Effect of Geometric Isomerism in Dinuclear Platinum Antitumor Complexes on DNA Interstrand Cross-Linking†

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Received February 2, 1999; Revised Manuscript Received May 12, 1999

ABSTRACT: The requirement for novel platinum antitumor drugs led to the synthesis of dinuclear bisplatinum complexes. To understand the molecular mechanisms underlying the biological activity of this new class of platinum cytostatics, modifications of natural DNA and synthetic oligodeoxyribonucleotide duplexes by dinuclear bisplatinum complexes with equivalent monofunctional coordination spheres, represented by the general formula $[\{cis\text{-}PtCl(NH_3)_2\}_2(H_2N-R-NH_2]^{2+}$ (1,1/c,c), in which R is a linear alkane chain, butane or hexane, were studied by various biochemical and molecular biology methods. The results indicated that the major adducts of 1,1/c,c complexes in DNA (~90%) were interstrand cross-links preferentially formed between guanine residues. Besides 1,2 interstrand cross-links (between guanine residues in neighboring base pairs), 1,3 or 1,4 interstrand cross-links were also possible. In the latter two long-range adducts, the sites involved in the cross-links were separated by one or two base pairs. 1,2, 1,3, and 1,4 interstrand cross-links were formed with a similar rate and were preferentially oriented in the 5′ → 5′ direction. In addition, the DNA adducts of these complexes inhibited DNA transcription in vitro. Thus, the binding of the 1,1/c,c complexes modifies DNA in a way that is distinctly different from the modification by the antitumor drug cisplatin. In addition, there are significant differences between the dinuclear 1,1/c,c and 1,1/t,t isomers. The results of this work are consistent with the hypothesis and support the view that platinum drugs that bind to DNA in a fundamentally different manner can exhibit different biological properties including the spectrum and intensity of antitumor activity. The intracellular DNA binding of the dinuclear compounds is compared to the results presented here. It has been suggested that differences in cross-link structure may be an important factor underlying their different biological efficiencies.

The clinical efficiency of cisplatin[1] [*cis*-diamminedichloroplatinum(II)] has led to the search for additional platinum complexes to overcome problems with acquired resistance and the toxic effects of the parent drug or to broaden its clinical spectrum of antitumor activity. From this work the somewhat less toxic analogue carboplatin [*cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II)] has been already introduced in the clinic. Cisplatin and carboplatin are known to form coordination complexes with base residues in DNA (*1*, *2*), which is a main pharmacological target of biological action of platinum complexes (*3*). As carboplatin differs from cisplatin only in the substitution of chloride leaving groups for the 1,1-cyclobutanedicarboxylato ligand, it is not surprising that it forms identical DNA adducts to cisplatin (*4*, *5*) and consequently shows the same spectrum of antitumor activity (*6*).

A logical strategy aimed at overcoming problems associated with chemotherapy by cisplatin or carboplatin is to rationally design platinum complexes that interact with DNA in a manner distinct from that of the parent drug. Dinuclear platinum complexes represent one such distinct class of anticancer agents (*7*). Within this class, formally bifunctional DNA-binding agents consist of two monofunctional platinum(II) spheres with the single chloride leaving group on each platinum linked by a variable-length diamine chain, $[\{PtCl(NH_3)_2\}_2(H_2N(CH_2)_nNH_2]^{2+}$ (*8*, *9*). The leaving chloride ligands are either cis (1,1/c,c) (Figure 1) or trans (1,1/t,t) to the linker. These dinuclear platinum complexes exhibit antitumor activity in vitro and in vivo comparable with that of cisplatin, but importantly they retain activity in acquired cisplatin-resistant cell lines (*10*, *11*). However, the 1,1/c,c

† This work was supported by the Grant Agency of the Czech Republic (Grants 305/99/0695, 307/97/P029, and 204/97/P028) and the Grant Agency of the Academy of Sciences of the Czech Republic (Grants A5004702 and A7004805). The authors also acknowledge support of this work by National Institutes of Health (RO1-CA78754), National Science Foundation (INT-9805552 and CHE-9615727), and The American Cancer Society (RPG89-002-11-CDD). The research of V.B. was supported in part by an International Research Scholar's award from the Howard Hughes Medical Institute.

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[1] Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II) {*cis*-$[PtCl_2(NH_3)_2]$}; [PtCl(dien)]Cl, chlorodiethylenetriamineplatinum(II) chloride {$[PtCl(H_2NCH_2CH_2NHCH_2CH_2NH_2)]Cl$}; DMS, dimethylsulfate; DPP, differential pulse polarography; EtBr, ethidium bromide; FAAS, flameless atomic absorption spectrophotometry; FPLC, fast protein liquid chromatography; r_b, the number of the molecules of platinum complex coordinated/nucleotide residue; r_i, the molar ratio of free platinum complex to nucleoside phosphates at the onset of incubation with DNA; t_m, DNA melting temperature; transplatin, *trans*-diamminedichloroplatinum(II) {*trans*-$[PtCl_2(NH_3)_2]$}.

10.1021/bi990245s CCC: $18.00
Published on Web 08/05/1999

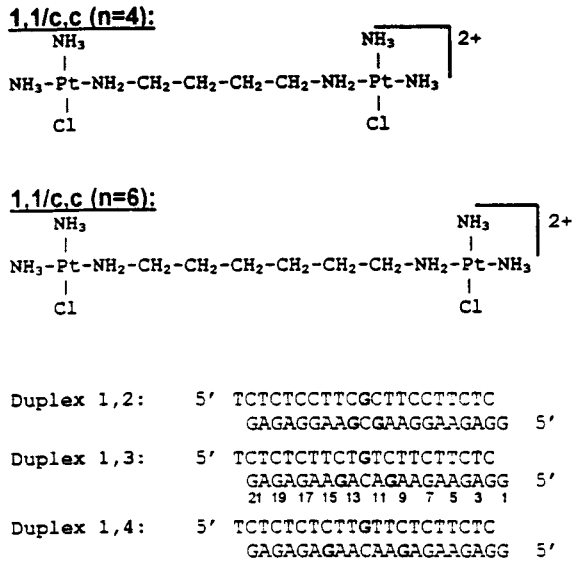

FIGURE 1: Structure of 1,1/c,c complexes, sequences, and abbreviations of the oligodeoxyribonucleotide duplexes investigated in this study. The top and bottom strands of each pair are designated top and bottom, respectively, in the text. For the duplex 1,3, the numbering of the nucleotide residues in the bottom strand is also shown.

