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## Chemistry and Structural Biology of 1,2-Interstrand Adducts of Cisplatin

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### 1. FREQUENCY

Shortly after the discovery of the cytostatic activity of cis-diamminedichloroplatinum(II) (cisplatin) (1) it was suggested that the mechanism of antitumor activity of cisplatin involves its binding to DNA (for review, see refs. 2–4). In addition, it was suggested that the mechanism of antitumor activity of cisplatin might be analogous to the biologic activity of bifunctional alkylating agents (such as nitrogen mustard), which also exhibit cytostatic effects (5). As the cytostatic effect of di-alkylating agents was generally accepted to be associated with their DNA interstrand crosslinking efficiency, the first hypotheses on the mechanism underlying the cytotoxicity of cisplatin in tumor cells were derived from its ability to form interstrand crosslinks in DNA (6). On the basis of this analogy, cisplatin was sometimes incorrectly called an “alkylating agent.”

Cisplatin is typically administered intravenously. Extracellular and extracellular fluids contain relatively high concentrations of chlorides, in which cisplatin exists in stable, i.e., nonreactive, dichloro form. Cisplatin is activated as

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it crosses cellular membranes. During this process, the concentration of chloride drops from approx 0.15 *M* to approx 4 mM, producing the kinetically more reactive mono-aqua and diaqua species (7).

When a solution of freshly dissolved cisplatin is added to linear, high-molecular-mass DNA, the drug loses one chloride ligand and preferentially forms on DNA a monofunctional adduct at guanine residues [at the N(7) atom]. The next step involves hydrolysis of the second chloride ion and closure of monofunctional adducts to bifunctional lesions. A typical experiment, in which DNA adducts of cisplatin were analyzed after a 16-h reaction in a cell-free medium at  $r_b = 0.002$  ( $r_b$  is the number of platinum atoms fixed per nucleotide) showed that the major adducts are 1,2-intrastrand crosslinks between purine residues (approx 90%). The minor adducts were interstrand crosslinks, 1,3-intrastrand adducts, and monofunctional adducts. Recent results showed that interstrand crosslinks represented 5–10% of the bound cisplatin (8–10). However, it was shown more recently (11) that in negatively supercoiled DNA of the plasmid pSP73 the frequency of interstrand crosslinks was noticeably higher than in the corresponding relaxed or linearized forms and increased with growing negative supercoiling. For instance, at low levels of platination of the naturally supercoiled plasmid DNA ( $r_b$  approx  $10^{-4}$ ), the frequency of interstrand crosslinks was approx 30% of all platinum adducts, i.e., considerably higher than in linear DNA.

Contradictory data have been published on DNA interstrand crosslinking by cisplatin in different cell lines treated with this drug. Whereas numerous studies have reported the correlation between interstrand crosslinking and the cytotoxicity of cisplatin (12–15), others have not (16,17).

## 2. SEQUENCE PREFERENCE

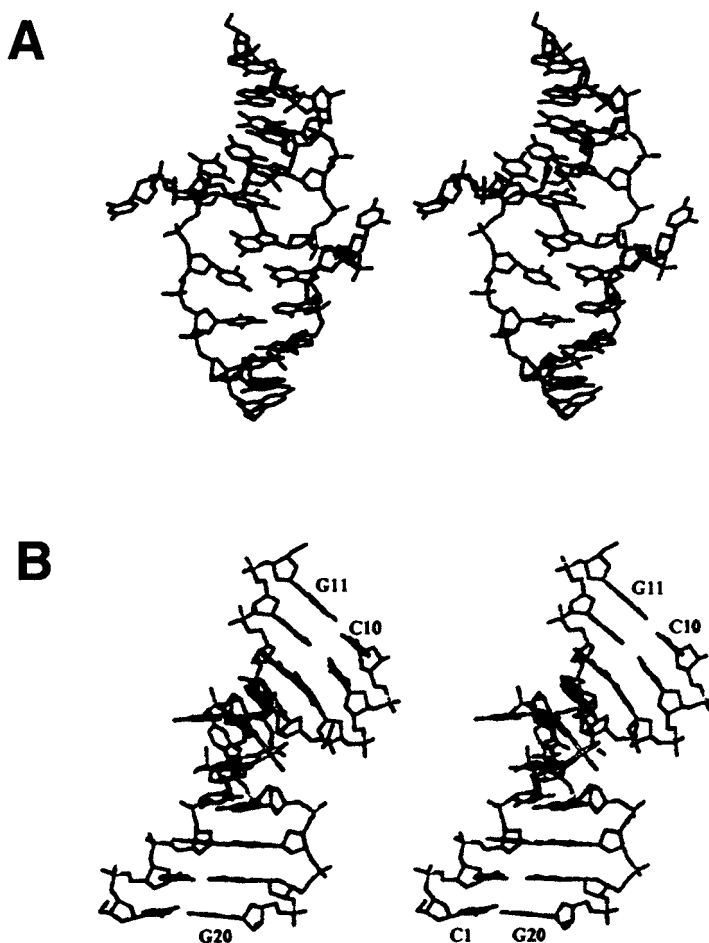
The mechanism by which interstrand crosslinks formed by cisplatin in DNA elicit their biologic responses remains unclear. To shed light on this question, these interstrand lesions have been intensively analyzed, although to a lesser extent than the more frequent DNA intrastrand adducts formed by cisplatin between neighboring base residues.

An important step in this analysis is to identify the sites in DNA at which cisplatin forms interstrand adducts. Initial studies employing in particular chromatographic analysis of DNA modified by cisplatin in cell-free media, which was subsequently hydrolyzed to monomeric nucleosides or various products containing cisplatin, revealed that DNA interstrand crosslinks of cisplatin occurred predominantly between N(7) atoms of guanine residues in opposite strands (18,19). This conclusion was further corroborated by the observation that in the reaction of the synthetic double-helical polydeoxyribonucleotide complex poly(dG-dC)·poly(dG-dC) (in which in each guanine strand alternates regularly with cytosine) with cisplatin, interstrand crosslinks

were only formed between guanine residues in neighboring base pairs (20). This result, however, did not allow us to distinguish whether the crosslink was preferentially oriented in the 3'→3' or 5'→5' direction, i.e., in the 5'-CG or 5'-GC sequences. The distance between the leaving (reactive) groups in cisplatin is approx 0.3 nm, so that two neighboring guanine residues on the opposite strands, either in 5'-CG or 5'-GC sequences, are the most likely sites on DNA for interstrand crosslinking by cisplatin (21). Manipulation of three-dimensional molecular models inferred that the 5'-CG sequence should be more capable of matching the interstrand crosslinking by cisplatin (19,22). This finding was surprising since the distance between two N(7) atoms of guanine residues in the 5'-CG sequence is larger (0.9 nm) than in the 5'-GC sequence (0.7 nm) (23).

