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Conformation, protein recognition and repair of DNA interstrand and intrastrand cross-links of Antitumor *trans*-[$PtCl_2(NH_3)$(thiazole)]

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ABSTRACT

Replacement of one ammine in clinically ineffective *trans*-[$PtCl_2(NH_3)_2$] (transplatin) by a planar N-heterocycle, thiazole, results in significantly enhanced cytotoxicity. Unlike 'classical' cisplatin {*cis*-[$PtCl_2(NH_3)_2$]} or transplatin, modification of DNA by this prototypical cytotoxic transplatinum complex *trans*-[$PtCl_2(NH_3)$(thiazole)] (*trans*-PtTz) leads to monofunctional and bifunctional intra or interstrand adducts in roughly equal proportions. DNA fragments containing site-specific bifunctional DNA adducts of *trans*-PtTz were prepared. The structural distortions induced in DNA by these adducts and their consequences for high-mobility group protein recognition, DNA polymerization and nucleotide excision repair were assessed in cell-free media by biochemical methods. Whereas monofunctional adducts of *trans*-PtTz behave similar to the major intrastrand adduct of cisplatin [J. Kasparkova, O. Novakova, N. Farrell and V. Brabec (2003) *Biochemistry*, 42, 792–800], bifunctional cross-links behave distinctly differently. The results suggest that the multiple DNA lesions available to *trans*-planaramine complexes may all contribute substantially to their cytotoxicity so that the overall drug cytotoxicity could be the sum of the contributions of each of these adducts. However, acquisition of drug resistance could be a relatively rare event, since it would have to entail resistance to or tolerance of multiple, structurally dissimilar DNA lesions.

INTRODUCTION

The clinical inactivity of *trans*-diamminedichloroplatinum(II) (transplatin) (Figure 1A) is a paradigm for the structure–activity relationships of platinum drugs. A structurally diverse set of transplatin analogues exhibiting different spectra of cytotoxicity, including activity in tumor cells resistant to cisplatin [*cis*-diamminedichloroplatinum(II)] (Figure 1A), have been identified by replacement of one or both NH_3 ligands (1–3). There is a large body of experimental evidence that the success of platinum complexes in killing tumor cells results from the formation of covalent adducts on DNA (4). Hence, it is reasonable to hypothesize that the reasons for the enhanced pharmacological properties and activation of bifunctional mononuclear *trans*-platinum(II) compounds is that they bind to DNA in a manner fundamentally different from that of the clinically ineffective 'parent' transplatin.

Cytotoxic *trans*-platinum(II) complexes whose DNA-binding modes have been intensively investigated include complexes of the general structure *trans*-[$PtCl_2(NH_3)$(L)], where L = planar amine such as pyridine, thiazole (Figure 1A) or quinoline (1). To understand the mechanism of cytotoxicity, biochemical and biophysical methods as well as molecular modeling techniques were used to study the modifications of natural, high molecular mass DNA by *trans*-[$PtCl_2(NH_3)$(thiazole)] (*trans*-PtTz) (Figure 1A) or *trans*-[$PtCl_2(NH_3)$(quinoline)] (*trans*-PtQ) (5–8). The adducts of these compounds preferentially terminate *in vitro* RNA synthesis in transcription mapping experiments at guanine residues and at similar sites as the adducts of cisplatin (5). DNA modified by these analogues is recognized by cisplatin-specific antibodies but not by transplatin-specific antibodies, suggesting that these newer analogues behave in some aspects similar to cisplatin. Importantly, the planar ligand in all or in

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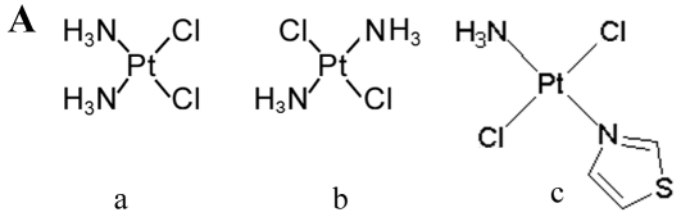

Figure 1. Structures of platinum compounds and sequences of the synthetic oligodeoxyribonucleotides with their abbreviations. (**A**) Structures: a, cisplatin; b, transplatin; and c, (*trans*-PtTz). (**B**) Sequences: the top and bottom strands of each pair in the figure are designated 'top' and 'bottom,' respectively, throughout. The boldface letters in the top and bottom strands of the duplexes indicate the platinated residues.

a significant fraction of covalent DNA adducts of these transplatin analogues is well positioned to interact with the duplex, presumably through stacking interactions.

Monofunctional DNA adducts formed by *trans*-PtTz and *trans*-PtQ are formed at a rate similar to that of transplatin (5). The rate of rearrangement to bifunctional adducts is relatively slow but similar to that observed for transplatin. In contrast to transplatin, however, the analogues with planar ligands form considerably more interstrand cross-links (CLs) (~30–40% after 48 h) with a much shorter half-time ($t_{1/2}$) ~5 h (5) [~12% interstrand CL is formed by transplatin after 48 h with $t_{1/2} > 11$ h (9)]. Further, the interstrand CLs are formed between guanine residues at the 5′-GC/5′-GC sites—formally equivalent to cisplatin but different from transplatin, which forms these adducts between complementary guanine and cytosine residues (9). A significant fraction of the monofunctional adducts persist over time (30–40% after ~48 h) so that 20–40% of the total adducts are intrastrand, presumably mainly 1,3 CLs (5). Thus, no one specific adduct, i.e. monofunctional or bifunctional interstrand and intrastrand CLs, represents a major lesion. Formation of one or all of these adducts may be relevant to the cytotoxicity of *trans*-[$PtCl_2(NH_3)$(planar amine)], meriting a detailed study of their impact on DNA conformation and some 'downstream' intracellular processes, such as DNA damage recognition by specific proteins, DNA polymerization and repair, which all play a crucial role in the mechanism of antitumor activity of 'classical' cisplatin (4).