complexes have been shown to be less efficient in overcoming cisplatin resistance than their 1,1/t,t counterparts in both murine leukemia and human ovarian cancer cell lines in vitro and in vivo (*7*, *11*, *12*). This situation represents a fundamental difference between mononuclear and dinuclear platinum chemistry and biology—in the mononuclear case cisplatin is active, while its direct isomer transplatin is antitumor-inactive. These differences in biological activity between dinuclear and mononuclear platinum complexes on one hand and the differences between the dinuclear compounds themselves have provided the impetus for the studies of molecular mechanisms underlying these differences.

The dinuclear platinum complexes, similarly to cisplatin or carboplatin, bind to DNA and inhibit DNA replication and transcription, which indicates that DNA modification by dinuclear platinum complexes plays an important role in the mechanism of their biological action (*13*). An initial study has already shown some significant differences in DNA modification in cell-free medium by individual dinuclear platinum complexes and cisplatin (*11*). 1,1/t,t isomers bound to DNA more readily than 1,1/c,c complexes. Both isomers ($n = 4$) unwound globally modified DNA by 10−12 °C, i.e., similarly as cisplatin. In contrast to cisplatin, which forms in DNA mainly 1,2 GG or AG intrastrand cross-links (ca. 90% in linear DNA), both dinuclear isomers formed in DNA preferentially interstrand cross-links, 1,1/c,c isomer being more efficient. 1,1/t,t complexes were also shown to form minor 1,2 GG intrastrand cross-links producing a flexible, nondirectional bend in DNA. Intrastrand cross-links were not observed in double-helical DNA if it was modified by 1,1/c,c. A more recent study (*14*) has investigated the reasons underlying the observed inability of 1,1/c,c complexes to form 1,2 GG intrastrand adduct with double-helical DNA. ^{1}H NMR spectroscopy of samples of very short single-stranded di- or tetranucleotides containing the GG sequence modified by 1,1/c,c provided evidence for restricted rotation around the 3′ G in single-stranded 1,2 GG intrastrand adducts of 1,1/c,c. This steric hindrance, not present in 1,2 GG intrastrand adducts of 1,1/t,t complexes, has been suggested to be responsible for the inability of 1,1/c,c complexes to form 1,2 GG intrastrand cross-links with sterically more demanding double-helical DNA.

Differences in antitumor activity, and especially differential activity in cisplatin-resistant cells, may be manifested through cellular pharmacology (different uptake and DNA platination) as well as in the nature of the DNA adducts formed by the two (1,1/c,c or 1,1/t,t) isomers. Studies for the pair of isomers [{*cis*- or *trans*-PtCl(NH$_3$)$_2$}$_2$(H$_2$N(CH$_2$)$_6$NH$_2$]$^{2+}$ show similar cellular accumulation in both cisplatin-sensitive and -resistant cell lines (*15*, *16*; J. D. Roberts, J. P. Peroutka, and N. Farrell, unpublished experiments). Likewise, overall DNA platination is not significantly different for cellular exposure to equimolar concentrations. Alkaline elution studies confirm that the 1,1/c,c isomer also is an extremely effective interstrand cross-linking agent inside cells, while the 1,1/t,t compound, surprisingly, is only somewhat more effective than cisplatin (*15*). Thus the relative order of cross-linking efficiency inside cells (1,1/c,c > 1,1/t,t > *cis*-DDP) mirrors that found for cell-free medium. Time-course experiments showed that a significant proportion of these interstrand cross-links are rapidly removed for the 1,1/c,c compounds, whereas those for the 1,1/t,t isomer remain constant over 24 h (*15*). These results suggest that the nature of the cross-links inside cells is different for the two isomers, a fact that may contribute to their differing ability to overcome cisplatin resistance.

The aim of this study was to continue in investigating DNA interactions of 1,1/c,c isomers in cell-free medium in order to answer further fundamental questions about the mechanism underlying antitumor activity of dinuclear platinum complexes. Previous studies have examined DNA binding for the $n = 4$ pair of isomers (*11*). Since chain length is an important determinant of cytotoxicity in dinuclear compounds (*12*), we sought to compare the effects of chain length on DNA-binding properties and the nature of the adducts formed. Particular attention was paid to the reactions of 1,1/c,c isomers resulting in interstrand cross-linking in double-helical DNA.

MATERIALS AND METHODS

Starting Materials. Cisplatin, *trans*-diamminedichloroplatinum(II) (transplatin), and chlorodiethylenetriamineplatinum(II) chloride {[PtCl(dien)]Cl} were synthesized and characterized in Lachema a.s. (Brno, Czech Republic). 1,1/c,c complexes ($n = 4$ or 6) were prepared and characterized as described earlier (*11*, *14*). If not stated otherwise, stock solutions of the platinum complexes (5×10^{-4} M in 10 mM $NaClO_4$) were prepared in the dark at 25 °C and stored for at least 7 days before they were used. Calf thymus DNA (42% G + C, mean molecular mass ca. 2×10^7) was also prepared and characterized as described previously (*17*, *18*). Plasmid pSP73KB (2455 bp) (*19*) was isolated according to standard procedures and banded twice in CsCl/ethidium bromide (EtBr) equilibrium density gradients. Restriction endonucleases *Eco*RI, *Hpa*I, and *Nde*I were purchased from New England Biolabs (Beverly, MA). T4 polynucleotide kinase and Klenow fragment of DNA polymerase I were from Boehringer Mannheim Biochemica (Mannheim, Germany). Riboprobe Gemini System II for transcription mapping containing SP6 and T7 RNA polymerases was pur-

chased from Promega (Madison, WI). Ethidium bromide (EtBr), NaCN, agarose, acrylamide, (bis)acrylamide, urea, dimethylsulfate (DMS), and formic acid were from Merck KgaA (Darmstadt, Germany). The radioactive products were from Amersham (Arlington Heights, IL).

