# Unique Structural Properties of DNA Interstrand Cross-links Formed by a New Antitumor Dinuclear Pt(II) Complex

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**RESULTS**

**Sequence specificity of interstrand cross-linking**
The top strands of the duplexes [1,2], [1,3], [1,4], and [1,6] (Figure 1A in the main text) contained pyrimidine residues and only one guanine residue in the middle (printed bold in Figure 1A in the main text) were reacted with **1** forming an unique monofunctional adduct of **1** at the central guanine residue. The complementary strands of these DNA duplexes contained two guanine residues symmetrical to the central cytosine residue. Thus, the guanine residue in the top strand containing the monofunctional adduct of **1** may be transformed to the 1,2-, 1,3-, 1,4-, or 1,6-GG interstrand cross-link in duplexes [1,2], [1,3], [1,4], or [1,6], respectively, or into interstrand adduct between guanine and complementary cytosine. The cross-link formed between the sites in adjacent base pairs is defined as 1,2-interstrand cross-link; if the sites involved in the cross-link are separated by 1, 2 and 4 base pairs, such DNA lesions are designated as 1,3-, 1,4- and 1,6-interstrand cross-links, respectively. Importantly, the closure of the monofunctional adducts formed in the top strands of the duplexes shown in Figure 1A in the main text may be transformed into the interstrand cross-links so that these cross-links may contain the guanine residue located on both sides of the central cytosine residue in the complementary (bottom) strands, *i.e.* in the 3′-3′ or 5′-5′direction.

The top strands of duplexes [1,2], [1,3], [1,4], and [1,6]  containing the monofunctional adduct of **1** were hybridized with their complementary strands in $NaClO_4$ (0.05 M ) at 37 °C as described in the section Material and methods. Kinetics of DNA interstrand cross-linking was determined by electrophoresis in a denaturing polyacrylamide (PAA) gel. The non-cross-linked duplex yielded only one band. New bands migrating more slowly occurred as a consequence of the continuing incubation. The intensity of this more slowly migrating band increased with the incubation time which was accompanied by a decrease in the intensity of the band corresponding to the non-cross-linked duplex.

The bands corresponding to the interstrand cross-linked duplexes were cut off from the gel, the duplexes were isolated (eluted from the bands and precipitated) and further characterized in order to determine base residues involved in the interstrand cross-links.

The samples of duplexes [1,2], [1,3], [1,4], and [1,6] interstrand cross-linked by **1**, in which the top strand was only 5′-end-labeled with $^{32}$P, were reacted with DMS, which does not react with platinated G residues because N-7 is no longer accessible (1,2). The platinum adducts were removed by NaCN (2,3), and then the samples were treated with piperidine. In the unplatinated duplexes, the central G residue in the top strands was reactive with DMS (Figure 3A in the main text, lanes NoPt), however it was no longer reactive in all four cross-linked duplexes. This observation confirms that the unique G residue in the top strands remained platinated and was involved in the interstrand cross-link contained in the single fraction of interstrand cross-linked duplexes (1,2).

In additional studies, the interstrand cross-linked duplexes in which the bottom strand was 5′-end labeled with $^{32}$P were examined (Figure 3B in the main text). The interstrand crosslinked duplexes were reacted with DMS. These samples were then further treated with NaCN to remove the adducts and finally also with piperidine. The treatment with piperidine of the control unplatinated duplexes resulted in cleavage at all G sites in the bottom strand (Figure 3B in the main text, lanes NoPt). If the cross-linked duplexes treated with DMS and subsequently NaCN were cleaved (Figure 3B in the main text, lanes Pt/CN), bands corresponding to all G residues were observed that had the same intensity as the corresponding bands seen for unplatinated duplexes, except for the G residues marked by arrows in Figure 3B in the main text. This result proves that these G residues were platinated and involved in the interstrand cross-links of **1**. Interestingly, the band corresponding to G12, G13, G14 and G16 in the bottom strand of interstrand crosslinked duplexes [1,2], [1,3], [1,4]

and [1,6], respectively, entirely disappeared, whereas the bands corresponding to other G residues in the bottom strand had the same intensity as the corresponding bands seen for the unplatinated duplex. This result implies that the interstrand cross-links were formed by 1 exclusively in the 3´-3´direction, independently on their length.

