DNA Interactions of Antitumor Cisplatin Analogs Containing Enantiomeric Amine Ligands

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ABSTRACT Modifications of natural DNA and synthetic oligodeoxyribonucleotide duplexes in a cell-free medium by analogs of antitumor cisplatin containing enantiomeric amine ligands, such as *cis*-[$PtCl_2$(RR-DAB)] and *cis*-[$PtCl_2$(SS-DAB)] (DAB = 2,3-diaminobutane), were studied by various methods of molecular biophysics and biophysical chemistry. These methods include DNA binding studies by pulse polarography and atomic absorption spectrophotometry, mapping of DNA adducts using transcription assay, interstrand cross-linking assay using gel electrophoresis under denaturing conditions, differential scanning calorimetry, chemical probing, and bending and unwinding studies of the duplexes containing single, site-specific cross-link. The major differences resulting from the modification of DNA by the two enantiomers are the thermodynamical destabilization and conformational distortions induced in DNA by the 1,2-d(GpG) intrastrand cross-link. It has been suggested that these differences are associated with a different biological activity of the two enantiomers observed previously. In addition, the results of the present work are also consistent with the view that formation of hydrogen bonds between the carbonyl oxygen of the guanine residues and the "quasi equatorial" hydrogen of the *cis* amine in the 1,2-d(GpG) intrastrand cross-link plays an important role in determining the character of the distortion induced in DNA by this lesion.

INTRODUCTION

Since the discovery of its anticancer activity, several new analogs of cisplatin [*cis*-diamminedichloroplatinum(II), *cis*-$PtCl_2(NH_3)_2$] have been synthesized and tested for biological activity. Some of these compounds are now considered potent anticancer drugs (Pasini and Zunino, 1987; Bloemink and Reedijk, 1996; Reedijk, 1996; O'Dwyer et al., 1999). Although the precise mechanism of antitumor action of platinum drugs is not completely understood, they are known to target DNA primarily by forming bifunctional adducts (Pinto and Lippard, 1985; Johnson et al., 1989). The anticancer activity displayed by cisplatin and its analogs is usually attributed to a unique type of intrastrand d(GpG) adduct with platinum cross-linking N7 atoms of neighboring guanine residues of DNA. It has been also shown that carrier amine ligands of cisplatin analogs appear to modulate the antitumor properties of this class of drugs. The antitumor activity is usually lost or diminished if the primary or secondary amines on platinum are replaced by tertiary amines (Sundquist and Lippard, 1990).

There are many possible roles for the carrier ligand of the platinum antitumor compounds. Hydrogen bonding between DNA and the carrier ligand could affect the initial attack of DNA by the drug and the type of DNA cross-linking (intra or interstrand). It is also reasonable to expect that the direction (5′ or 3′) of closure of monofunctional DNA adducts (formed in the first step of their binding to DNA) into cross-links is affected by hydrogen bonding. In addition, the carrier ligand may also affect biodistribution, and recognition of DNA adducts by repair enzymes, regulatory and/or DNA-binding proteins.

The biological activity of platinum complexes with enantiomeric amine ligands such as *cis*-[$PtCl_2$(RR-DACH)] and *cis*-[$PtCl_2$(SS-DACH)] (DACH = 1,2-diaminocyclohexane) and other enantiomeric pairs has been intensively investigated (Kidani et al., 1978; Noji et al., 1981, 1983; Coluccia et al., 1986, 1991; Fanizzi et al., 1987; Pasini and Zunino, 1987; Giannini and Natile, 1991; Vickery et al., 1993; Fenton et al., 1997). For instance, the DACH carrier ligand has been shown to significantly affect the ability of platinum-DNA adducts to block essential processes such as replication and transcription (Page et al., 1990). Also importantly, *cis*-[$PtCl_2$(N-N)] complexes with N-N = DACH or 1,2-diaminopropane (DAB) having an S configuration at the asymmetric carbon atoms were markedly more mutagenic toward several strains in *Salmonella typhimurium* than their R isomers (Fanizzi et al., 1987). Hence, although the asymmetry in the amine ligand in these platinum complexes did not involve the coordinated nitrogen atom, but rather an adjacent carbon atom, a dependence of the biological activity on the configuration of the amine was observed.

The major DNA adduct of cisplatin and its analogs is an intrastrand d(GpG) cross-link (Sherman and Lippard, 1987; Bloemink and Reedijk, 1996). It has been demonstrated that this cross-link adopts an *anti*, *anti* head-to-head (HH) conformation in both single- and double-stranded DNA. However, it has been speculated in numerous reports that this conformer equilibrates with other forms that interconvert too rapidly for separate characterization by NMR spectros-

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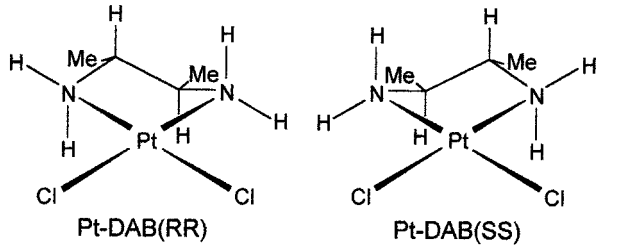
copy (Den Hartog et al., 1982; Neumann et al., 1984; Kline et al., 1989; Mukundan et al., 1991; Berners-Price et al., 1996, 1997; Van Boom et al., 1996). The dynamic character of the platinum-DNA adduct makes establishing a correlation between its stereochemistry and the configuration of the carrier ligand difficult.

In the present work modifications of DNA by *cis*-[$PtCl_2$(DAB)] enantiomers (Fig. 1) in cell-free media were investigated by using various techniques of molecular biophysics. The goal of these studies was to contribute to understanding how the chirality at the carbon atoms of the carrier ligand in cisplatin analogs can affect their biological activity. *cis*-[$PtCl_2$(DAB)] isomers were chosen for the studies described in the present paper as the representatives of platinum drugs with enantiomeric amine ligands because the effect of different chirality at the carbon atoms on the biological activity was most pronounced in the case of these compounds. The effect of the configuration of the carrier diamine in *cis*-[$PtCl_2$(DAB)] complexes with R, R and S, S configurations at the asymmetric carbons [these complexes are abbreviated as Pt-DAB(RR) and Pt-DAB(SS), respectively] was investigated. A schematic representation of the puckering of the chelate rings in Pt-DAB(RR) or Pt-DAB(SS) isomers is shown in Fig. 1.



d(TGGT)d(ACCA)(20-DSC): 5′-TCTCCTTCTGGTCTCTTCTC-3′
3′-AGAGGAAGACCAGAGAAGAG-5′

d(TGGT)d(ACCA)(20): 5′-CCTCTCCTTGGTCTCCTTCT-3′
3′- GAGAGGAACCAGAGGAAGAG-5′

d(TGGT)d(ACCA)(21): 5′-CCTCTCCTTGGTCTCCTTCTC-3′
3′- GAGAGGAACCAGAGGAAGAGG-5′

d(TGGT)d(ACCA)(22): 5′-CCTCTCCTTGGTCTCCTTCTCT-3′
3′- GAGAGGAACCAGAGGAAGAGAG-5′

d(TGGT)d(ACCA)(23): 5′-CCTCTCCTTGGTCTCCTTCTCTC-3′
3′- GAGAGGAACCAGAGGAAGAGAGG-5′

FIGURE 1 Structures of Pt-DAB(RR) and Pt-DAB(SS), and sequences of the synthetic oligodeoxyribonucleotides used in the present study with their abbreviations. The top and bottom strands of each pair are designated top and bottom, respectively, in the text. The bold letters in top strands indicate the location of the intrastrand cross-link after modification of the oligonucleotides by Pt-DAB complexes in the way described in the Experimental section.

MATERIALS AND METHODS

Starting materials

Cisplatin was synthesized and characterized in Lachema (Brno, Czech Republic). Pt-DAB(RR) and Pt-DAB(SS) complexes were prepared and characterized as described previously (Fanizzi et al., 1987). The stock solutions of the platinum complexes (5×10^{-4} M in 10 mM $NaClO_4$) were prepared in the dark at 25°C. Calf thymus (CT) DNA (42% G + C, mean molecular mass $\sim 2 \times 10^7$ Da) was also prepared and characterized as described previously (Brabec and Paleček, 1970, 1976). Plasmid pSP73KB [2455 bp (Lemaire et al., 1991)] was isolated according to standard procedures and banded twice in CsCl/EtBr equilibrium density gradients. The synthetic oligodeoxyribonucleotides were synthesized and purified as described previously (Brabec et al., 1992). Restriction endonucleases, T4 DNA ligase, Klenow fragment of DNA polymerase I, and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). Riboprobe Gemini System II for transcription mapping containing SP6 and T7 RNA polymerases was purchased from Promega (Madison, WI). Ethidium bromide, agarose, acrylamide, bis(acrylamide), and NaCN were from Merck KgaA (Darmstadt, Germany). Dimethyl sulfate (DMS), $KMnO_4$, diethylpyrocarbonate (DEPC), KBr, and $KHSO_5$ were from Sigma, Prague. [γ-^{32}P]ATP and [α-^{32}P]dATP were from Amersham (Arlington Heights, IL).

