Activation of Trans Geometry in Bifunctional Mononuclear Platinum Complexes by a Piperidine Ligand

MECHANISTIC STUDIES ON ANTITUMOR ACTION*§

Jana Kasparekova‡‡, Olga Novakova‡, Victoria Marini‡, Yousef Najajreh‡‡*, Dan Gibson‡‡*, Jose-Manuel Perez‡‡, and Viktor Brabec‡

From the ‡Institute of Biophysics, Academy of Sciences of the Czech Republic, CZ-61265 Brno, Czech Republic, §Department of Medicinal Chemistry and Natural Products, School of Pharmacy and the **David R. Bloom Center for Pharmacy, The Hebrew University of Jerusalem, Jerusalem 91120, Israel, and ¶Departamento de Química Inorgánica, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain

A paradigm for the structure-pharmacological activity relationship of bifunctional platinum antitumor drugs is that the trans isomer of antitumor cisplatin (transplatin) is clinically ineffective. To this end, however, several new complexes of the trans structure have been identified that exhibit cytotoxicity in tumor cells that is even better than that of the analogous cis isomers. We reported recently (Kasparekova, J., Marini, V., Najajreh, Y., Gibson, D., and Brabec, V. (2003) Biochemistry 42, 6321–6332) that the replacement of one ammine ligand by the heterocyclic ligand, such as piperidine, piperazine, or 4-picoline in the molecule of transplatin resulted in a radical enhancement of its cytotoxicity. We examined oligodeoxyribonucleotide duplexes bearing a site-specific cross-link of the transplatin analogue containing the piperidine ligand by biochemical methods. The results indicate that in contrast to transplatin, trans-(PtCl2(NH3)(piperidine)) forms stable 1,3-intrastrand cross-links in double-helical DNA that distort DNA and are not readily removed from DNA by nucleotide excision repair system. Hence, the intrastrand cross-links of trans-(PtCl2(NH3)(piperidine)) could persist for a sufficiently long time, potentiating its toxicity toward tumor cells. trans-(PtCl2(NH3)(piperidine)) also forms in DNA minor interstrand cross-links that are similar to those of transplatin so that these adducts appear less likely candidates for genotoxic lesion responsible for antitumor effects of trans-(PtCl2(NH3)(piperidine)). Hence, the role of structurally unique intrastrand cross-links in the antitumor effects of transplatin analogues in which one ammine group is replaced by a heterocyclic ligand may predominate.

The more widespread clinical applicability of cis-diaminedichloroplatinum(II) (cisplatin)† (Fig. 1A) and its analogue cis-diamminecyclobutanedicarboxylatoplatinum(II) is limited to a relatively narrow range of tumors (1–3); some tumors have natural resistance to these platinum drugs, whereas others develop resistance after the initial treatment. Cisplatin also has limited solubility in aqueous solution and is administered intravenously. There are also significant problems in terms of inducing severe side effects (especially kidney damage and vomiting/nausea). The drawbacks coupled with cisplatin and cis-diamminecyclobutanedicarboxylatoplatinum(II) toxicity have been the impetus for the development of improved platinum drugs.

The paradigm for structure-pharmacological activity relationship of platinum complexes is that the trans isomer of cisplatin (transplatin) (Fig. 1A) is clinically ineffective. To this end, however, several new complexes of the trans structure have been identified that exhibit an enhanced cytotoxicity in tumor cell lines, such that cytotoxicity is equivalent or even better than that of the analogous cis isomers and, indeed, cisplatin itself (for reviews, see Refs. 4–6). Examples of such new antitumor transplatinum compounds are (i) analogues containing planar amine ligand of general structure trans-(PtCl2(NH3)L), where L represents planar amine such as quinoline or thiazole; (ii) analogues containing iminoether groups of the general formula trans-(PtCl2(E-iminoether)2) (trans-EE); and (iii) analogues with asymmetric aliphatic ligands and trans-(PtCl2(NH3)L), where L represents cyclohexylamine.