A convincing conclusion about the bases preferentially involved in the interstrand crosslinks formed by cisplatin under physiologic conditions (in a cell-free medium) was obtained with the aid of transcriptional footprinting of the platinum adducts on DNA. Recent work has shown that the *in vitro* RNA synthesis by bacterial RNA polymerases on DNA templates containing several types of bifunctional adducts of platinum complexes can be prematurely terminated at the site or in the proximity of adducts (10,24). The resulting RNA transcripts are separated using high-resolution sequencing gels, and quantification of blocked transcripts is performed by conventional autoradiography or by a phosphorimaging technique. Importantly, monofunctional DNA adducts of cisplatin and several other platinum(II) complexes {for instance, those of chlorodiethylenetriamineplatinum(II) chloride,  $[\text{PtCl}(\text{NH}_3)_3]\text{Cl}$ , or the trans isomer of cisplatin (transplatin)} are incapable of terminating RNA synthesis (10,24). This assay was applied to a restriction fragment (212 base pairs) modified by cisplatin, which contained only interstrand adducts. This was achieved by treatment of the cisplatin-modified fragment with sodium cyanide at slightly alkaline pH. It was shown that at pH 8.3 and 37°C treatment with NaCN could remove approx 90% of intrastrand crosslinks and monofunctional adducts formed by cisplatin in double-helical DNA, whereas interstrand crosslinks were more stable under these conditions. (Only approx 15% of all interstrand adducts were removed.) Transcriptional footprinting using this template revealed that cisplatin-interstrand crosslinks were formed in linear DNA between two guanine residues, preferentially in the 5'-GC sequence. This finding was further confirmed by assays employing dimethyl sulphate and formic acid as chemical probes of platinum binding to N(7) atoms of adenine and guanine residues in DNA.

Interstrand crosslinks of cisplatin can be formed in negatively supercoiled DNA with a higher frequency than in linear DNA (11) (*see above*). However, the topology of DNA does not affect only the amount of interstrand crosslinks. Transcriptional footprinting of interstrand crosslinks formed by cisplatin in nega-



**Fig. 1.** Structure of *cis*-(NH<sub>3</sub>)<sub>2</sub>Pt:[d(C<sub>1</sub>C<sub>2</sub>T<sub>3</sub>C<sub>4</sub>G\*<sub>5</sub>C<sub>6</sub>T<sub>7</sub>C<sub>8</sub>T<sub>9</sub>C<sub>10</sub>)·d(G<sub>11</sub>A<sub>12</sub>G<sub>13</sub>A<sub>14</sub>G\*<sub>15</sub>C<sub>16</sub>G<sub>17</sub>A<sub>18</sub>G<sub>19</sub>G<sub>20</sub>)], where G\*<sub>5</sub> and G\*<sub>15</sub> are guanine residues on opposite strands of DNA crosslinked by cisplatin at the N7 position. Stereoviews of the atomic model. (A) View showing the minor groove and the bound platinum residue. (B) View after a 90° rotation around a vertical axis. Reproduced with permission from *ref.* 32.

tively supercoiled DNA revealed that these lesions were formed at both 5'-GC and 5'-CG sequences, which was in contrast to linear DNA (11). Thus, DNA topology also controls sequence preferences of cisplatin to form this type of DNA adduct.

Interestingly, interstrand crosslinks between guanine and cytosine residues were formed by cisplatin in the synthetic polydeoxyribonucleotide complex poly(dG)·poly(dC). In this double-helical DNA, one strand contains only gua-

nine residues, whereas the other strand contains cytosine residues (25). This type of interstrand crosslink was also formed in a short oligodeoxyribonucleotide duplex (20 base pairs) at its central sequence 5'-TCGT/5'-ACGA (Vrana et al., unpublished results). However, this interstrand adduct of cisplatin was formed only very slowly ( $t_{1/2} > 48$  h at 37°C), which was consistent with the inability to detect crosslinks of this type in natural linear DNA randomly modified by cisplatin at 37°C (22).

### 3. STRUCTURE

In B-DNA the distance between the N(7) atoms of the opposite guanine residues in the 5'-GC sequence [i.e., between the sites at which cisplatin preferentially forms interstrand crosslinks (*see above*)] are separated by 0.7 nm. On the other hand, the crosslinking reaction by cisplatin requires a distance of DNA binding sites of about 0.3 nm (26,27). It is therefore evident that DNA interstrand crosslinking by cisplatin requires a distortion of the canonical B-DNA conformation.

A double-stranded oligodeoxyribonucleotide (22 base pairs) containing a single interstrand crosslink of cisplatin within the central sequence 5'-TGCT/5'-AGCA was analyzed by using gel electrophoresis, chemical probes of DNA conformation, and molecular modeling (28). The electrophoretic mobility of multimers obtained by ligation of crosslinked oligonucleotide duplexes was anomalously slow. It was concluded that the interstrand adducts at GC sites bent DNA more than the 1,2-G,G intrastrand adduct. Chemical probing by hydroxylamine showed that cytosine residues complementary to the platinated guanines were largely exposed to the solvent, whereas the flanking adenines and thymines were not reactive with diethyl pyrocarbonate and osmium tetroxide, respectively. These results indicated that the distortion induced by the crosslink formation was localized at the platinated GC/CG base pairs and no local denaturation took place in the vicinity of the platination site. These conclusions were corroborated by molecular mechanics modeling, which yielded two different lowest energy structures. Both structures were similar to B-DNA on the 3' and 5' sites of the adduct and exhibited a lack of stacking interactions of the cytosine residues complementary to platinated guanines. The two structures differed by the magnitude of bending at the platinated site toward the major groove; the bending angles were 24° and 57°, respectively. The bending angle of the latter model corresponded well with the angle determined from the gel electrophoresis experiment.