Platination reactions

CT DNA and plasmid DNAs were incubated with the platinum complex in 10 mM $NaClO_4$ at 37°C for 48 h in the dark if not stated otherwise. The number of molecules of the platinum compound bound per nucleotide residue (r_b values) were determined by flameless atomic absorption spectrophotometry (FAAS) or by differential pulse polarography (DPP) (Kim et al., 1990). The oligonucleotides were allowed to react with the platinum compounds, and repurified as described previously (Brabec et al., 1992). Briefly, the oligonucleotides synthesized on an Applied Biosystems solid-phase synthesizer were purified by ion-exchange FPLC. The single-stranded oligonucleotides (the top strands in Fig. 1) were reacted in stoichiometric amounts with Pt-DAB(RR) or Pt-DAB(SS). The platinated oligonucleotides were purified by FPLC. It was verified by platinum FAAS and by the measurements of the optical density that the modified oligonucleotides contained one platinum atom. It was also verified using DMS footprinting of platinum on DNA (Lemaire et al., 1991; Brabec and Leng, 1993) that in the platinated top strands the N7 position of both neighboring guanines was not accessible for reaction with DMS. Briefly, platinated and nonmodified top strands (5′-end-labeled with ^{32}P) were reacted with DMS. DMS methylates the N7 position of guanine residues in DNA, producing alkali-labile sites (Maxam and Gilbert, 1980). However, if N7 is covalently bound to platinum, it cannot be methylated. The oligonucleotides were then treated with hot piperidine and analyzed by denaturing polyacrylamide gel electrophoresis. For the nonmodified oligonucleotides, shortened fragments due to the cleavage of the strand at the two methylated guanine residues were observed in the gel. However, no such bands were detected for the platinated oligonucleotides. These results indicate that one Pt-DAB molecule was coordinated to neighboring guanine residues, forming the 1,2-d(GpG) intrastrand cross-link. The platinated strands were allowed to anneal with nonplatinated complementary strands (the bottom strands in Fig. 1) in 50 mM NaCl plus 1 mM Tris · HCl with 0.1 mM EDTA, pH 7.4. FPLC purification and FAAS measurements were carried out on a Pharmacia Biotech FPLC System with a MonoQ HR 5/5 column and a Unicam 939 AA spectrometer equipped with a graphite furnace, respectively.

DNA transcription by RNA polymerases in vitro

Transcription of the (*NdeI*/*Hpa*I) restriction fragment of pSP73KB DNA with SP6 and T7 RNA polymerases and electrophoretic analysis of tran-

scripts was performed according to the protocols recommended by Promega [Promega Protocols and Applications, 43-46 (1989/90)] and previously described in detail (Lemaire et al., 1991; Brabec and Leng, 1993).

Interstrand cross-link assay

If not stated otherwise, Pt-DAB(RR) or Pt-DAB(SS) at varying concentrations were incubated with 2 μg pSP73KB DNA after it had been linearized by *Eco*RI. The platinated samples were precipitated by ethanol and the linear duplexes were then analyzed for DNA interstrand cross-links in the same way as described in several recent papers (Farrell et al., 1990; Lemaire et al., 1991; Brabec and Leng, 1993). The linear duplexes were first 3′-end-labeled by means of a Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. The samples were deproteinized by phenol, precipitated by ethanol, and the pellet was dissolved in 18 μl of 30 mM NaOH with 1 mM EDTA, 6.6% sucrose, and 0.04% bromophenol blue. The amount of interstrand cross-links was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1.5%). After the electrophoresis was completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified by means of a Molecular Dynamics Phosphor Imager (Storm 860 system with ImageQuant software). The frequency of interstrand cross-links, F (the number of interstrand cross-links per adduct), was calculated as $F = XL/4910 \cdot r_b$ (pSP73KB plasmid contained 4910 nucleotide residues). XL is the number of interstrand cross-links per one molecule of the linearized DNA duplex which was calculated assuming Poisson distribution of the interstrand cross-links as $XL = -\ln A$, where A is the fraction of molecules running as a band corresponding to the non-cross-linked DNA (Farrell et al., 1990).

Differential scanning calorimetry

Excess heat capacity (ΔC_p) versus temperature profiles for the thermally induced transitions of d(TGGT)/d(ACCA)(20-DSC) duplex unmodified or containing a unique 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) or Pt-DAB(SS) were measured by using a VP-DSC Calorimeter (Microcal, Northampton, MA). In these experiments the heating rate was 60°C/h. Transition enthalpies (ΔH) and entropies (ΔS) were calculated from the areas under the experimental ΔC_p versus T and the derived $\Delta C_p/T$ versus T curves, respectively, by using the ORIGIN version 4.1 software (Microcal, Northampton, MA). The oligonucleotide duplexes at the concentration of 5 μM were dissolved in the buffer containing 10 mM sodium cacodylate (pH 7.2), 100 mM NaCl, 10 mM $MgCl_2$, and 0.1 mM EDTA. The samples were vacuum-degassed before the measurement. The formation of 1:1 complexes between the top and bottom strand of d(TGGT)/d(ACCA) nonmodified or containing the cross-link was verified by recording UV absorbance mixing curves at 25°C (Poklar et al., 1996). It was also verified in the same way as described in the previous paper (Poklar et al., 1996) that the melting transition of both the platinated and nonmodified duplexes were fully reversible.

Chemical modifications

The chemical probing of the conformation of the platinated oligonucleotide duplexes with the aid of NaCN was performed as described previously (Schwartz et al., 1990; Boudný et al., 1992). The top strand of the d(TGGT)/d(ACCA)(20) nonmodified or containing the Pt-DAB intrastrand cross-link was 5′-end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase before it was annealed with its complementary (bottom) nonlabeled strand. The oligonucleotide duplexes were treated with 0.2 M NaCN in 20 mM Tris · HCl, pH 8.3, and control nonmodified samples were run on a denaturing 24% polyacrylamide/8M urea gel.

The modifications by $KMnO_4$, DEPC, and $KBr/KHSO_5$ were also performed as described previously (Brabec et al., 1993; Bailly et al., 1994; Ross and Burrows, 1996; Bailly and Waring, 1997). The top or bottom strand of the d(TGGT)/d(ACCA)(20) was 5′-end-labeled with [γ-^{32}P]ATP before it was annealed with its complementary nonlabeled strands. In the case of the platinated oligonucleotides, platinum was removed after reaction of the DNA with the probe by incubation with 0.2 M NaCN (alkaline pH) at 45°C for 10 h in the dark.

Ligation and electrophoresis of oligonucleotides

Nonplatinated single strands (bottom strands in Fig. 1) were 5′-end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Then they were annealed with their phosphorylated complementary strands [nonplatinated or containing 1,2-d(GpG) intrastrand cross-links of Pt-DAB compounds]. Nonplatinated and intrastrand cross-link containing duplexes were allowed to react with T4 DNA ligase. The resulting samples along with ligated nonplatinated duplexes were subsequently examined on 8% native polyacrylamide [mono:bis(acrylamide) ratio = 29:1] electrophoresis gels. Other details of these experiments were as described in previously published papers (Koo et al., 1986; Bellon and Lippard, 1990).

