DNA lesions was examined by using the plasmid incision assay (Roberts et al., 1989). Superhelical PM2 DNA was treated with Pt(dien), *cis*-DDP, and the 1,1/t,t and 2,2/c,c bis(platinum) complexes to give approximately 10–15 lesions per molecule. These damaged substrates were incubated with the UvrABC subunits in the presence of ATP. The amount of incision was monitored by the conversion of supercoiled DNA to nicked open circle by agarose gel electrophoresis. The UvrABC complex incised DNA that had been treated with all four complexes (data not shown).

The fact that the conformational distortions of purely monodentate adducts of Pt(dien) are recognized by the UvrABC complex is of interest because these lesions are not cytotoxic. This result has been recently observed by other workers (Page et al., 1990). In this regard, it is pertinent to note that the UvrABC complex also recognizes DNA adducts induced by *trans*-DDP in supercoiled DNA (Beck et al., 1985). Thus, efficient (toxic) and inefficient (nontoxic) lesions are incised by this repair enzyme.

Sequence Specificity of Bis(platinum) Complexes. The principal sites of attack of *cis*-DDP on DNA are adjacent guanine/guanine or adenine/guanine bases forming intrastrand cross-links. The preference for guanine may be important in those cases where runs of contiguous guanines are implicated in regulation and transcription such as the SV40 promoter (Gidoni et al., 1985). The binding of *cis*-DDP is, however, somewhat sequence and conformation specific and not all runs of guanines are platinated (Hemminki & Thilly, 1988; Marrot & Leng, 1989; Tullius & Lippard, 1981; Scovell et al., 1983). The synthesis of bis(platinum) complexes was motivated in part by the postulate that a greater conformational distortion might be induced by the presence of two platinum atoms close to each other on the polynucleotide, as distinct from the essentially random nature of monomer binding. Further, bis(platinum) complexes also offer the possibility of targeting larger sequences and reacting at sites inaccessible to *cis*-DDP. Previous results using the UvrABC nuclease fragment incision assay showed similar sequence specificity of the 2,2/c,c complex to *cis*-DDP but with some unique incision sites such that bis(platinum) adducts either involve additional bases or oth-

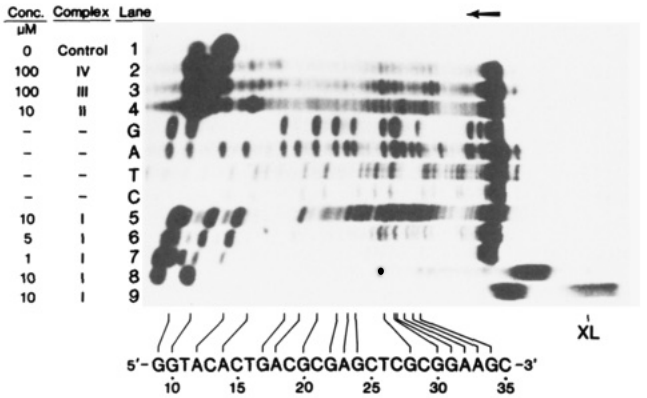


FIGURE 6: Sequence specificity of the bis(platinum) complexes. The 49-bp duplex that had been 5′-labeled with ^{32}P, on the strand, of which the sequence is given, was treated with various platinum compounds. Following digestion with the exonuclease activity of T4-polymerase the DNA was treated with KCN to remove all bound platinum (lanes 1–7). The resulting samples were analyzed by electrophoresis on a 12% polyacrylamide sequencing gel. The dried gel was used for autoradiography. Lane 8 contains DNA that had been treated with 10 μM 1,1/t,t and treated with exonuclease, but not with KCN. Lane 9 contains DNA that was only treated with 10 μM 1,1/t,t. Different amounts of DNA were loaded in each lane in order to achieve the relatively same exposures. This is most evident in lanes 8 and 9. I = 1,1/t,t, II = 2,2/c,c, III = Pt(dien), IV = *cis*-DDP. A, G, T, and C refers to the A + G, G, T + C, and C Maxam and Gilbert sequencing reactions. The arrow indicates the direction of electrophoresis. XL = DNA interstrand cross-links.

erwise produce alterations which affect the UvrABC incision patterns (Roberts et al., 1989).