Another study (29) was performed by using gel electrophoresis of ligated oligodeoxyribonucleotides that differed in the central platinated sequences (5'-TGCT/5'-AGCT, 5'-AGCT/5'-AGCT, and 5'-CGCT/5'-AGCG). The results of this study were interpreted to mean that the interstrand crosslink formed by cisplatin in the GC/GC sequences induced bending of the duplex axis at the plati-

nated site by approximately  $45^\circ$  and unwinding by  $79^\circ$ , the distortion being independent of the flanking base residues. It should be noted, however, that the unwinding was not compatible with the simple model based on the N(7)-Pt-N(7) bond localized in the major groove, which would rather lead to overwinding of the DNA double helix at the platination site (30).

A novel solution structure of the cisplatin-induced interstrand crosslink of DNA was proposed on the basis of  $^1\text{H}$  nuclear magnetic resonance measurements (NMR) in two laboratories nearly simultaneously (30,31). The experiments were carried out with short duplexes, deoxyribonucleotide decamers [(5'-CATAG\*CTATG)<sub>2</sub> (30) or 5'-CCTCG\*CTCTC/5'-GAGAG\*CGAGG (31)], which were treated with an aqueous solution of cisplatin to obtain a single interstrand crosslink between two central opposite guanine residues (denoted in the sequences by asterisks). Even though the central sequences of the two duplexes were different, the general features of the solution structures were in a good agreement.

The NMR data were used to assign the imino and nonexchangeable protons. Several irregularities in the crosslinked base pairs and their immediate neighbor pairs indicated that the structure of the central region of the duplex had features not present in B-DNA. Evidence was obtained that the crosslinked deoxyriboguanosine residues were not paired with hydrogen bonds to the complementary deoxyribocytidines, which were located outside the duplex and not stacked with other aromatic rings. All other base residues were paired. Furthermore, the spectra indicated an unusual glycosidic angle for the crosslinked deoxyriboguanosines and a close proximity between the platinated guanine and the flanking neighbor base pair.

Solution structures were calculated and refined by molecular dynamics and energy minimization (30). The refined structures corresponded closely to the features derived from the NMR spectra. The two crosslinked guanines adopted a head-to-tail arrangement and were stacked with flanking neighbor base pairs. An entirely unforeseen feature was that the *cis*-diammineplatinum(II) bridge resided in the minor groove, and not in the major groove as reported earlier (28). Also surprising was the finding that the double helix was locally reversed to a left-handed, Z-DNA-like form. The change of the helix sense and the extrusion of deoxyribocytidine residues (complementary to the platinated deoxyriboguanosine residues) from the duplex resulted in the helix unwinding by approximately  $80^\circ$  [precisely by  $87^\circ$ , as determined by Huang et al. (30), or  $76^\circ$ , as determined by Paquet et al. (31)] relative to B-DNA. This angle is in a good agreement with the value deduced from electrophoretic measurements (29). It was demonstrated by an electrophoretic retardation technique that the helix axis was bent at the crosslinked site toward the minor groove (30). The bending angle determined by Huang et al. (30) ( $20^\circ$ ) was smaller than that re-

ported by Paquet et al. (31) or determined by electrophoresis measurements (29) ( $40^\circ$  and  $45^\circ$ , respectively).

The crosslinking reaction of cisplatin requires that the N(7) atoms of guanines in opposite strands be brought closer to a distance that is less than half that in B-DNA. Both spin relaxation measurements and molecular dynamic calculations demonstrated that in solution at room temperature the glycosidic angle in B-DNA could vary within  $\pm 28^\circ$ . It has been suggested that such restricted diffusion around the glycosidic angle induces large coupled motions of the backbone and deoxyribose conformation that can bring the bases closer (31).

Quite recently, the crystal structure of a double-stranded DNA decamer containing a single interstrand crosslink of cisplatin formed between opposite guanine residues in the 5'-GC sequence was solved (Figure 1) (32).  $47^\circ$  for double-helix bending towards the minor groove and  $70^\circ$  for unwinding were found. The major conformational distortions were located at the level of the adduct and did not extend over the flanking nucleotide residues. The platinum residue protruded in the minor groove of the DNA duplex and the N7 atoms of the crosslinked guanine residues localized initially in the major groove of B-DNA moved in the minor groove. The minor groove was enlarged to  $>1.0$  nm and the planar character of the platinum coordination remained preserved. The structure also exhibited a network of ordered water molecules forming a cage which surrounded the platinum atom and the crosslinked guanine residues.

#### 4. ISOMERIZATION

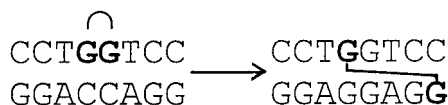
A local distortion of the canonical conformation of DNA due to the formation of interstrand crosslinks by cisplatin can occur because the DNA conformation is dynamic. Double-helical DNA exists in solution in various transient and distorted conformations, which differ in the extent of, for instance, base pair opening, the duplex unwinding, and the bending of the duplex axis (32*a,b*). In addition, accessibility of the binding sites, conformation of the duplex (its geometry), nucleotide sequence, electrostatic potential, flexibility, and the formation of transient reactive species can affect the DNA binding mode of cisplatin, but each to a different extent. Interestingly, the reactivity of the monofunctional adduct of cisplatin (which is formed in the first step of bidentate binding of cisplatin to DNA) to close to bifunctional adducts in double-helical DNA was sequence dependent (33). The half-times of closure of the monofunctional adduct of cisplatin at guanine residues were 14 and 3 h if this adduct was formed in the sequence 5'-TGCT and 5'-AGCT, respectively. In both cases, only the interstrand crosslinks were formed. This is particularly interesting for the sequence 5'-AGCT, since the monofunctional adduct at the

guanine residue reacted preferentially with the guanine residue in the opposite strand and not with the neighboring adenine residue on the 5' site of the same strand to form one of the major adducts of cisplatin, 1,2-AG-intrastrand crosslink. In addition, it was shown using an assay based on the DNA cleavage by exonuclease III that in a double-helical DNA fragment (49 base pairs) randomly modified by cisplatin the drug formed in the sequence 5'-CGCGGG an unexpected interstrand crosslink, rather than the expected intrastrand adduct at a GG site (34). These results have indicated that in some sequences of double-helical DNA, in which cisplatin could form both a 1,2-intrastrand adduct between purine residues and an interstrand adduct between opposite guanine residues in the neighboring base pairs, cisplatin preferentially forms the interstrand crosslink.