RESULTS

DNA binding

Solutions of double-helical CT DNA at a concentration of 32 μg/ml were incubated with Pt-DAB(RR) or Pt-DAB(SS) at r_i values of 0.01 in 10 mM $NaClO_4$ at 37°C (r_i is defined as the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA). At various time intervals an aliquot of the reaction mixture was withdrawn and assayed by DPP for the amount of platinum bound to DNA (r_b) (Kim et al., 1990). The amount of platinum coordinated to DNA increased with time (not shown). After ~24 h, all molecules of Pt-DAB(RR) or Pt-DAB(SS) present in the reaction mixtures were coordinated to DNA [exhaustive dialysis of the samples of DNA treated with Pt-DAB(RR) or Pt-DAB(SS) against platinum-free background solution (10 mM $NaClO_4$) did not affect the amount of the platinum bound to DNA]. In these binding reactions both enantiomers coordinated to DNA with approximately the same rate, which indicates that isomerism in the non-leaving ligand of Pt-DAB compounds does not significantly affect the rate of the coordination of platinum moiety to natural double-helical DNA. The binding of Pt-DAB(RR) or Pt-DAB(SS) to CT DNA was also quantified in the following way. Aliquots of the reaction withdrawn at various time intervals were quickly cooled on an ice bath and then exhaustively dialyzed against 10 mM $NaClO_4$ at 4°C to remove free (unbound) platinum compound. The content of platinum in these samples was determined by FAAS. Results identical to those obtained using the DPP assay were obtained. Thus, DNA binding of Pt-DAB compounds resulted within <24 h in their complete coordination, which made it possible to prepare easily and precisely the samples of natural DNAs or their fragments modified by these compounds at a preselected r_b value.

In vitro transcription of DNA containing platinum adducts

In vitro RNA synthesis by RNA polymerases on DNA templates containing several types of bifunctional adducts of platinum complexes can be prematurely terminated at the level or in the proximity of adducts (Corda et al., 1991, 1992; Lemaire et al., 1991; Brabec and Leng, 1993; Brabec et al., 1994; Nováková et al., 1995; Žaludová et al., 1997). Importantly, monofunctional DNA adducts of several platinum complexes are unable to terminate RNA synthesis.

Cutting of pSP73KB DNA (Lemaire et al., 1991) by *Nde*I and *Hpa*I restriction endonucleases yielded a 212-bp fragment (a substantial part of its nucleotide sequence is shown in Fig. 2 *B*). This fragment contained convergent T7 and SP6 RNA polymerase promoters [in the upper and lower strands, respectively, close to its 3′-ends (Fig. 2 *B*)]. The experiments were carried out using this linear DNA fragment, modified by Pt-DAB(RR), Pt-DAB(SS), or cisplatin at $r_b = 0.01$, for RNA synthesis by T7 and SP6 RNA polymerases (Fig. 2 *A*, lanes RR, SS or cisDDP, respectively). RNA synthesis on the template modified by the platinum complexes yielded fragments of defined sizes, which indicates that RNA synthesis on these templates was prematurely terminated. The major stop sites produced by both Pt-DAB complexes were identical to those produced by cisplatin, and the corresponding bands produced by both enantiomers and cisplatin on the autoradiogram had similar intensity. The sequence analysis revealed that the major bands resulting from termination of RNA synthesis by the adducts of Pt-DAB(RR), Pt-DAB(SS), and cisplatin were identical and appeared at G sites and in a considerably less extent at A sites. These G and A sites were mostly contained in GG or AG sites, which are preferential DNA binding sites for untargeted cisplatin. Taken together, the results of the transcription mapping experiments suggest that base sequence selectivity of Pt-DAB(RR), Pt-DAB(SS), and cisplatin are similar.

Interstrand cross-linking

The experiments of the present work were carried out to compare the amounts of the interstrand cross-links formed by Pt-DAB(RR) or Pt-DAB(SS) in linear DNAs. We used in these experiments pSP73KB plasmid (2455 bp) modified by Pt-DAB complexes after it had been linearized by *Eco*RI (*Eco*RI cuts only once within pSP73KB plasmid). The samples were analyzed for the interstrand cross-links by agarose gel electrophoresis under denaturing conditions.

An electrophoretic method for precise and quantitative determination of interstrand cross-linking by platinum complexes in DNA was described previously (Farrell et al., 1990; Lemaire et al., 1991; Brabec and Leng, 1993). Upon electrophoresis under denaturing conditions, 3′-end-labeled strands of linearized pSP73KB plasmid containing no inter-

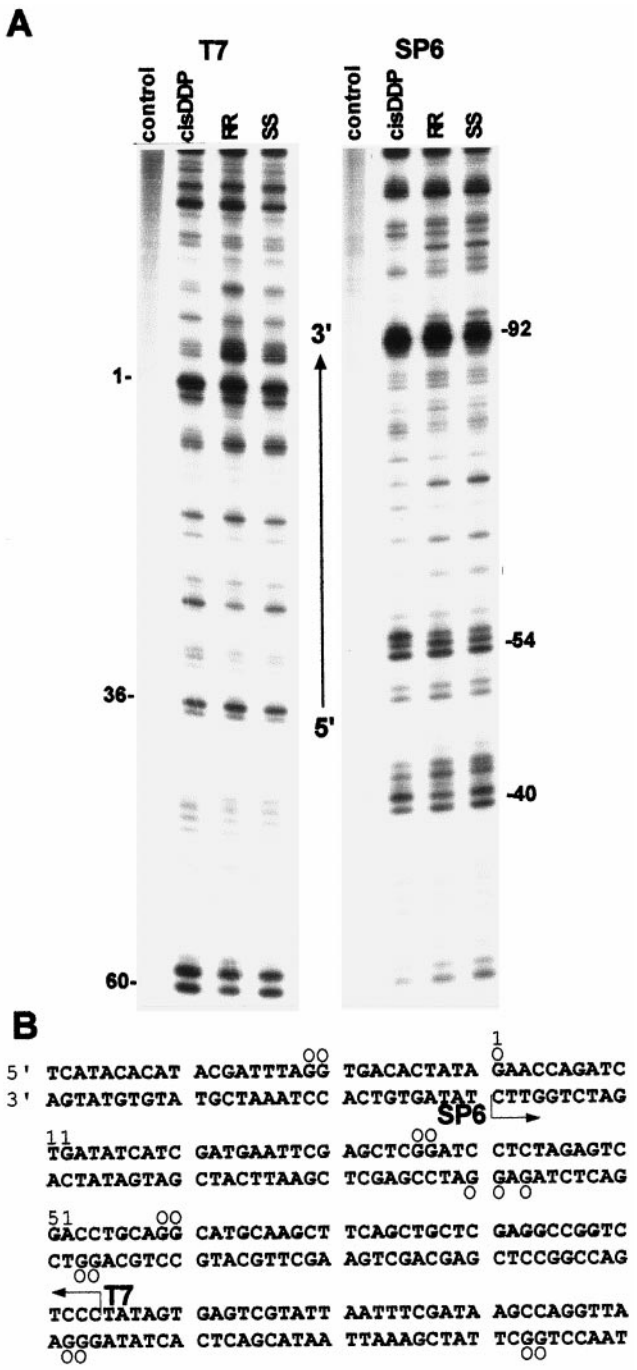


FIGURE 2 Inhibition of RNA synthesis by T7 (left) and SP6 (*right*) RNA polymerases on the *Nde*I/*Hpa*I fragment of pSP73KB plasmid modified by platinum complexes. (*A*) Autoradiogram of 6% polyacrylamide/8 M urea sequencing gel. *Lanes:* control, nonmodified template; cisDDP; RR; SS, the template modified by cisplatin; Pt-DAB(RR), Pt-DAB(SS) at $r_b = 0.01$, respectively. (*B*) Schematic diagram showing the portion of the nucleotide sequence of the template (upper) strand of the *Nde*I/*Hpa*I fragment used to monitor inhibition of RNA synthesis by platinum complexes. The arrows indicate the start of the T7 or SP6 RNA polymerases. (○), major stop signals (from *A*) for DNA modified by Pt-DAB(RR). The numbers correspond to the nucleotide numbering in the sequence map of the pSP73KB plasmid.

strand cross-links migrate as a 2455-nucleotide single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species. The bands corresponding to more slowly migrating interstrand-cross-linked fragments were noticed if either Pt-DAB complex was used to modify DNA at r_b as low as 3×10^{-4} (Fig. 3 *A*). The intensity of the more slowly migrating band increased with the growing level of the modification. The radioactivity associated with the individual bands in each lane was measured to obtain estimates of the fraction of non-cross-linked or cross-linked DNA under each condition. The frequency of interstrand cross-links (the amount of interstrand cross-links per one molecule of Pt-DAB complex coordinated to DNA) was calculated using the Poisson distribution in combination with the r_b values and the fragment size (Farrell et al., 1990) (for details see also Materials and Methods).

