# Recognition of DNA Interstrand Cross-Links of *cis*-Diamminedichloroplatinum(II) and Its *trans* Isomer by DNA-Binding Proteins†

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ABSTRACT: Recognition and processing by cellular proteins of DNA modified by platinum complexes have been suggested to be relevant to the mechanism of their antitumor activity. Platinum complexes form on DNA various mono- and bifunctional adducts. It has already been described by other authors that intrastrand cross-links formed on DNA by antitumor *cis*-diamminedichloroplatinum(II) (cisplatin) between neighboring purine residues are recognized by several DNA-binding proteins. In contrast, these proteins do not recognize the intrastrand cross-links formed on DNA by cisplatin or its clinically ineffective *trans* isomer (transplatin) between nonadjacent base residues. An eventuality heretofore not addressed is that DNA interstrand cross-links (ICLs) of platinum compounds may be recognized by and bound to DNA-binding proteins. DNA probes of 110 base pairs (bp) were constructed containing five equally spaced ICLs of cisplatin or transplatin. These ICLs were formed at specific sites at which these adducts are preferentially formed in natural DNA. Gel electrophoresis mobility shift and competition assays with these probes were used to investigate the specific recognition and binding of the calf thymus HMG1 protein to the DNA ICLs of both platinum isomers. The ICL of antitumor cisplatin was recognized by and bound to the HMG1 protein with a similar affinity as the 1,2-intrastrand d(GpG) cross-link of this drug. The protein binding to the ICL is selective for the DNA modification by cisplatin, but not by chemotherapeutically inactive transplatin. In further experiments, 22-bp duplexes containing a single ICL of cisplatin or transplatin were used to investigate whether DNA interstrand cross-linked by cisplatin or transplatin is a substrate for cleavage by bacteriophage T4 endonuclease VII. We show that the DNA duplex containing the ICL of either platinum isomer was precisely cleaved in both strands by this DNA debranching enzyme. The duplex containing the ICL of transplatin, however, was cleaved markedly less efficiently. A comparison of the data presented here and those published by these and other authors earlier reveals no apparent correlation between the bending and/or unwinding angles introduced in DNA by the single platinum adducts and their recognition by the DNA-binding proteins. On the other hand, these data suggest that both DNA-binding proteins utilize kinked, unwound, platinated DNA as a basis for recognition and binding, but that these processes are hindered if the formation of the platinum-induced DNA lesion is accompanied by a local denaturation and/or an increased flexibility of the duplex around the adduct.

*cis*-Diamminedichloroplatinum(II) (cisplatin)[1] and its analogues are successful chemotherapeutic drugs employed in the treatment of several human malignancies (Drobník, 1983; Loehrer & Einhorn, 1984). The anticancer activity of cisplatin is generally accepted to involve coordination to base residues in DNA [for general reviews, see Reedijk (1987), Johnson et al. (1989), Lepre and Lippard (1990), and Leng and Brabec (1994)]. The resulting adducts block DNA and RNA synthesis (Johnson et al., 1989; Bruhn et al., 1990) and induce programmed cell death (Eastman, 1993). The *trans* isomer of cisplatin (transplatin) also coordinates to bases in DNA and blocks replication and transcription but is clinically ineffective (Drobník, 1983; Reedijk, 1987; Johnson et al., 1989; Leng & Brabec, 1994). Thus, the antitumor activity of cisplatin cannot be explained solely on the basis of its activity to coordinate to DNA bases.

The two isomers form various adducts on DNA. Cisplatin adducts in linear DNA include ca. 90% 1,2-intrastrand d(GpG) or d(ApG) cross-links. 1,3-d(GpNpG) intrastrand cross-links, interstrand cross-links (ICLs), and monofunctional adducts represent minor damage to linear DNA by cisplatin (Eastman, 1987). Transplatin forms on DNA mainly monofunctional adducts, 1,3-intrastrand cross-links, and ICLs (Eastman, 1987; Bancroft et al., 1990). The formation of the platinum adducts results in conformational alterations in DNA. Differences in these conformational distortions produced by active and inactive isomers indicate that the antitumor efficacy of cisplatin is associated with a unique conformational motif on DNA. Its recognition and processing have been suggested to be relevant to the mechanism of antitumor activity of cisplatin (Toney et al., 1989; Chao et al., 1991; Hughes et al., 1992; Pil & Lippard, 1992). Consequently, the interactions of DNA damage by cisplatin or transplatin with cellular proteins have been extensively studied (Chu & Chang, 1990; Fujiwara, 1990;

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[1] Abbreviations: bp, base pair; cisplatin, *cis*-diamminedichloroplatinum(II); HMG, high mobility group; ICL, interstrand cross-link; $r_b$, the ratio of platinum atoms fixed per nucleotide; TE, Tris-EDTA; transplatin; *trans*-diamminedichloroplatinum(II).

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Chao et al., 1991; Bruhn et al., 1992, 1993; Hughes et al., 1992; Pil & Lippard; 1992; Brown et al., 1993; Chow et al., 1994; Treiber et al., 1994; Locker et al., 1995). Recently, it was discovered that some nuclear proteins bind specifically to DNA modified by cisplatin, but much less to DNA modified by clinically ineffective transplatin.

The proteins so far identified as cisplatin-damaged DNA-binding proteins are those with high mobility group (HMG) domain motifs (Bruhn et al., 1992; Hughes et al., 1992). These chromosomal proteins found in higher eukaryotes are believed to play a role in transcription, cell differentiation, and assembly of chromatin structure (Grosschedl et al., 1994). Another protein that has been found to recognize specifically DNA adducts of cisplatin is deoxyribonuclease enzyme T4 endonuclease VII (Murchie & Lilley, 1993). This bacteriophage T4-encoded protein cleaves branched DNA structures and is regarded as a repair enzyme (Kemper & Garabett, 1981; Duckett et al., 1988; Murchie & Lilley, 1993). DNA recognition properties of these proteins have been characterized with respect to 1,2-intrastrand d(GpG) and d(ApG) cross-links of cisplatin and 1,3-intrastrand d(GpNpG) cross-links of both cisplatin and transplatin. It was discovered that these proteins recognize the 1,2-intrastrand cross-links of cisplatin (Donahue et al., 1990; Murchie & Lilley, 1993; Pil & Lippard, 1992). In contrast, 1,3-intrastrand cross-links of both isomers are recognized much less efficiently. It has been proposed (Bruhn et al., 1992, 1993; Pil & Lippard, 1992; Murchie & Lilley, 1993; Chow et al., 1994; Treiber et al., 1994) that DNA unwinding and bending induced by platinum adducts provide the conformational motif recognized by platinum-damaged DNA-binding proteins.