Another example of the higher stability of an interstrand adduct in comparison with the 1,2-intrastrand adduct of cisplatin is the isomerization of a 1,2-G,G-intrastrand crosslink in double-helical DNA into a more stable interstrand crosslink. This isomerization was observed in several DNA duplexes. It was shown recently (35) that the adjacent guanine bases in the 1,2-intrastrand crosslink formed by cisplatin in double-helical DNA were rolled toward one another by 49° and that one possible consequence of this roll is the strain induced in the Pt-N(7) bonds, which may labilize the platinum-guanine linkages. NMR analysis of the short oligodeoxyribonucleotide duplex (8 base pairs) whose upper strand, CCTG\*G\*TCC, contained a single intrastrand adduct of cisplatin at the central GG sequence (designated in the sequence by asterisks) clearly revealed transformation of intrastrand to interstrand adduct, which was promoted by the presence of the nucleophilic chloride ion in the medium (36). The new crosslink was formed between 5'G\* and the 5' terminal guanine residue of the complementary strand; (Fig. 2).

In the initially formed 1,2-intrastrand crosslinked structure, N(7) of the 5' terminal guanine residue of the complementary strand was 0.12 nm away from the platinum atom, but there was probably enough flexibility at the end of the duplex to facilitate the intramolecular isomerization. The finding that 1,2-intrastrand adducts of cisplatin can isomerize to interstrand crosslinks was reinforced by investigation of the palindromic dodecamer 5'-d(GACCATATG\*G\*TC) containing a 1,2-intrastrand crosslink between guanine residues (designated in the sequence by asterisks). Using NMR analysis (37), it was observed that during incubation of this platinated oligonucleotide various reactions took place and one resulted in the formation of the crosslinks between guanine residues belonging to two duplexes. Another example of this transformation was its induction by irradiation with 300–350 nm of light. Short restriction fragments (123 or 82 base pairs) were modified by cisplatin, irradiated with 300–350 nm of light, and analyzed by gel electrophoresis under denaturing conditions (38). This analysis revealed a considerably increased amount of interstrand

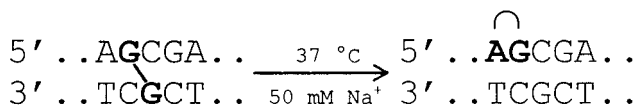




**Fig. 2.** Example of the isomerization of a 1,2-GG-intrastrand adduct of cisplatin into an interstrand cross-link (36).

crosslinks in comparison with control, nonirradiated platinated samples. Similarly, irradiation of a short oligodeoxyribonucleotide duplex (15 base pairs) containing a single 1,2-GG-intrastrand crosslink of cisplatin in the center resulted in interstrand crosslinked DNA. It was suggested (38) that, starting with a 1,2-GG-intrastrand adduct in double-helical DNA, irradiation with 300 nm of light resulted in photodissociation of one of the purine ligands, producing a reactive intermediate in which the vacant site was filled with a more labile ligand, presumably a water molecule. This labile ligand could be displaced by a nucleobase in the opposite DNA strand, producing a DNA interstrand crosslink. Another likely pathway would involve photosubstitution of one of the ammine ligands of cisplatin, a possibility supported by the observation that DNA containing a  $[\text{Pt}(\text{NH}_3)_3]^{2+}$  adduct also formed interstrand crosslinks upon irradiation.

It was also shown recently (39) that in short oligodeoxyribonucleotide duplexes (10 and 20 base pairs) containing a central sequence AGCGA/TCGCT the interstrand crosslink formed by cisplatin between guanine residues in the opposite strands in the 5'-GC sequence was labile at 37°C and rearranged into an intrastrand crosslink (Fig. 3). Importantly, this isomerization took place considerably less readily in the longer duplex. ( $t_{1/2}$  values were 29 h and 5 d for the isomerization in the short and long oligonucleotide duplexes, respectively.) We extended these studies to the oligonucleotide duplex, containing a single interstrand crosslink of cisplatin formed in the same central sequence, incorporated by ligation approximately into the center of a linearized plasmid DNA (2464 base pairs). We found (Brabec et al., unpublished results) that the interstrand crosslink was stable at 37°C for at least 2 weeks without any sign of decrease of the amount of interstrand crosslinked molecules. In addition, we prepared a sample of linearized plasmid DNA (2464 base pairs) that was randomly modified by cisplatin so that approximately 50% of the DNA molecules contained one interstrand crosslink. This sample was incubated for 2 weeks at 37°C, but the amount of interstrand crosslinked molecules, detected using gel electrophoresis under denaturing conditions, remained unchanged. Thus, the rearrangement of interstrand crosslinks into intrastrand adducts in DNA modified by cisplatin is specific only for some short oligonucleotide duplexes and should not be generalized to natural, high-molecular-mass DNA. The effect of isomerization observed



**Fig. 3.** Rearrangement of the interstrand crosslink of cisplatin into intrastrand adduct in short oligonucleotide duplexes (39).

in very short oligonucleotide duplexes was consistent with the labile Pt-N(7)(guanine) coordination bond involved in the interstrand crosslink of cisplatin. If this bond was ruptured in the case of an interstrand crosslinked short duplex, a monofunctional adduct was formed, which might considerably decrease the melting temperature of the short duplex, particularly if the base residue on the 5' side of the guanine monoadduct was a pyrimidine base (40–42). In this way the strands in a very short duplex separate, and the interstrand crosslink could not be reformed. Then it was not surprising that the transient monofunctional adduct closed into the intrastrand crosslink if the neighboring base on the 5' side of the platinated guanine was adenine or guanine. Thus, the isomerization of interstrand crosslinks of cisplatin into intrastrand crosslinks appears to be not only specific for very short oligonucleotide duplexes but probably also sequence dependent.

In the past, the stability of the cisplatin intrastrand and interstrand adducts was not systematically studied. It is well known that the Pt-N(7)(guanine) bond can be reverted by stronger nucleophilic ligands such as  $\text{CN}^-$  (24, 43–45), but it is still not entirely clear how stable this bond is in individual types of cisplatin adducts. In this chapter (*see above*) we have summarized several examples of the metastability of both intrastrand and interstrand crosslinks, although understanding the detailed mechanisms involved in the processes by which the Pt-N(7)(guanine) bond is ruptured requires further studies.