As summarized in Fig. 3 *B*, both Pt-DAB complexes showed a relatively low but significant interstrand cross-linking efficiency in linear DNA (at $r_b = 0.001$, approximately 6%). There was no significant difference between the yields of DNA interstrand cross-linking by Pt-DAB enantiomers. Thus, these results indicate that the interstrand cross-links are only minor adducts in double-helical DNA modified by Pt-DAB complexes which are formed with a similar frequency like the same lesions of cisplatin. This observation is consistent with an idea that the spectrum of adducts produced on DNA by the two Pt-DAB compounds is similar for each enantiomer and similar to that reported for cisplatin (Brabec and Leng, 1993; Vrána et al., 1996) or for other analogs such as *cis*-[$PtCl_2$(DACH)] complexes (Boudný et al., 1992; Brabec, unpublished results).

Taken together, the results of the interstrand cross-linking assay and transcription mapping experiments are consistent with the idea that the replacement of NH_3 nonleaving ligands in cisplatin by the DAB carrier ligand in both enantiomeric forms (RR or SS) has not significantly altered base sequence selectivity of the parent platinum drug or the spectrum of its DNA adducts.

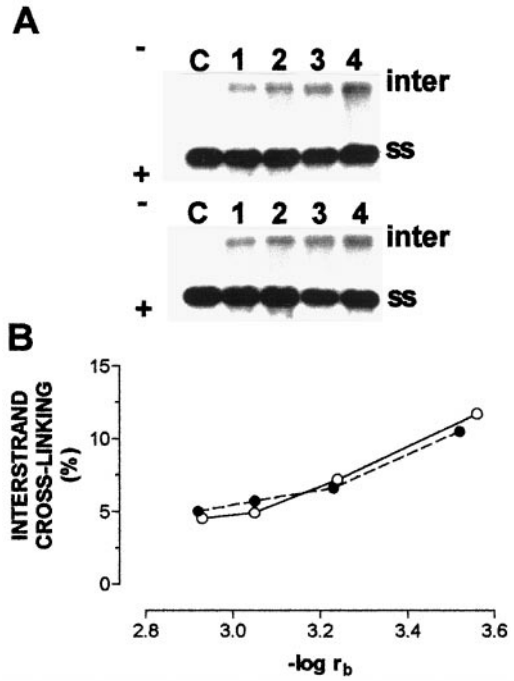


FIGURE 3 The formation of interstrand cross-links by Pt-DAB compounds in linearized pSP73KB plasmid. (*A*) Autoradiograms of a denaturing 1.5% agarose gel of linearized DNA, which was 3′-end-labeled; the interstrand cross-linked DNA appears as the top bands migrating on the gels more slowly than the single-stranded DNA (contained in the bottom bands); the plasmid linearized by *Eco*RI was incubated with Pt-DAB(RR) (*top*) or Pt-DAB(SS) (*bottom*) for 48 h at 37°C. *Lanes:* C, control, nonmodified DNA ($r_b = 0$); 1, $r_b = 3 \times 10^{-4}$; 2, $r_b = 6 \times 10^{-4}$; 3, $r_b = 9 \times 10^{-4}$; 4, $r_b = 1.2 \times 10^{-3}$. (*B*) Dependence on r_b of the percentage of interstrand cross-links per adduct [interstrand cross-linking (%)] formed by Pt-DAB(RR) (●) or Pt-DAB(SS) (○) in linearized DNA within 48 h. Data measured in triplicate varied on average ±3% from their mean.

Conformational changes produced in double-helical DNA by the site-specific d(GpG) intrastrand cross-link

The major DNA lesion of cisplatin and its simple analogs with different carrier amines is the 1,2-d(GpG) intrastrand adduct (Jennerwein et al., 1989; Page et al., 1990 and vide supra). The goal of our further work was to establish whether the steric structure of the non-leaving group of platinum DAB enantiomers could influence the distortions induced in DNA by the formation of the 1,2-d(GpG) intrastrand cross-link. We directed our further studies on establishing distortions and other biophysical properties of oligodeoxyribonucleotide duplexes containing a single, site-specific 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) or Pt-DAB(SS).

Differential scanning calorimetry

A calorimetric technique was used to characterize the influence of the 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) or Pt-DAB(SS) on the thermal stability and energetics of the site-specifically platinated 20-mer DNA duplex. Such thermodynamic data can reveal how the platinum adduct influences duplex stability, a property that has been shown to play a significant role in the mechanism of antitumor activity of platinum drugs. Recently, calorimetric and spectroscopic techniques were used to characterize the influence of the 1,2-d(GpG) intrastrand cross-link on the thermal stability and energetics of a 20-mer DNA duplex site-specifically modified by cisplatin (Poklar et al., 1996). We expanded these studies on the oligodeoxyribonucleotide duplex containing unique 1,2-d(GpG) site-specific intrastrand adducts of the Pt-DAB(RR) or Pt-DAB(SS) complexes.

Fig. 4 shows DSC melting profiles (ΔC_p versus *T*) for the parent, nonmodified 20 bp duplex d(TGGT(/d(ACCA)(20-

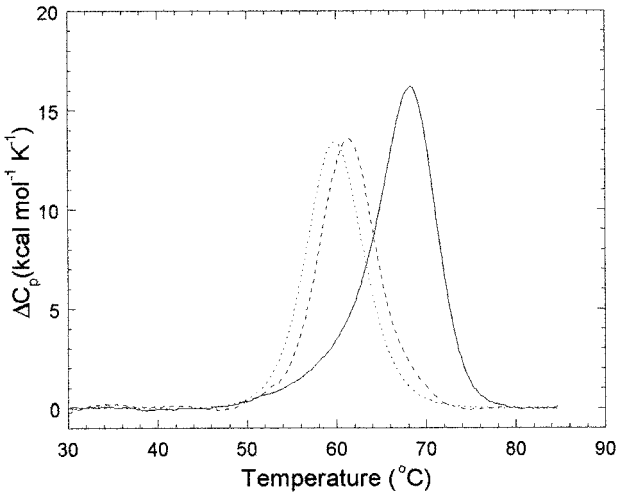


FIGURE 4 DSC thermograms for the d(TGGT)/d(ACCA)(20-DSC) duplex nonmodified (*solid curve*), containing the 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) (*dashed curve*), or Pt-DAB(SS) (*dotted curve*). The duplex concentrations were 5 μM, and the buffer conditions were 10 mM sodium cacodylate (pH 7.2), 100 mM NaCl, 10 mM $MgCl_2$, and 0.1 mM EDTA.

DSC) (*solid curve*) and the same duplex containing single 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) (*dashed curve*) or Pt-DAB(SS) (*dotted curve*). These curves were analyzed as described in Material and Methods to obtain the results listed in Table 1. Inspection of these thermodynamic parameters reveals a number of interesting features. First, cross-link formation of Pt-DAB(RR) and Pt-DAB(SS) reduced the duplex thermal stability by 6.9°C and 8.6°C, respectively. Second, cross-link formation by Pt-DAB(RR) and Pt-DAB(SS) resulted in a large increase of the enthalpy of duplex formation by 36 and 38 kcal/mol, respectively. In other words, the intrastrand cross-link of Pt-DAB enantiomers enthalpically destabilizes the duplex relative to their nonmodified counterpart. Third, cross-link formation by Pt-DAB(RR) and Pt-DAB(SS) resulted in a substantial increase in duplex transition entropy of 104 or 107 cal/K.mol ($T\Delta S$ = 31.0 or 31.9 kcal/mol at 25°C), respectively. In other words, the intrastrand cross-link of both DAB enantiomers entropically stabilizes the duplex. Thus, the 36 or 38 kcal/mol enthalpic destabilization of the duplex due to the cross-link of Pt-DAB(RR) or Pt-DAB(SS), respectively is partially, but not completely, compensated by the entropic cross-link-induced stabilization of the duplex of 31 or 32 kcal/mol at 25°C, respectively. The net result of these enthalpic and entropic effects is that 1,2-d(GpG) intrastrand cross-link formation by Pt-DAB(RR) or Pt-DAB(SS) at 25°C induces a decrease in duplex thermodynamic stability ($\Delta\Delta G_{25}$) of 5.0 or 7.6 kcal/mol, respectively, with this destabilization being enthalpic in origin. In this respect, the intrastrand cross-link of Pt-DAB(SS) was more effective than that of its RR counterpart.