Platination Reactions. If not stated otherwise, calf thymus and plasmid DNAs were incubated with platinum complex in 10 mM $NaClO_4$, pH 6, at 37 °C for 48 h in the dark. The number of the molecules of platinum complex coordinated per nucleotide residue (r_b values) was determined by flameless atomic absorption spectrophotometry (FAAS) or by differential pulse polarography (DPP) (*20*).

Oligonucleotides and Their Platination. The oligodeoxyribonucleotides (Figure 1) were synthesized, purified, allowed to react with the platinum compounds, and repurified as described previously (*21*). Briefly, the oligonucleotides synthesized on an Applied Biosystems solid-phase synthesizer were purified by ion-exchange FPLC with a 0.2−0.7 M NaCl gradient. The single-stranded oligonucleotides (the top strands in Figure 1) were reacted in stoichiometric amounts with monoaquomonochloro derivatives of 1,1/c,c complexes ($n = 4$ or 6) generated by allowing these complexes to react with 0.9 molar equiv of $AgNO_3$. The platinated oligonucleotides were again purified by FPLC. It was verified by platinum FAAS and by measurements of optical density that the modified oligonucleotides contained two platinum atoms. It was also verified by DMS footprinting of platinum on DNA (*19*, *22*) that in the platinated top strands the N7 position of the central G was not accessible for reaction with DMS. The platinated strands were allowed to anneal with unplatinated complementary strands in 0.4 M NaCl (pH 7.4) at 25 °C for 2 h. After dialysis against 0.1 M $NaClO_4$ for 4 h at 4 °C, the samples were incubated for 24 h in the dark at 37 °C if not stated otherwise. The duplexes containing the interstrand cross-links were separated on 12% polyacrylamide/8 M urea denaturing gel, the bands corresponding to interstrand cross-linked duplexes were cut off from the gel, eluted, precipitated by ethanol, and dissolved in 50 mM NaCl. The sites involved in interstrand cross-links were deduced from Maxam−Gilbert footprinting experiments (*19*, *22*−*24*). Other details can be found in the text.

DNA Transcription by RNA Polymerases in Vitro. Transcription of the (*Nde*I/*Hpa*I) restriction fragment of pSP73KB DNA with SP6 or T7 RNA polymerase and electrophoretic analysis of transcripts were performed according to the protocols recommended by Promega [Promega Protocols and Applications, 43−46 (1989/90)] and previously described in detail (*19*, *22*).

Circular Dichroism. CD spectra of DNA modified by the platinum complexes were recorded at 25 °C in 10 mM $NaClO_4$ on a Jasco spectropolarimeter, model J720.

Fluorescence Measurements. These measurements were performed on a Shimadzu RF 40 spectrofluorophotometer with a 1 cm quartz cell. Fluorescence measurements of DNA modified by platinum in the presence of EtBr were performed at an excitation wavelength of 546 nm, and the emitted fluorescence was analyzed at 590 nm. The fluorescence intensity was measured at 25 °C in 0.4 M NaCl to avoid secondary binding of EtBr to DNA (*25*). The concentrations were 0.01 mg/mL for DNA and 0.04 mg/mL for EtBr, which corresponded to the saturation of all intercalation sites of EtBr in DNA (*25*, *26*).

DNA Melting. The melting curves of DNAs were recorded by measuring the absorbance at 260 nm. The melting curves of unplatinated or platinated DNA were recorded in 10 mM $NaClO_4$. The value of the melting temperature (t_m) was determined as the temperature corresponding to a maximum on the first-derivation profile of the melting curves. The t_m values could be thus determined with an accuracy of ±0.3 °C.

Other Methods. UV spectra were measured with a Beckman DU-8 spectrophotometer. FAAS measurements were carried out on a Unicam 939 AA spectrometer equipped with a graphite furnace. For FAAS analysis, DNA was first exhaustively dialyzed against 0.2 M NaCl at 4 °C.

RESULTS

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 (*22*, *27*−*31*). Interestingly, monofunctional DNA adducts of several platinum complexes are unable to terminate RNA synthesis (*19*, *22*, *30*).

Cutting of pSP73KB DNA (*19*) by *Nde*I and *Hpa*I restriction endonucleases yielded a 212-bp fragment (a substantial part of its nucleotide sequence is shown in Figure 2B). This fragment contained convergent T7 and SP6 RNA polymerase promoters [in the upper and lower strands, respectively, close to its 3′-ends (Figure 2B)]. The experiments were carried out with this linear DNA fragment, modified by cisplatin, transplatin, [PtCl(dien)]Cl, or 1,1/c,c ($n = 4$,6) (shown only for $n = 4$) at $r_b = 0.005$, for RNA synthesis by T7 and SP6 RNA polymerases (Figure 2A, lanes cisDDP, transDDP, dienPt, and 1,1/c,c). RNA synthesis on the fragment modified by cisplatin, transplatin, and 1,1/c,c complexes yielded fragments of defined sizes, which indicates that RNA synthesis on these templates was prematurely terminated. The major stop sites produced by both 1,1/c,c complexes were identical and the corresponding bands on the autoradiogram had similar intensity. The sequence analysis has revealed that the main bands resulting from termination of RNA synthesis by 1,1/c,c adducts preferentially appeared one or half nucleotide preceding G sites (a total of 26 major termination sites corresponding to stronger bands were evaluated; 23 preceded G, 2 A, and 1 C residues). Importantly, the termination sites on the DNA template produced by the adducts of 1,1/c,c complexes were similar for $n = 4$ and 6 but considerably different from those produced under identical conditions by the adducts of cisplatin (cf. lane cisDDP in Figure 2A) and also of 1,1/t,t complexes (see Figure 7 in ref *27*).

