DNA Binding by Antitumor *trans*-[$PtCl_2(NH_3)$(thiazole)]. Protein Recognition and Nucleotide Excision Repair of Monofunctional Adducts†

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ABSTRACT: Antitumor effects of *cis*-diamminedichloroplatinum(II) (cisplatin) and the clinical inactivity of its trans isomer (transplatin) have been considered a paradigm for the classical structure−activity relationships of platinum drugs. However, several new analogues of transplatin which exhibit a different spectrum of cytostatic activity including activity in tumor cells resistant to cisplatin have been recently identified. Analogues containing the planar amine ligand of the general structure *trans*-[$PtCl_2(NH_3)$(L)], where L = planar amine, represent an example of such compounds. DNA is believed to be the major pharmacological target of platinum compounds. To contribute to the understanding of mechanisms underlying the activation of trans geometry in transplatin analogues containing planar amine ligands, various biochemical and biophysical methods were employed in previous studies to analyze the global modifications of natural DNA by *trans*-[$PtCl_2(NH_3)$(L)]. These initial studies have revealed some unique features of the DNA binding mode of this class of platinum drugs. As the monofunctional lesions represent a significant fraction of stable adducts formed in DNA by bifunctional antitumor *trans*-platinum compounds with planar ligands, we analyzed in the present work short DNA duplexes containing the single, site-specific monofunctional adduct of a representative of this class of platinum drugs, antitumor *trans*-[$PtCl_2$-(NH_3)(thiazole)]. It has been shown that, in contrast to the adducts of monodentate chlorodiethylenetriamineplatinum(II) chloride or [$PtCl(NH_3)_3$]Cl, the monofunctional adduct of *trans*-[$PtCl_2(NH_3)$(thiazole)] inhibits DNA synthesis and creates a local conformational distortion similar to that produced in DNA by the major 1,2-GG intrastrand CL of cisplatin, which is considered the lesion most responsible for its anticancer activity. In addition, the monofunctional adducts of *trans*-[$PtCl_2(NH_3)$(thiazole)] are recognized by HMGB1 domain proteins and removed by the nucleotide excision repair system similarly as the 1,2-GG intrastrand CL of cisplatin. The results of the present work further support the view that the simple chemical modification of the structure of an inactive platinum compound alters its DNA binding mode into that of an active drug and that processing of the monofunctional DNA adducts of the *trans*-platinum analogues in tumor cells may be similar to that of the major bifunctional adducts of "classical" cisplatin.

There is a large body of experimental evidence that the success of platinum complexes in killing tumor cells results from their ability to form on DNA various types of covalent adducts (*1*−*3*). Hence, in the search for new platinum antitumor drugs the hypothesis that platinum compounds which bind to DNA in a manner fundamentally different from that of *cis*-diamminedichloroplatinum(II) (cisplatin)[1] will have altered pharmacological properties has been tested. This concept has already led to the synthesis of several new unconventional platinum antitumor compounds that violate the original structure−activity relationships; the advance to the clinic of the novel trinuclear compound BBR3464 validates the hypothesis (*4*). The clinical inactivity of transplatin is considered a paradigm for the classical structure−activity relationships of platinum drugs, but to this end several new analogues of transplatin which exhibit a different spectrum of cytostatic activity including activity in tumor cells resistant to cisplatin have been identified (*3*, *5*−*7*).

The antitumor *trans*-platinum(II) complexes whose DNA binding mode has been already intensively investigated also include analogues containing a planar amine ligand of the

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[1] Abbreviations: bp, base pair; carboplatin, *cis*-diamminecyclobutanedicarboxylatoplatinum(II); CFE, cell-free extract; cisplatin, *cis*-diamminedichloroplatinum(II); CL, cross-link; DEPC, diethyl pyrocarbonate; DMS, dimethyl sulfate; HMG, high-mobility group; HMGB1a, domain A of HMGB1 protein; HMGB1b, domain B of HMGB1 protein; FAAS, flameless atomic absorption spectrophotometry; FPLC, fast protein liquid chromatography; KF, Klenow fragment of *Escherichia coli* DNA polymerase I deficient in 3′→5′ proofreading exonuclease activity; NER, nucleotide excision repair; PAA, polyacrylamide; [PtCl(dien)]Cl, chlorodiethylenetriamineplatinum(II) chloride; RP-HPLC, reversed-phase high-pressure liquid chromatography; *trans*-PtTz, *trans*-[$PtCl_2(NH_3)$(thiazole)].