We have recently reported (7–9) that the replacement of one ammine ligand by the heterocyclic ligand, such as piperidine (pip) (Fig. 1A), piperazine, or 4-picoline in the molecule of transplatin results in a radical enhancement of its activity in tumor cell lines both sensitive and resistant to cisplatin. For instance, the IC50 (the concentration of the compound that afforded 50% cell killing) of trans-(PtCl2(NH3)(pip)) in the A2780 cell line was more than 40 times lower than that of transplatin. Since DNA is considered the major pharmacological target of antitumor platinum drugs (10), it appears important that due to this replacement, the analogues of transplatin alter the properties of DNA in a markedly different way than the parent compound. This is an intriguing finding, because the.

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† To whom correspondence should be addressed. Tel.: 420-541517174; Fax: 420-541240499; E-mail: jana@ibp.cz.

‡‡ The abbreviations used are: cisplatin, cis-diaminedichloroplatinum(II); transplatin, trans-diaminedichloroplatinum(II); pip, piperi-
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EXPERIMENTAL PROCEDURES

Chemicals—trans-(PtCl₂(NH₃)(pip)) or 1,2-GG intrastrand CL of cisplatin were prepared in the top strand (the duplexes in Fig. 1B) containing two guanine (G) residues in the top strand were prepared as described (9, 19, 20). The interstrand cross-linked duplexes were also prepared and characterized in the same way as described previously (21, 22).

Inhibition of DNA Polymerization—The duplexes containing single, intrastrand CL of cisplatin, transplatin, or trans-(PtCl₂(NH₃)(pip)) in the top strand (the duplexes in Fig. 1B) contained two guanine (G) residues in the top strand prepared as described (9, 19, 20). The interstrand cross-linked duplexes were also prepared and characterized in the same way as described previously (21, 22).

Measurements of Platinum Accumulation in CHI/ir Cells and Determination of Platinum Binding to DNA in Culture Cells—All details of these experiments were recently published (17, 18).

Platinations of Oligonucleotides—The duplexes containing single, intrastrand CL of cisplatin, transplatin, or trans-(PtCl₂(NH₃)(pip)) in the top strand (the duplexes in Fig. 1B) contained two guanine (G) residues in the top strand were prepared as described (9, 19, 20). The interstrand cross-linked duplexes were also prepared and characterized in the same way as described previously (21, 22).

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same replacement in the molecule of anticancer cisplatin (Fig. 1A) results in a reduced activity of the drug in both sensitive and resistant cell lines (for instance, the IC₅₀ measured in A2780 cell line was 12 times higher than that of cisplatin (9)). Thus, the latter observation represents an additional example of activation of trans geometry in bifunctional mononuclear platinum(II) compounds, although the reasons for this activation have not been completely clarified.

Preliminary mechanistic studies using the analogues of transplatin containing piperidine, piperezine, or 4-picoline nonleaving ligand (9) suggest that one strategy how to activate trans geometry in platinum anticancer drugs. These results are of fundamental importance, because the clinical ineffectivity of transplatin has been proposed to be associated with a low stability of its intrastrand CLs in double-helical DNA and in general with its reduced capability to form in double-helical DNA bifunctional adducts (12, 13). Because structural details of individual DNA adducts formed by these analogues of transplatin are not yet available, it remains uncertain how these CLs affect conformation of DNA and how these alterations are further processed in the cells. Therefore, in order to shed light on the mechanism that underlies activity of transplatin analogues containing a heterocyclic ligand we examine in the present work in detail short oligodeoxyribonucleotide duplexes containing single, site-specific intrastrand or interstrand CL of the transplatin analogue containing the nonplanar piperidine ligand (Fig. 1A). The piperidine analogue of transplatin was chosen as the representative of this class of new platinum compounds. This choice was made because the changes in the activity in cancer cell lines and some features of its DNA binding mode in a cell-free medium due to the replacement of one ammine ligand by the heterocyclic group in the parent compound were most pronounced (9). We investigated how the CLs affect the local conformation of DNA (in particular, bending and unwinding) and how these adducts are stable in double-helical DNA and further processed by some cellular components in cell-free media.

Antitumor activity of platinum compounds is also affected by the factors that do not operate directly at the level of DNA adducts. Among these factors are also those that affect the amount of platinum complex that can reach target DNA in cancer cells by changing the cell accumulation of the complexes. Therefore, we also compared the cellular uptake of transplatin and its analogue containing the piperidine ligand and determined the amount of platinum bound to DNA in the cells treated with these platinum compounds.