Conformational Alterations Studied by Circular Dichroism. CD spectra of calf thymus DNA modified by 1,1/c,c complex ($n = 6$) to various r_b values are shown in Figure 3A. The intensity of the positive CD band of DNA at about 280 nm is increased with the growing level of the DNA modification up to $r_b = 0.05$. A further increase of the modification resulted in a gradual decrease of the intensity of this band. The 1,1/c,c complex ($n = 6$) was somewhat less efficient at enhancing the CD band at ∼280 nm than the 1,1/c,c complex ($n = 4$) (Figure 3B). In addition, the

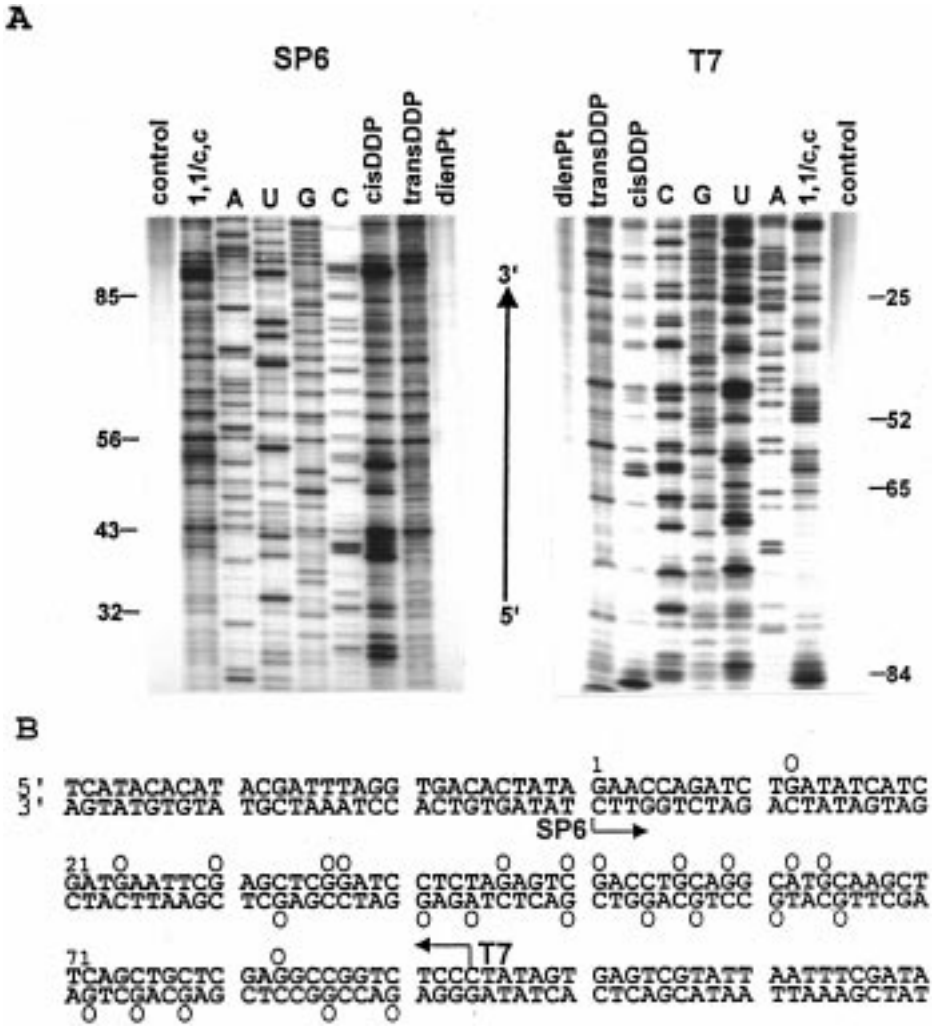

FIGURE 2: Inhibition of RNA synthesis by SP6 and T7 RNA polymerases on the *Nde*I/*Hpa*I fragment of pSP73KB plasmid containing adducts of 1,1/c,c complex ($n = 4$), cisplatin, transplatin, or [PtCl(dien)]Cl. (A) Autoradiograms of 6% polyacrylamide/8 M urea sequencing gels showing inhibition of RNA synthesis by SP6 (left) or T7 RNA polymerases (right) on the *Nde*I/*Hpa*I fragment containing adducts of 1,1/c,c complex ($n = 4$), cisplatin, transplatin, or [PtCl(dien)]Cl. Lanes: control, nonplatinated template; cisDDP, DNA modified by cisplatin at $r_b = 0.01$; transDDP, DNA modified by transplatin at $r_b = 0.01$; dienPt, DNA modified by [PtCl(dien)]Cl at $r_b = 0.01$; 1,1/c,c, DNA modified by 1,1/c,c ($n = 4$) at $r_b = 0.005$; A, C, G, and U, chain-terminated marker RNAs. The numbers correspond to the nucleotide sequence numbering of panel B. (B) Schematic diagram showing the portion of the sequence used to monitor inhibition of RNA synthesis by platinum complexes. The arrows indicate the start of SP6 or T7 RNA polymerases, which used as template the bottom or upper strand of *Nde*I/*Hpa*I fragment of pSP73KB DNA, respectively. O indicates the sites one or half nucleotide behind stop signals from panel A, lanes 1,1/c,c, respectively. The numbers correspond to the nucleotide numbering in the sequence map of pSP73KB plasmid.

changes in CD spectra of DNA induced by 1,1/c,c complexes (Figure 3A) are also consistent with DNA unwinding that has already been demonstrated (*11*) in negatively supercoiled DNA modified by 1,1/c,c complexes by nondenaturing agarose gel electrophoresis. Thus, 1,1/c,c complexes appear to change the conformation of calf thymus DNA in a manner qualitatively similar to cisplatin or 1,1/t,t complexes—including changes indicative of the transition of B-DNA into its A-form (*27*, *32*, *33*). Previous results had confirmed the ability of dinuclear complexes to induce the B → Z transition in poly(dG·dC)-poly(dG·dC) (*11*)—the conformational changes induced by both dinuclear isomers would appear to be dependent on the sequence studied.