Site-specific adducts containing one defined lesion give more information on the structural details of 'global' DNA binding. The results to date indicate that DNA conformational changes of *trans*-[$PtCl_2(NH_3)$(planar amine)] are relatively independent of the nature of the planar amine. To examine the consequences of specific adducts containing planar amines we have used *trans*-PtTz as a suitable representative as it is water-soluble and formally exhibits antitumor, albeit moderate, activity (1). Monofunctional DNA adducts of *trans*-PtTz, in contrast to monodentate [PtCl(dien)]Cl or [$PtCl(NH_3)_3$]Cl, inhibit DNA synthesis and create a local conformational distortion similar to that produced by the 1,2-GG intrastrand CL of cisplatin, which is considered the lesion most responsible for its anticancer activity (8). In addition, *trans*-PtTz monofunctional adducts are recognized by HMGB1 domain proteins and are removed by the nucleotide excision repair (NER) system similar to the 1,2-GG intrastrand CL of cisplatin.

This paper describes the analysis of short duplexes containing the single, site-specific interstrand and 1,3-intrastrand CLs of *trans*-PtTz to obtain a more complete picture on the DNA modifications produced by this compound. The conformational changes, subsequent consequences with respect to protein recognition, DNA polymerization and repair compared to cis and transplatin are highly dependent on the nature of the adduct. The interstrand CL of *trans*-PtTz affects DNA conformation and its recognition by high mobility group (HMG) domain proteins differently from the interstrand CLs formed by the parent transplatin, but similarly, although in a lesser extent, as the interstrand CL of cisplatin. In addition, the results indicate that in contrast to transplatin, *trans*-PtTz forms long-lived 1,3-intrastrand CLs that distort DNA conformation in double-helical DNA, but are weakly repaired.

MATERIALS AND METHODS

Chemicals

trans-PtTz was prepared according to the previously published methods (10). Cisplatin and transplatin were obtained from Sigma. The stock solutions of platinum compounds were prepared at the concentration of 5×10^{-4} M in 10 mM $NaClO_4$ and stored at 4°C in the dark. The synthetic oligodeoxyribonucleotides (Figures 1B, 5C and 6) were purchased from VBC-Genomics (Vienna, Austria) and were purified as described previously (11). Restriction endonucleases, Klenow fragment of *Escherichia coli* DNA polymerase I deficient in 3′→5′ proofreading exonuclease activity (KF^-), and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). Reverse transcriptase of human immunodeficiency virus type 1 (RT HIV-1) was from Amersham Pharmacia Biotech, Nonidet N P-30 was from Fluka (Prague, Czech Republic). Acrylamide, bis(acrylamide), urea and NaCN were from Merck. Radioactive products were from Amersham Biosciences. ATP was from Roche Applied Science.

Platination of oligonucleotides

The duplexes containing single, intrastrand CL of cisplatin, transplatin or *trans*-PtTz in the top strand [the duplexes in Figure 1B that contained two guanine (G) residues in the top strand] were prepared as described previously (8,12,13). The interstrand cross-linked duplexes were also prepared as described previously (7,9,14).

Gel-mobility shift assay

The 5′ end labeled 20 bp oligonucleotide duplexes with blunt ends containing the central sequences in the top strands TGCT, TGTGT and TGGT (these duplexes were identical to TGCT, TGTGT and TGGT duplexes shown in Figure 1B except that overhanging nucleotides at 3′ ends were paired with their complementary nucleotides) either unplatinated (controls) or containing the central platinum CL were used and their reaction with HMG-domain proteins was performed and analyzed as described previously (15). The electrophoresis was performed for 50 min at 4°C, gels were dried and visualized by using the FUJIFILM bio-imaging analyzer and the radioactivities associated with bands were quantified with the AIDA image analyzer software. Competition experiments performed to determine apparent dissociation constants, $K_{D(app)}$, were performed as described previously (15). $K_{D(app)}$ values were estimated in the manner described in Ref. (15). Each $K_{D(app)}$ is the average of at least two measurements.

Nucleotide excision assay

The 148 bp substrates containing single, central intrastrand or interstrand CL were assembled from three oligonucleotide duplexes as described previously (16,17). Oligonucleotide excision reactions were performed in cell-free extracts (CFEs) prepared from the HeLa S3 and CHO AA8 cell lines as described previously (18). These extracts were kindly provided by J. T. Reardon and A. Sancar (University of North Carolina, Chapel Hill, NC). *In vitro* repair was measured with excision assay using these CFEs and 148 bp linear DNA substrates (see above) as described previously (18).

Inhibition of DNA polymerization

The 30mer or 23mer templates (Figure 5) containing a single 1,3-GTG intrastrand CL of *trans*-PtTz or 1,2-GG intrastrand CL of cisplatin were prepared in the same way as described above. The 17mer or 8mer DNA primers (the sequence of the 17mer primer is shown in Figure 6) were complementary to the 3′ termini of the 23mer or 30mer templates, respectively. The DNA substrates (1.5×10^{-7} M) were formed by annealing templates and 5′ end-labeled primers at a molar ratio of 3:1. All experiments using RT HIV-1 were performed at 37°C in a volume of 50 µl in a buffer containing 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM KCl, 3 mM DTT, 0.1% Nonidet N P-30, 100 µM dATP, 100 µM dCTP, 100 µM dGTP, and 100 µM TTP and 1.0 U of RT HIV-1. The experiments with KF^- were performed at 25°C using the same volume, 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM DTT, 50 µM BSA/ml; the nucleoside triphosphates were at a concentration of 25 µM and 0.5 U of KF^- was used. Reactions were terminated by the addition of EDTA, so that its resulting concentration was 20 mM, and heating at 100°C for 30 s. Products were resolved by denaturing 24% polyacrylamide (PAA)/8 M urea gel and then visualized and quantified by using the FUJIFILM bioimaging analyzer and AIDA image analyzer software. Other details were published previously (19,20).