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in vitro repair was measured with excision assay using these CFEs and 148-bp linear DNA substrates (see above) in the same way as described previously (26).

RESULTS

Cellular Platinum Complex Uptake—Platinum complexes of novel structure may have an altered pharmacology associated with drug uptake that may affect their cytotoxicity. Therefore, we examined first how the replacement of the ammine group in transplatin by piperidine ligand affects its accumulation in tumor cell lines.

Results in CH1cisR cells (this cancer cell line was among those used for testing activity of the novel transplatin analogues in which trans-(PtCl2(NH3)(pip)) exhibited a markedly enhanced activity in comparison with transplatin (9)) show that the replacement of the ammine group by the piperidine ligand considerably reduces the amount of platinum associated with the cells (Fig. 2A). Given that significantly less trans-(PtCl2(NH3)(pip)) appears to be accumulated in cells, the question arises as to how the piperidine ligand affects the binding of transplatin analogues to DNA in cells. Results in Fig. 2B demonstrate that trans-(PtCl2(NH3)(pip)) binds to DNA in CH1cisR cells treated with this transplatin analogue more slowly and to a lesser extent than the parent complex. This is in contrast to the data showing that transplatin and trans-(PtCl2(NH3)(pip)) bind to DNA in a cell-free medium with approximately the same rate (9). Hence, it is reasonable to suggest that the slower binding rate and the lesser binding level of trans-(PtCl2(NH3)(pip)) to DNA in culture cells is mainly a consequence of its lower intracellular concentration.

Fig. 2 shows that the markedly enhanced cytotoxicity of trans-(PtCl2(NH3)(pip)) in comparison with transplatin (9) is not due to either enhanced cellular accumulation or higher levels of DNA platination in the cells. Since the cytotoxicity cannot be correlated with the levels of DNA modification, it is intuitively appealing to suggest that the enhanced cytotoxicity may be related to the nature of the adducts that are formed with the DNA and to the effects of the altered DNA properties on downstream cellular events.
Intrastrand Cross-links—Unlike cisplatin, due to steric reasons, transplatin and its analogues cannot form intrastrand CLs in double-helical DNA between adjacent base residues. The trans compounds can cross-link two bases on the same strand only if they are separated by at least one intervening base, forming mostly 1,3-GTGG intrastrand CLs (where N represents adenine, cytosine, or thymine). These adducts formed by the antitumor analogues of transplatin, in which the ammine group was replaced by the heterocyclic ligand, are stable in double-helical DNA and represent major DNA adducts of this class of antitumor trans compounds. It was, therefore, of great interest to examine how single, site-specific 1,3-GTG intrastrand CL of trans-(PtCl2(NH3)(pip)) affects configuration of double-helical DNA and what are some subsequent “downstream” effects of this type of DNA damage, such as recognition by some damaged DNA-binding proteins and repair capacity for this lesion of eukaryotic nucleotide excision repair (NER) system.

Important structural motifs induced in DNA by antitumor platinum compounds that play a significant role in the mechanism underlying their antitumor activity are the bending and unwinding of the helix axis (27). For DNA intrastrand adducts of cisplatin and transplatin, the structural details responsible for bending and unwinding have been elucidated (28, 29). In this work we further performed studies on the bending and unwinding induced by single, site-specific intrastrand CL of trans-(PtCl2(NH3)(pip)) using electrophoretic retardation as a quantitative measure of the extent of planar curvature.

The oligodeoxyribonucleotide duplexes TGTGT(19–22) (19–22 bp long, whose sequences were identical or similar to that of the duplex TGTGT (21) shown in Fig. 1B; the 19- and 20-bp duplexes had one or two marginal C-G pairs deleted, respectively, whereas one additional T-A pair was added to one end in the 22-bp duplex) were used for the bending and unwinding studies of the present work. The ligation products of these unplatinated or CL-containing duplexes were analyzed on native PAA electrophoresis gel. Experimental details of these studies are given in our recent reports (20, 30, 31). The DNA bending toward the minor groove and unwinding due to one 1,3-intrastrand adduct of trans-(PtCl2(NH3)(pip)) has been found 30° ± 2 and 8° ± 2°, respectively (the direction of the bend was determined using the duplex TGTGT + (A/T)20 (32) (Fig. 1B) in the same way as in our recent papers (21, 32, 33)). Moreover, the ligation of the 21- and 22-bp duplexes containing 1,3-intrastrand CL of trans-(PtCl2(NH3)(pip)) resulted in the formation of circles, suggesting that the 1,3-intrastrand CLs of this transplatin analogue increased the flexibility of the double helix (29, 34).