**DNA unwinding and bending**

For DNA adducts of conventional cisplatin, the structural details responsible for bending and subsequent protein recognition have recently been elucidated (4,5). The role of the intrinsic bending and unwinding of DNA by DNA adducts of cisplatin has been recognized as being important mediator of its antitumor effects. Therefore, it was also of considerable interest to examine how the formation of interstrand adduct of 1 affected the 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 interstrand cross-links of 1 formed in oligodeoxyribonucleotide duplexes between guanine residues in the 5'-CTTG/5'-CAAG sequence, i.e. 1,4-interstrand cross-link formed in 3´-3´direction. As in the previous studies (6-8) we used electrophoretic retardation as a quantitative measure of the extent of planar curvature to analyze the bending and unwinding induced by the single, site-specific 1,4-interstrand crosslink formed by 1.

The oligodeoxyribonucleotide duplexes [1,4]-19-23 (the sequences of which are shown in Figure 1B in the main text) were used in these studies. All sequences were designed to leave a 1 bp overhang at their 5'-ends in the 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. The ligation products of these unplatinated or 1,4-interstrand cross-link-containing duplexes were analyzed on a native PAA electrophoresis gel. A representative autoradiogram of electrophoresis gels revealing resolution of the ligation products of unplatinated 19–23-bp duplexes or containing a unique 1,4-GG interstrand cross-link of 1 in is shown in Figure S1A.
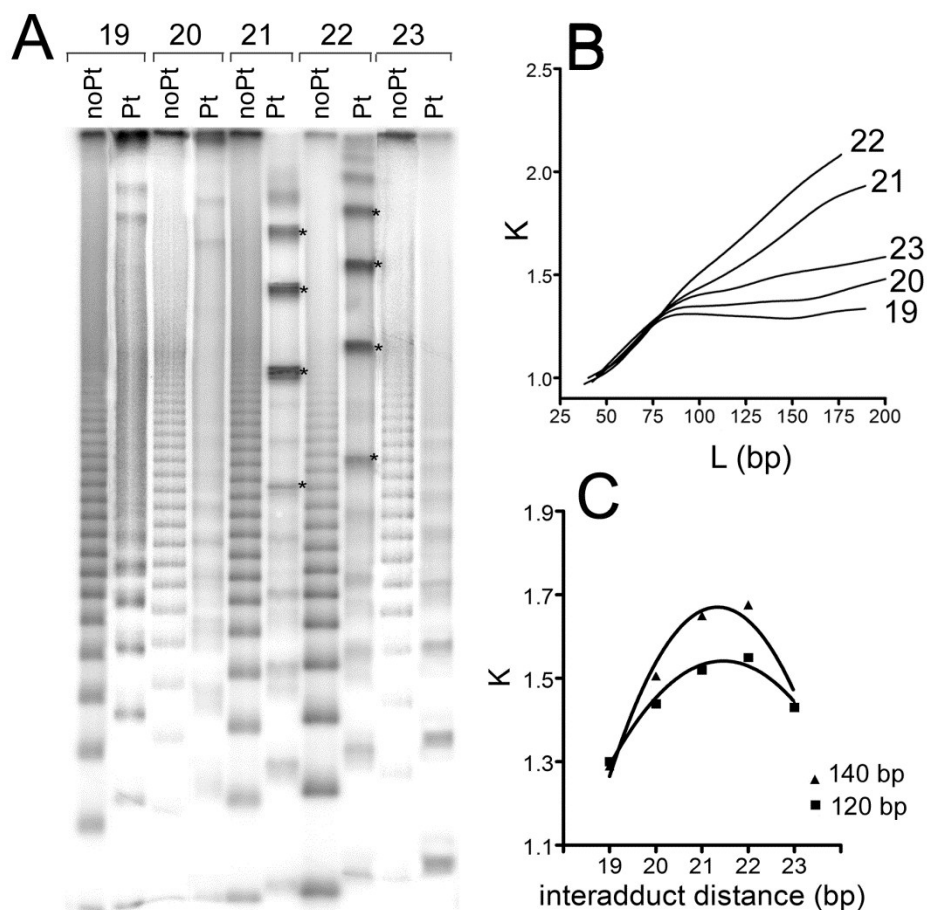
**Figure S1.** Mobility of the ligation products of duplexes [1,4]-19–23 containing a single, site-specific interstrand cross-link of **1** on 8% PAA gel. A. Autoradiogram of the ligation products. The duplexes contained a unique 1,4-interstrand cross-link formed by **1** between the central G residue in the top strand and the G residue in the bottom strand oriented in the 3´-3´direction. Pt lanes, interstrand cross-linked duplexes; NoPt lanes, unplatinated duplexes. B. Plots showing the relative mobility K versus sequence length curves for oligomers 19–23 bp long denoted as 19, 20, 21, 22, and 23, respectively. C. Plot showing the relative mobility K versus interadduct distance in bp for oligomers 19–23 bp long with a total length of 120 bp (squares) and 140 bp (triangles). The experimental points represent the average of three independent electrophoresis experiments. The curve represents the best fit of these experimental points to the equation $K = ad^2 + bd + c$ (9).