1,2-Intrastrand cross-links are the most prevalent DNA adducts made by cisplatin in linear DNA. This type of adduct cannot be formed by clinically ineffective transplatin for steric reasons. Considerable attention has therefore turned to the study of 1,2-intrastrand cross-links of cisplatin. Nevertheless, the nature of the DNA lesion (or lesions) responsible for antitumor effects and an adequate mechanistic rationale for the antitumor efficacy of platinum drugs still remain elusive.

Platinum drugs also make DNA ICLs. Their role in the biological effects of platinum compounds has also not been conclusively determined. Interestingly, the DNA ICLs of cisplatin and its *trans* isomer are different. Cisplatin preferentially forms the ICLs between guanine residues in the 5′-d(GpC)-3′ sequences (Hopkins et al., 1991; Lemaire et al., 1991), while transplatin preferentially forms these lesions between guanine and complementary cytosine residues (Brabec & Leng, 1993). In addition, these adducts are formed with a different rate and induce in DNA distinct conformational alterations (Sip et al., 1992; Brabec & Leng, 1993; Brabec et al., 1993; Malinge et al., 1994). Of interest also is the observation that the conformational distortions induced in DNA by ICLs and intrastrand adducts are different for both isomers.

To characterize further the nature of the platinated DNA conformational motif recognized by platinum-damaged DNA-binding proteins, the experiments were carried out with DNA duplexes containing defined ICLs of cisplatin or transplatin. In this study, we find that the DNA ICL of cisplatin is efficiently bound to HMG1 protein or cleaved by T4 endonuclease VII, but that the same type of lesion made by transplatin is not.

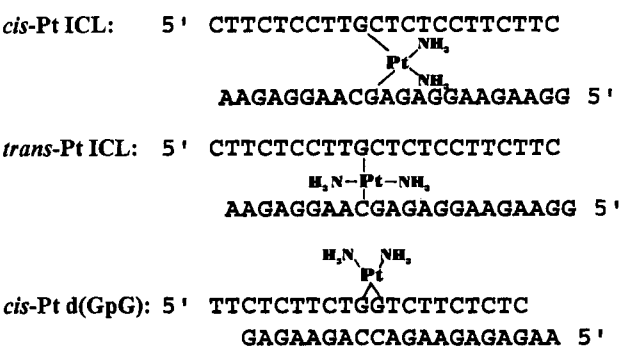


FIGURE 1: Duplex deoxyribooligonucleotides containing specific platinum adducts. The 22- or 20-base pyrimidine-rich oligonucleotides designated as top strands are shown with their complementary bottom strands. The duplexes contain a single interstrand cross-link of cisplatin (*cis*-Pt ICL) or transplatin (*trans*-Pt ICL) between guanine residues or guanine and complementary cytosine residues, respectively, or a 1,2-intrastrand d(GpG) cross-link of cisplatin [*cis*-Pt d(GpG)].

## MATERIALS AND METHODS

*Materials.* The Klenow fragment of *Escherichia coli* polymerase I, bacteriophage T4 DNA ligase, and poly(dG-dC)·poly(dG-dC) were from Boehringer Mannheim Biochemicals. Polynucleotide kinase was purchased from New England Biolabs. Cisplatin and transplatin were from Lachema, Brno (Czech Republic). Oligonucleotides were purchased from BioVendor, Brno (Czech Republic). Calf thymus DNA was isolated and characterized as described in the previous paper (Brabec & Paleček, 1976). [$\alpha$-$^{32}$P]dCTP, [$\alpha$-$^{32}$P]dGTP, and [$\gamma$-$^{32}$P]ATP were from Amersham. HMG1 protein was a generous gift of Dr. M. Štros from the Institute of Biophysics in Brno (Czech Republic); it was isolated from calf thymus under non-denaturing conditions and purified and stored as described previously (Štros & Vorlíčková, 1990; Štros et al., 1994). T4 endonuclease VII was from an overexpressing strain of *E. coli* and was kindly provided by professor D. M. J. Lilley from the University of Dundee (United Kingdom); it was also prepared and purified as described previously (Murchie & Lilley, 1993). Acrylamide, bis(acrylamide), urea, and NaCN were from Merck.

*Construction of Deoxyribooligonucleotide Duplexes Containing Specific Platinum−DNA Adducts.* Oligonucleotides 22 or 20 bases in length from a commercial source were purified, allowed to react with the platinum compound, and annealed with the complementary strands as described previously (Brabec et al., 1992, 1993). Briefly, the oligonucleotides were first purified by ion-exchange FPLC. The single-stranded oligonucleotides (the top strands in Figure 1) were reacted with cisplatin or transplatin, and the platinated oligonucleotides were again purified by FPLC. It was verified (Lemaire et al., 1991; Brabec et al., 1993) that the oligonucleotides contained one platinum atom that was bound at the single guanine residue or at the single d(GG) site in the case of the duplex designated in Figure 1 as *cis*-Pt d(GpG). The platinated strands were allowed to anneal with nonplatinated complementary strands (the bottom strands in Figure 1) and dialyzed against 0.1 M $NaClO_4$ at 4 °C. Complementary oligonucleotides were constructed such that, when annealed to the adducted single-stranded