In order to determine whether the 1,1/t,t complex has different sequence specificity, as compared to *cis*-DDP or 2,2/c,c, we have studied the interactions of these compounds with DNA duplexes of defined sequence. As mentioned above, our previous experiments with the 2,2/c,c compound and the UvrABC complex suggested differences in the sequence specificity, although the exact position of the DNA adducts could not be determined from these experiments (Roberts et al., 1989). The 3′ → 5′ exonuclease activity of T4-DNA polymerase has been used previously to determine the sites of reaction for a number of DNA damaging agents (Panigrahi & Walker, 1990; Royer-Pokora et al., 1981). The 3′ → 5′ exonuclease activity of T4 polymerase has been shown to stop one or two bases prior to the modified nucleotide. A uniquely 5′-labeled 49-bp duplex was treated with either 1,1/t,t, 2,2/c,c, Pt(dien), or *cis*-DDP and then subjected to exonuclease digestion by T4-DNA polymerase. The products were analyzed by electrophoresis on a 12% polyacrylamide sequencing gel under denaturing conditions. Since, at the concentration used, the bis(platinum) complexes resulted in a high frequency of DNA cross-links, which would result in a complicated gel pattern, it was essential to remove all the platinum adducts prior to electrophoresis. The digested DNA was therefore treated with KCN followed by extensive dialysis to remove all the platinum adducts (Ushay et al., 1981). The results of such an experiment are shown in Figure 6. The analysis is based on the fact that the T4-exonuclease product (with a 3′-OH-terminal group) will run 1.5–2 bases slower than the Maxam–Gilbert fragment (with a 3′-P-terminal group) of the same length and the assumption that the enzyme stops one nucleotide 3′ to the platinum adduct.

Examination of this gel reveals several interesting features regarding the reactivities of these four structurally related platinum compounds. First, specific stop sites that are common to all four compounds can be seen. For example, the bands at position 13 and 14 are a result of adducts at G_9G_{10}. A second type of sequence that is reactive only to compounds with monodentate coordination spheres [the 1,1/t,t and Pt(dien)] can be observed by bands migrating between positions 36 and 37, corresponding to adducts at $A_{33}G_{34}$. A third class of stop sites appear to be unique to the bis(platinum) interstrand cross-linking agents. An example of this can be seen for the band that migrates between A_{32} and A_{33}, and which therefore corresponds to a stop site at $G_{28}C_{29}$. Since C residues are not as readily attacked as G or A sites (Marrot & Leng, 1989), a plausible and more reasonable explanation of this stop site is that it corresponds to an interstrand cross-link at $G_{28}G'_{29}$ (in which the G_{28} is cross-linked to the guanine opposite to C_{29}). Finally, there are unique stop sites that are due solely to the 1,1/t,t complex I. One such site clearly observable is an $A'_{15}G_{16}$ cross-link (from the band migrating at $C_{19}G_{20}$). The cluster of bands migrating between A_{23} and C_{27} correspond to a number of unique binding sites for the 1,1 complex in the C_{19} (or G'_{19}) to G_{22} region. This latter region is a likely candidate for interstrand cross-links involving G'_{19} and G'_{21}. These results may be summarized as

stop site/adduct	complex
G_9G_{10}	all complexes
$A_{33}G_{34}$	1,1/t,t Pt(dien) only
$G_{28}C_{29}$	bis(Pt) only
$T_{15}G_{16}$	1,1/t,t only
C_{19}–G_{22}	1,1/t,t only

These results extend our previous findings and demonstrate that DNA sequences that are not reactive with *cis*-DDP are bound by bis(platinum) species. Interestingly, both CGCG regions (19–23 and 26–29) present in this 49-mer oligonucleotide are preferentially reactive with the bis(platinum) compounds. Further, the sequence specificity is different for 1,1/t,t in comparison to that of 2,2/c,c.