RESULTS

Structural consequences of interstrand cross-linking

Unwinding and bending. The structural details responsible for bending and unwinding of cisplatin and transplatin–DNA interstrand adducts have been elucidated recently (21). Therefore, it was of interest to compare the bending and unwinding induced by a single, site-specific interstrand CL of *trans*-PtTz. Multimers of the oligonucleotide platinated at a single site may show strong anomalies in their electrophoretic mobilities as a consequence of coherent addition in-phase platinum-induced bends. The maximum anomalies are observed if the match between the sequence repeat and the helix screw is optimized (22). In addition, if the interplatinum distance in these multimers is systematically varied in polymers containing the adduct, the multimer that migrates most slowly gives the optimal phasing for cooperative bending, from which the degree of unwinding can be determined (23). Thus, electrophoretic migration of the multimers formed from the series of oligonucleotide duplexes of different lengths, which contain a single, site-specific platinum adduct can be evaluated in terms of a quantitative measure of the extent of planar curvature and unwinding (13,24–26).

Oligodeoxyribonucleotide duplexes TGCT (20–23) (20–23 bp long) were used for these studies. The sequences were identical or similar to that of the duplex TGCT (21) shown in Figure 1B; the sequence of the duplex TGCT (20) had one marginal C·G base pair deleted, whereas one or two additional TA base pairs were added to one or two ends in the duplexes TGCT (22) or TGCT (23), respectively. The ligation products of the unplatinated or CL-containing duplexes were analyzed on native PAA electrophoresis gels (Figure 2A). The DNA unwinding of the *trans*-PtTz interstrand adduct was

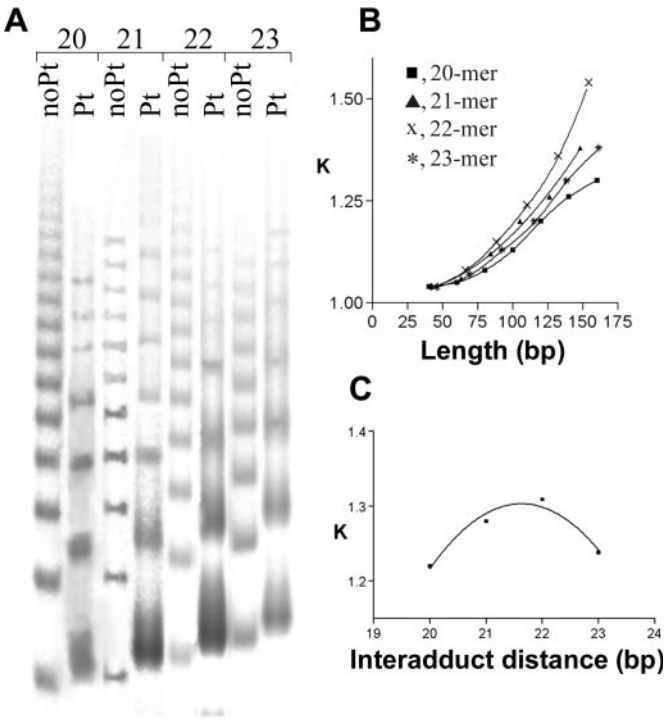

Figure 2. DNA bending. The mobility of the ligation products of 20–23 bp duplexes containing single, site-specific interstrand CL of *trans*-PtTz formed between guanine residues in the central sequence 5′-TGCT/5′-AGCA in an 8% PAA gel. (**A**) Phosphorimage of the ligation products. Lanes: NoPt, unplatinated duplexes; Pt, duplexes containing the CL. The sequence of the 21 bp duplex TGCT (21) is shown in the Figure 1B. The sequences of the 20mer, 22mer and 23mer duplexes are described in the text. (**B**) Plots showing the relative mobility *K* versus sequence length curves for the oligomers 20–23 bp long containing the CL. (**C**) Plot showing the relative mobility *K* versus interadduct distance in bp for the oligomers 20–23 bp long containing the CL with a total length of 126 bp. The experimental points represent the average of three independent electrophoresis experiments. The curves represent the best fit of these experimental points to the equation $K = ad^2 + bd + c$ (23).