The findings on the instability of DNA adducts of cisplatin and their isomerization observed *in vitro* may have biologic implications. It has been suggested (36) that the same reactions, which involve rupture of the Pt-N(7)(guanine) bond, can be promoted *in vivo* by other nucleophilic biologic ligands, such as sulphhydryl or amine groups of proteins. In addition, if the Pt-N(7)(guanine) bond is broken at one place and reformed at another place, it might also be hypothesized that the platinum atom can “migrate” on the DNA duplex. It can also be speculated that the rate of isomerization of DNA adducts of cisplatin could be affected by DNA conformation or topology, which can be locally changed during genetic processes involved in the mechanisms underlying the antitumor activity of the drug. Thus, the frequency of the individual types of the cisplatin adducts could be locally and transiently altered in DNA in the cell nucleus during some phase (or phases) of the cell cycle and could be different from that found in DNA randomly modified in cell-free media.

## 5. RECOGNITION BY SPECIFIC PROTEINS

Studies on the mechanisms underlying antitumor activity of cisplatin often employ inactive compounds such as, e.g., the *trans* isomer of cisplatin (transplatin). In this approach, one searches for differences between active and inactive compounds, which may be responsible for their different pharmacologic effects. DNA serves as a template for polymerases that copy the genetic code and transcribe it into messenger RNA. Important results were obtained when the effect of cisplatin and transplatin on replication of chromosomal DNA was examined by using African green monkey CV-1 cells infected by simian virus 40 (46). The results of these studies indicated that: (1) equal numbers of cisplatin and transplatin lesions per nucleotide residue produced the same degree of inhibition of DNA replication; (2) the cellular uptake of cisplatin and transplatin was equal; (3) a time-course analysis of platinum binding to DNA in CV-1 cells treated with the two platinum isomers revealed that cisplatin adducts continuously accumulated on the DNA, whereas transplatin adducts did not. On the basis of these results, it has been suggested that the DNA adducts formed by the two isomers in cells might be differentially processed and that cellular components, presumably cellular proteins, must exist that interact with specific platinum lesions on DNA.

Gel mobility shift assays revealed the presence of proteins in mammalian cellular extracts that bind specifically to DNA modified by antitumor cisplatin and its direct analogs, but not by inactive transplatin or monofunctional chlorodiethylenetriamineplatinum(II) chloride (47–50). One class of the proteins, which bind selectively to DNA modified by cisplatin, was identified as proteins containing a high mobility group (HMG) domain, HMG1 and HMG2 proteins (51–53). These proteins belong to architectural chromatin proteins that play some kind of structural role in the formation of functional higher order protein/DNA or protein/protein complexes (54). Other bacterial and mammalian proteins were also identified that bind selectively to cisplatin adducts (reviewed recently in ref. 55), but in most studies the recognition of platinated DNA by HMG-domain proteins was investigated.

Unfortunately, the initial studies were only aimed at recognition by these proteins of 1,2- and 1,3-intrastrand crosslinks and monofunctional adducts, and no attention was paid to interstrand crosslinks of cisplatin. These studies were performed with oligodeoxyribonucleotide probes containing defined and unique platinum adducts. It was found (48,53) that HMG-domain proteins bound selectively to the 1,2-GG or AG adducts of cisplatin, but not to the 1,3-intrastrand crosslinks and monofunctional adducts. At the same time, HMG-domain proteins were reported not to bind to 1,3-intrastrand crosslink and monofunctional adducts of transplatin (48,53).

Later we extended these studies to DNA interstrand crosslinks produced by cisplatin or transplatin by using oligodeoxyribonucleotide probes containing only one type of interstrand crosslink (the cisplatin crosslink between opposite

guanine residues in the 5'-GC sequence or transplatin crosslink between guanine and complementary cytosine, i.e., the crosslinks located at the sites where interstrand adducts of these platinum compounds are formed preferentially) (56). It was found that mammalian HMG1 protein bound to the interstrand crosslink of cisplatin with a similar affinity as to the 1,2-GG-intrastrand crosslink. On the other hand, no binding of the HMG1 protein to the probe containing transplatin interstrand crosslink was noted.

Another protein that has been tested for its specific recognition of DNA interstrand adducts of cisplatin and transplatin was an enzyme with deoxyribonuclease activity, T4 endonuclease VII. This bacteriophage T4-encoded protein cleaves branched DNA structures, most notably four-way junctions, and is regarded as a repair enzyme (57,58). Again, DNA recognition properties of this protein were characterized initially only with respect to 1,2-GG or AG intrastrand crosslinks of cisplatin and the 1,3-GNG crosslink of both cisplatin and transplatin (58). It was discovered that T4 endonuclease VII recognized 1,2-intrastrand crosslinks of cisplatin, whereas 1,3-intrastrand crosslinks of both isomers were recognized much less efficiently. In later studies (56), we used oligodeoxyribonucleotide duplexes (22 base pairs) containing a single, site-specific interstrand crosslink of cisplatin or transplatin to investigate whether DNA interstrand crosslinked by either platinum isomer was a substrate for cleavage by T4 endonuclease VII. It was shown that the DNA duplex containing a single interstrand crosslink of cisplatin was precisely cleaved in both strands by this DNA-debranching enzyme with an efficiency similar to that observed with DNA containing a single 1,2-GG-intrastrand adduct or a four-way junction. In contrast, the duplex containing the interstrand crosslink of clinically ineffective transplatin was cleaved considerably less efficiently, a property shared by HMG-domain proteins. Thus, T4 endonuclease VII recognized interstrand crosslinks of cisplatin and transplatin like HMG1 protein. The cleavage of branched DNA structures is highly specific in that the enzyme leaves two or three nucleotides to the 3' side of the point of strand exchange (57). Interestingly, both strands of the duplex containing an interstrand crosslink of cisplatin were cleaved at approximately the same rate, and the sites of cleavage by T4 endonuclease VII were positioned symmetrically to the crosslink, two nucleotides on the 3' side of the platinated guanine residues in both strands (56). This result was in favor of a symmetric distortion induced in both strands of DNA by the interstrand crosslink of cisplatin. Such symmetry of conformational distortions is apparent from the experiments describing equal chemical reactivity of both opposite cytosine residues in the 5'-GC sequence in which the interstrand crosslink of cisplatin was formed and has been confirmed by the structural model of this lesion based on NMR analysis (28,30,31). The results describing cleavage of interstrand crosslinked DNA by T4 endonuclease VII, in conjunction with the fact that this enzyme interacts

with four-way junction DNA, suggest that T4 endonuclease VII recognizes DNA that is distorted in a particular way, such as DNA that contains mutually inclined DNA helical segments.