Further studies of the present work were focused on analysis of the distortion induced by the 1,3-intrastrand CL of trans-(PtCl2(NH3)(pip)) by chemical probes of DNA conformation. The duplex TGTGT (Fig. 1B) containing a single, site-specific adduct was treated with several chemical agents that are used for monitoring the existence of conformations other than canonical B-DNA. These agents included KMnO4, diethyl pyrocarbonate, and bromine. They react preferentially with single-stranded DNA and distorted double-stranded DNA (34, 35). We used for this analysis exactly the same methodology described in detail in our recent papers dealing with DNA adducts of various antitumor platinum drugs (21, 31, 34, 36, 37). Therefore, these experiments are only described in more detail in the Supporting material (see “Chemical Probes” and Fig. S2). The results schematically summarized in Fig. 3A indicate that 1,3-intrastrand CL of trans-(PtCl2(NH3)(pip)) induces in DNA the distortion that extends over at least 6 bp and is localized mainly at the base pair between the platinated G residues and the base pair on its 5' side.

It has been demonstrated that various DNA secondary structures have significant effects on processivity of a number of prokaryotic, eukaryotic, and viral DNA polymerases (38, 39). Interestingly, with DNA templates containing site-specifically added adducts of various platinum compounds, a number of prokaryotic and eukaryotic DNA polymerases were blocked but could also traverse through platinum adducts, depending on their character and conformational alterations induced in DNA. It is therefore of great interest to examine whether DNA polymerases, processing DNA substrates containing either the 1,3-intrastrand CL of trans-(PtCl2(NH3)(pip)) or 1,2-GG intrastrand CL of cisplatin, could reveal potential differences in conformational alterations imposed on DNA by these two adducts. We investigated in the present work DNA polymerization using the templates site-specifically modified by trans-(PtCl2(NH3)(pip)) or cisplatin by two DNA polymerases, which differ in processivity and fidelity. In the first series of our experiments, we used Klenow fragment of DNA polymerase I as a model enzyme frequently used in the studies aimed at understanding the processes in which nucleic acid polymerases take part.

We constructed the 8-mer/23-mer primer-template duplexes TGTGT(KF) and TGGT(KF) (Fig. 4A) unplatinated or containing either 1,3-GTG intrastrand CL of trans-(PtCl2(NH3)(pip)) in the central TGTGT sequence or 1,2-GG intrastrand CL of cisplatin in the central TGTGT sequence. The first 8 nucleotides on the 3' terminus of the 23-mer template strand were complementary to the nucleotides of the 8-mer primer, and the 3' guanine involved in the 1,3-GTG CL of trans-(PtCl2(NH3)(pip))
or in 1,2-GG CL of cisplatin on the template strand was located at its 13th position from the 3' terminus (Fig. 4A). After annealing the 8-nucleotide primer to the 3' terminus of the un-platinated or platinated template strand (positioning the 3'-end of the primer five bases before the adduct in the template strand), we examined DNA polymerization through the single

![Diagram of primer extension activity of exonuclease-deficient Klenow fragment of DNA polymerase I (A) and reverse transcriptase from the human immunodeficiency virus type 1 (B).](image)