DNA Melting. The general properties of 1,1/c,c complexes suggest that their DNA binding modes, in particular the character and frequency of their DNA adducts and resulting conformational alterations, might be significantly different from those of mononuclear platinum(II) complexes. Also importantly, the bifunctional coordination of one 1,1/c,c molecule may introduce up to 4+ charges into the DNA molecule in contrast to only 2+ charges introduced by the coordination of mononuclear platinum(II) complexes.

Calf thymus DNA was modified by 1,1/c,c compounds ($n = 4$ or 6) to various r_b (0−0.06) in 10 mM $NaClO_4$ [at 37 °C for 48 h so that no free molecules of the platinum complexes (not coordinated to DNA) remained in the solution]. The DNA melting curves were measured directly in 10 mM $NaClO_4$. The modification of DNA by 1,1/c,c complexes resulted in a considerable increase of melting temperature of DNA (t_m) (Figure 4). The modification by longer aliphatic linker 1,1/c,c ($n = 6$), resulted in a more pronounced enhancement of t_m (~21 °C at $r_b = 0.05$) in comparison with 1,1/c,c ($n = 4$) (~18 °C at $r_b = 0.03$). Interestingly, under identical experimental conditions mononuclear cisplatin lowers t_m of DNA (*34*). Dinuclear 1,1/t,t isomers also increase t_m, but the maximum enhancement observed was only 8 °C (*27*). The observation that the t_m values of DNA modified by 1,1/c,c complexes are so

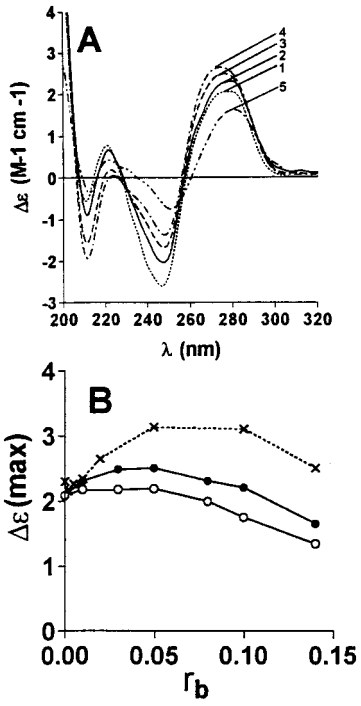

FIGURE 3: CD spectroscopy of calf thymus DNA modified by platinum complexes. CD spectra were recorded for DNA in 10 mM $NaClO_4$. (A) CD spectra of DNA modified by 1,1/c,c ($n = 6$). Curves: 1, control (nonmodified) DNA; 2, $r_b = 0.01$; 3, $r_b = 0.03$; 4, $r_b = 0.05$; 5, $r_b = 0.14$. (B) Changes in CD spectra of DNA at λ_{max} around 280 nm (at the wavelength at which the maximum of the positive CD band around 280 nm occurred) induced by the binding of 1,1/c,c ($n = 4$) (○), 1,1/c,c ($n = 6$) (●), and cisplatin (×) plotted as a function of r_b.

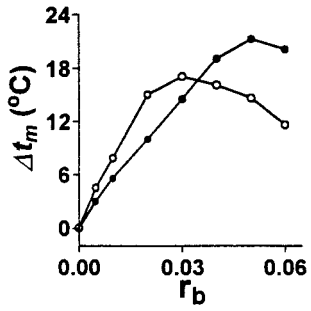

FIGURE 4: Melting of calf thymus DNA modified by 1,1/c,c complexes. DNA was modified to various r_b values in 10 mM $NaClO_4$ at 37 °C for 48 h and the melting temperature of DNA was measured in the same medium. (○) 1,1/c,c ($n = 4$); (●), 1,1/c,c ($n = 6$). Δt_m is defined as the difference between the t_m values of platinated and nonmodified DNAs.

radically increased probably is not only associated with a higher electrostatic contribution of the positive charges from platinum moieties of the adducts of dinuclear complexes in comparison with cisplatin. The adducts of dinuclear 1,1/t,t isomers, which introduced into the DNA molecule the same charge as the adducts of 1,1/c,c isomers, increased t_m considerably less. Thus, the melting behavior of DNA modified by 1,1/c,c complexes might be rather consistent with the formation of a high amount of interstrand cross-links, which also stabilize the duplex. The formation of long-range interstrand cross-links is also supported by the studies showing that the 1,1/c,c−DNA adducts markedly affect ethidium bromide fluorescence (Figure 5). In agreement with our previous observations (*27*), these results are consistent with the formation of long-range DNA cross-links of 1,1/c,c complexes formed between nucleotide residues separated by several base pairs. These findings along with the previous observation (*11*) demonstrating a high frequency of interstrand cross-links formed by 1,1/c,c complexes in a short synthetic oligonucleotide duplex (49 base pairs) prompted us to investigate DNA interstrand cross-linking by 1,1/c,c complexes in more detail.

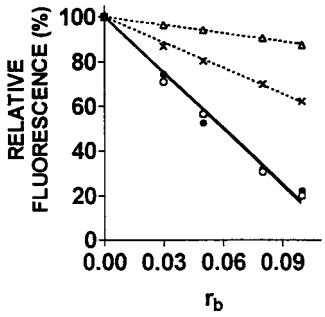

FIGURE 5: Ethidium bromide fluorescence of calf thymus DNA modified by platinum complexes. DNA was modified to various r_b values in 10 mM $NaClO_4$ at 37 °C for 48 h. (×) cisplatin; (△) [PtCl(dien)]Cl; (○) 1,1/c,c ($n = 4$); (●) 1,1/c,c ($n = 6$).