counterparts, they formed duplexes containing one- or two-base 5′-overhangs at both ends (Figure 1). The duplexes designated in Figure 1 as *cis*-Pt ICL or *trans*-Pt ICL were still purified by FPLC in an alkaline gradient. Using this denaturing gradient, non-interstrand cross-linked strands were eluted as 20- or 22-base single strand, whereas the interstrand cross-linked strands were eluted later in a single peak as a higher molecular mass species. This single peak was only collected so that the samples of the interstrand cross-linked duplexes contained no single-stranded molecules. The location of the ICLs between a guanine residue in the top strand and the base in the complementary strand was verified as described previously (Lemaire et al., 1991; Brabec & Leng, 1993). In the experiments with the HMG1 protein, the duplexes were 5′-end labeled with [$\gamma$-$^{32}$P]ATP by polynucleotide kinase and purified from unincorporated ATP on a Sephadex G50 column. Platinated and control (non-platinated) duplexes were then 5′-end phosphorylated with nonradioactive ATP and purified on the Sephadex column, and T4 DNA ligase was added at a concentration of 10 000 units/mL. The samples were incubated at 4 °C for 16 h. Double-stranded oligonucleotides 110 or 100 bp in length (containing five identical 22- or 20-bp oligonucleotide units shown in Figure 1) were then eluted, purified from native polyacrylamide gels, and resuspended in TE to 5000 cpm/mL. For quantitation, the gel was stained with 0.5 mg of ethidium bromide/mL and the amount of oligonucleotide duplex was determined in comparison with a known quantity of standard double-stranded oligonucleotide using a SepraScan 2001 image processing system for gel quantitation (ISS-Enprotech, Natich, MA). In the experiments with T4 endonuclease VII, the platinated or nonplatinated (control) duplexes were used without subsequent ligation by T4 ligase. The duplexes were 3′-end labeled with [$\alpha$-$^{32}$P]dGTP or [$\alpha$-$^{32}$P]dCTP in the top or bottom strands, respectively, by the Klenow fragment of DNA polymerase I. The quantification of these duplexes was performed after gel electrophoresis as described above.

*Modification of Poly(dG-dC)·poly(dG-dC).* The polynucleotide complex was dissolved in 10 mM $NaClO_4$. Cisplatin dissolved in the same medium was added to obtain $r_b = 0.045$. The reaction mixture was incubated at 37 °C for 48 h. It was verified by differential pulse polarography (Kim et al., 1990) that after this incubation all platinum was coordinated to the polynucleotide complex.

*Electrophoretic Gel Mobility Shift Assay.* The study of HMG1−DNA binding complexes was carried out as described by Bianchi et al. (1989) with small modifications. Three nanomolar radiolabeled oligonucleotide probes 110 or 100 bp in length (concentration is related to the content of the 110- or 100-bp duplex) that contained the adducts shown in Figure 1 or that were nonmodified were incubated in the presence of 50 or 100 nM HMG1 protein and 0.2 mg of unlabeled, sonicated calf thymus DNA/mL for 15 min at 0 °C (ice bath) in binding buffer [150 mM NaCl, 10 mM $MgCl_2$, 20% glycerol, 0.2 mg of bovine serum albunim/mL, 10 mM HEPES-OH (pH 7.9), and 1 mM DTT] in a final volume of 10 $\mu$L. The protein−DNA complexes were then resolved on a 7 % polyacrylamide gel [29:1 acrylamide/*N*,*N*′-methylenebis(acrylamide)]. The samples were electrophoresed at 4 °C using the electrophoresis buffer containing 0.045 M Tris−borate and 1 mM EDTA, pH 8.0, and autoradiographed.

*Competition Assay.* Competition assays were performed by adding various amounts of unlabeled competitor poly(dG-dC)·poly(dG-dC) to the binding reactions of the gel mobility shift assay before the 15-min incubation step. These reactions contained 30 nM HMG1 protein, but no sonicated calf thymus DNA or another competitor. The competitor poly(dG-dC)·poly(dG-dC) was either nonmodified or modified with cisplatin at $r_b = 0.045$.

*T4 Endonuclease VII Digestion of Platinated Oligonucleotides 22 or 20 bp in Length.* If not stated otherwise, 1 pmol of oligonucleotide duplex was cleaved with 1.5 units of endonuclease VII in digestion buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, and 100 $\mu$g of bovine serum albumin/mL) in a final volume of 15 $\mu$L for 20 min at 10 °C. A unit of T4 endonuclease VII activity was defined in the same way as in the paper of Murchie and Lilley (1993). To facilitate fine mapping of the cleavage sites of T4 endonuclease VII on the DNA duplexes, the products were treated with 0.2 M NaCN (alkaline pH) for 12 h at 40 °C. Then the reaction mixtures were loaded directly onto a 24% denaturing polyacrylamide gel containing 8 M urea or 15% non-denaturing polyacrylamide gel. The radiolabeled top or bottom strands of the nonplatinaned duplexes were also sequenced by chemical degradation using dimethyl sulfate (G specific), hydrazine (C + T specific), or formic acid (A + G specific). The kinetics of T4 endonuclease VII cleavage of cross-linked oligonucleotide duplexes were measured for 1 pmol of DNA cleaved with 3 units of the enzyme in a volume of 15 $\mu$L under the conditions stated above at 20 °C. Two-microliter samples were removed at various time intervals, and the reaction was terminated by addition of a 30-fold excess of 80% ethanol and 10 mM EDTA and freezing at −70 °C. At the end of the kinetic measurement, the ethanol was removed by vacuum centrifugation, and the platinum was displaced with NaCN *(vide supra)*. The samples were electrophoresed in 24% denaturing polyacrylamide gel. After electrophoresis was completed, the bands corresponding to the cleaved and uncleaved duplex were cut off and their radioactivity was quantified on an LKB Wallac 1410 Betaspectrometer (Finland). The extent of cleavage was assessed as the ratio of the radioactivity of cleaved to total DNA ($F$). Cleavage rate constants were calculated in the same way as in the paper of Murchie and Lilley (1993), i.e., by linear regression from plots of $1 + \ln(1 - F)$ versus time.

## RESULTS

*Interactions of Platinated DNA with HMG1 Protein.* The interactions of the HMG1 protein with DNA ICLs of cisplatin and its *trans* isomer were investigated by means of gel mobility shift experiments with synthetic oligonucleotide duplexes (Figure 1). In these experiments, the duplexes 22 bp in length were modified so that they contained a single, defined interstrand adduct. These duplexes were radiolabeled and ligated to multimers. After the ligation reaction, the DNA probes composed of five duplex oligonucleotide units were purified. Thus, these 110-bp DNA probes contained five identical ICLs regularly separated by identical 22-bp segments. Similar DNA probes of such length were already used in the previous work (Donahue et al., 1990) to demonstrate a specific binding of the DNA damage-recognition proteins to DNA adducts of cisplatin. These proteins, identified as the HMG domain proteins (Pil & Lippard,