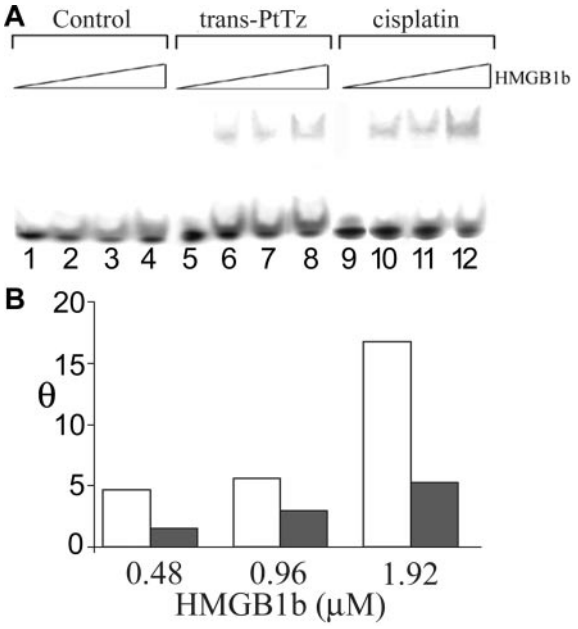

Figure 3. Recognition of interstrand CLs by HMGB1b protein. Gel-mobility shift assay analysis of the titration of the 20 bp duplexes containing the single, site-specific CLs of *trans*-PtTz or cisplatin with HMGB1b protein. (**A**) Phosphorimage: lanes 1–4, unplatinated TGCT (20) duplex; lanes 5–8, TGCT (20) duplex-containing interstrand CL of *trans*-PtTz; lanes 9–12, TGCT (20) duplex-containing interstrand CL of cisplatin. The duplexes were at the concentration of 20 nM. Lanes 1, 5, 9: no protein added; lanes 2, 6 and 10: 0.48 μM HMGB1b added, respectively; lanes 3, 7 and 11: 0.96 μM HMGB1b added, respectively; and lanes 4, 8 and 12: 1.92 μM HMGB1b added, respectively. (**B**) Plot of Θ values (ratio of protein-bound duplex to total duplex) obtained from gel-shift experiments. Open bars, interstrand CL of cisplatin and closed bars, interstrand CL of *trans*-PtTz.

found to be 20 ± 2°, considerably higher than that induced by the interstrand CL of transplatin [12°; (12)], but lower than that induced by the interstrand CL of cisplatin (76–80°). Moreover, the interstrand CL bends DNA by ∼22 ± 2° toward the minor groove [the direction of the bend was determined using the duplex [TGCT(A/T)$_5$] (32) (Figure 1B) (27)]. Thus, the bending is similar to those afforded by the interstrand CL of cisplatin or transplatin using the same experimental procedure [20–40° or 20°, respectively (21)]. Interestingly, in contrast to the cisplatin or transplatin interstrand CLs, the ligation of duplexes with *trans*-PtTz interstrand CLs did not result in the formation of circles, suggesting that the flexibility of the double helix is not increased (12,28).

Chemical probes of DNA conformation. The duplex TGCT (21) (Figure 1B) containing a single, site-specific interstrand CL of *trans*-PtTz was treated with several reagents that act as chemical probes for the existence of conformations other than canonical B-DNA (Figure S1 in Supplementary Data), as described previously (12). The interstrand CL induces in DNA a distortion extending over at least 4 bp and localized mainly at the base pairs containing the platinated G residues.

Recognition by HMG domain proteins. An important feature of the mechanism of antitumor activity of several platinum drugs (depending on the cell type) is that their adducts are recognized by proteins containing HMG domains (3,29–31). For instance, the major 1,2-GG intrastrand CL of cisplatin and the monofunctional adducts of *trans*-PtTz are readily recognized by HMGB1 domains A and B (8,32) and the interstrand CL of cisplatin by the HMGB1 domain B (33). In contrast, the cisplatin 1,3-GNG intrastrand CL and DNA modified by transplatin or monodentate [PtCl(dien)]Cl or [PtCl(NH$_3$)$_3$)]Cl, are not recognized by these cellular proteins (3,29,30). The interactions of the rat HMGB1 domain A (HMGB1a) and HMGB1 domain B (HMGB1b) with the 20 bp duplex TGCT (20) containing a single, site-specific interstrand CL of *trans*-PtTz were investigated using a gel-mobility shift assay and binding was detected by retardation of the migration of the radiolabeled 20 bp probes through the gel (Figure 3) (33,34). Consistent with previous reports (15), a shifted band upon incubation of the duplex containing 1,2-GG intrastrand CL of cisplatin with both HMGB1a and HMGB1b was observed (data not shown) indicating that both proteins recognize the duplex containing this adduct. The HMGB1a exhibited under the same experimental conditions no binding to the 20 bp duplex when unplatinated or containing the interstrand CL of either cisplatin [consistent with previous results (33)] or *trans*-PtTz (data not shown). The HMGB1b exhibited under the same experimental

conditions no binding to the unplatinated 20 bp duplex (Figure 3, lanes 1–4), but a shifted band upon incubation of the interstrand CLs of cisplatin (Figure 3A, lanes 9–12) or *trans*-PtTz (Figure 3A, lanes 5–8) was clearly observed indicating that the protein recognizes the duplex containing these interstrand CLs. Thus, HMGB1b, but not HMGB1a, protein exhibited affinity for the interstrand CL of *trans*-PtTz which was similar, although somewhat less pronounced, compared with that for the interstrand CL of cisplatin (Figure 3B).

Nucleotide excision repair. In mammalian cells, the NER pathway is an important mechanism for the removal of bulky, helix-distorting DNA adducts, such as those generated by various chemotherapeutics, including cisplatin (3,35,36). Efficient repair of 1,2-GG or 1,3-GTG intrastrand CL of cisplatin and no excision repair of interstrand CLs of cisplatin have been reported from various NER systems, including human and rodent excinucleases (3,37). The results in Figure 4A, lanes 4 and 8, demonstrating NER by the rodent excinuclease, are consistent with these reports. The major excision fragment contains 28 nt, and other primary excision fragments are 23–27 nt in length. No excision products were, however, detected for the interstrand CL of *trans*-PtTz under identical conditions where the cisplatin 1,2- or 1,3-intrastrand CLs were readily removed by both rodent and human excinuclease (shown in Figure 4A, lane 12 for the CL treated with rodent excinuclease).