Sequence Specificity of Interstrand Cross-Linking. We have designed a series of synthetic oligodeoxyribonucleotide duplexes, their sequences and abbreviations are shown in Figure 1. The pyrimidine-rich top strands of these duplexes only contained one G in the center (printed in boldface type in Figure 1). These top strands were modified by 1,1/c,c complexes so that they contained a single monofunctional adduct of 1,1/c,c ($n = 4$ or 6) at this central G site. The duplexes were also designed in such a way that their bottom (complementary) strands contained G in different positions symmetrically to the single central C (complementary to the platinated G in the top strand). In this way, the G residue in the top strand with the monofunctionally attached 1,1/c,c complex could close to 1,2, 1,3, or 1,4 GG interstrand cross-links in the duplex 1,2, 1,3, or 1,4, respectively. Interstrand cross-links (1,2) are formed between G sites in neighboring base pairs, whereas in 1,3 and 1,4 interstrand cross-links, the platinated G sites are separated by one or two base pairs, respectively. G sites in the bottom strands involved in these interstrand cross-links are also printed in boldface type in the Figure 1. The nucleotide sequences of the duplexes were also designed in the way that these interstrand cross-links could close to G in the bottom strands located on both sides of the central C residue, i.e., in the 5′ → 5′ or 3′ → 3′ direction. The orientation of the interstrand cross-link in the 5′ → 5′ or 3′ → 3′ direction can be explained with the aid of the sequence of the duplex 1,3. For instance, the 1,3 GG interstrand cross-link oriented in the 5′ → 5′ direction is that formed in the duplex 1,3 between the central G in the top strand and G8 in the bottom strand, whereas the same cross-link oriented in the 3′ → 3′ direction is that between the central G in the top strand and G11 in the bottom strand.

The monoadducted top strands of the duplexes 1,2, 1,3, or 1,4 were hybridized with their complementary strands, and the hybrids were incubated in 0.1 M $NaClO_4$ at 37 °C. The aliquots were withdrawn at various time intervals and analyzed by gel electrophoresis under denaturing conditions. As shown in Figure 6 for the duplex 1,3 modified by 1,1/c,c ($n = 4$), one band was only observed for the non-cross-linked duplex. The subsequent incubation resulted in new bands

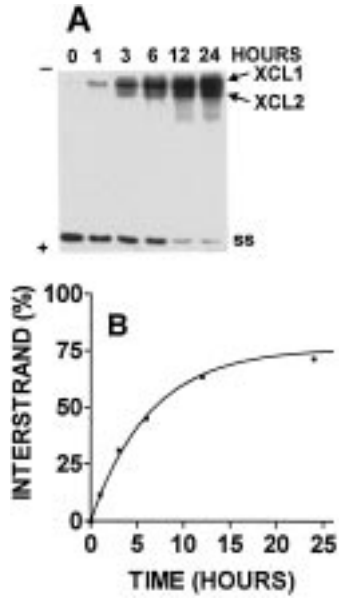

FIGURE 6: Kinetics of the interstrand cross-link formation in the duplex 1,3 by 1,1/c,c complex. The duplex was formed by mixing the bottom strand of the duplex 1,3 with the complementary upper strand uniquely monoadducted by the 1,1/c,c ($n = 4$) at the central G residue at 37 °C. (A) Autoradiogram of a 12% polyacrylamide/8 M urea denaturing gel of the duplex whose bottom strand was ^{32}P end-labeled. The cross-linking reaction was stopped by adjusting the NaOH concentration to 10 mM and cooling the samples to −70 °C. XCL1 and XCL2 designate bands corresponding to major interstrand cross-linked fractions (see also the text). (B) Percentage of interstrand cross-linking calculated from the ratio of the sum of the intensities of the bands corresponding to the fragments containing an interstrand cross-link to the sum of the intensities of all bands (corresponding to the non-cross-linked and the cross-linked oligonucleotides).

migrating markedly more slowly. Their intensity increased with the incubation time with a concomitant decrease in the intensity of the band corresponding to the non-cross-linked duplex. This observation can be interpreted to mean that interstrand cross-links were formed. From the ratio of the sum of intensities of all bands corresponding to cross-linked duplexes and the sum of intensities of all bands, the percentage of interstrand cross-links was calculated (Figure 6B). The half-time of this interstrand cross-linking reaction was about 7 h. A similar result was obtained for the duplex 1,2, whereas the half-time found for the duplex 1,4 was slightly lower (~6 h, not shown). The kinetics of the interstrand cross-linking reactions in all three duplexes were approximately the same for both 1,1/c,c complexes ($n = 4$ and 6).

The cross-linking reactions in the duplexes 1,2, 1,3, and 1,4 resulted in two major bands in the autoradiogram, designated as XCL1 and XCL2 (shown for the duplex 1,3 in Figure 6A). After a 24-h reaction period, the individual major bands XCL1 and XCL2 corresponding to the interstrand cross-linked duplexes were cut off from the gel, and the duplexes were isolated and further characterized by Maxam−Gilbert footprinting (*22*, *23*, *35*, *36*).

The samples of the duplexes 1,2, 1,3, and 1,4 eluted from the bands XCL1 and 2 in which the upper strand was only 5′-end-labeled with ^{32}P were reacted with DMS. The adducts were removed by NaCN (*11*, *19*, *24*) and then the sample was treated with piperidine. In the unplatinated duplexes, the central G in the top strands was reactive with DMS (not