**Fig. 4.** Primer extension activity of exonuclease-deficient Klenow fragment of DNA polymerase I (A) and reverse transcriptase from the human immunodeficiency virus type 1 (B). The experiments were conducted using the 8-mer/23-mer (A) or 17-mer/30-mer primer-template (B) duplexes for the times indicated. Lanes 1–5, undamaged template; lanes 6–10, the template containing 1,2-GG intrastrand CL of cisplatin; lanes 11–15, the template containing 1,3-GTG intrastrand CL of trans-(PtCl₂(NH₃)(pip)). The strong pause sites opposite the platinated guanines were marked 12 and 13 in A or 20, 21, and 22 in B.
1,3-CL of trans-(PtCl₂(NH₃)₂(pip)) or 1,2-intrastrand CL of cisplatin on the template by KF – in the presence of all four deoxyribonucleoside 5’-triphosphates. The reaction was stopped at various time intervals, and the products were analyzed using a sequencing gel (Fig. 4A). Polymerization using the template containing the CL of cisplatin proceeded rapidly up to the nucleotide preceding and at the sites opposite the CL, such that the 12- and 13-nucleotide products accumulated to a significant extent (shown in Fig. 4A, lanes 6–10). There was only a slight accumulation of larger DNA intermediates, whereas no intermediate products were seen with the 23-mer control template as the full-length product was being formed (shown in Fig. 4A, lanes 1–5). The full-length products were also noticed with the 23-mer template containing the CL of cisplatin, although in a smaller amount. This result is in agreement with previously published work (40) in which T7 DNA polymerase and RT HIV-1 were used and confirms that 1,2-GG intrastrand CL of cisplatin inhibits DNA synthesis (38), but translesion synthesis may occur. In contrast, under the same experimental conditions, DNA polymerization by KF – using the template containing the 1,3-intrastrand CL of trans-(PtCl₂(NH₃)₂(pip)) proceeded up to the nucleotide preceding and at the site opposite the 3’ G involved in the CL (Fig. 4A, lanes 11–15). There was almost no accumulation of shorter and larger DNA intermediates, and importantly, no full-length products accumulated. This result indicates that the character of the 1,3-GTG intrastrand CLs of trans-(PtCl₂(NH₃)₂(pip)) and alterations induced in DNA by this adduct are distinctly different from the features of the major adduct of cisplatin so that the adducts of trans-(PtCl₂(NH₃)₂(pip)) could potentially impede elongation of DNA to a higher extent than the major adducts of cisplatin (Fig. 4A).

We have also examined the effects of the 1,3-intrastrand CL of trans-(PtCl₂(NH₃)₂(pip)) on polymerization by RT HIV-1. This enzyme also possesses DNA template-dependent DNA polymerase activity but relatively low processivity and fidelity (41). In these studies, elongation of the 17-mer/30-mer primer-template duplexes was tested. As is demonstrated in Fig. 4B, we confirmed also by using this DNA polymerase showing a different mechanism underlying its catalytic activity than KF – that the 1,3-intrastrand CL of trans-(PtCl₂(NH₃)₂(pip)) constitutes a fairly strong block to DNA synthesis catalyzed by both DNA polymerases. Since there is a high degree of structural and sequence conservation of the domains among eukaryotic, prokaryotic, and viral polymerases (42), insights gleaned from studies of the KF – and RT HIV-1 should be also applicable to other DNA polymerases (43–45). Hence, the repercussion of stronger inhibition of DNA polymerization by the 1,3-intrastrand CL of trans-(PtCl₂(NH₃)₂(pip)) in comparison with the major adduct of cisplatin adds a new dimension to the impact of the activated trans geometry in platinum compounds on biological processes, possibly including replication or DNA repair.

An important feature of the mechanism that underlies the antitumor activity of cisplatin and its analogues is that the major adducts of these drugs (1,2-GG intrastrand CLs) are recognized by proteins containing HMG domains (27). Importantly, DNA modified by transplatin or monodentate platinum(II) compounds, such as chlorodihydrinetriniaminoplatinum(II) chloride or (PtCl(NH₃)₂)Cl, is not recognized by these cellular proteins. We examined whether also the replacement of the ammine group in transplatin by piperidine (resulting in the enhancement of cytotoxicity in tumor cell lines) also affects affinity of HMG-box proteins to the intrastrand adduct of this transplatin analogue. The interactions of the rat HMGB1 domain A (HMGB1a) and HMGB1 domain B (HMGB1b) with the 1,3-GTG intrastrand CLs of trans-(PtCl₂(NH₃)₂(pip)) were investigated using a gel mobility shift assay (46, 47). In these experiments (described in more detail in Supplemental Mate-
These results are consistent with the view that the low efficiency of the mammalian NER system to recognize this type of platinum damage.