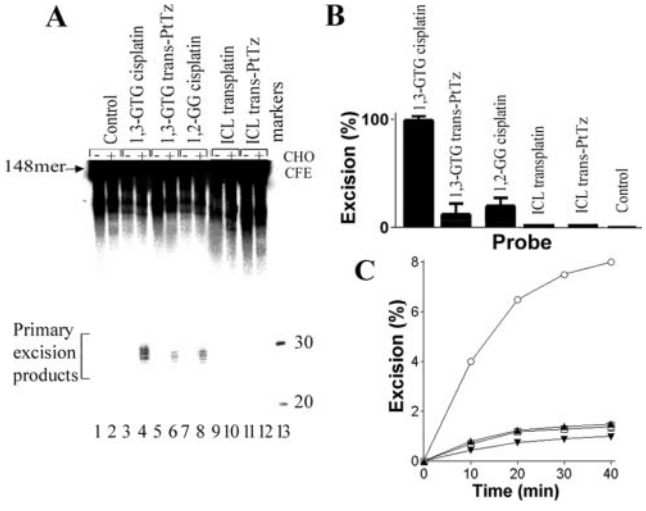

Figure 4. Excision of the intrastrand and interstrand CLs of platinum complexes by rodent excinuclease. (**A**) Phosphorimage. The substrates were incubated with CHO AA8 CFE and subsequently treated overnight with NaCN before analysis in 10% PAA/8 M urea denaturing gel. Lanes 1 and 2, control, unplatinated substrate; lanes 3 and 4, the substrate containing the 1,3-GTG intrastrand CL of cisplatin; lanes 5 and 6, the substrate containing the 1,3-GTG intrastrand CL of *trans*-PtTz; lanes 7 and 8, 1,2-GG intrastrand CL of cisplatin; lanes 9 and 10, the substrate containing the interstrand CL (ICL) of transplatin; lanes 11 and 12, the substrate containing the interstrand CL of *trans*-PtTz; lanes 1, 3, 5, 7, 9 and 11, no extract added; lanes 2, 4, 6, 8, 10 and 12, the substrates were incubated with CHO AA8 CFE for 40 min at 30°C. Lane 13, the 20 and 30 nt markers. (**B**) Quantitative analysis of removal of the adducts. The columns marked as 1,3-GTG cisplatin, 1,3-GTG trans-PtTz, 1,2-GG cisplatin, ICL transplatin, ICL *trans*-PtTz and Control are for 1,3-GTG intrastrand CL of cisplatin, 1,3-GTG intrastrand CL of *trans*-PtTz, 1,2-GG intrastrand CL of cisplatin, the interstrand CL of transplatin, the interstrand CL of *trans*-PtTz and unplatinated substrate, respectively. The radioactivity associated with the fragments excised from the duplex containing the 1,3-GTG intrastrand CL of cisplatin was taken as 100% and corresponded to 8% excision. Data are the average of two independent experiments performed under the same conditions; bars indicate the range of excision. (**C**) Quantitative analysis of the kinetic experiment. Removal of 1,3-GTG intrastrand CL of cisplatin (open circles), 1,3-GTG intrastrand CL of *trans*-PtTz (inverted closed triangle), 1,2-GG intrastrand CL of cisplatin (closed triangles) and monofunctional adduct of *trans*-PtTz (open squares).

Structural consequences of intrastrand CL

Steric constraints prevent the formation of transplatin intrastrand CLs between adjacent base residues in double-helical DNA. The *trans* compounds can, however, cross-link two bases on the same strand separated by at least one intervening base, forming mostly 1,3-GNG intrastrand CLs (where N = A, C, G or T).

Stability of the 1,3-GNG intrastrand CLs. The 1,3-GNG intrastrand CL (N = any nucleotide) of transplatin is stable in single-stranded DNA under physiological conditions. Pairing of single-stranded DNA containing this 1,3-GNG intrastrand adduct with the complementary DNA sequences results in rearrangement into interstrand CLs (38). The stability of 1,3-GNG intrastrand CLs of *trans*-PtTz was investigated using 20mer oligodeoxyribonucleotides (the top strands of the duplexes CGCGC, TGCGT and TGTGT shown in Figure 5C) that were radioactively labeled at their 5′ ends and platinated so that they contained single and central, site-specific 1,3-GCG or GTG intrastrand CLs, respectively. The single-stranded oligonucleotides containing this CL or the corresponding duplexes were incubated in 0.2 M $NaClO_4$ at 37°C. At various time intervals, aliquots were withdrawn and analyzed by gel electrophoresis under denaturing conditions. Only 2–5% of these intrastrand CLs were transformed into interstrand CLs after 24 h (Figure 5B). In contrast, 70% of the transplatin 1,3-intrastrand CLs in the duplex TGTGT were transformed into interstrand CLs after 24 h [Figure 5A and B and (39)]. Further, the 1,3-GNG intrastrand adduct of *trans*-PtTz in the single-stranded oligonucleotides was inert over a long period of time (>5 days) (data not shown).

Unwinding and bending. Bending and unwinding studies of the 1,3-intrastrand CL were performed in the oligodeoxyribonucleotide duplexes TGTGT (19–22) (19–22 bp long) (12,13). The sequences were identical or similar to that of the duplex TGTGT (21) shown in Figure 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. The DNA bending toward the minor groove and unwinding owing to one 1,3-intrastrand adduct of *trans*-PtTz were measured at 40 ± 2 and 15 ± 2°, respectively [the direction of the bend was determined using the duplex $[TGTGT(A/T)_5]$ (32) (Figure 1B) as described previously (27)]. Moreover, the ligation of the duplexes containing 1,3-intrastrand CL of *trans*-PtTz did not result in the formation of circles, suggesting that these 1,3-intrastrand CLs did not increase the flexibility of the double helix (12,28).