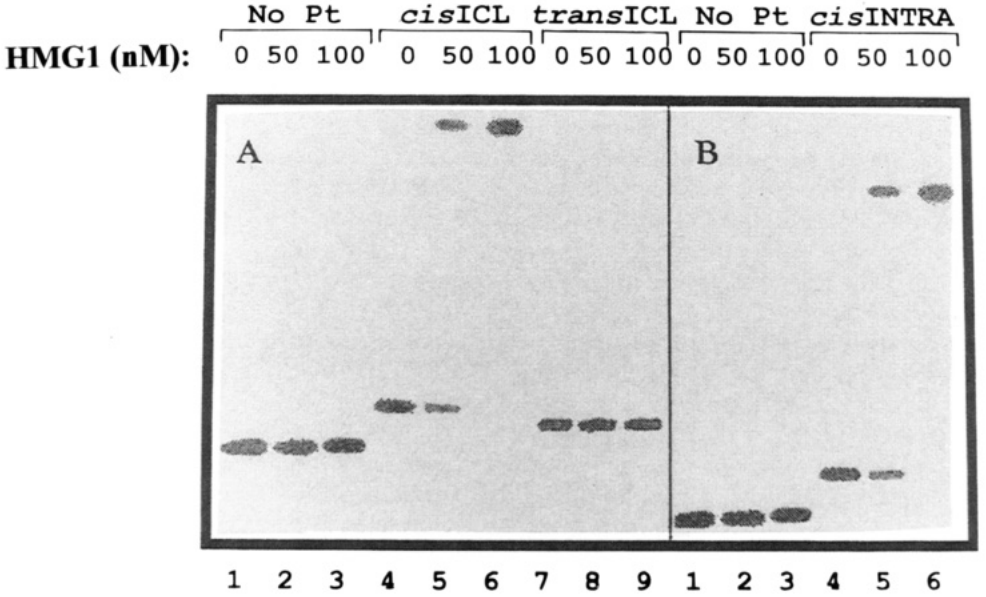


FIGURE 2: HMG1 protein binds to the interstrand cross-link (ICL) formed by cisplatin but not to that formed by transplatin (see Figure 1). (A) Electrophoretic gel mobility shift assay with the ICL of cisplatin (lanes 4−6) or transplatin (lanes 7−9). Double-stranded DNA fragments, 110 bp in length, were prepared from the 5′-end-labeled duplexes formed by complementary oligonucleotides 22 bases in length. The duplexes either were unmodified (lanes 1−3) or contained the ICL of cisplatin or transplatin. These 110-bp DNA probes at a concentration of 3 nM were incubated in the absence (lanes 1, 4, and 7) or the presence of 50 nM (lanes 2, 5, and 8) and 100 nM (lanes 3, 6, and 9) HMG1 protein. Unmodified, unlabeled calf thymus DNA was used as a nonspecific competitor at a concentration of 0.2 mg/mL (lanes 1−9). (B) Gel mobility shift assay with the 1,2-intrastrand d(GpG) cross-link of cisplatin shown for comparative purposes. DNA fragments, 100 bp in length (Figure 1), were prepared from oligonucleotides 20 bases in length that either were unmodified (lanes 1−3) or contained the d(GpG) adduct of cisplatin (lanes 4 and 5). These 100-bp DNA probes at a concentration of 3 nM were incubated in the absence (lanes 1 and 4) or the presence of 50 nM (lanes 2 and 5) or 100 nM (lanes 3 and 6) HMG1 protein. Unmodified, unlabeled calf thymus DNA was used as a nonspecific competitor at the concentration of 0.2 mg/mL (lanes 1−6).

1992), were found to bind probes similar to those used in this work, which were at least 88 or more base pairs long (Donahue et al., 1990). The probe size limitation has been suggested (Donahue et al., 1990) to reflect a minimum requirement for a flanking nucleic acid domain in order for protein binding to occur.

The binding of the HMG1 protein to these DNA probes in the presence of a 2000-fold excess of unlabeled nonspecific calf thymus competitor DNA was detected by retardation of the migration of the radiolabeled 110-bp probe through the gel (Donahue et al., 1990; Pil & Lippard, 1992) (Figure 2A). There is substantial specific binding of the HMG1 protein to the DNA probe containing the ICLs of antitumor cisplatin as evidenced by the presence of a slower migrating band not seen in the cases where the same duplex was analyzed in the absence of the HMG1 protein. For comparative purposes, the binding of the HMG1 protein to the 100-bp probe composed of five 20-bp oligonucleotide units, each containing a unique 1,2-intrastrand d(GpG) cross-link of cisplatin [designated in Figure 1 as *cis*-Pt d(GpG)], was investigated under identical experimental conditions (Figure 2B). This probe readily bound the HMG1 protein with approximately the same affinity as the 110-bp probe containing the ICLs of cisplatin. In contrast, no binding of the protein occurred under identical experimental conditions in the cases where the same 110-bp DNA probe was not platinated (Figure 2A, lanes 2 and 3) or contained the ICLs formed by clinically ineffective transplatin (Figure 2A, lanes 8,9).

Further evidence for specific binding of the HMG1 protein to the DNA ICL of cisplatin was obtained by using a synthetic double-stranded polynucleotide, poly(dG-dC)·poly(dG-dC), modified by cisplatin as a competitor. It has been shown (Rahmouni & Leng, 1987) that, in poly(dG-dC)·poly(dG-dC), only cross-links between two guanine residues are formed. This implies that cisplatin does not form in this polynucleotide complex 1,2-intrastrand cross-links which are recognized by the HMG1 protein (Pil & Lippard, 1992; Huang et al., 1994). In addition, mainly ICLs are formed in the reaction of cisplatin with poly(dG-dC)·poly(dG-dC) (Rahmouni & Leng, 1987). As minor adducts in this reaction, 1,3-intrastrand d(GpCpG) cross-links could be also formed, but these DNA lesions of cisplatin are not recognized by the HMG1 protein (Pil & Lippard, 1992; Huang et al., 1994). Thus, considering the fact that the DNA ICLs of cisplatin are recognized by the HMG1 protein, it may be expected that cisplatin-modified poly(dG-dC)·poly(dG-dC) should be an effective competitor of the DNA probes for binding of the HMG1 protein. Competitive binding experiments were performed using unlabeled poly(dG-dC)·poly(dG-dC) which either was nonplatinated or was modified by cisplatin at $r_b = 0.045$. As shown in Figure 3A (lanes 3−5), binding of the HMG1 to the labeled 110-bp probe containing five ICLs was effectively competed by increasing quantities of unlabeled poly(dG-dC)·poly(dG-dC) modified by cisplatin (the modification was performed to the extent corresponding to the level of the modification of the 110-bp probe). The band representing specific binding to 110-bp oligonucleotide containing the ICLs of cisplatin could be competed away with a ~100-fold excess of unlabeled poly(dG-dC)·poly(dG-dC) modified by cisplatin at $r_b = 0.045$. By contrast, unlabeled nonplatinated poly(dG-dC)·poly(dG-dC) did not compete at the same ~100-fold excess for binding of the HMG1 protein with this labeled interstrand cross-linked probe (Figure 3A, lanes 6−8). This result indicates that poly(dG-dC)·poly(dG-dC) modified by cisplatin