A significant retardation was observed for the multimers of 19-23/bp platinated duplexes (Figure S1A), whereas there was no significant retardation observed for the 16 bp duplex (not shown). The K factor is defined as the ratio of calculated to actual length. The variation of the K factor versus sequence length obtained for multimers of the duplexes 16 and 19–23 bp long and containing the unique 1,4-GG interstrand cross-link of **1** is shown in Figure S1B. Maximum retardation was observed for the 22-bp cross-linked duplex. This observation suggests that the natural 10.5-bp repeat of B-DNA and that of DNA perturbed by the interstrand cross-link of **1** are different as a consequence of DNA unwinding (9).

The unwinding angle was calculated from the exact helical repeat of the 1,4-interstrand cross-linked duplexes (9). The maximum of this curve constructed for the interstrand cross-inked duplexes with a total length of 140 bp (Fig. S2C) were determined to be 21.53 ± 0.05 bp. Total sequence lengths other than 140 bp (150 and 120 bp) were examined and gave identical results. To convert the interadduct distance in base pairs corresponding to the curve maximum into a duplex unwinding angle in degrees, the value was compared with that of the

helical repeat of B-DNA, which is $10.5 \pm 0.05$ bp (10,11). The difference between the helical repeat of B-DNA and DNA containing the 1,4-interstrand cross-link of **1** is therefore $((21.53 \pm 0.05) - 2(10.5 \pm 0.05)) = 0.53 \pm 0.10$ bp. There are $360°/10.5$ bp, so the DNA unwinding due to one 1,4-interstrand adduct of **1** is $18 \pm 3°$.

The evaluation 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 (Figure S1C) characteristic of bending. Quantitation of the bend angles of the 1,4-interstrand cross-links of **1** was performed as described previously (8,12-14) utilizing the following empirical equation (Equation 1)

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

where L represents the length of a particular oligomer with relative mobility K, and RC is the curvature relative to DNA bending induced at the tract of A residues (A tract) (15,16). Application of Equation 1 to the 110-, 132-, and 154-bp multimers of the 22-bp oligomer containing the single 1,4-interstrand cross-link of **1** leads to a mean curvature of $0.77 \pm 0.08$ relative to the A tract. The average bend angle per helix turn can be calculated by multiplying the relative curvature by the absolute value of the A tract bend of $20°$ (7,16). The results indicate that the bends induced by the 1,4-GG interstrand cross-links formed by **1** is $31 \pm 4°$.

The mobility of a linear DNA molecule through a native PAA gel is directly proportional to its end-to-end distance. DNA multimers of identical length and number of bend units, but with differently phased bends, have different end to- end distances. The DNA bends must be spaced evenly and phased with the DNA helical repeat in order to add constructively. Such constructively phased bends add in plane to yield short end-to-end distances and the most anomalous gel migration. If the ends come close enough together, they may close covalently upon reaction with DNA ligase to form small DNA circles. Produced in ligations of monomers investigated in this work were also some separate bands arising from these small DNA circles, which migrate close to the top of the gel (see the bands marked by asterisks in Fig. S2A as an example). The highest tendency to yield DNA circles was observed for the 21- or 22-bp multimers, a fact confirming a close match between the 21- and 22-bp sequence repeats and the helix screw (8,15,17).