Chemical probes of DNA conformation. The single, site-specific 1,3-GTG intrastrand CL in the duplex TGTGT (21) induces a distortion extending over at least 6 bp and is localized mainly at the base pair between the platinated G residues and the base pairs on its 5′ side (Figure S2 in Supplementary Data).

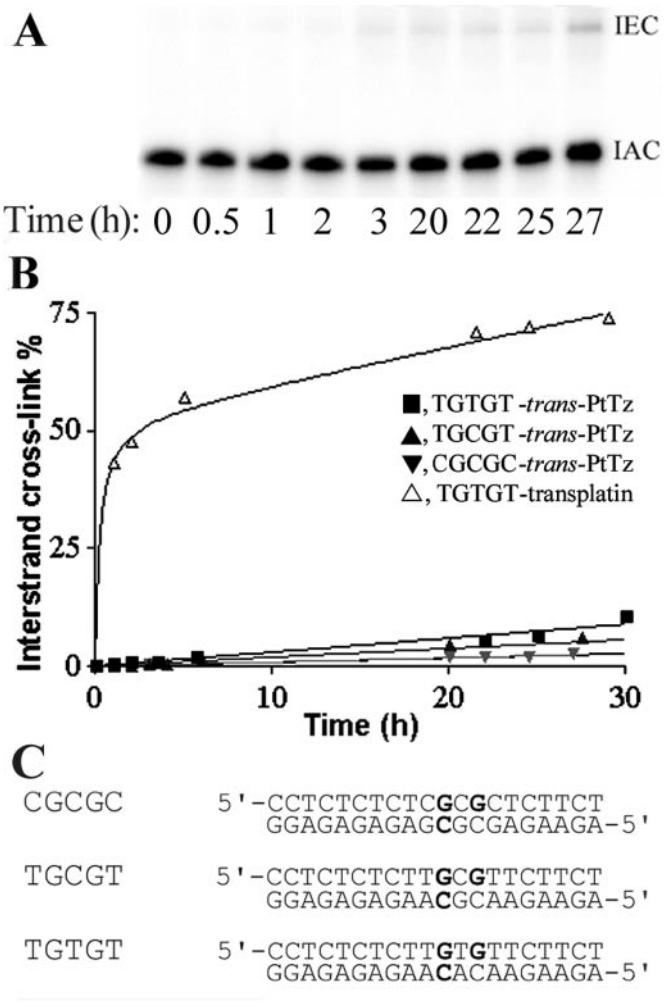

Figure 5. Rearrangement of the 1,3-intrastrand CLs formed by *trans*-PtTz and transplatin in the 20 bp duplexes into the interstand CLs. The samples of the 20 μM duplexes TGTGT, TGCGT and CGCGC containing the single, site-specific 1,3-intrastrand CL were incubated at 37°C in 0.2 M $NaClO_4$, 5 mM Tris–HCl buffer (pH 7.5) and 0.1 mM EDTA; at various time intervals, the aliquots were withdrawn and analyzed by electrophoresis in 12% PAA/8 M urea gel. (**A**) Phosphorimage of the gel of the duplex TGTGT modified by *trans*-PtTz radioactively labeled at the 5′ end of its top strand. Incubation times in hours are indicated under each lane. Lane 0 refers to the 5′ end labeled single-stranded top (platinated) strand. The upper bands corresponding to more slowly migrating species contain the interstrand cross-linked oligonucleotides (ICLs) whereas the bottom bands corresponding to more rapidly migrating species contain the intrastrand cross-linked oligonucleotides (IACs). (**B**) Plots of the percentages of interstrand CL versus time in TGTGT, TGCGT and CGCGC duplexes. These percentages were calculated from the ratio of the radioactivity associated with the interstrand cross-linked product to the total radioactivity loaded in each lane (multiplied by 100). Data for the plot demonstrating the rearrangement of the 1,3-intrastrand CL formed by transplatin in the duplex TGTGT were identical to those published previously (39). For other details, see the text. (**C**) The nucleotide sequences of the duplexes TGTGT, TGCGT and CGCGC.

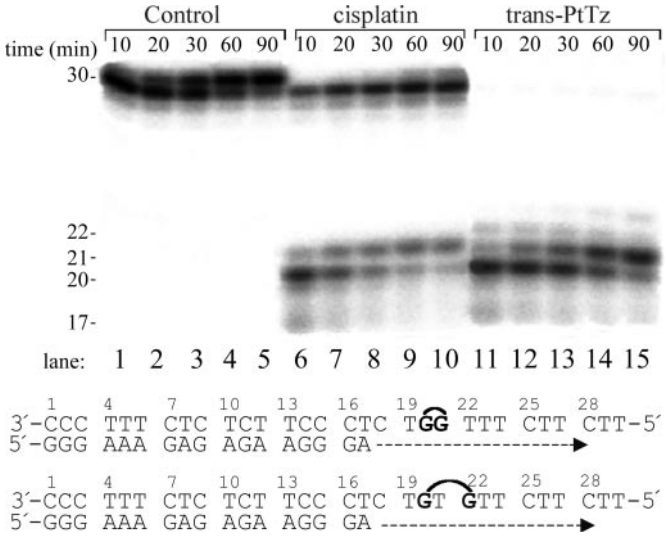

Figure 6. Primer extension activity of RT-HIV1. The experiments were conducted using the 17mer/30mer primer–template duplexes (their nucleotide sequences are shown at the bottom of this figure) for the times indicated. Lanes 1–5, undamaged template; lanes 6–10, the template containing 1,2-GG intrastrand CL of cisplatin; lanes 11–15, the template containing 1,3-GTG intrastrand CL of *trans*-PtTz. The strong pause sites opposite the platinated guanines were marked 20 and 21.