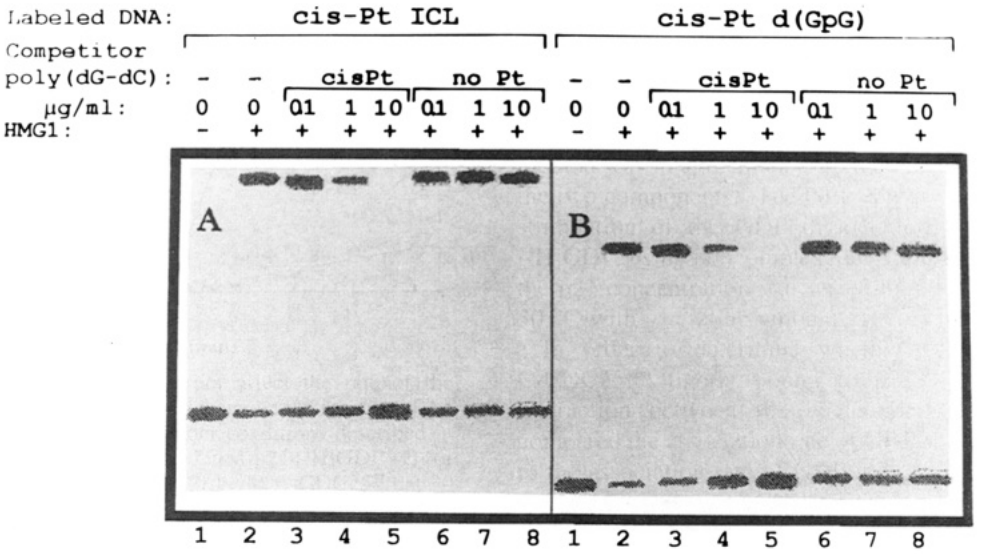


FIGURE 3: Binding of the HMG1 protein to the interstrand or 1,2-intrastrand cross-link of cisplatin competed with poly(dG-dC)•poly(dG-dC) modified by cisplatin. Labeled 110- or 100-bp DNA probes containing the interstrand or intrastrand cross-links of cisplatin, respectively (Figure 1), were incubated at a concentration of 3 nM in the presence of 30 nM HMG1 protein and 0.1−10 µg of unlabeled poly(dG-dC)•poly(dG-dC)/mL, which was modified by cisplatin at $r_b = 0.045$ (lanes 3−5) or remained nonplatinated (lanes 6−8). Binding in the absence of the competitor is shown in lanes 2. Lanes 1 show migration of the platinated probes in the absence of the HMG1 protein. The binding to the interstrand cross-linked DNA probe is shown in panel A, whereas the binding to the intrastrand cross-linked probe is shown in panel B.

readily binds HMG1 protein, thus providing additional support for the conclusion that this protein effectively binds to the DNA ICLs of cisplatin.

In order to compare the binding affinity of the HMG1 protein to the 1,2-intrastrand cross-link and the ICL of cisplatin, a DNA probe was also prepared so that it contained 1,2-intrastrand d(GpG) cross-links of cisplatin. This probe was prepared by ligation of five 20-bp oligonucleotide units, each containing a single central d(GpG)/d(CpC) sequence at which the intrastrand cross-link of cisplatin was formed (Figure 1), so that this DNA probe was 100 bp in length. As expected, this radiolabeled intrastrand cross-linked probe readily bound the HMG1 protein in the presence of a 2000-fold excess of unlabeled nonspecific calf thymus competitor DNA (Figure 2B, lanes 5 and 6). Importantly, this probe was also effectively competed by unlabeled poly(dG-dC)•poly(dG-dC) modified by cisplatin at $r_b = 0.045$ (Figure 3B, lanes 3−5). Competition for binding was complete when a ~100-fold excess of the platinated competitor was added to the binding reaction mixture. On the other hand, non-platinated, unlabeled poly(dG-dC)•poly(dG-dC) at the same excess did not compete with this labeled intrastrand cross-linked DNA probe (Figure 3B, lanes 6−8). These data indicate that there is no marked difference in the binding affinity of the HMG1 protein to the interstrand and 1,2-(dGpG) intrastrand cross-links formed on double-stranded DNA by cisplatin.

*Interactions of Platinated DNA with T4 Endonuclease VII.* In further experiments, we have studied the recognition of DNA containing ICLs of cisplatin or transplatin by the DNA debranching enzyme T4 endonuclease VII. This protein exhibits recognition capability to intrastrand cross-links of cisplatin and its *trans* isomer similar to that of HMG1 protein (Murchie & Lilley, 1993). We investigated the cleavage by the enzyme of the same double-stranded oligonucleotides [containing a unique ICL of either platinum isomer or 1,2-intrastrand d(GpG) cross-link of cisplatin], as in the experiments with the HMG1 protein (Figure 2), but not ligated to multimers. The duplexes were 3′ radiolabeled in the top or bottom strands and incubated with T4 endonuclease VII. The products were then treated with NaCN at alkaline pH to displace the platinum adduct and analyzed by electrophoresis on denaturing polyacrylamide gel. The treatment with NaCN prior to electrophoresis was necessary in order to restore regular mobility in the gel of the fragments that would remain, after cleavage of the duplex by T4 endonuclease VII, cross-linked to the complementary strand.

The cleavage of the interstrand cross-linked duplexes occurred for both platinum isomers (Figure 4, lanes 3 and 6 in both panels). It has been verified that the nonplatinated duplex is not cleaved by the enzyme and that no cleavage occurred in the absence of T4 endonucleaseVII under identical conditions. The cleavage reactions were restricted to a single phosphodiester bond 3′ to the location of the cross-link (Figure 4, lanes 3 and 6). Importantly, the duplex interstrand cross-linked by cisplatin was cleaved markedly more efficiently than that cross-linked by transplatin. In addition, the efficiency of cleavage of different strands within one interstrand cross-linked molecule, as judged from this experiment, was approximately the same for both isomers. Analogous results were obtained if the cleavage reactions were performed at various temperatures (2−40 °C, data not shown). The rates of cleavage reactions were lowered with decreasing temperature, but the locations of the cleavage sites and the higher cleavage rates for duplexes containing the ICL of the *cis* isomer remained unchanged.