The direction of the bend was determined by using the duplex [1,4](A/T tract) (the sequence is shown in Figure 1B), which also contained, besides the single interstrand cross-link of **1**, the $(A–T)_5$ tract located "in phase" from the cross-link (the center of cross-link and the center of the A tract were separated by 11 bp), in the same way as in our previous articles (18,19). In the cross-linked multimers, the cross-links or the A tracts were separated by 32 bp, corresponding to about three helical turns after incorporation of the estimated $18°$ of unwinding at the lesion (see above). The cross-linked multimers of duplex [1,4](A/T tract) migrated on the gel in all cases more rapidly than their unplatinated counterparts (data not shown). Hence, the effective bend of the helix axis at the center of the 1,4-interstrand cross-links of **1** is in the opposite direction from that at the center of the A tract, i.e. the 1,4-GG interstrand cross-links formed by **1** in both the 3´-3´ directions bend DNA toward the major groove.

**Chemical probes of DNA conformation**

To further characterize the distortions induced in DNA by interstrand cross-links of **1**, the 22-bp oligonucleotide duplex containing the site-specific 1,4 interstrand cross-link of **1** was treated with several chemical agents that are used as tools for monitoring the existence of conformations other than canonical B-DNA. These agents included $KMnO_4$, bromine, or DEPC as probes for thymine, cytosine, and adenine/guanine residues, respectively. These probes react, under the conditions used, with base residues in single stranded DNA and distorted double-stranded DNA but not with the base residues in intact, double-stranded

DNA. For this analysis, we used exactly the same methodology as in our recent studies dealing with DNA adducts of various antitumor platinum drugs. Thus, the details of this experiment can be found in those articles (8,20,21) and here the results are discussed briefly. Representative gels showing piperidine-induced specific strand cleavage at $KMnO_4$-modified, $KBr/KHSO_5$-modified, and DEPC-modified bases in the 22-bp duplexes that were unplatinated or contained a single interstrand cross-link of **1** are demonstrated in Figures S2A and B.
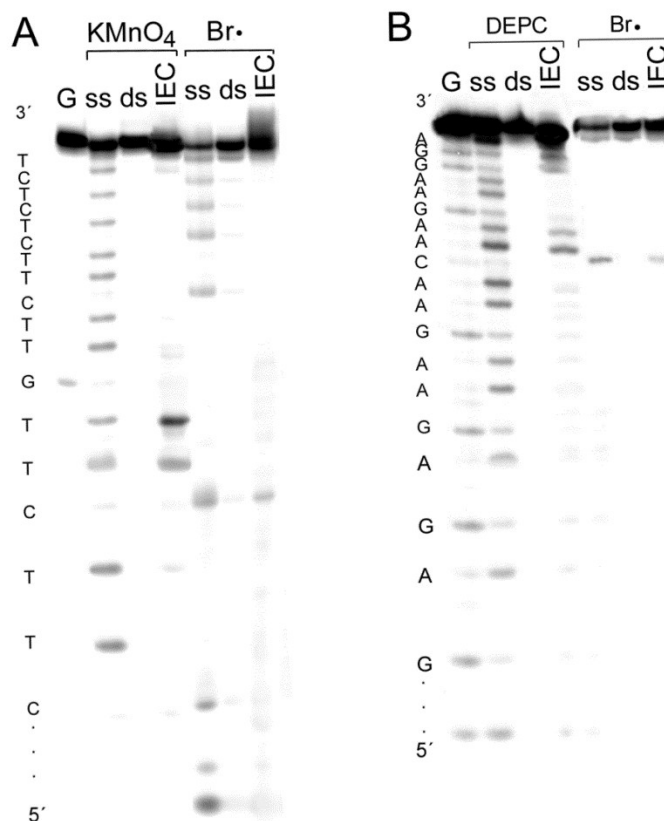


**Figure S2.** Chemical probes of DNA conformation. Shown are the results from piperidine-induced specific strand cleavage at $KMnO_4$- and $KBr/KHSO_5$-modified (A), and DEPC-and and $KBr/KHSO_5$ modified (B) bases in duplex [1,4]-21 unplatinated or containing a single 1,4-GG interstrand cross-link of **1**. The top (A) or bottom (B) strands of the oligomers were 5′-end-labeled. IEC lanes, the duplex interstrand cross-linked by **1**; ds lanes, unplatinated duplex; ss lanes, unplatinated single strand; G lanes, a Maxam-Gilbert-specific reaction for the unplatinated duplex.