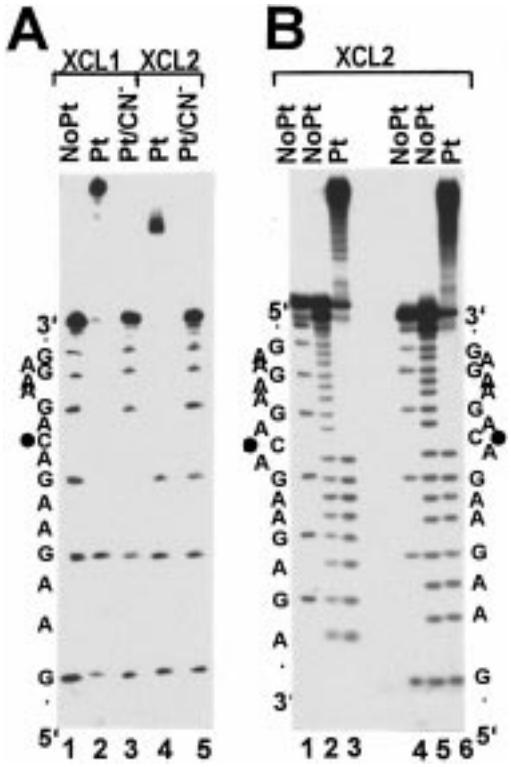

FIGURE 7: Maxam−Gilbert footprinting experiments. Autoradiograms of denaturing 24% polyacrylamide/8 M urea gels of the products of the reaction between DMS (guanine-specific reactions) or formic acid (purine-specific reactions) and the duplex 1,3 containing an interstrand cross-link of 1,1/c,c ($n = 4$). (A) Reaction with DMS. The bottom strand of the duplex was 5′-end-labeled. Lanes: 1, unplatinated duplex; 2, duplex containing interstrand cross-link, fraction XCL1; 3, same as in lane 2 but with platinum removed by NaCN after modification by DMS; 4, duplex containing interstrand cross-link, fraction XCL2; 5, same as in lane 4 but with platinum removed by NaCN after modification by DMS. (B) Reaction with formic acid (or with DMS in the case of the unplatinated duplex); the samples were not treated with NaCN. The bottom strand of the duplex is 3′-end- (lanes 1−3) or 5′-end- (lanes 4−6) labeled. Lanes: 1 and 4, unplatinated duplex, reaction with DMS; 2 and 5, unplatinated duplex, reaction with formic acid; 3 and 6, duplex containing interstrand cross-link, fraction XCL2. For definition of the fractions XCL1 or XCL2, see Figure 6. The nucleotide sequence of the bottom strand of the duplex 1,3 is shown on each panel; the closed circles designate C opposite the platinated G in the top strand.

shown). It was no longer reactive in all three cross-linked duplexes. This observation confirms that the unique G in the upper strands remained platinated and was involved in the interstrand cross-link contained in both major fractions of interstrand cross-linked duplexes (*22*, *23*).

In further studies the fractions XCL1 and 2 of the duplexes 1,2, 1,3, and 1,4 in which the bottom strand was 5′-end-labeled with ^{32}P were examined. Results of a typical experiment, in which the location of nucleotides involved in the interstrand cross-links of 1,1/c,c complex ($n = 4$) in the fractions XCL1 and 2 of the duplex 1,3 was determined, are shown in Figure 7A. The duplexes XCL1 and 2 were reacted with DMS, which does not react with platinated G because the N7 position is no longer accessible (*22*, *23*). One-half of these samples were still treated with NaCN to remove the adducts. Both samples (one treated with NaCN and the other not subjected to a cyanide reversal step) were subsequently treated with piperidine. The treatment with piperidine of the control, unplatinated duplex resulted in the

Table 1: Summary of DNA Binding of 1,1/c,c Complexes and Comparison with DNA Binding of Cisplatin and 1,1/t,t Complexes in 10 mM $NaClO_4$

	cisplatin	1,1/t,t ($n = 6$)	1,1/c,c ($n = 6$)
half-time of binding (min)	~120	40	~120
sequence preference	GG, AG	G	G
CD at 275 nm	increase	increase	increase
decrease of EtBr fluorescence	medium	strong	strong
unwinding angle/adduct (deg)	13	10−12	10−12
melting temperature	decrease	increase (max. 8 °C)	increase (max. 21 °C)
intrastrand adducts between neighboring purines (%)	~90	<25	no
interstrand cross-linking			
% ICL/adduct	~6	~75	87−95
long-range interstrand adducts	no	yes	yes
half-time (h)	4 h	1 h	6−7 h

cleavage at all G sites in the bottom strand (lane 1 in Figure 7A). If the XCL1 fraction of the duplex 1,3 not treated with NaCN was cleaved, G residues up to the position 7 from the 5′-end were only detected at the expected positions. G residues more distant from the 5′ end were not detected because they were cross-linked to the upper strand (Figure 7A, lane 2). If NaCN was used to remove platinum from DMS-treated duplexes, all G residues were found at the expected positions except G10 (Figure 7A, lane 3). This result proves that G10 was platinated and involved in the interstrand cross-link in the duplex XCL1. It implies that one of the major interstrand adducts formed by the 1,1/c,c complex in the duplex 1,3 was the 1,3 GG interstrand adduct formed at the G site in the bottom strand in the direction to its 5′ end.

The site in the bottom strand involved in the interstrand cross-link contained in the fraction XCL2 of the duplex 1,3 was also determined by means of DMS footprinting (Figure 7A, lanes 3 and 4). Analysis of these results shows that the cross-linking site in the bottom strand could be an A residue in position 11 or higher or C12.

To distinguish among these eventualities the XCL2 sample, in which the bottom strand was 3′- or 5′-end-labeled with ^{32}P, was reacted with formic acid. This agent is known to cleave DNA duplexes at the level of the unplatinated purine residues [platinated purine residues are more resistant to the formic acid-catalyzed depurination (*37*)]. In this case (Figure 7B) the samples were not subjected to a cyanide reversal step before piperidine treatment. The analysis of the results in Figure 7B indicates that neither of the A residues (in position 11 or higher) was involved in this cross-link. Thus, another interstrand cross-link formed in the duplex 1,3 by the 1,1/c,c complex was formed between the central G in the top strand and its complementary C residue.

The same results were obtained if the duplex 1,3 contained an interstrand cross-link of 1,1/c,c complex, $n = 6$ (not shown), so that the sites of interstrand cross-linking by 1,1/c,c complexes were not affected by the length of the linker chain.

The sites involved in the two major fractions containing interstrand cross-links of 1,1/c,c complexes were also identified by the same approach for the duplexes 1,2 and 1,4. The results showed that the XCL2 fraction always contained the interstrand cross-link formed between the central G site in the top strand and its complementary C. The fraction XCL1 of the duplexes 1,2 or 1,4 contained the 1,2 or 1,4 GG interstrand cross-link, respectively. These adducts were also formed at the G site in the bottom strand preferentially in the direction to its 5′ end (oriented in the 5′ → 5′ direction).