Recognition by HMG domain proteins. Using the same experimental approach as for the *trans*-PtTz interstrand CL, neither HMGB1a nor HMGB1b was found to bind the 20 bp probe containing the intrastrand CL of *trans*-PtTz (data not shown).

Nucleotide excision repair. Importantly, excision repair substrates containing the site-specific *trans*-PtTz 1,3-GTG intrastrand CL were repaired by both human and rodent excinucleases, but with a markedly lower efficiency than the intrastrand CLs of cisplatin (shown in Figure 4A, lane 6, and in Figure 4B and C for the adduct repaired by rodent excinuclease).

Inhibition of DNA polymerization. DNA secondary structures have significant effects on processivity of a number of prokaryotic, eukaryotic and viral DNA polymerases. The character of DNA templates containing site-specific platinum adducts dictate whether prokaryotic and eukaryotic DNA polymerases are blocked or can traverse through the lesion. DNA polymerization using the templates site-specifically modified by *trans*-PtTz or cisplatin by RT HIV-1 was investigated to reveal the potential differences in conformational alterations imposed on DNA by these two adducts. We constructed the 17mer/30mer primer–template duplexes TGTGT and TGGT (Figure 6) unplatinated or containing either the 1,3-GTG intrastrand CL of *trans*-PtTz or the 1,2-GG intrastrand CL of cisplatin, respectively. The first 17 nt on the 3′ terminus of the 30mer template strand were complementary to the nucleotides of the 17mer primer, and the 3′ guanine involved in the 1,3-GTG CL of *trans*-PtTz or in 1,2-GG CL of cisplatin on the template strand was located at its 20th position from the 3′ terminus (Figure 6). After annealing the 17 nt primer to the 3′ terminus of the unplatinated or platinated template strand (positioning the 3′ end of the primer three bases before the adduct in the template strand), we examined DNA polymerization through the unique CLs on the template by RT HIV-1 in the presence of all four deoxyribonucleoside 5′-triphosphates. The reaction was stopped at various time intervals, and the products analyzed using a sequencing gel (Figure 6). Polymerization using the template containing the CL of cisplatin proceeded rapidly up to the nucleotides at

the sites opposite the CL, such that the 20 and 21 nt products accumulated to a significant extent (shown in Figure 6, lanes 6–10). There was only a slight accumulation of larger DNA intermediates, whereas no intermediate products were seen with the 30mer control template as the full-length product was being formed (shown in Figure 6, lanes 1–5). The full-length products were also noticed with the 23mer template containing the CL of cisplatin, although in a smaller amount. The results are in agreement with previously published work (40) using T7 DNA polymerase and RT HIV-1 and confirm that the 1,2-GG intrastrand CL of cisplatin inhibits DNA synthesis, but translesion synthesis may occur. In contrast, under the same experimental conditions, DNA polymerization by RT HIV-1 on the *trans*-PtTz 1,3-intrastrand CL template proceeded up to the nucleotide at the site opposite the 3′ G involved in the CL (Figure 6, 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 1,3-GTG intrastrand CLs of *trans*-PtTz impede elongation of DNA to a greater extent than the major adducts of cisplatin (Figure 6).

The effects of the 1,3-intrastrand CL of *trans*-PtTz on polymerization by KF^- were also examined. This enzyme possesses template-dependent DNA polymerase activity but relatively better processivity and fidelity (41). In these studies, elongation of the 8mer/23mer primer–template duplexes was tested (data not shown). The *trans*-PtTz 1,3-intrastrand CL constitutes a fairly strong block to DNA synthesis catalyzed by both DNA polymerases. The high degree of structural and sequence conservation of the domains among eukaryotic, prokaryotic and viral polymerases (42), suggests that the results from studies of the RT HIV-1 and KF^- should be also applicable to other DNA polymerases (43,44). Hence, the stronger inhibition of DNA polymerization by the *trans*-PtTz 1,3-intrastrand CL reflects an important difference in biological processes of replication or DNA repair in comparison with the major adduct of cisplatin.

DISCUSSION

Transplatinum complexes with sterically hindered ligands [planar N-heterocycles; iminoethers (45), aliphatic amines (46) and aliphatic heterocycles, such as piperazine (39)] display significantly increased cytotoxicity over the 'parent' *trans*-$[PtCl_2(NH_3)_2]$ compound. *trans*-PtTz is a representative example of the structural class of cytotoxic compounds of general formula *trans*-$[PtCl_2(L)(L')]$ containing at least one planar amine. The planar ligand clearly has significant consequences for the DNA conformational changes induced by these molecules. It appears likely from the work published to date that all planar ligands will have similar effects. The cytotoxicity of transplatinum complexes containing planar ligands is characterized by values similar to cisplatin and the retention of activity in cisplatin-resistant cells. A distinct profile of cytotoxicity in the NCI tumor cell line panel is also observed (47). It is therefore of interest to consider how the DNA binding of the various adducts summarized in Table 1 may contribute to these biological properties.

Unlike 'classical' cisplatin or transplatin, modification of DNA by *trans*-PtTz leads to monofunctional and bifunctional intra and interstrand CLs in roughly equal proportions. The bending of ~34° toward the major groove and local unwinding of 12–13° of the monofunctional adduct are uncannily similar to those of the 1,2-intrastrand CL of cisplatin and as a consequence the lesion is susceptible to NER and recognized by HMG-family proteins (8). Thus, other downstream biological effects of the *trans*-PtTz monofunctional adduct are also likely to be similar to those induced by cisplatin.