Physical studies on oligonucleotides modified by *cis*-DDP have been particularly instructive in elucidating the structural features of the adducts and the conformational alterations induced. One goal of these studies is to develop a model for the structure of the bis(platinum) interstrand cross-link. In this respect we studied the interaction of the complexes with the self-complementary 12-mer sequence 5′-TTTTGGCCAAAA-3′. The sequence has the advantages that it will self-anneal and also contains the *Hae*III restriction enzyme site. This GGCC site is a potential reactive site for both intra- (GG) and interstrand (GG′) cross-links and is also a useful probe for the differences between the monodentate and bidentate bis(platinum) complexes. Sensitivity to the *Hae*III digestion is also a good estimate of the amount of duplex in solution. Digestion of the 12-mer with *Hae*III at 20 °C for 1 h resulted in >90% digestion (data not shown). The 12 base pair duplex was incubated with either Pt(dien), *cis*-DDP, 1,1/t,t, or 2,2/c,c complexes for 1 h at 20 °C, followed by electrophoresis in 20% polyacrylamide gels under denaturing conditions. This gel system allows the resolution of several different clusters of DNA bands representing structurally discrete DNA lesions, Figure 7. These bands are interpreted in the following manner: nonadducted 12-mer, Pt(dien) monoadducts (region a), Pt(dien) diadducts in which two Pt(dien) molecules are linked to two different bases (region b), bis(platinum) 1,1/t,t monoadducts (region c), bis(platinum) 1,1/t,t diadducts in which one Pt of two different bis(Pt) complexes is linked to two different bases (region d), and 1,1/t,t interstrand cross-links (region e). The latter species are apparent after overexposure of the gel (not shown). It is possible that region d represents 1,1/t,t intrastrand cross-links in which each Pt atom of the bis(platinum) complex is linked

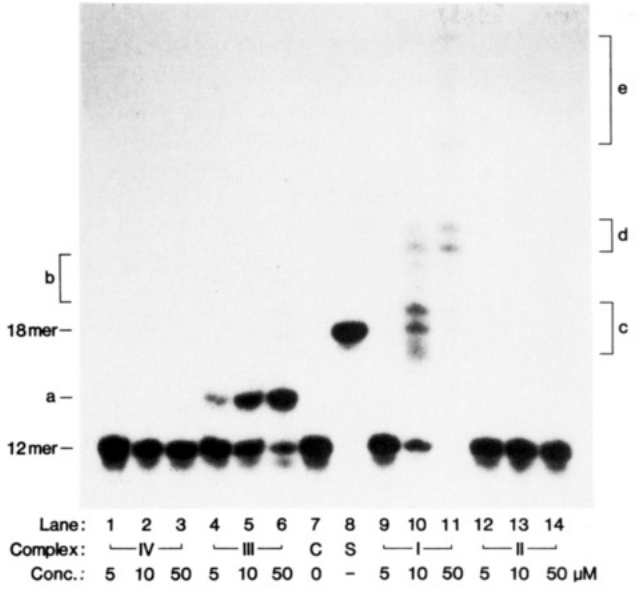


FIGURE 7: Formation of platinum–DNA adducts using a 12-bp duplex. The 12-mer 5′-TTTTGGCCAAAA-3′, which had been 5′-labeled and allowed to self-anneal, was treated with the indicated concentrations of the four platinum complexes, where I = 1,1/t,t, II = 2,2/c,c, III = Pt(dien), and IV = *cis*-DDP. C is a nontreated control sample, and S is a 18-mer, which was run as a standard marker. Regions a and b indicate the position of migration of the 12-mer-containing Pt(dien) mono- and diadducts, respectively. c, d, and e = 12-mer-containing mono- and diadducts and cross-links of 1,1/t,t. Overexposure reveals bands at e.

to bases (G or A) on the same strand. Although molecular modeling studies suggest that this type of lesion is sterically less favored than interstrand cross-link formation, we cannot at present rule out the possibility that some of the bands in region d may be intrastrand cross-links from one bis(platinum) molecule.