DISCUSSION

The parameters of DNA binding of 1,1/c,c complexes in cell-free medium are compared with those of 1,1/t,t complexes and cisplatin in Table 1. The binding of both 1,1/c,c $n = 4$ and $n = 6$ is essentially identical and thus the geometry of the ligands around the platinum atoms in dinuclear complexes principally affects the interactions with DNA. The 1,1/c,c complexes bind to DNA with a similar rate as cisplatin but less readily than their 1,1/t,t counterparts. Overall alterations in DNA conformation induced by global modifications by 1,1/c,c or 1,1/t,t complexes or cisplatin evaluated by CD spectroscopic or DNA unwinding analyses (*37*) are similar. In contrast, the effects of the adducts of these complexes on the thermal stability of DNA is distinctly different, very likely due to different efficiency of these platinum drugs in forming interstrand cross-links in DNA. The markedly different efficiency of cisplatin and dinuclear platinum complexes in forming interstrand adducts appears to be a major difference in the DNA binding modes of these two types of platinum drugs, which might be relevant to their different antitumor effects. In addition, almost all modifications by 1,1/c,c complexes, which are more effective interstrand cross-linking agents than their 1,1/t,t counterparts, result in interstrand cross-links. Thus, there are considerable differences among DNA binding modes of all three types of antitumor platinum complexes (1,1/c,c, 1,1/t,t, and cisplatin), which may underlie their different antitumor activity.

An intriguing feature of the biological activity of the 1,1/c,c compounds is that they are antitumor-active but less effective at overcoming cisplatin resistance than 1,1/t,t compounds (*7*, *12*). This feature is observed in both murine leukemia and human ovarian tumor cell lines. First, high cytotoxicity means that interstrand cross-links are definitively capable of producing highly cytotoxic lesions, since these are the only and unique lesions produced by these compounds. Thus, the spectrum of adducts capable of cell killing is expanded beyond those commonly produced by cisplatin, such as the intrastrand cross-link. The 1,1/t,t compounds produce a mixture of interstrand and long-range intrastrand cross-links and are also antitumor-active (*15*). In animal studies the 1,1/c,c is if anything more potent than the 1,1/t,t isomer (*7*). However, the 1,1/c,c isomer is less effective than 1,1/t,t in overcoming cisplatin resistance.

To contribute to the detailed mechanistic understanding of these antitumor effects we have studied in the present

work in detail the sequence specificity and the character of the major DNA adducts of 1,1/c,c complexes. Almost all adducts (~90%) on the template DNA globally modified by 1,1/c,c complexes and used in the transcription mapping experiments were interstrand cross-links. Thus, the results of transcription mapping experiments indicate that 1,1/c,c complexes preferentially form interstrand adducts between G sites in DNA. Further studies of site-specific interstrand adducts formed in synthetic oligonucleotide duplexes have revealed that 1,1/c,c complexes form various types of GG interstrand cross-links; in addition to 1,2 cross-links, 1,3 and 1,4 interstrand cross-links are also possible. Importantly, the interstrand cross-links between G sites are preferentially oriented in the 5′ → 5′ direction (Figure 7). The reasons for this preference in the orientation of DNA interstrand cross-links of 1,1/c,c complexes are unknown. The rate of interstrand cross-linking by 1,1/c,c complexes in 1,2, 1,3, and 1,4 duplexes is not very different, which suggests that all the three types of interstrand cross-links are formed with a similar preference.

Transcription mapping experiments with natural DNA template have also revealed two A residues (~8%) as the minor sites at which 1,1/c,c complexes form adducts (Figure 2B, positions 44 and 62). However, the A residues are not among the sites involved in the major interstrand cross-links formed in the synthetic oligonucleotide duplexes although the A sites were accessible for the formation of 1,2, 1,3, or 1,4 GA cross-links in the duplexes tested in the study. It seems reasonable to suggest that A residues are not involved in major interstrand cross-links of 1,1/c,c complexes but rather in some type of minor noninterstrand adducts. Transcription mapping experiments (Figure 2) were performed with two RNA polymerases. T7 RNA polymerase could be used for mapping the adducts in the top strand shown in Figure 2B, while SP6 polymerase could be used for the adducts in the bottom strand. In this way, the template DNA contained a short sequence of ca. 40 base pairs in which the binding sites of 1,1/c,c complexes could be identified simultaneously in both strands. The identification of these sites is consistent with the formation of 1,2 or other long-range G,G interstrand cross-links of 1,1/c,c complexes oriented in the 5′ → 5′ direction.

The results of the experiments with synthetic oligonucleotide duplexes have indicated that DNA interstrand cross-links of 1,1/c,c complexes formed between G and complementary C residues are also possible. However, a C residue has been found among 26 identified sites to which 1,1/c,c complexes are coordinated in natural DNA (used as the template in transcription mapping experiments) only once (Figure 2, position 50). This C residue was complementary to the G residue, which was also platinated. Thus, the interstrand cross-link between G and complementary C by 1,1/c,c complexes is possible but apparently is not a frequent adduct in natural DNA with a random sequence. Nevertheless, the overall results indicate that the population of cross-linked structures is more diverse than for the 1,1/t,t isomer. Likewise, there is again little difference between the chain lengths, suggesting that the structural differences between interstrand cross-links formed by 1,1/t,t and 1,1/c,c isomers is an intrinsic property of the geometry—the diamine linker is cis to the site of platination and may be the source of steric constraints. Perhaps the steric demands of the diamine bridge cis to the binding sites make the lesions more susceptible to removal; thus despite the high concentration, cells adapted to DNA repair such as cisplatin-resistant cells may more easily remove the 1,1/c,c rather than the 1,1/t,t adducts. Thus, differences in cross-link structure may underlie their different biological activities. These aspects are under investigation.

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BI990245S