**Interstrand Cross-linking**—Frequent, although not major, adducts formed by bifunctional antitumor analogues of transplatin containing a heterocyclic ligand are interstrand CLs (9, 60). Interestingly, transplatin forms these CLs preferentially between G and complementary C residues (19), whereas quite surprisingly its analogues, such as trans-(PtCl₂(NH₃)(thiazole)) (i.e. the trans complexes containing a planar heterocyclic ligand) form these CLs between G residues in the 5’-GC/5’-GC sequences (22) (i.e. “cisplatin-like” interstrand CLs) (61). Therefore, it was of interest to unambiguously identify the residues involved in the interstrand CL formed by trans-(PtCl₂(NH₃)(pip)). Diaminedichloroplatinum(II) complexes react with DNA in a two-step process (62). Monofunctional adducts are formed preferentially at N-7 atoms of G residues. These lesions subsequently close to bifunctional CLs (intrastand and/or interstrand). Considering this fact, we have designed synthetic oligodeoxyribonucleotide duplexes (duplexes CGC, TGC, and TGT in Fig. 1B). The pyrimidine-rich top strands of these duplexes contain a unique G residue at which the monofunctional adduct of transplatin or its piperidine analogue was formed. Thus, the choice of this nucleotide allowed for a cross-linking study under competitive conditions (i.e. interstrand CLs were in principle possible: in the CGC duplex, between the central G in the top strand and either complementary C or adjacent 5’ or 3’ Gs on the opposite strand; in the case of the TGC duplex, between the central G in the top strand and complementary C or adjacent 5’ G in the bottom strand; in the case of the TGT duplex, between the central G in the top strand and complementary C. The top strands of the duplexes containing the monofunctional adduct of transplatin or trans-(PtCl₂(NH₃)(pip)) were hybridized with their complementary (bottom), 5’-end ³²P-labeled strands. The mixtures were incubated at 37 °C in 0.1 M NaClO₄, and the aliquots were withdrawn at various time intervals and subjected to gel electrophoresis under denaturing (strand-separat-
The interstrand adducts formed by transplatin or cisplatin were also prepared and analyzed using both human and rodent excinuclease. No excision products were detected under conditions when 1,2- or 1,3-intrastrand CLs were readily excised (shown in Fig. 5A, lanes 10 and 12 for the CLs treated with rodent excinuclease).

**DISCUSSION**

The results of the present work (Fig. 2) demonstrate that the replacement of one of the ammine group in transplatin results in the reduced cellular accumulation of this drug in comparison with the parent compound, which, however, does not correlate with its markedly enhanced activity in the cancer cell lines (9). It is therefore reasonable to expect that there are other biochemical factors dominating the mechanism of action of transplatin analogues containing a heterocyclic nonleaving ligand, such as piperidine in tumor cells. DNA is a major pharmacological target of platinum compounds (10). Hence, among these factors might be also those associated with the modulation of the platinum-DNA interaction, with subsequent effects on further “downstream” effects of damaged DNA, such as for instance repair capacity for the platinum-DNA lesions (11).

The results of the present work were focused on analysis of the distortion induced by the interstrand CL of trans-(PtCl₂(NH₃)(pip)) by chemical probes of DNA conformation also in the same way as described in the present work for 1,3-intrastrand CL (see above). The results described in more detail in the Supplemental Material (see “Chemical Probes” and Fig. S3) and summarized in Fig. 3. B and C, indicate that this adduct induces in DNA the distortion that extends over at least 5 bp and is localized mainly at the platinated base pairs. The distortion induced by the CL of trans-(PtCl₂(NH₃)(pip)) was stronger than that induced by the parent compound.

We also examined how the interstrand CL of trans-(PtCl₂(NH₃)(pip)) is recognized by DNA-binding proteins, such as HMGB1a domain and RPA and XPA proteins using the same experimental approach as demonstrated in the present work for 1,3-intrastrand CL of this transplatin analogue. The data indicate that HMGB1a, HMGB1b (Fig. S4, lanes 11 and 12), and RPA proteins (Fig. 6A) do not bind the probe containing the interstrand CL of trans-(PtCl₂(NH₃)(pip)). XPA exhibited affinity to the probe containing this lesion, but it was markedly lower than that to the probe containing the 1,2-GG intrastrand CL of cisplatin (~50%) (Fig. 6B).