Surprisingly, the 1,1/t,t compound gave rise to very few interstrand cross-linked species, although these are seen at higher concentrations and longer exposures (region e). One possible explanation is that under the conditions used in these experiments formation of a monoadduct may destabilize the duplex sufficiently to cause melting of the two strands such that formation of the interstrand cross-link is not favored. Alternatively, the specific sequence chosen for these studies may be inherently refractory to the formation of DNA cross-links. Future studies with these and other short DNA duplexes will allow a systematic characterization of how DNA interstrand cross-links form and the steric factors of the bis(platinum) compounds that affect their formation. The results again confirm that the 1,1/t,t complex is more reactive than its 2,2/c,c analogue. In agreement with this, we found that *Hae*III digestion was inhibited by the 1,1/t,t complex at a concentration of 10 μM, whereas the 2,2/c,c at 50 μM did not inhibit *Hae*III digestion. The fact that DDP is not reactive with this sequence is in agreement with the above-mentioned nonrandom binding of this molecule to GG sequences (Hemminki & Thilly, 1988; Marrot & Leng, 1989; Tullius & Lippard, 1981; Scovell et al., 1983).

Discussion

Cytotoxicity. Both bis(platinum) complexes are active in *cis*-DDP-sensitive and -resistant cell lines but the pattern is intriguingly different. The bis(platinum) complex with monodentate coordination spheres is relatively more efficient at overcoming acquired cisplatin resistance of cells than the bidentate complex. Thus, the structural feature of the interstrand cross-link is sufficient, by itself, in overcoming *cis*-DDP resistance. Conversely, the 1,1/t,t complex is not as cytotoxic as the 2,2/c,c chloride complexes in the sensitive cell line, where the structural feature of the intrastrand cross-link is necessary. The 2,2/c,c chloride complexes themselves are generally equally as cytotoxic as *cis*-DDP itself, but rarely more so, in *cis*-DDP-sensitive lines. The results point toward a rational approach to overcomining *cis*-DDP resistance at the molecular level and the eventual development of platinum compounds active against tumor types hitherto refractive to the parent cisplatin.

A preliminary interpretation, and a working hypothesis for further complex development, of the cytotoxicity results reported here may be obtained by considering that the 2,2/c,c complex contains many cis-DDP-like lesions along with other lesions including interstrand cross-links. The combination of adducts is responsible for the overall spectrum of activity. These *cis*-DDP lesions may be discrete; i.e., the second platinum atom need not necessarily be bound in any fashion to DNA. For the 1,1/t,t complex I, interstrand cross-linking dominates and the activity in the resistant line is increased. The results strongly imply that the interstrand cross-link of a bis(platinum) complex is an effective cytotoxic lesion in a cis-DDP-resistant cell. Preliminary experiments using L1210 cells have confirmed the formation of interstrand cross-links in genomic DNA upon treatment with these bis(platinum) complexes. A full analysis, which is presently under way, must take into account pharmacokinetic factors such as uptake, intracellular Pt/DNA values, and DNA–protein cross-links before any definitive comparisons can be made.

Nature of the Interstrand Cross-Link. The DNA-binding studies presented here point to some general differences between the bis(platinum) complexes, which may be relevant to the interpretation of the cytotoxicity data. Interstrand cross-linking by a 1,1 bis(platinum) complex is more efficient, and sequence specificity is also different, than for structurally analogous 2,2 species. A fundamental difference in the nature of the cross-links formed by both types of bis(platinum) complex is that in the case of monodentate coordination spheres, the platinum atoms of the complex are "saturated" upon the formation of the cross-link. In the 1,1/t,t case the initial binding of the complex to DNA will give a monoadduct with one free platinum end. These monoadduct intermediates may go on to form cross-links and the efficiency of formation will be dictated by the nature of the diamine backbone and the near-neighbor sequence. The monoadduct intermediate is, however, still very flexible and may present a steric block to more monoadduct platination. There is perfectly free rotation of not only the diamine backbone around the *adducted* platinum square plane but the monoadducted platinum coordination sphere also has free rotation around both the Pt–purine and platinum–diamine nitrogen bonds. This is simply the opposite of the statement that, in the absence of steric effects of the amine groups, there is no restricted rotation of the purine around the Pt–purine bond in complexes such as *cis*-$[Pt(amine)_2(purine)_2]^{2+}$ (Inagaki et al., 1988).