Chemical probing of conformational distortions

Cyanide ions can rapidly remove cisplatin and its analogs from double-helical oligonucleotides containing 1,2-d(GpG) intrastrand cross-links of these platinum compounds. It has been shown (Schwartz et al., 1990; Boudný et al., 1992) that the kinetics of the reaction between cyanide ions and the d(GpG) intrastrand cross-link of cisplatin and its analogs is strongly dependent on the DNA conformation. The samples of d(TGGT)/d(ACCA) containing the intrastrand cross-link of Pt-DAB(RR) or Pt(DAB)(SS) were treated with a large excess of cyanide ions. At various times, aliquots were withdrawn and analyzed by gel electrophoresis under denaturing conditions (Schwartz et al., 1990; Boudný et al., 1992) (Fig. 5, *A* and *B*). As judged by the disappearance of the starting products (upper bands), cyanide ions were considerably less reactive with the double-stranded oligonucleotide containing the intrastrand cross-link of Pt-DAB(SS) as compared with that containing the same adduct of Pt-DAB(RR) (Fig. 5 *C*). It has been shown that the rate of removal of the bound platinum residues decreases when the distortion induced by the Pt-d(GpG) adduct is larger (Schwartz et al., 1990; Boudný et al., 1992). Thus, the results shown in Fig. 5 support the idea that the 1,2-d(GpG) intrastrand cross-link of Pt-DAB(SS) induces in

TABLE 1 Thermodynamic parameters for formation of the oligonucleotide duplex d(TGGT)/d(ACCA)(20-DSC) nonplatinated or containing the 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) and Pt-DAB(SS) determined by DSC

Duplex	T_m, °C	$\Delta H°$, kcal/mol duplex	$\Delta S°$, cal/K · mol duplex	$\Delta G°_{25}$,* kcal/mol duplex
no Pt	68.3	−151 ± 8	−446 ± 23	−18.0 ± 1.9
Pt-DAB(RR)	61.4	−115 ± 7	−342 ± 19	−13.0 ± 1.3
Pt-DAB(SS)	59.7	−113 ± 8	−339 ± 18	−10.3 ± 1.3

Calorimetric measurements were conducted as described in the text. The $\Delta H°$ and $\Delta S°$ values are averages derived from three independent experiments, with the indicated errors corresponding to the average deviation from the mean.
*$\Delta G°_{25}$ is the free energy of duplex formation at 25°C, as determined using the equation

$$\Delta G°_{25} = \Delta H° - (298.15)\Delta S° \quad (2)$$

The indicated uncertainties reflect the maximum possible errors in $\Delta G°_{25}$ that result from the corresponding uncertainties noted above in $\Delta H°$ and $\Delta S°$, as propagated through Eq. 2.

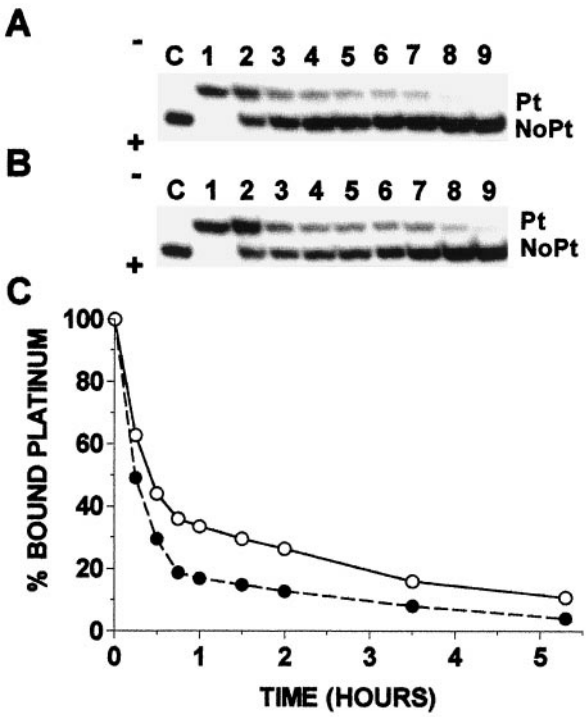


FIGURE 5 Autoradiograms of a denaturing 24% polyacrylamide gel of the products of the reaction between cyanide ions and the oligonucleotide duplex d(TGGT)/d(ACCA)(20) containing a single 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) (*A*) or Pt-DAB(SS) (*B*). The platinated samples at the concentration of 3×10^{-6} M were incubated at 37°C and in 0.2 M NaCN, 20 mM Tris · HCl adjusted at pH 8.3 by addition of HCl. At various times, the samples were precipitated with ethanol, washed three times with ethanol, and electrophoresed. *Lanes:* C, control, nonmodified duplex; 1–9 correspond to the following times (in hours) of incubation of the platinated duplex with NaCN: 1, 0.0; 2, 0.25; 3, 0.5; 4, 0.75; 5, 1.0; 6, 1.5; 7, 2; 8, 3.5; 9, 5.3. The top strand of the d(TGGT)/d(ACCA) was ^{32}P-end-labeled at the 5′ end. (*C*) The dependence of the amount of platinum coordinated to the oligonucleotide duplex d(TGGT)/d(ACCA)(20) containing single intrastrand cross-link of Pt-DAB(RR) (●) or Pt-DAB(SS) (○) on the time of incubation with NaCN. The experimental conditions were the same as in *A* and *B*.

DNA a larger conformational distortion than its RR counterpart. These results are also consistent with the DSC analysis (Fig. 4 and Table 1), which indicated a larger thermal and thermodynamic destabilization induced in DNA by the 1,2-d(GpG) intrastrand cross-link of Pt-DAB(SS) in comparison with the cross-link of Pt-DAB(RR).

To further characterize the distortion induced in DNA by intrastrand cross-links of Pt-DAB(RR) or Pt-DAB(SS), the d(TGGT)/d(ACCA)(20) containing the 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) or Pt-DAB(SS) was treated with several chemical agents that are used as tools for monitoring the existence of conformations other than canonical B-DNA. These agents include $KMnO_4$, DEPC, and bromine. They react preferentially with single-stranded DNA and distorted double-stranded DNA (Nielsen, 1990; Brabec et al., 1993; Bailly et al., 1994; Ross and Burrows, 1996; Bailly and Waring, 1997).

$KMnO_4$ is hyperreactive with thymine residues in single-stranded nucleic acids and in distorted DNA as compared to B-DNA (McCarthy et al., 1990; McCarthy and Rich, 1991; Bailly et al., 1994; Bailly and Waring, 1997). $KMnO_4$ reacted with no residue within the nonplatinated duplex (Fig. 6*A*, *left*, *lane* ds). All thymine residues were strongly reactive in the nonplatinated single-stranded top oligonucleotide (Fig. 6 *A*, *left*, *lane* ss). The duplex containing the intrastrand cross-link of Pt-DAB(RR) showed strong reactivity of the 5′ and 3′ thymine residues adjacent to the adduct (Fig. 6 *A*, *left*, *lane* RR). A weak but significant reactivity was also observed for the second 5′ thymine residue adjacent to the cross-link. The duplex containing the intrastrand cross-link of Pt-DAB(SS) showed strong reactivity of the two neighboring 5′ thymine residues in the top strand adjacent to the adduct, and a weak but significant reactivity was also observed for the 3′ thymine residue adjacent to the cross-link (Fig. 6 *A*, *left*, *lane* SS). No reactivity between $KMnO_4$ and the residues in the bottom strand of the cross-linked duplexes was apparent.

DEPC carbetoxylates purines at the N(7) position. It is hyperreactive with unpaired and distorted adenine residues in DNA and with left-handed Z-DNA (Herr, 1985; Johnston and Rich, 1985; Bailly et al., 1994; Bailly and Waring, 1997). Adenine and guanine residues within the nonplatinated single-stranded oligonucleotides (top and bottom) readily reacted with DEPC (shown for the bottom strand in Fig. 6 *A*, *center*, *lane* ss). No reactivity of adenine and guanine residues was observed within the nonplatinated double-stranded oligonucleotide (shown in Fig. 6 *A*, *center* for the bottom strand, *lane* ds). Within the double-stranded oligonucleotide containing the intrastrand cross-link of Pt-DAB(RR) or Pt-DAB(SS), three base residues in the bottom strand became reactive (Fig. 6 *A*, *center*, *lanes* RR *and* SS): these are readily identified as the three adenine residues complementary to the reactive thymine residues of the top strand. Importantly, a strong reactivity with DEPC was only observed for adenine residues complementary to strongly reactive thymine residues, whereas adenine residues complementary to weakly reactive thymine residues also reacted with DEPC only weakly.