Excision repair substrates containing a site-specific interstrand CL of trans-(PtCl₂(NH₃)(pip)) or transplatin were also prepared and analyzed using both human and rodent excinuclease. No excision products were detected under conditions when 1,2- or 1,3-intrastrand CLs were readily excised (shown in Fig. 5A, lanes 10 and 12 for the CLs treated with rodent excinuclease).

**Fig. 7. Hydroxyl radical footprinting of interstrand cross-links.** Shown is an autoradiogram of denaturing 24% PAA/8 M urea gel of the products of the reaction between hydroxyl radicals and the duplex TGC either unmodified or containing an interstrand CL of trans-(PtCl₂(NH₃)(pip)). The top (A) or bottom (B) strand was 5′-end-labeled. noPt lane, unincubated duplex; transPt lane, the duplex containing interstrand CL of transplatin; trans-pip lane, the duplex containing interstrand CL of trans-(PtCl₂(NH₃)(pip)); G lane, a Maxam-Gilbert-specific reaction for the unplatinated duplex. For other details, see “Results.”
stable intrastand CLs, and this property of transplatin has been related to its clinical inefficiency (13, 68). We have demonstrated in our recent work (9) that the replacement of one ammine ligand in "classical" transplatin by piperidine, pipera-
zine, or 4-picoline ligand results in a distinctively enhanced
stability of the 1,3-GNG intrastrand CLs formed by these com-
ponents in several sequence contexts in short oligodeoxyribo-
nucleotide duplexes. This result correlates with the markedly
enhanced activity of these transplatin analogues in tumor cell
lines.

The characteristics of this most frequent 1,3-intrastrand CL
of trans-(PtCl2(NH3)(pip)) are summarized and compared with
the interstrand CLs of this compound and transplatin and
1,2-GG intrastrand CL of cisplatin in Table I. There are several
reports (12, 26, 29, 69) describing properties of short oligodeoxy-
ribonucleotide duplexes containing single, site-specific 1,3-
intrastrand CLs of transplatin. These CLs were, however,
formed in the sequence TGTGT in which these adducts are
unable in double-helical DNA and readily isomerize in inter-
strand CLs (9, 67). Thus, no reliable data on 1,3-intrastrand
CLs of transplatin in double-helical DNA are available so that
a comparison of the 1,3-intrastrand CLs of trans-
(PtCl2(NH3)(pip)) and transplatin is impossible.

The bending experiments were carried out with the oligode-
rinucleotide duplexes containing the unique intrastand CL of trans-(PtCl2(NH3)(pip)) in their central sequence. The phasing assay has revealed that the 1,3-GTG intrastand CL results in a directional bending of helix axis (30° toward the
minor groove) and a relatively small duplex unwinding (8°). In
addition to these bending and unwinding effects, the 1,3-intra-
strand CLs formed by trans-(PtCl2(NH3)(pip)) create rather
extensive local conformational distortions revealed by the
chemical probes extending over 6 bp (Fig. 3A).

It has been suggested (46, 70) that HMG domain proteins
play a role in sensitizing cells to cisplatin. It has been shown
that HMG domain proteins recognize and bind to DNA CLs
formed by cisplatin between bases in neighboring base pairs
(46, 70, 71). The molecular basis for this recognition is still not
entirely understood, although several structural details of the
1:1 complex formed between HMG domain and the duplex
containing 1,2-GG intrastand CL of cisplatin were recently
ecluciated (46). The details of how the binding of HMG domain
proteins to cisplatin-modified DNA sensitizes tumor cells to
cisplatin are also still not completely resolved, but possibilities
such as shielding cisplatin-DNA adducts from excision repair
or that these proteins could be recruited from their native
transcriptional regulatory function have been suggested (67, 70,
72, 73) as clues for how these proteins are involved in the
antitumor activity.