With bidentate coordination spheres, formation of a cross-link may proceed in one of two limiting ways: an interstrand cross-link is initially formed followed by subsequent closure of the intrastrand cross-links on each strand or alternatively an intrastrand cross-link could be formed first, which is followed by the interstrand cross-link formation upon reaction of the second platinum atom. If this latter situation occurs, the subsequent chelate formation would restrict considerably the flexibility of the free platinum moiety because there is now no longer any possibility of rotation of the *di-*

adducted platinum square plane about the Pt–purine or Pt–diamine nitrogen bonds. In contrast to the 1,1 complex, therefore, this more rigid structure may not prevent further access of Pt atoms to the DNA but interstrand cross-link formation may now be restricted considerably.

Conformational Changes on DNA. The conformational changes induced by bis(platinum) complexes are due, either independently or in combination, to the type of lesions formed or the nature of the sequences attacked. The spectrum of DNA damage induced by the 2,2/c,c complexes is the sum of interstrand and intrastrand cross-links. In principle, the 1,1/t,t complex may form monoadducts, intrastrand cross-links by binding of both Pts to the same strand, and "pure" interstrand cross-links. As stated, molecular models show that the intrastrand cross-link is sterically more demanding than the interstrand cross-link. Future studies with a complex such as $[Pt(NH_3)_3\{H_2N(CH_2)_4\}PtCl(NH_3)_2]^{3+}$ (containing one monodentate and one platinum–tetraamine coordination sphere) will resolve the contribution of monodentate coordination of a bis(platinum) complex to the overall DNA conformational change.

A major contributing factor to conformational distortion of platinated DNA in *cis*-DDP adducts is the alteration of the sugar conformation with the deoxyribose ring of the 5′-purine adopting the N (C3′-endo) conformation. It is therefore likely that the same situation will occur for bis(platinum) complexes (Y. Qu and N. Farrell, unpublished results). Pt(dien) induces large changes in the circular dichroism of poly(dG-dC)·poly(dG-dC) (Tullius et al., 1983). Further, only Pt(dien), and not cis-DDP, can facilitate the switch from B → Z DNA (Malfoy et al., 1981). The dramatic decrease in the negative band of CT DNA upon binding of the monodentate bis(platinum) complexes could reflect the initiation of the B → Z conformational transition, especially if $(CG)_n$ sequences are preferentially attacked. In this case, the alteration of the glycosyl bond angles on both strands from the anti form (B DNA) to the syn (Z DNA) within those sequences recognized to be B → Z junctions may be very efficient in inducing the overall conformational transition. The sequence specificity of the 1,1/t,t complex may, therefore, be critical to its distortion of the DNA structure. Other explanations of the unusual CD spectrum of DNA modified by the 1,1/t,t complex are also possible. For instance mitomycin C causes inversion of the CD spectrum without inducing the Z conformation (Tomasz et al., 1983).

Conclusions. We are now in a position to examine how interstrand cross-links form, what steric factors on the bis(platinum) complexes affect this formation, how the sequence specificity may be altered from that of the simple monomers, and whether this conformational change is a feature of DNA binding by similar complexes with activity in *cis*-DDP-resistant cell lines.

The general structure for monodentate bis(platinum) complexes (and thus for complexes with enhanced activity in *cis*-DDP-resistant cell lines) is in fact $[\{PtCl(L)(L')\}_2H_2N(CH_2)_nNH_2]^{2+}$. In the present case L = L′ = NH_3, but, in principle, variation of L, L′, the diamine backbone, and geometry allows scope for design of molecules with considerably greater and different sequence selectivity than the parent *cis*-DDP. The interstrand cross-linking of bis(platinum) complexes makes them much more analogous to the classic alkylating agents than monomeric species such as *cis*-DDP. In this respect it is noteworthy that structural modification of the alkylating agents may affect the sequence specificity (Hartley et al., 1988). It would be of considerable clinical importance if the novel DNA adducts and/or sequence specificity led to a broader spectrum of antitumor action.