Bromination of cytosine and formation of piperidine-labile sites are observed when two simple salts, KBr and $KHSO_5$, are allowed to react with single-stranded or distorted double-stranded oligonucleotides (Ross and Burrows, 1996). The reaction proceeds via generation of Br_2 in situ, which reacts selectively with the 5,6 double bond to add Br and OH, respectively. H_2O is then eliminated to give 5-bromodeoxycytidine, which is susceptible to depyrimidination under basic conditions. All cytosine residues within the nonplatinated top and bottom strands of d(TGGT)d(ACCA) were readily reactive (shown for the bottom strand in Fig. 6 *A*, *right*, *lane* ss). No reactivity of these residues was ob-

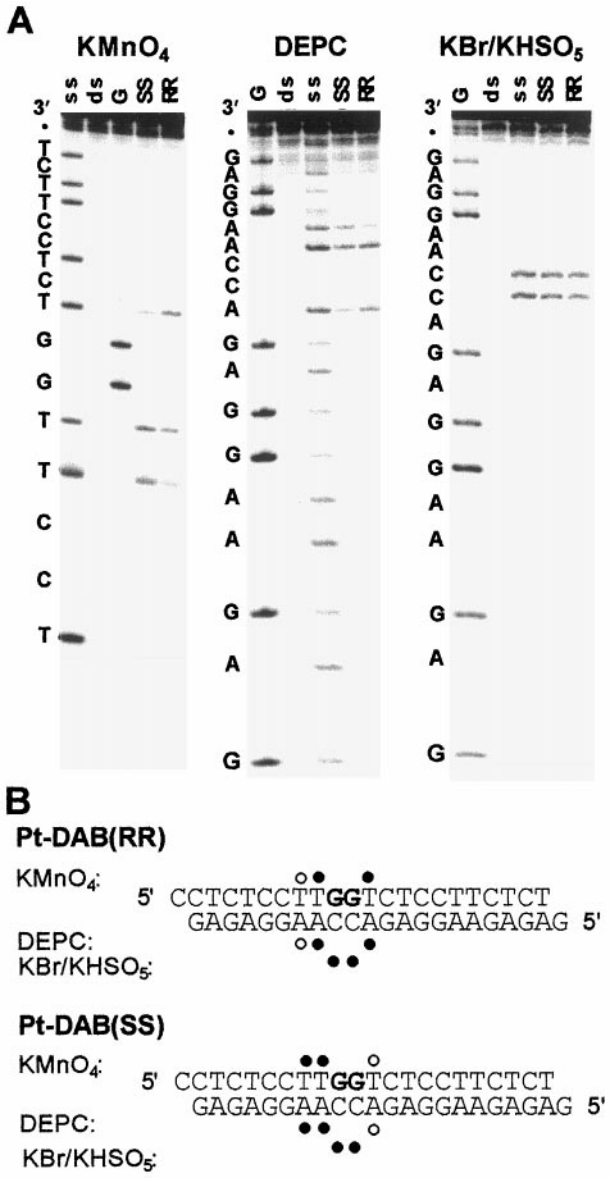


FIGURE 6 (*A*) Piperidine-induced specific strand cleavage at $KMnO_4$-modified (*left*), diethylpyrocarbonate-modified (*center*), and $KBr/KHSO_5$-modified (*right*) bases in nonplatinated and platinated d(TGGT)/d(ACCA)(20). The oligomers were 5′-end-labeled at their top strands in the case of the modification by $KMnO_4$ or at their bottom strands in the case of the modification by DEPC or $KBr/KHSO_5$. $KMnO_4$ (*left*): the lane ss is relative to the nonplatinated top strand. The lane ds is relative to the nonplatinated duplex. The lane G is a Maxam-Gilbert specific reaction for the nonplatinated duplex that had only top strand end-labeled. The lanes SS and RR are relative to the duplex containing Pt-DAB(SS) or Pt-DAB(RR) 1,2-d(GpG) intrastrand cross-link, respectively. DEPC (*center*): The lane G is a Maxam-Gilbert specific reaction for the nonplatinated duplex that had only bottom strand end-labeled. The lane ds is relative to the nonplatinated duplex. The lane ss is relative to the nonplatinated bottom strand. The lanes SS and RR are relative to the duplex containing Pt-DAB(SS) or Pt-DAB(RR) 1,2-d(GpG) intrastrand cross-link, respectively. $KBr/KHSO_5$ (*right*): The lane G is a Maxam-Gilbert specific reaction for the nonplatinated duplex. The lane ds is relative to the nonplatinated duplex. The lane ss is relative to the nonplatinated bottom strand that had only bottom strand end-labeled. The lanes SS and RR are relative to the duplex containing Pt-DAB(SS) or Pt-DAB(RR) 1,2-d(GpG) intrastrand cross-link, respectively, that had only bottom strand end-labeled. (*B*) Summary of the reactivity of chemical probes; ● and ○ designate strong or weak reactivity, respectively.

served within the nonplatinated duplex (shown for the bottom strand in Fig. 6 *A*, *right*, *lane* ds). Cytosine residues in the bottom strand complementary to the platinated guanine residues in the top strand containing the intrastrand cross-links of Pt-DAB(RR) or Pt-DAB(SS) were, however, strongly reactive.

The results obtained with chemical probes are summarized in Fig. 6 *B*. The pattern and degree of reactivity toward the chemical probes was different for the two Pt-DAB isomers indicating different character of the conformational distortion. In addition, the results suggest that the conformational distortion extends over at least five basepairs around the cross-link.

DNA unwinding and bending

Among the alterations of secondary and tertiary structure of DNA to which it may be subject, the role of intrinsic bending and unwinding of DNA is increasingly recognized as being potentially important in regulating replication and transcription functions through specific DNA-protein interactions. For cisplatin adducts, the structural details responsible for bending and subsequent protein recognition have recently been elucidated (Ohndorf et al., 1999; Zamble and Lippard, 1999). Given the recent advances in our understanding of the structural basis for the bending of DNA caused by cisplatin, it is of considerable interest to examine how the character of a carrier amine in the 1,2-d(GpG) intrastrand adduct affects conformational properties of DNA, such as bending and unwinding. In this work we further performed studies on the bending and unwinding induced by single, site-specific intrastrand cross-links of Pt-DAB enantiomers formed in oligodeoxyribonucleotide duplexes between neighboring guanine residues.

The top strands of the oligonucleotide duplexes examined in the present work were designed to contain only one high-affinity platinum binding site, the two adjacent guanine bases of the intrastrand cross-link (Fig. 1). All sequences were designed to leave a 1 nucleotide overhang at their 5′-ends in double-stranded form. These overhangs facilitate polymerization of the monomeric oligonucleotide duplexes by T4 DNA ligase in only one orientation, and maintain a constant interadduct distance throughout the resulting multimer. Autoradiograms of electrophoresis gels revealing resolution of the ligation products of 20–23-bp duplexes containing a unique 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) or Pt-DAB(SS) are shown in Fig. 7. A significant retardation was observed for the multimers of all platinated duplexes. Decreased gel electrophoretic mobility

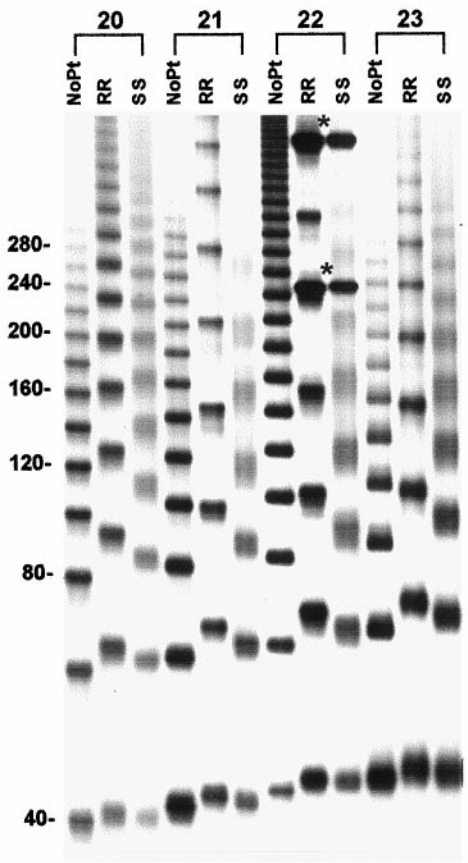


FIGURE 7 Autoradiograms of the ligation products of double-stranded oligonucleotides d(TGGT)d(ACCA)(20–23) containing a unique 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) or Pt-DAB(SS) separated on an 8% polyacrylamide gel (lanes RR and SS, respectively). Nonplatinated oligomers, lanes NoPt.