In order to confirm that both strands of the duplex containing the ICL of cisplatin or transplatin were cleaved at approximately the same rate, non-denaturing gel electrophoresis with the same incubation with T4 endonuclease VII (as in the experiment shown in Figure 4) was performed. The duplex containing the ICL of cisplatin or transplatin was 3′-end labeled in the top or bottom strand, treated with T4 endonuclease VII, and analyzed by non-denaturing gel electrophoresis (Figure 5). In the first series of experiments, the products of the cleavage were not treated with NaCN to

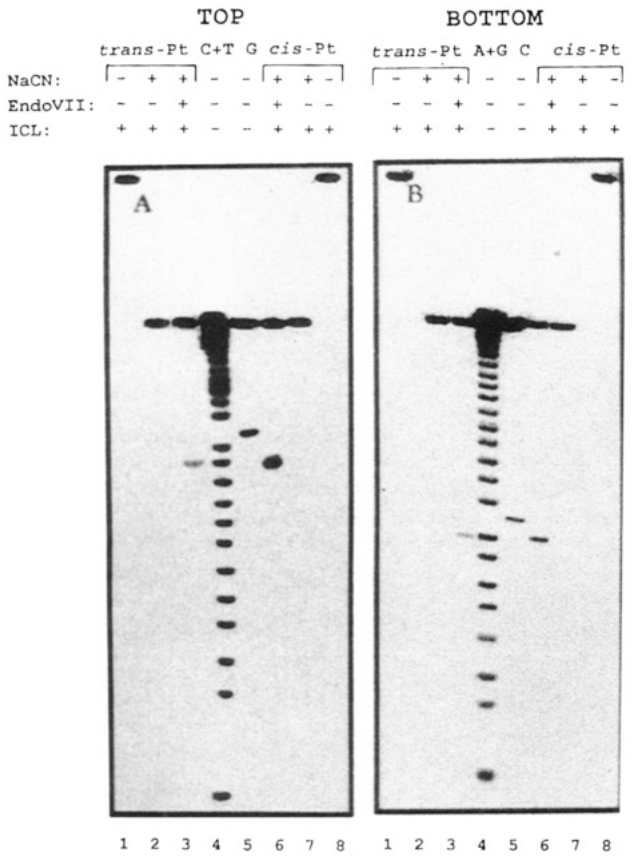


FIGURE 4: Comparison of the cleavage of DNA containing interstrand cross-links (ICLs) of cisplatin or transplatin by T4 endonuclease VII by electrophoresis in denaturing polyacrylamide gel. Complementary oligonucleotides 22 bases in length (Figure 1) were used to prepare the duplexes, which were subsequently modified so that they contained a single ICL of transplatin (lanes 1−3) or cisplatin (lanes 6−8). After 3′-end labeling of the top (A) or bottom (B) strands, the duplexes were analyzed by electrophoresis without (lanes 1 and 8) or with (lanes 2 and 7) subsequent treatment with NaCN. The interstrand cross-linked duplexes were also cleaved with T4 endonuclease VII for 15 min at 15 °C and subsequently treated with NaCN (lanes 3 and 6). Lanes 4 and 5 in panel A contain C+T and G sequence markers, whereas the same lanes in panel B contain A+G and C sequence markers.

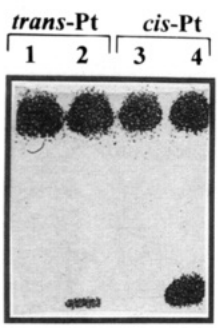


FIGURE 5: Comparison of the cleavage of DNA containing interstrand cross-links (ICLs) of cisplatin or transplatin by T4 endonuclease VII by electrophoresis in non-denaturing polyacrylamide gel. The duplexes containing a single ICL of transplatin (lanes 1 and 2) or cisplatin (lanes 3 and 4) were the same as in Figure 4. After 3′-end labeling at the top strand, the duplexes were cleaved with T4 endonuclease VII in the same way as in Figure 4 and were either directly analyzed by electrophoresis (lanes 1 and 3) or were, before electrophoresis, still treated with NaCN (lanes 2 and 4).

remove the ICLs. A product was only visible that migrated as the noncleaved duplex containing the ICL (Figure 5, lanes 1 and 3). In this case, the double-stranded cleavage could not produce shorter fragments migrating more rapidly

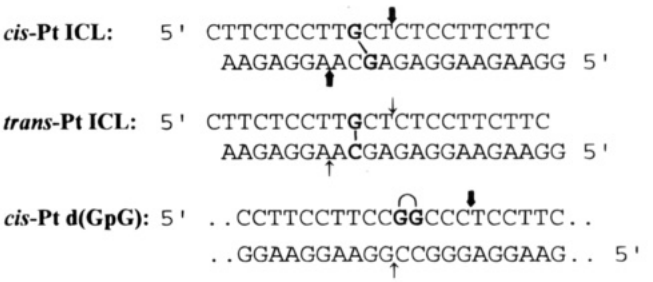


FIGURE 6: Sites of cleavage of DNA containing the ICLs of cisplatin or transplatin by T4 endonuclease VII. The bases involved in the platinum cross-link are highlighted in boldface type. The positions of cleavage of the interstrand cross-linked DNA duplexes are indicated by arrows; the more efficient cleavages are indicated by larger arrows. For comparative purposes, the positions of cleavage of the DNA duplex containing the 1,2-intrastrand d(GpG) cross-link, determined by other authors (Murchie & Lilley, 1993), are also shown.

because the strands in the duplex were still cross-linked. However, if the same product of the cleavage reaction was still treated with NaCN to remove the platinum ICL, a single band was detected corresponding to the duplex fragment that migrated faster than the noncleaved duplex also treated with NaCN (Figure 5, lanes 2 and 4). Such behavior was observed for the duplexes containing the single ICL of both platinum isomers. The fraction of the radioactivity associated with the band corresponding to this double-stranded cleavage was identical to that associated with the band corresponding to the cleavage of the top or bottom strand and analyzed by denaturing gel electrophoresis.