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DNA Topoisomerase II Is Required for Formation of Mitotic Chromosomes in Chinese Hamster Ovary Cells: Studies Using the Inhibitor 4′-Demethylepipodophyllotoxin 9-(4,6-*O*-Thenylidene-β-D-glucopyranoside)†

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ABSTRACT: To study the biochemical processes which DNA topoisomerase II carries out in mammalian cells, which have not been identified, we have examined the effects on chromosome replication in Chinese hamster ovary cells of an agent which traps molecules of topoisomerase II when they are covalently integrated into DNA during their reaction. This agent, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-thenylidene-β-D-glucopyranoside) (VM-26), targets this enzyme specifically according to a compelling body of evidence. Using synchronously growing cells, we found that VM-26 at a cytotoxic concentration (0.08 μM) did not affect DNA replication during the S phase. The formation of mitotic chromosomes was delayed by 4 h, and its rate was reduced thereafter, causing a delay in mitosis of >14 h in 65% of the cells; in some cells, the chromatin was aberrantly condensed, forming diffuse chromosomes or particles. Chromosome formation was completely inhibited at 0.32 μM VM-26. DNA fragments derived from topoisomerase II molecules covalently integrated in DNA and trapped by VM-26 were detected by FIGE analysis in the G2 period, but not during the S phase. The delay of chromosome formation appeared to be caused by two factors: first, a delay in the completion of DNA replication, because progress of some cells to mitosis after removal of VM-26 was prevented by aphidicolin, an inhibitor of DNA polymerases α and δ; and second, a delay of chromosome formation in cells which had apparently completed DNA replication. The observations reported here show that topoisomerase II carries out reactions which are essential for formation of mitotic chromosomes. They are compatible with a model in which topoisomerase II functions both during the completion of DNA replication and in a subsequent process which, by analogy with VM-26-sensitive steps in simian virus 40 DNA replication, may be the topological conversion of a series of replicated DNA loops or domains into two linear chromatid-length DNA molecules.

DNA topoisomerase II (Cozzarelli, 1980; Gellert, 1981; Wang, 1985; Vosberg, 1985; Maxwell & Gellert, 1986) carries out reactions which are essential for segregation of the chromosomes of simian virus 40 (SV40)[1] and of yeast. In SV40, its activity is required for the decatenation of progeny DNA molecules by passing one double-stranded daughter molecule across the second; except during replication of the terminal ≃250 bp region, which may occur simultaneously with decatenation (Varshavsky et al., 1983; Snapka et al., 1988), topoisomerase II is not required for DNA replication (Snapka, 1986; Richter et al., 1987; Yang et al., 1987; Richter & Strausfeld, 1988; Snapka et al., 1988) but can replace topoisomerase I in this process (Yang et al., 1987). In yeast, topoisomerase II is essential for the segregation of chromosomes at mitosis (DiNardo et al., 1984; Uemura & Yanagida, 1984, 1986; Uemura et al., 1987), but, as in SV40, it is not required for DNA replication although it can replace topoisomerase I in this process (Uemura & Yanagida, 1984, 1986). The prokaryotic homologue of topoisomerase II, DNA gyrase, is, in contrast, essential both for DNA replication and for chromosome segregation in *Escherichia coli* (Kreuzer & Cozzarelli, 1979; Snyder & Drlica, 1979; Steck & Drlica, 1984).

The biochemical processes which topoisomerase II carries out during the replication of the chromosomes of mammalian

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[1] Abbreviations: bp, base pair(s); CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; FIGE, field inversion gel electrophoresis; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; kbp, kilobase pair(s); LMP, low melting point; SV40, simian virus 40; VM-26, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-thenylidene-β-D-glucopyranoside).

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