may result from a decrease in the DNA end-to-end distance (Koo and Crothers, 1988). Various platinum(II) complexes have been shown to form DNA adducts that decrease gel mobility of DNA fragments due to either stable curvature of the helix axis or increased isotropic flexibility (Rice et al., 1988; Bellon and Lippard, 1990; Leng, 1990; Brabec et al., 1993; Huang et al., 1995; Malinge et al., 1995). DNA multimers of identical length and number of stable bend units, but with differently phased bends, have different end-to-end distances. The DNA bends of a multimer must be, therefore, spaced evenly and phased with the DNA helical repeat in order to add constructively. Such constructively phased bends add in plane, yielding short end-to-end distances and the most retarded gel migration. In other words, gel electrophoresis of multimers of oligonucleotide duplexes, which only differ in length and contain a stable curvature induced by the same platinum adduct, should exhibit a phase effect, i.e., the maximum retardation should be observed for the multimers having the bends in phase with the helix screw. In contrast, the normal electrophoretic mobility should be observed for the multimers having the bends separated by a half-integral number of DNA turns. The *K* factor is defined as the ratio of calculated to actual length. The calculated length is based on a multimer's mobility, and is obtained from a calibration curve constructed from the mobilities of nonplatinated multimers. The variations of the *K* factor versus sequence length obtained for multimers of the duplexes 20–23 bp long and containing the unique 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) or Pt-DAB(SS) are shown in Fig. 8 *B*. Maximum retardation was observed for the 22-bp duplex containing the adduct of Pt-DAB(RR) (Fig. 8 *B, left*). This observation suggests that the natural 10.5-bp repeat of B-DNA and that of DNA perturbed by the Pt-DAB(RR) intrastrand cross-link are different as a consequence of DNA unwinding (Bellon et al., 1991). Similarly, in the case of the duplex containing the adduct of Pt-DAB(SS), maximum retardation was observed for the 21-bp duplex, but the 22-bp curve had only a slightly smaller slope, whereas the 20-bp curve differed more pronouncedly (Fig. 8 *B, right*). This asymmetry is also consistent with a significant DNA unwinding due to the formation of the cross-link by the SS enantiomer.

The exact helical repeat of the intrastrand cross-linked duplex and from it the unwinding angle were calculated by interpolation with the use of the *K* versus interadduct distance curve as described in the previous paper for intrastrand adducts of cisplatin (Bellon et al., 1991). The maximum of these curves constructed for the duplexes

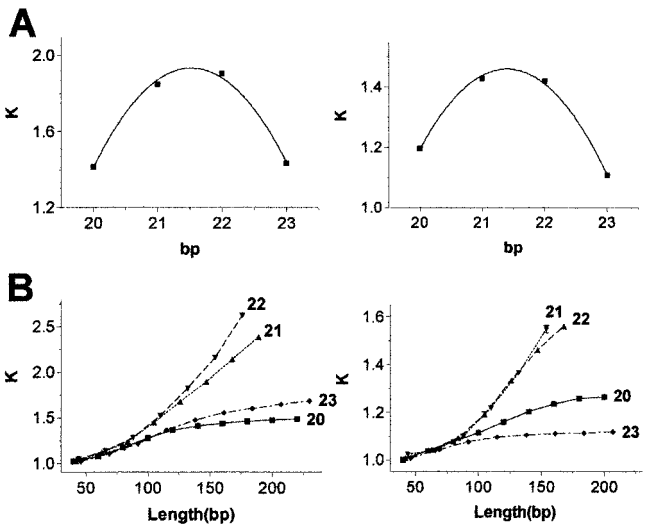


FIGURE 8 (*A*) Plots showing the relative mobility *K* versus interadduct distance in bp for the oligomers d(TGGT)/d(ACCA)(20–23) modified by Pt-DAB(RR) (*left*) or Pt-DAB(SS) (*right*) with a total length of 140 bp. The experimental points represent the average of three independent electrophoresis experiments. The curves represent the best fit of these experimental points to the equation $K = ad^2 + bd + c$ (Bellon et al., 1991). (*B*) Plots showing the relative mobility *K* versus sequence length curves for the oligomers d(TGGT)/d(ACCA)(20–23), denoted respectively as 20, 21, 22, and 23.

intrastrand cross-linked by Pt-DAB(RR) or Pt-DAB(SS) with a total length of 140 bp (Fig. 8 *B*) were determined to be 21.57 ± 0.01 or 21.44 ± 0.04 bp. Total sequence lengths other than 140 bp were examined and gave identical results. To convert the interadduct distance in basepairs corresponding to the curve maximum into a duplex unwinding angle in degrees, the value is compared with that of the helical repeat of B-DNA, which is 10.5 ± 0.05 bp (Wang, 1979; Rhodes and Klug, 1980). The difference between the helical repeat of B-DNA and the DNA containing intrastrand cross-link of Pt-DAB(RR) or Pt-DAB(SS) complex, therefore, is [(21.57 ± 0.01) − 2(10.5 ± 0.05)] = 0.57 ± 0.06 bp or [(21.44 ± 0.04) − 2(10.5 ± 0.05)] = 0.44 ± 0.09 bp, respectively. There are 360°/10.5 bp, so the DNA unwinding due to one intrastrand adduct of Pt-DAB(RR) or Pt-DAB(SS) is 20 ± 2° or 15 ± 3°, respectively. These unwinding angles are considerably greater than that found for the 1,2-d(GpG) intrastrand cross-link of cisplatin (13°) using the same experimental procedure (Bellon et al., 1991). One plausible explanation of this observation might be associated with an additional contribution to unwinding associated with interaction of the DAB moiety with the duplex upon covalent binding of platinum. In a similar way, large unwinding angles (~19°) produced by cisplatin tethered to intercalators were explained (Keck and Lippard, 1992).

The appreciation of the relationship between interadduct distance and phasing for self-ligated multimers composed of the identical number of monomeric duplexes (bend units) resulted in a bell-shaped pattern (Fig. 8 *B*) characteristic for bending (Rice et al., 1988; Bellon and Lippard, 1990; Leng, 1990; Brabec et al., 1993; Huang et al., 1995; Malinge et al., 1995). The quantitation of the bend angle of the intrastrand cross-links of Pt-DAB(RR) or Pt-DAB(SS) complexes was performed in the way described previously (Rice et al., 1988; Bellon and Lippard, 1990; Leng, 1990; Brabec et al., 1993; Huang et al., 1995; Malinge et al., 1995) utilizing the empirical equation

$$K - 1 = (9.6 \times 10^{-5}L^2 - 0.47)(\mathrm{RC})^2 \qquad (1)$$

where L represents the length of a particular oligomer with relative mobility K and RC the curvature relative to a DNA bending induced at the tract of six adenines (A_6 tract) (Rice et al., 1988). Application of Eq. 1 to the 132- or 154-bp multimers of the 22-bp oligomers containing the single intrastrand cross-link of Pt-DAB(RR) leads to curvatures of 0.87, relative to an A_6 tract. Similarly, the application of Eq. 1 to the 126- or 147-bp multimers of the 21-bp oligomers containing the single intrastrand cross-link of Pt-DAB(RR) leads to curvatures of 0.57, relative to an A_6 tract. The average bend angle per helix turn can be calculated by multiplying the relative curvature by the absolute value of an A_6 tract bend [20° (Bellon and Lippard, 1990; Koo et al., 1990)]. The results indicate that the bends induced by the intrastrand cross-link of Pt-DAB(RR) or Pt-DAB(SS) are ~35° or 24°, respectively. That these bands were oriented toward the major groove of DNA was verified in the same way as in the previously published paper (Huang et al., 1995). Other details of the calculations of the unwinding and bending angles are given in the previously published papers (Rice et al., 1988; Bellon and Lippard, 1990; Leng, 1990; Brabec et al., 1993; Huang et al., 1995; Malinge et al., 1995).

Also produced in ligations of monomers investigated in this work were separate bands arising from small DNA circles that migrate close to the top of the gel (see the bands marked by asterisk in Fig. 7 as example). The highest tendency to yield DNA circles was observed for the 22-bp multimers confirming a close match between the 22-bp sequence repeat and the helix screw (Ulanovsky et al., 1986; Rice et al., 1988).