The sites of cleavage by T4 endonuclease VII of interstrand cross-linked oligonucleotides are shown in Figure 6. These sites on the duplex cross-linked by cisplatin are positioned symmetrically to the ICL, two nucleotides 3′ from the platinated guanine residues in both strands. This result is in favor of a symmetrical distortion induced in both strands of DNA by the ICL of cisplatin. Such symmetry of conformational distortions has already been suggested in the work of Sip et al. (1992). These authors observed identical chemical reactivity of both cytosine residues in the d(GpC)/d(GpC) sequence in which the ICL of cisplatin was formed. The duplex containing the ICL of transplatin was also cleaved in the pyrimidine-rich strand two nucleotides 3′ from its platinated guanine residue. However, the complementary strand was only cleaved one nucleotide 3′ from its platinated base (cytosine) involved in this cross-link. This result supports the view that there are differences in the distortions of the top and bottom strands induced by the ICL of transplatin. The dissymmetry of the conformational alterations in the top and bottom strands around the ICL of transplatin is consistent with the results of analysis of this DNA lesion by means of chemical probes of DNA conformation and molecular mechanics modeling (Brabec et al., 1993).

Our results (Figure 4) suggest that the duplex containing the ICL of cisplatin is a better substrate for T4 endonuclease VII than the duplex containing the ICL of transplatin. To explore this finding further, we measured the kinetics of the cleavage of the two substrates in the top strands at 20 °C. This temperature was chosen to use identical conditions as in the same kinetic experiment performed with the duplex containing intrastrand cross-links of cisplatin (Murchie & Lilley, 1993). The results are shown in Figure 7. The

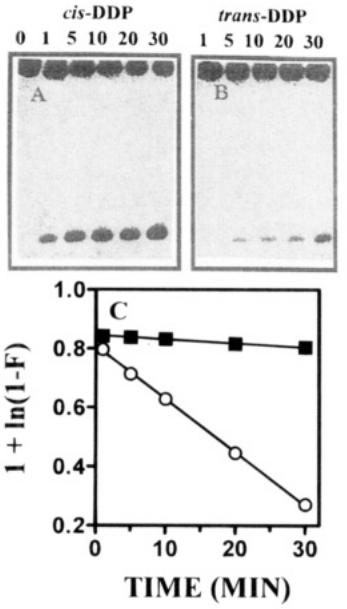


FIGURE 7: Kinetics of cleavage by T4 endonucleaseVII of DNA containing interstrand cross-links (ICLs) of cisplatin or transplatin. The duplexes containing a single ICL of cisplatin (A) or transplatin (B) were the same as in Figure 4. After 3′-end labeling of the top strand, the duplexes were cleaved with T4 endonuclease VII in the same way as in Figure 4, but at 20 °C, and before electrophoresis were still treated with NaCN. The samples were removed at the time intervals indicated (in minutes) and analyzed by electrophoresis in denaturing polyacrylamide gel. The data are relative to the top strands of the interstrand cross-linked duplexes. (C) Semilogarithmic plots of the fraction of uncleaved DNA against time for duplexes containing ICLs of cisplatin (○) or transplatin (■). The data for these plots were obtained from the experiments shown in panels A and B.

duplexes containing the ICL of cisplatin or transplatin were cleaved with a rate constant of $(-3.9 \pm 0.4) \times 10^{-4}$ or $(-1.7 \pm 0.3) \times 10^{-5}$ $s^{-1}$, respectively. The results show clearly that the ICL of cisplatin is a much better substrate than is the ICL of transplatin. In addition, the value of the rate constant obtained for the duplex containing the ICL of cisplatin was close to that obtained for the similar DNA probe, but containing the 1,2-intrastrand d(GpG) cross-link of cisplatin under similar conditions ($-3.2 \times 10^{-4}$ $s^{-1}$) (Murchie & Lilley, 1993).

The previous experiments (Murchie & Lilley, 1993) demonstrating that DNA containing 1,2-intrastrand cross-links is a good substrate for precise cleavage by T4 endonuclease VII were performed with duplexes 32 bp in length. Therefore, we have also verified that the position of cleavage sites and kinetics of the cleavage are not influenced if the duplexes are shortened to ~20 bp. The cleavage of the duplex designated in Figure 1 as *cis*-Pt d(GpG) yielded results (not shown) that were identical to those reported recently for the 32-bp duplex. Thus, the character and the rate of the cleavage of the duplexes containing single platinum adducts located approximately in the center of the duplex are not influenced by the length of the duplexes at least in the range of 18−32 bp.

## DISCUSSION

The present results demonstrate that the DNA ICL of antitumor cisplatin is specifically recognized by and bound to two damaged DNA-binding proteins of different types, mammalian HMG1 protein and bacteriophage T4 endonuclease VII. In contrast, the same DNA lesion formed by clinically ineffective transplatin is not recognized or is recognized markedly less efficiently by these proteins. It has been suggested (Bruhn et al., 1992, 1993; Murchie & Lilley, 1993; Pil & Lippard, 1992; Treiber et al., 1994) that distortions such as bending and/or unwinding induced in DNA by platinum complexes are important for the recognition and binding capability of the platinum-damaged DNA-binding proteins. The DNA bending and unwinding angles produced by the DNA adducts of platinum(II) so far tested for their recognition by the HMG1 protein or T4 endonuclease VII are summarized in Table 1. These data show no apparent correlation between bending and/or unwinding induced in DNA by the platinum adducts and their fixation to the damaged DNA-binding proteins. For instance, DNA bending and unwinding by the interstrand and 1,2-intrastrand cross-links of cisplatin are markedly different, but these DNA adducts are readily recognized by and bound to the damaged DNA-binding proteins used in this work with an approximately similar affinity (Figures 2−4, 7). On the other hand, 1,2- and 1,3-intrastrand cross-links of cisplatin unwind and bend DNA to a similar extent (although it is not known whether the bend of the 1,3-intrastrand cross-link is also directed toward the major groove of the DNA double helix as it is in the case of the 1,2-intrastrand cross-link), but their binding to the HMG1 protein is markedly different (Pil & Lippard, 1992; Huang et al., 1994). There is, however, increasing evidence indicating that a local deformation of the DNA helix, including DNA bending, at the site of its interaction with the damaged DNA-binding proteins is significant for the recognition and binding. Therefore, the comparison shown in Table 1 suggests that another structural factor or factors on DNA, besides DNA bending and