Table 1. Summary and comparison of basic characteristics of DNA CLs of *trans*-PtTz(*t*-PtTz), transplatin and cisplatin[a]

	1,3-Intrastrand CL of *t*PtTz	Interstrand CL of *t*PtTz	Monofunctional adduct of *t*PtTz	Interstrand CL of transplatin	Interstrand CL of cisplatin	1,2-Intrastrand CL of cisplatin
Frequency (%)	20–40[b]	30–40[b]	30–40[b]	~12[c]	~6[c]	~90[d]
Reactivity of chemical probes (bp)	6	4	2[e]	4[f]	ND	ND
DNA bending	40° toward minor groove	22° toward minor groove	34° toward major groove[e]	~20° toward minor groove[f,g]	40–45° toward minor groove[h]	32–34° toward major groove[i]
DNA unwinding	15°	20°	12°[e]	~12°[f,g]	76–79°[h]	13°[i]
HMGB1a recognition	≫1.5 μM	≫1.5 μM	38.5 nM[e]	ND[j]	≫1.5 μM	30.8 nM[e]
HMGB1b recognition	≫30 μM	13.40 μM	2.05 μM[e]	ND[j]	4.60 μM	1.85 μM
Translesion synthesis	No	ND	20%[e]	ND	ND	6%[e]
NER by eukaryotic excinuclease (% excision)[k]	1.0	No	1.4[e]	no[l,m]	no[m]	1.5[e]

ND, not determined.
[a]If not stated otherwise, the data are from this work. The 1,3-intrastrand CL of transplatin is not included because these adducts are unstable in double-helical DNA and readily isomerize to interstrand CLs (38,39).
[b]Data are from Ref. (5,7).
[c]Data are from Ref (9).
[d]Data are from Ref (52).
[e]Data are from Ref (8).
[f]Data are from Ref (12).
[g]Data are from Ref (53).
[h]Data are from Ref. (54,55).
[i]Data are from Ref (23).
[j]Data are only available for recognition by full-length rat HMGB1, which indicated no recognition (56).
[k]Data taken from Figure 4; the substrates were incubated with CHO AA8 CFE for 40 min at 30°C.
[l]Data are from Ref (19).
[m]Data are from Ref (19).

The steric effect of the planar ligand results in the formation of bifunctional interstrand CLs between adjacent guanines in a 5′-GC/5′-GC bp. The DNA bending and unwinding are quantitatively significantly smaller than those of the analogous cisplatin interstrand CL. Nevertheless, similar to that adduct but in contrast to that of transplatin, the *trans*-PtTz interstrand CL is a weak substrate for HMGB1b protein recognition and is not repaired in NER assays.

Transplatin does not form stable 1,2-intrastrand CLs in double-helical DNA (38) and this property has been related to its clinical inefficiency (48,49). Replacement of one ammine by a thiazole ligand results in a distinctively enhanced stability of the 1,3-GNG intrastrand CLs in short oligoeoxyribonucleotide duplexes (Figure 5). Despite the similarity in bending to that of the 1,2-GG intrastrand CL of cisplatin no recognition of the *trans*-PtTz 1,3-intrastrand CL by HMG-domain proteins was observed. A plausible explanation may be that the bending owing to the *trans*-PtTz 1,3-intrastrand CL is in the opposite direction than that of the 1,2-intrastrand CL of cisplatin, thus preventing DNA bending toward the major groove required for its accommodation in the complex with HMGB1 protein (8,32).

Several reports have demonstrated that NER is a major mechanism contributing to cisplatin resistance (18,37,50). The 1,3-intrastrand CL of *trans*-PtTz is not removed as readily by excision repair as the cisplatin intrastrand adducts (Figure 4). The repercussion of stronger inhibition of DNA polymerization by the 1,3-intrastrand CL of *trans*-PtTz in comparison with the major adduct of cisplatin (Figure 6) adds a new dimension to the impact of the activated *trans* geometry in platinum compounds on biological processes, possibly including replication or DNA repair. However, it is not surprising that the interstrand CL of *trans*-PtTz is not excised in the assays we have used. Excision repair factors, most notably XPF-ERCC1, are involved in the interstrand CL repair, but there are no published data describing cell extracts proficient in the interstrand CL removal when assayed in a way similar to that described in the present work.

The cytotoxicity profile of *trans*-PtTz may be reasonably attributed to the structural consequences of DNA damage and the variety of adducts or lack of dominance of one specific adduct may correlate with the unusual profile seen. Thus, while long-lived monofunctional adducts simulate the biological consequences of cisplatin, the bifunctional adducts will induce cellular effects unique from the clinical drug. HMG-domain proteins sensitize cells to cisplatin (32,51), possibly owing to shielding of cisplatin–DNA adducts from excision repair or recruitment of these proteins from their native transcriptional regulatory function (3,15,30,51). For the subset of *trans*-PtTz bifunctional adducts with weak or no HMG binding these hypothetical pathways will not operate. Thus, a certain proportion of adducts will circumvent the cellular control induced for cisplatin and indeed other DNA-damaging agents and such circumvention may be reflected as a different cytotoxicity profile.

The multiple DNA lesions available to *trans*-planaramine complexes may all contribute substantially to their cytotoxicity so that the overall drug cytotoxicity could be the sum of the contributions of each of these adducts. However, an intriguing scenario consistent with the cytotoxicity profile different from cisplatin may be that that acquisition of drug resistance is a relatively rare event, since it would have to entail resistance to or tolerance of multiple, structurally dissimilar DNA lesions.

A long-term goal of our investigations is to place the cytotoxicity of metal-based compounds into the context of molecular pathways leading to tumor cell death. We believe that the significance of the results of the present work consists in improvement of the theoretical background needed for the design of new anticancer metal-based drugs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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