DISCUSSION

The results of the present work (Figs. 2 and 3) are consistent with the view that the replacement of NH_3 nonleaving ligands in cisplatin by the DAB carrier ligand in both enantiomeric forms (RR or SS) changes neither the spectrum and frequency of DNA adducts nor the sequence selectivity of DNA binding of the parent drug. Thus, these features of DNA binding mode of Pt-DAB compounds are unlikely to be associated with the different biological activity of these platinum compounds. A possible explanation for the different biological activity of Pt-DAB enantiomers can be associated with the different conformational distortions induced in DNA by the adducts of these compounds and their different processing in the cell. To test this hypothesis, the experiments described in the present work were carried out.

Thermal and thermodynamical stability of duplexes containing single, site-specific 1,2-d(GpG) intrastrand adduct of either Pt-DAB enantiomer (this cross-link is the major DNA adduct of cisplatin and its direct analogs) and the resistance of this adduct to NaCN treatment demonstrate that the lesions formed by Pt-DAB(SS) were more effective at inducing overall destabilization of the duplex and global conformational alterations than those formed by Pt-DAB(RR). This result is consistent with the idea and supports the hypothesis that the enhancement of mutagenic activity of Pt-DAB(SS) compound is associated with an increase of the thermodynamical destabilization of the duplex and the character of the overall or global conformational alteration induced by this platinum compound in DNA.

The character of global conformational distortions and alterations of the overall stability of the double-helical DNA induced by its damage are determined by the sum of individual contributions from various features of the damage. Some of these individual features may result in stabilization

of the duplex, others may lower its stability. An important feature of the local conformational distortion induced by the 1,2-d(GpG) adduct of cisplatin and its analogs is bending of the duplex axis (Bellon and Lippard, 1990; Takahara et al., 1996; Gelasco and Lippard, 1998). The results of the present work indicate that bending and unwinding angles due to the Pt-DAB(SS) cross-link are smaller than those due to the cross-link of Pt-DAB(RR). However, the overall destabilization of the duplex due to the cross-link of Pt-DAB(SS) is greater than that due to the cross-link of Pt-DAB(RR). It was suggested recently (Poklar et al., 1996) that helical bending induced by the 1,2-d(GpG) intrastrand cross-link of cisplatin thermodynamically stabilized the duplex. A crude estimate has indicated that helical bending due to cisplatin-1,2-d(GpG) cross-link contributed ~6.4 kcal/mol toward stabilization of the global duplex structure. Thus, helical bending induced by the d(GpG) intrastrand cross-link has been suggested (Poklar et al., 1996) to partially compensate destabilization due to the formation of this adduct. With this qualification in mind, we suggest that one reason why the intrastrand cross-link of Pt-DAB(SS) globally destabilizes the DNA duplex more efficiently than the same cross-link of the RR enantiomer is also associated with a lower efficiency of the adduct of the SS enantiomer to contribute toward stabilization of the global duplex structure associated with the bending.

Another conformational parameter of the distortion induced by the formation of the 1,2-d(GpG) intrastrand cross-links of Pt-DAB compounds determined in the present work was unwinding of the double helix (lowering of the number of basepairs per a helical turn). The adduct of the RR enantiomer was slightly more effective in DNA unwinding than the cross-link of its SS counterpart. The energetics of DNA unwinding can be crudely estimated using the same approach as that used to calculate the free energy required to twist a DNA fragment 12 bp long containing a single 1,2-(GpG) intrastrand adduct of cisplatin about its helix axis (Bellon et al., 1991). The free energy of unwinding of only 0.29 kcal/mol was calculated assuming the unwinding angle 13° (Bellon et al., 1991). The same calculations were performed, assuming unwinding angles of 20° for the cross-link of Pt-DAB(RR), 15° for the cross-link of Pt-DAB(SS) (vide supra), and local twisting within the fragment 20 bp long [the fragment 20 bp long was taken for these calculations because energetics of the duplex of this length containing the single, site-specific intrastrand d(GpG) cross-link of Pt-DAB enantiomers was characterized in the present work (Fig. 4 and Table 1)]. These approximate calculations gave free energies of unwinding of only 0.42 and 0.23 kcal/mol, respectively. If this rough estimate is justified, then it seems reasonable to suggest that unwinding due to the intrastrand cross-link of Pt-DAB compounds contributes to the efficiency of these platinum complexes to affect the overall stability of the duplex only in a very small extent. More detailed proposals as to the exact nature of the effect of unwinding induced in DNA by the 1,2-d(GpG) intrastrand cross-link of Pt-DAB compounds must await the results of further experiments.

The structural perturbation caused by d(GpG) intrastrand cross-links of cisplatin has been subjected to numerous NMR investigations, which were recently reviewed (Ano et al., 1999). In these adducts, the polynucleotide chain confines the guanine to a head-to-head (HH) arrangement (Sherman and Lippard, 1987; Bloemink and Reedijk, 1996). The preferential orientations of the guanines in the HH conformation are those with one guanine close to perpendicular to the coordination plane (Ø = 100–110°) and the other rather tilted and forming a hydrogen bond between its carbonyl oxygen atom and the NH_3 group in *cis* position (Ø = 50–60°) (Kozelka et al., 1992). The tilting will be greater for hydrogen bond formation between the carbonyl oxygen of the guanine residue and a "quasi equatorial" hydrogen of the *cis* amine (Grabner et al., 1998). Molecular models indicate that one "quasi equatorial" amino proton of the Pt-DAB(SS)-[d(GpG)] cross-link is close to O(6)-5′ (carbonyl oxygen of the 5′-guanosine) and that one "quasi equatorial" amino proton of Pt-DAB(RR)-[d(GpG)] is close to O(6)-3′ (carbonyl oxygen of the 3′-guanosine). Consequently, the formation of the Pt-DAB(SS)-d(GpG) cross-link is expected to give greater tilt of the guanine residue on the 5′ side, and therefore the greater distortion on the 5′ side of the cross-link. In contrast, formation of the Pt-DAB(SS)-d(GpG) cross-link should result in the greater tilt for the guanine residue on the 3′ side, so that the distortion should be greater on the 3′ side of the cross-link.

These assumptions are in good agreement with the results of the experiments in which structural changes in DNA induced by the single, site-specific 1,2-d(GpG) intrastrand cross-link of Pt-DAB(RR) or Pt-DAB(SS) were investigated by studying the effect of this cross-link on the reactivity of $KMnO_4$ and DEPC toward DNA. $KMnO_4$ and DEPC are complementary probes capable of revealing the location of AT basepairs, the secondary structure of which has been perturbed by the cross-link. Importantly, these chemical probes do not represent measures of basepair disruption as they can proceed even if the basepairing is maintained (Bailly et al., 1994). Formation of adducts by these probes requires out-of-plane attack by the electrophile so that they will be sterically hindered by stacking of neighboring basepairs. Thus, $KMnO_4$ and DEPC are essentially probes of base stacking (Bailly et al., 1994) so that they are particularly suitable for proving distortions induced by the cross-links of Pt-DAB enantiomers predicted above. If the reactivity of the two chemical probes with the duplexes containing the cross-links of Pt-DAB(RR) or Pt-DAB(SS) is compared (Fig. 6), the duplex containing the cross-link of Pt-DAB(RR) shows considerably stronger reactivity of the AT basepair whose thymine residue is adjacent to the adduct on its 3′ side. In contrast, the duplex containing the intrastrand adduct of Pt-DAB(SS) shows

stronger reactivity of the two probes of the AT basepair on the other side of the adduct (particularly the second AT basepair 5′ to the adduct). Thus, the results obtained with the aid of these chemical probes highlight the importance of hydrogen bond formation between the carbonyl oxygen of the guanine residue and a "quasi equatorial" hydrogen of the *cis* amine as discussed above.

In conclusion, the excellent agreement between the previsions and the results of the present work demonstrates the potentiality of the techniques of molecular biophysics in highlighting very fine structural modifications, such as those promoted on DNA by enantiomeric Pt-DAB compounds. Importantly, the configuration of the asymmetric carbons in these complexes dictates the conformation of the chelate ring (*δ-gauche* and *λ-gauche* for the SS and RR isomer, respectively) and thus the "axial" or "equatorial" disposition of the hydrogen atoms on the coordinated nitrogen atoms. Also importantly, formation of hydrogen bonds between the carbonyl oxygen of the guanine residue and the "quasi equatorial" hydrogen of the *cis* amine determines propagation of the distortion of double-helical structure either on the 3′ or on the 5′ side of the cross-linked bases, depending on the configuration of the carrier ligand.

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