Table 1: Conformational Distortions Induced in DNA by Platinum Adducts and Their Recognition by HMG1 Protein and T4 Endonuclease VII

| DNA adduct type[a] | bending angle (deg) | unwinding angle (deg) | denaturation, flexibility | recognition | |
|---|---|---|---|---|---|
| | | | | HMG1 | T4endoVII |
| *cis*-DDP 1,2-intra | 32−34[b,c] | 13[d] | (−)[e−g] | (+)[h,i] | (+)[j] |
| *cis*-DDP 1,3-intra | 35[c] | 23[d] | (+)[k−m] | (−)[h,i] | ND |
| *cis*-DDP inter | 45[n] | 79[n] | (−)[n] | (+)[o] | (+)[o] |
| *trans*-DDP 1,3-intra | hinge joint[d] | 6−13[d] | (+)[k] | (−)[h] | (−)[j] |
| *trans*-DDP inter | 26[p] | 12[p] | (+)[p] | (−)[o] | (~)[o] |

[a] *cis*-DDP 1,2-intra = 1,2 intrastrand cross-link of cisplatin; *cis*-DDP 1,3-intra = 1,3-intrastrand cross-link of cisplatin; *cis*-DDP inter = ICL of cisplatin; *trans*-DDP 1,3-intra = 1,3-intrastrand cross-link of transplatin; *trans*-DDP inter = ICL of transplatin. [b] Rice et al. (1988). [c] Bellon and Lippard (1990). [d] Bellon et al. (1991). [e] Brabec et al. (1990). [f] den Hartog et al. (1985). [g] Sherman and Lippard (1987). [h] Pil and Lippard (1992). [i] Huang et al. (1994). [j] Murchie and Lilley (1993). [k] Anin and Leng (1989). [l] Marrot and Leng (1989). [m] van Houte and van Gerderen (1994). [n] Malinge et al. (1994). [o] This work. [p] Brabec et al. (1993).

unwinding, control the process of the binding of the platinated DNA to the HMG1 or T4 endonuclease VII.

The conformational change induced in DNA by the single platinum adducts, which has also been systematically investigated, is a local denaturation of the duplex and its reduced rigidity (Table 1). Interestingly, the local denaturation and/or enhanced flexibility have been observed in DNA containing the 1,3-intrastrand cross-links of both platinum isomers and the ICL of transplatin. Neither of these DNA adducts is recognized and bound to the proteins containing HMG boxes. The cleavage by T4 endonuclease VII of the duplexes containing the adducts which induce in DNA a local denaturation and/or flexibility was only examined in the case of the adducts of transplatin. Nevertheless, no cleavage (Murchie & Lilley, 1993) or only a slight cleavage (Figures 4, 5, and 7) of the duplexes containing these adducts has been observed. On the other hand, the ICL or 1,2-intrastrand cross-link of cisplatin has been readily recognized by the HMG1 protein or T4 endonuclease VII. However, these cisplatin adducts have been found to induce in DNA non-denaturational distortions, and/or no enhancement of the duplex flexibility [detected by an undirectional bending (Bellon et al., 1991)] has been observed (Table 1). Thus, these observations are consistent with the idea and support the hypothesis that local denaturation and increased flexibility impede recognition of bending and unwinding induced in DNA by platinum adducts by the damaged DNA-binding proteins.

Several HMG domain proteins, including HMG1 protein, have been shown to recognize prebent DNA, but also to bend linear (nonplatinated) DNA by sequence-nonspecific interactions (Grosschedl et al., 1994). In addition, not only do the HMG domain proteins recognize prebent, cisplatin-modified DNA, but also, in the presence of these proteins, the platinated DNAs are bent significantly more than in their absence (Chow et al., 1994). The preferential binding of the HMG1 protein to prebent DNA has been suggested to reflect a reduced energy requirement in the binding step (Bracale et al., 1994). Importantly, some HMG domain proteins have been shown to induce a bend that has a precise directionality rather than to simply increase the flexibility of the DNA (Chow et al., 1994; Grosschedl et al., 1994). Thus, the formation of the complex between the DNA-binding protein and damaged DNA may require contacts or linkages of the specific groups in the two biomacromolecules. Precedent suggests that these contacts could be difficult to constitute if the specific groups in DNA are contained in denatured base pairs or in more flexible segments of DNA. Such groups may have more freedom to adopt various geometries in comparison with the groups in rigid, non-denatured DNA segments. In other words, the reduced rigidity of the DNA double helix around the platinum adduct could decrease the probability that the groups in DNA capable of specific contacts with the DNA-binding protein occur in the positions favorable for these specific interactions. In support of this argument, the HMG proteins have a lower affinity for cisplatin adducts in flexible, single-stranded DNA than for the same lesions in the rigid, double-helical DNA molecule (Marples et al., 1994).

Our results may also lend deeper insight into the molecular mechanisms of biological activity of antitumor cisplatin. The differences in antitumor effectiveness of cisplatin and its *trans* isomer has been often related to the fact that transplatin cannot form in DNA 1,2-intrastrand cross-links, which are major adducts formed in linear DNA by cisplatin. It was shown recently (Lemaire et al., 1991; Brabec & Leng, 1993; Brabec et al., 1993; Malinge et al., 1994) that there are also differences in the nature and amount of the DNA ICLs of the two isomers including differences in alterations induced by these lesions in DNA conformation. This paper shows differences in the recognition of the DNA ICLs of cisplatin and transplatin by the two damaged DNA-binding proteins. Taken together, it seems reasonable to suggest that different clinical effectiveness of cisplatin and its *trans* isomer may also be a consequence of the differences in the character and recognition by specific proteins of their DNA ICLs.

Finally, the formation of nucleic acid ICLs of platinum complexes has been suggested as a promising procedure for the irreversible binding of antisense oligonucleotides to their target nucleotide sequences (Dalbiès et al., 1994). The results of this work show that the nature and character of nucleic acid ICLs of platinum noticeably affect their binding to nucleic acid-binding proteins. Among these proteins belong also those which facilitate nucleic acid repair or tolerance of modified nucleic acids (Vaisman & Chaney, 1995). Thus, the binding of such proteins to the sites at which antisense oligonucleotide is cross-linked to the target nucleotide sequence could substantially influence their repair and/or tolerance. Therefore, a success of the antisense oligonucleotide therapy exploiting the interstrand cross-linking capability of platinum complexes could also be dependent on how these ICLs are recognized by the nucleic acid-binding proteins. Further investigations of the recognition and fixation of the nucleic acid ICLs to nucleic acid-binding proteins are warranted to contribute to a rational approach to the use of nucleic acid adducts of platinum in the antisense strategy.

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