It has been suggested (52,53,58–60) that distortions such as bending and/or unwinding at the site of platination induced in DNA by platinum complexes are important for the recognition and affinity of the platinum-damaged DNA binding proteins. The data so far available show no clear correlation between the magnitude of bending and/or unwinding induced in DNA by the individual types of the platinum adducts and the resulting fixation of HMG1 or T4 endonuclease VII (Table 1).

On the other hand, these data strongly support the view that the platinum-damaged DNA binding proteins utilize kinked, unwound, platinated DNA as a basis for recognition and binding. The 1,2-intrastrand adduct of cisplatin bends DNA toward the major groove (35,61,62–73) whereas the interstrand crosslink of this drug bends DNA toward the minor groove (30–32). The recognition of both these lesions by HMG1 and T4 endonuclease VII implies that the DNA bending at the platinated site is recognized by HMG-domain proteins or T4 endonuclease VII independently of whether it is directed toward the major or minor groove. An important feature of the interstrand crosslink of cisplatin is that the crosslinked guanine residues are moved into the minor groove where the *cis*-diammineplatinum(II) bridge is then located (30–32). The observation that HMG-domain proteins readily bind to the DNA interstrand crosslinks of cisplatin also implies that the presence of the *cis*-diammineplatinum(II) bridge in the minor groove represents no marked sterical hindrance for the binding of these proteins to DNA. This is an interesting observation since generally HMG-domain proteins bind to their recognition sequences in the minor groove.

The 1,3-GNG-intrastrand adduct of cisplatin is not recognized by HMG1 or T4 endonuclease VII even though the bending and unwinding induced in DNA by this adduct are rather similar to those induced by the 1,2-GG-intrastrand adduct (which is recognized when formed in double-helical DNA) (Table 1). This suggests that there are another factors controlling recognition of platinum adducts by the DNA binding proteins. At present the factors that hinder the binding of these proteins to the platinum adducts capable of bending and unwinding of DNA are unknown. Some data on local conformation of DNA around the individual types of platinum adducts (Table 1) are consistent with the hypothesis that the recognition of DNA bending and/or unwinding induced by these lesions might be obscured, if the formation of the platinum-induced DNA lesion is accompanied by a local denaturation and/or by a lowered rigidity of the duplex around the adduct. This hypothesis is supported by the fact that the HMG-domain proteins have a considerably lower affinity for cisplatin adducts in flexible, single-stranded DNA than for the same lesions in the rigid, double-helical DNA (74) and that considerably decreased levels of binding are

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

<i>DNA adduct type<sup>a</sup></i>	<i>Bending angle (deg)</i>	<i>Unwinding angle (deg)</i>	<i>Denaturation, flexibility</i>	<i>Recognition</i>	
				<i>HMG1</i>	<i>T4endoVII</i>
Cisplatin 1,2-intra	32–34, <sup>b,c</sup> 58, <sup>d</sup> 78 <sup>e</sup>	13, <sup>f</sup> 21, <sup>d</sup> 25 <sup>e</sup>	(–) <sup>c,f–i</sup>	(+) <sup>j,k</sup>	(+) <sup>l</sup>
Cisplatin 1,3-intra	35 <sup>c</sup>	23 <sup>f</sup>	(+) <sup>m–o</sup>	(–) <sup>j,k</sup>	ND
Cisplatin inter	20, <sup>p</sup> 40, <sup>q</sup> 45, <sup>r</sup> 47 <sup>s</sup>	70, <sup>s</sup> 76, <sup>q</sup> 79, <sup>r</sup> 87 <sup>p</sup>	(–) <sup>r</sup>	(+) <sup>t</sup>	(+) <sup>t</sup>
Transplatin 1,3-intra	hinge joint <sup>f</sup>	6–13 <sup>f</sup>	(+) <sup>m</sup>	(–) <sup>j</sup>	(–) <sup>l</sup>
Transplatin inter	26 <sup>u</sup>	12 <sup>u</sup>	(+) <sup>u</sup>	(–) <sup>t</sup>	(~) <sup>t</sup>

<sup>a</sup>cisplatin 1,2-intra = 1,2-intrastrand crosslink of cisplatin; cisplatin 1,3-intra = 1,3 intrastrand crosslink of cisplatin; cisplatin inter = interstrand crosslink of cisplatin; transplatin 1,3 intra = 1,3-intrastrand crosslink of transplatin; transplatin inter = interstrand crosslink of transplatin.

<sup>b</sup>Rice et al., 1988 (61).

<sup>c</sup>Bellon and Lippard, 1990 (62).

<sup>d</sup>Yang et al., 1995 (63).

<sup>e</sup>Gelasco and Lippard, 1998 (35).

<sup>f</sup>Bellon et al., 1991 (64).

<sup>g</sup>Brabec et al., 1990 (65).

<sup>h</sup>den Hartog et al., 1985 (66).

<sup>i</sup>Sherman and Lippard, 1987 (67).

<sup>j</sup>Pil and Lippard, 1992 (53).

<sup>k</sup>Huang et al., 1994 (68).

<sup>l</sup>Murchie and Lilley, 1993 (58).

<sup>m</sup>Anin and Leng, 1989 (69).

<sup>n</sup>Marrot and Leng, 1989 (70).

<sup>o</sup>van Garderen and van Houte, 1994 (71).

<sup>p</sup>Huang et al., 1995 (30).

<sup>q</sup>Paquet et al., 1996 (31).

<sup>r</sup>Malinge et al., 1994 (29).

<sup>s</sup>Coste et al., 1999 (32).

<sup>t</sup>Kašpárková and Brabec, 1995 (56).

<sup>u</sup>Brabec et al., 1993 (72).

observed when a cisplatin-damaged double-helical DNA substrate contains a single-strand break in the phosphodiester backbone (75). It was suggested (56) that the formation of the complex between the DNA binding protein and platinated DNA might require contacts or linkages of the specific groups in both interacting biomacromolecules. It cannot be excluded that these contacts or linkages could be difficult to constitute if the specific groups in DNA were 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 same groups in rigid DNA segments. In other words, the reduced rigidity of the DNA duplex around the platinated site could decrease the probability that the groups in DNA capable of specific contacts with the DNA binding protein occur in positions favorable for these specific interactions. Further studies are warranted to reveal all factors involved in the recognition of DNA adducts of platinum compounds by DNA binding proteins.