An important structural motif recognized by HMG domain
proteins on DNA containing the major 1,2-GG intrastand CL
of cisplatin is a stable, directional bend of the helix axis toward
the major groove. As demonstrated in the present work (Table
I) the 1,3-GTG intrastand CL of trans-(PtCl2(NH3)(pip)) bends
the helix axis almost as efficiently as the intrastand CLs of
cisplatin (29, 74). However, no recognition of DNA intrastand
CL of trans-(PtCl2(NH3)(pip)) by HMG1 proteins was ob-
erved in the present work. A plausible explanation of this
observation may be that the binding due to the 1,3-intrastrand
CL of trans-(PtCl2(NH3)(pip)) is in the opposite direction from
that due to the 1,2-intrastrand CL of cisplatin. Hence, it is
possible that trans-(PtCl2(NH3)(pip)) in the 1,3-intrastrand
CL prevents the DNA bending toward the major groove required
for its accommodation in the complex with HMG1 protein.
Thus, from the results of the present work, it is clear that the
data intrastand CLs of antitumor trans-(PtCl2(NH3)(pip))
are not a substrate for recognition by HMG domain proteins (Fig.
S4). From these considerations and from the fact that also
intrastand CLs of trans-(PtCl2(NH3)(pip)) are not recognized
by HMG domain proteins, we could conclude that the mecha-
nism of antitumor activity of trans-(PtCl2(NH3)(pip)) does not
involve recognition of its DNA adducts by HMG domain pro-
teins as a crucial step, in contrast to the proposals for cisplatin
and its direct analogues (70).

Several reports have demonstrated (26, 52, 53) that NER is
a major mechanism contributing to cisplatin resistance. The
examinations of excision repair of 1,3-intrastrand CL of trans-
(PtCl2(NH3)(pip)) have revealed that these adducts cannot be
removed so readily by excision repair as intrastand adducts of
antitumor cisplatin (Fig. 5). Hence, the intrastand CLs of bifunc-
tional trans-(PtCl2(NH3)(pip)) would not have to be
shielded by damaged DNA recognition proteins, such as those
containing HMG domains, as efficiently as 1,2-intrastrand CLs
of cisplatin to prevent their repair. It is reasonable to suggest
that intrastand CLs of trans-(PtCl2(NH3)(pip)) could persist
for a sufficiently long time even without being shielded by
HMG box proteins, which would potentiate its toxicity toward
tumor cells sensitive to this drug.

| Summary and comparison of basic characteristics of DNA cross-links of trans-(PtCl2(NH3)(pip)) (trans-pip), transplatin, and cisplatin |
|---------------------------------------------------------------|
| Frequency (%) | 1,3-Intrastrand CL of trans-pip | Interstrand CL of trans-pip | Interstrand CL of transplatin | 1,3-Intrastrand CL of cisplatin |
| Reactivity of chemical probes (bp) | ~60° | 26° | ~12° | ~90° |
| DNA bending (degrees) | ~30° toward minor groove | ~26–30° toward minor groove | ~20° toward minor groove | ~32–34° toward major groove |
| DNA unwinding (degrees) | –8 | –20 | –12° | 13° |
| HMGB1 recognition | No | No | No | Strong |
| RPA recognition | Weak | No | No | Medium |
| XPA recognition | No | Weak | Weak | Medium |
| NER by eukaryotic excinuclease | Weak | No | No | Medium |
| Translesion DNA synthesis | No | No | No | ND |

* Data from Ref. 9.
* Data from Ref. 19.
* Data from Ref. 75.
* Determined by gel electrophoresis.
* Data from Ref. 34.
* Data from Ref. 66.
* Data from Ref. 29.
* Data from Ref. 71.
* Data from Ref. 76.
* ND, not determined.
trans-(PC16Cl2(NH2)(pip)) also forms in DNA minor strand CLs. Their basic characteristics are also summarized in Table I and compared with those of transplatin. The properties of interstrand CLs of trans-(PC16Cl2(NH2)(pip)) and clinically ineffective transplatin investigated in the present work are very similar. The only more pronounced difference consists in a higher efficiency of trans-(PC16Cl2(NH2)(pip)) to form this type of the adduct. Despite this difference, the interstrand CLs remain minor adducts of trans-(PC16Cl2(NH2)(pip)). Hence, the interstrand CLs are probably less likely candidates for genotoxic lesion responsible for antitumor effects of this compound. Nonetheless, the cytotoxic effects of trans-(PC16Cl2(NH2)(pip)) may realistically be due to a cumulative effect of the structurally heterogeneous adducts produced by this drug, but the role of structurally unique intrastrand CLs in the antitumor effects of transplatin analogues in which one ammine group is replaced by a heterocyclic ligand may predominate.

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