$KMnO_4$ is hyperreactive with thymine (T) residues in single stranded nucleic acids and in distorted DNA compared with B-DNA (22-24). The interstrand cross-linked duplex showed strong and medium reactivity for the two T residues in a center of the adduct (shown in Figure S2A, panel $KMnO_4$, IEC lane).

Bromination of C residues 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 (25). No reactivity of these residues was observed within the unplatinated duplexes (shown for the top and bottom strands of the duplex in Figures S2A,B, lanes Br•, ds). Within the double-stranded duplex containing the interstrand cross-link of **1**, the C residues complementary to the G residues involved in the cross-link in both strands were slightly reactive (shown for the top and bottom strands of duplex in Figures S2A and B, respectively, IEC lane).

DEPC carbetoxylates purines at N-7. It is hyperreactive with unpaired and distorted adenine (A) residues in DNA and with left-handed Z-DNA (26-28). Within the double-stranded oligonucleotides containing the interstrand cross-link, three base residues in the bottom strand of duplex became reactive (Figure S2B, panel DEPC, IEC lane). The strongly reactive residues were readily identified as the two A residues complementary to the strongly reactive T residues of the top strand.

**DNA melting**

Melting-temperature measurements by UV absorption spectrophotometry were conducted to characterize the thermal consequences of modifying and constraining DNA through a single, site-specific 1,4-GG interstrand cross-link of **1** (Table 1 in the main text).

The unconstrained (unplatinated) duplex [1,4] denatures in a bimolecular reaction to form two single strands. As a consequence, the melting of the unplatinated duplex was dependent on the overall oligonucleotide concentration. By contrast, the duplex containing the interstrand cross-link melted in a concentration-independent manner to a denatured state, which is consistent with the expectation that the molecularity had been reduced from bimolecular to monomolecular due to the presence of the interstrand cross-link. Thus, the observed $\Delta T_m$ values are apparently affected by the fact that the original bimolecular oligomer system became monomolecular as a consequence of the cross-linking. Upon introduction of a single platinum interstrand cross-link, the change in the $T_m$ value can result not only from the change in the molecularity of the system but also from a different mechanism of melting transition. We tried to dissect these two components of the observed cross-link-induced shift in thermal stability. To estimate how much of the observed $\Delta T_m$ difference results from the change in molecularity, we used an approach based on examination of the thermal transition of the hairpin (duplex [1,4]-loop, Figure 1B in the main text). The stem duplex of this hairpin structure contained two complementary nucleotide sequences identical to those of the top and bottom strands of duplex [1,4]; however, these strands were still linked by a short single-stranded loop composed of five thymine residues. The thymine residues in this short loop behave as denatured single strands and should contribute little to the transition energetics of an adjacent stem duplex (29,30). Hence, it is reasonable to expect that the hairpin [1,4]-loop melts in the same way as the duplex [1,4] but in a monomolecular, concentration-independent reaction (30). In this way, if the $\Delta T_m$ value is calculated as the difference between the values of $T_m$ for the interstrand cross-linked duplex [1,4] and the nonplatinated hairpin dupklex [1,4]-loop, one "corrects" for the concentration dependence of the $T_m$ value of duplex [1,4] and obtains the change in the $T_m$ value corresponding to effects other than the change in molecularity of the system. Examination of the thermal melting of the nonmodified hairpin [1,4]-loop revealed that its melting temperature was 80.3 °C, that is, a value by 11.5 °C higher than that found for duplex [1,4]. Hence, this value can be taken in the first approximation as a "reduced" concentration-independent melting temperature for the nonmodified duplex [1,4]. This "reduced" $T_m$ value of the unplatinated duplex is significantly different from the $T_m$ values of the cross-linked duplexes. Formation of the 1,4-GG interstrand cross-link in DNA by **1** in the 3'-3'direction decreased the thermal stability of DNA by 6.6 °C (Table 1 in the main text). Thus, the overall impact of a single 1,4-GG interstrand cross-link of **1** should not be associated only with the change in molecularity of the duplex system; instead, another mechanism also has to be involved in lowering the thermal stability of the duplex (31), presumably through conformational alterations.

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