The cisplatin-damaged DNA binding proteins apparently occur in nature for other purposes than for specific recognition of platinum adducts in DNA, since platinum compounds do not belong to natural components of our environment. The capability of cisplatin-damaged DNA to bind DNA binding proteins, which may have a fundamental relevance to the antitumor activity of cisplatin and its simple antitumor analogs, is probably a coincidence when the formation of some platinum adducts in double-helical DNA adopts a structure that mimics the recognition signal for these proteins.

## 6. REPAIR

The mechanism by which DNA binding proteins might mediate cisplatin cytotoxicity has not yet been elucidated, although several models have been proposed (76). For example, specific binding of HMG-domain proteins to cisplatin-modified DNA could shield the adducts from nucleotide excision repair, which is one of the many cellular defense mechanisms involved in elimination of the toxic effects of cisplatin (77–79). This type of repair includes removal of the damaged base by hydrolyzing phosphodiester bonds on both sides of the lesion. It was found (80) using a reconstituted system containing highly purified nucleotide excision repair factors that 1,2- and 1,3-intrastrand crosslinks were efficiently repaired. Importantly, this repair of the 1,2-, but not 1,3-intrastrand crosslink was blocked upon addition of an HMG-domain protein. This is consistent with the observation that HMG-domain proteins bind to 1,2- and not 1,3-intrastrand crosslinks and also with the “shielding” model (*see above*).

An *in vitro* excision repair of a site-specific cisplatin interstrand crosslink was also studied (80) using mammalian cell-free extracts containing HMG-domain proteins at the levels that were not sufficient to block excision repair of the 1,2-intrastrand adducts. Repair of the interstrand crosslink formed by cis-

platin between opposite guanine residues in the 5'-GC sequence was not detected. Similarly, in cell strains derived from patients with Fanconi's anemia nucleotide excision repair of cisplatin-interstrand crosslinks was not observed (81,82), although nucleotide excision repair can readily occur in these cells. Fanconi's anemia cells were described as being extremely sensitive to crosslinking agents so that their noticeably high sensitivity to cisplatin was explained by the inability of these cells to repair cisplatin interstrand crosslinks (83). On the other hand, repair of these lesions was detected with the aid of a repair synthesis assay, which measured the amount of new DNA synthesized after the damage removal in whole cell extracts (84). In this way, however, the repair could also result from a mechanism different from that of nucleotide excision.

The pathways for the repair of DNA interstrand crosslinks of cisplatin and other genotoxic agents in mammalian cells are poorly defined. DNA interstrand crosslinks pose a special challenge to repair enzymes because they involve both strands of DNA and therefore cannot be repaired using the information in the complementary strand for resynthesis. So far most of the studies have been performed using bacterial cells. Based on the genetic and biochemical evidence from bacterial systems, it is thought that interstrand crosslinks are eliminated from DNA by the combined actions of excision repair and recombination systems. Recently, the activities of various human cell extracts and purified human excinuclease on a duplex containing a site-specific interstrand crosslink of psoralen have been tested (85). It was found that, in contrast to monoadducts, which were removed by dual incisions bracketing the lesion, the interstrand crosslink also caused dual incisions, but both were 5' to the crosslink in one of the two strands. The result of this dual incision was a 22- to 28-nucleotide-long gap immediately 5' to the crosslink. This gap was suggested to act as a recombinogenic signal to initiate interstrand crosslink removal. On the other hand, there is also a recombination-independent pathway capable of repairing nitrogen mustard interstrand crosslinks, but not psoralen interstrand crosslink (86). It has been suggested that not all interstrand crosslinks are repaired comparably, which might have an influence on the relative ability of each one to be repaired and contribute to the cytotoxicity. Further work is required to find a mechanism effective in the repair of interstrand crosslinks of cisplatin.

## **7. COMPARISON WITH CLINICALLY INEFFECTIVE TRANSPLATIN**

Cisplatin and transplatin exhibit distinctly different antitumor activities in spite of their very similar chemistry. An argument for substantiating the view that DNA interstrand crosslinks of cisplatin are unlikely candidates of antitumor effects of this drug was also based on the observations that clinically ineffective transplatin does not form a considerably lower amount of interstrand



**Table 2**  
**Characteristics of Interstrand Crosslinks Formed in DNA by Cisplatin and Transplatin in Cell-Free Media**

	<i>Cisplatin</i>	<i>Transplatin</i>
Quantity after 48 h <sup>a</sup>	~6% in linearized plasmid DNA <sup>b,c</sup>	~12% in linearized plasmid DNA <sup>b</sup>
Rate of the interstrand cross-linking <sup>a</sup>	t <sub>1/2</sub> = 4 h <sup>b,c</sup>	t <sub>1/2</sub> > 11 h <sup>b</sup>
Bases preferentially involved in the interstrand cross-links	5'-GC- <sup>d</sup> \ -CG-5'	-G- <sup>b</sup>   -C-
Bending at the site of platination	20–47° towards minor groove <sup>e</sup>	26° towards major groove <sup>f</sup>
Unwinding of the duplex at the site of platination	70–87° <sup>e</sup>	12° <sup>f</sup>
Some characteristics of the helix distortion	Pt in the minor groove, in the platinated sequence Z-DNA-like form and G not H-bonded with complementary C, which are extrahelical, cross-strand base-base stacking <sup>e</sup>	Nondenaturational, distortion extending over approx. 4 bp around the crosslink, platinated guanosine in <i>syn</i> conformation, duplex locally flexible <sup>f</sup>
Recognition by DNA binding proteins, <sup>e,g</sup>	Yes	No

<sup>a</sup>Estimated in linearized plasmid (2464 base pairs) in 10 mM NaClO<sub>4</sub> at 37°C.

<sup>b</sup>Brabec and Leng, 1993 (10).

<sup>c</sup>Vrána et al., 1996 (11).

<sup>d</sup>Lemaire et al., 1991 (24).

<sup>e</sup>See Table 1 and Sections 3 and 5 of the present article.

<sup>f</sup>Brabec et al., 1993 (72).

<sup>g</sup>Recognition by HMG1 and T4 endonuclease VII proteins.

crosslinks in DNA than cisplatin. The acceptance of this argument would, however, require us to admit that, for instance, the structural or conformational properties of the interstrand crosslinks formed by cisplatin and transplatin in DNA are identical. Here we summarize the data on cisplatin and transplatin interstrand crosslinks formed in DNA in cell-free media (Table 2) with the goal of showing how these lesions are structurally different.

The data in Table 2 show that there are considerable differences not only in the base residues preferentially involved in the interstrand crosslinks of cisplatin and its *trans* isomer, but also in the rate of their formation and confor-

mational alterations induced by the two interstrand adducts. Also importantly, the interstrand crosslinks of cisplatin are recognized by DNA binding proteins (HMG-domain proteins and T4 endonuclease VII), whereas those of transplatin are not (*see above*). Taken together, it cannot be excluded that the different clinical effectiveness of cisplatin and its *trans* isomer may also be associated with the differences in their DNA interstrand crosslinks.

## 8. FINAL REMARKS

The interstrand crosslink was the first bifunctional adduct of cisplatin detected *in vitro* and *in vivo* more than 25 years ago, and it was initially considered the main lesion associated with the antitumor effect of cisplatin. However, later much more attention was paid to the more frequent intrastrand crosslinks formed by cisplatin between neighboring purine residues, but the relative antitumor efficacy of intrastrand and interstrand crosslinks still remains unknown. In the following text we present several examples consistent with the hypothesis and supporting the view that interstrand crosslinks of cisplatin, although they are not the major adducts, play an important role in the mechanisms underlying the antitumor activity of cisplatin, or that at least the possibility of a contribution of interstrand crosslinks to these mechanisms cannot be ruled out.

1. Interstrand crosslinks of cisplatin strongly inhibit DNA transcription elongation by prokaryotic and eukaryotic RNA polymerases (87).

2. Increased gene-specific DNA repair efficiency of interstrand but not intrastrand crosslinks of cisplatin is associated with resistance of cells to the drug (88–91).

3. Interferon- $\alpha$  significantly increases the sensitivity to cisplatin of glioblastoma cells strongly resistant to the cytotoxic effect of this drug. Importantly, interferon- $\alpha$  alone is not cytotoxic in this cell line, but increases considerably the magnitude of cisplatin-induced DNA interstrand crosslinks (92).

4. Antitumor fludarabine nucleoside and arabinosyl-2-fluoroadenine (efficient DNA replication inhibitors) and topoisomerase I inhibitors synergistically enhance cisplatin-induced cytotoxicity *in vitro*, and the synergism parallels the inhibition of removal of cellular cisplatin-induced DNA interstrand crosslinks (93–95).

In general, interstrand crosslinks formed by various compounds of biologic significance are more inhibitory to DNA replication and transcription because of the damage sustained by both complementary strands, and the resultant severe blockages imposed on DNA-dependent polymerases. In addition, interstrand crosslinks are more difficult to repair, probably requiring both nucleotide excision and recombinational repair, and thus leading to greater cytotoxicity than that expected for monoadducts or intrastrand lesions (which can be repaired solely by nucleotide excision). The fact that interstrand crosslinks of cisplatin are more difficult to repair also suggests that cytotoxic effects of

these lesions could rely less on the recognition by and binding of DNA binding proteins, as they probably have to in the case of 1,2-intrastrand crosslinks. In addition, the importance of DNA interstrand crosslinks of cisplatin is reinforced since some regulatory sequences associated with proliferation are known to contain a high number of GC sequences at which cisplatin just preferentially forms interstrand crosslinks.

Exploring new structural classes of platinum antitumor drugs resulted in the discovery of dinuclear *bis*(platinum) complexes with equivalent coordination spheres, represented by the general formula  $[\text{PtCl}(\text{NH}_3)_2]_2(\text{H}_2\text{N-R-NH}_2)]^{2+}$ . (R is a linear alkane chain.) To date, the properties of *trans*- $[\text{PtCl}(\text{NH}_3)_2]_2(\text{H}_2\text{N-R-NH}_2)]^{2+}$  and *cis*- $[\text{PtCl}(\text{NH}_3)_2]_2(\text{H}_2\text{N-R-NH}_2)]^{2+}$  have been reported, including their high activity in vitro and in vivo in both murine and human tumor cell lines resistant to cisplatin (96–98). Importantly, these novel platinum drugs, structurally different from cisplatin, exhibit a spectrum of antitumor activity different from that of cisplatin, and also the major DNA adducts formed by these dinuclear platinum compounds are interstrand crosslinks (99). Moreover, these interstrand adducts are formed at sites that differ from those preferentially involved in the interstrand crosslinking by cisplatin, and conformational distortions induced in DNA by the interstrand crosslinks of dinuclear platinum compounds are markedly distinct from those induced by interstrand and intrastrand crosslinks of cisplatin (99).

Furthermore, in contrast to ineffective transplatin, platinum(II) complexes of the types *trans*- $[\text{PtCl}_2\text{L}_2]$  and *trans*- $[\text{PtCl}_2(\text{NH}_3)\text{L}]$  (L = planar N-donor) exhibit greatly enhanced cytotoxicity, including activity in cisplatin-resistant tumor cells (100). Interestingly, formal substitution of an  $\text{NH}_3$  ligand in transplatin by a planar base, such as quinoline, considerably enhances the interstrand crosslinking efficiency of these transplatin derivatives (100,101). The interstrand adduct formed by both *trans*- $[\text{PtCl}_2(\text{NH}_3)(\text{quinoline})]$  was identified as a 1,2-GG-interstrand crosslink in the sequence 5'-GC, thus being formally equivalent to that of cisplatin rather than transplatin (Neplechová, Kašpárková, Brabec, Bierbach and Farrell, submitted for publication). This result is unique and represents the first demonstration of alteration of a DNA binding site of an inactive drug into a DNA adduct characteristic of an active drug by simple chemical modification of the drug structure. The most intriguing finding proved to be the fact that DNA randomly platinated by *trans*- $[\text{PtCl}_2(\text{NH}_3)(\text{quinoline})]$  is recognized by cisplatin-specific antibodies that exhibit a strict requirement for 1,2-intrastrand crosslinked DNA (101). Thus, one or several unique adducts of the drug produce changes in DNA conformation that efficiently mimic the most frequently formed cisplatin-DNA adduct.

Understanding the formation of DNA interstrand crosslinks of different platinum antitumor compounds is a challenge, not only from the mechanistic view, but also from the therapeutic one. Comprehension of the physiologic roles of

DNA interstrand crosslinks of antitumor platinum compounds in functions of tumor cells requires integration of inorganic chemistry, molecular and cell biology and pharmacology. A coordinated effort in the research of interstrand crosslinks induced by genotoxic agents is under way to resolve the remaining problems of their structures, cellular processing, and biologic significance.

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