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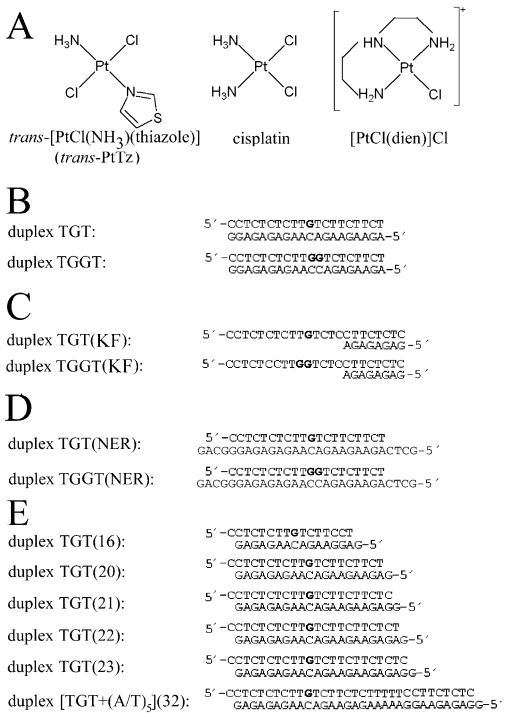

FIGURE 1: Structures of platinum complexes (A) and sequences of the synthetic oligodeoxyribonucleotides used in the present study with their abbreviations (B−E). The top and bottom strands of each pair are designated top and bottom, respectively, in the text. The bold letter in the top strands of all duplexes except TGGT and TGGT(NER) indicates the location of the monofunctional adduct of *trans*-PtTz. The bold letters in the top strands of the duplexes TGGT and TGGT(NER) indicate the location of the intrastrand CL after modification of the duplexes by cisplatin.

general structure *trans*-[$PtCl_2(NH_3)(L)$], where L = planar amine such as quinoline or thiazole (*5*), and *trans*-[$PtCl_2L_2$], where L = pyridine or thiazole, for example (*8*). To contribute to the understanding of mechanisms underlying the antitumor activity of these transplatin analogues, various biochemical and biophysical methods as well as molecular modeling techniques have been employed to study the modifications of natural, high molecular mass DNA by antitumor *trans*-[$PtCl_2(NH_3)$(thiazole)] (*trans*-PtTz) (Figure 1A) or *trans*-[$PtCl_2(NH_3)$(quinoline)] (*9*). These compounds bind monofunctionally to DNA with a rate similar to that of transplatin. The overall rate of the rearrangement to bifunctional adducts is also similar to that observed in the case of DNA modification by transplatin; i.e., it is relatively slow (*10*). In contrast to transplatin, however, the analogues containing the planar ligand form considerably more interstrand cross-links (CLs) (~30−40% after 48 h) with a much shorter half-time ($t_{1/2}$ = ~5 h) (*9*, *11*) [~12% interstrand CL is formed by transplatin after 48 h with $t_{1/2}$ > 11 h (*12*)]. However, a significant fraction of the adducts formed by *trans*-[$PtCl_2(NH_3)$(quinoline)] or *trans*-PtTz remains monofunctional even after long reaction times (after 48 h ca. 30−40% adducts remain monofunctional) (*9*, *11*).

Additional work has shown that *trans*-[$PtCl_2(NH_3)$-(quinoline)] and *trans*-PtTz preferentially form DNA interstrand CLs between guanine residues at the 5′-GC/5′-GC sites (*9*). Thus, DNA interstrand cross-linking by these transplatin analogues is formally equivalent to that by antitumor cisplatin but different from clinically ineffective transplatin, which preferentially forms these adducts between complementary guanine and cytosine residues (*13*). These results have shown for the first time that the simple chemical modification of structure of an inactive platinum compound alters its DNA binding mode into that of an active drug.

The adducts of the transplatin analogues containing the planar ligand terminate in vitro RNA synthesis in transcription mapping experiments preferentially at guanine residues and at similar sites as the adducts of cisplatin (*9*). Interestingly, DNA modified by the *trans*-platinum compounds containing the planar ligand is recognized by cisplatin-specific antibodies and not by transplatin-specific antibodies, which suggests that these transplatin analogues behave in some respects like cisplatin (*9*).

Importantly, the planar ligand in all or in a significant fraction of DNA adducts of these transplatin analogues, in which platinum is coordinated by base residues, is well positioned to interact with the duplex. Models for both monofunctional adducts and bifunctional interstrand CLs have proposed (*9*, *14*) that the combination of monofunctional covalent binding and a stacking interaction between the planar ligand and the DNA bases can produce a kink in the duplex.

Further investigations, which are described in the present work, have focused on the analysis of short duplexes containing the single, site-specific monofunctional adduct of *trans*-PtTz. This compound exhibits antitumor activity (*5*) so that it is a suitable representative of antitumor *trans*-platinum compounds with planar ligands. It has been shown that the monofunctional adduct of *trans*-PtTz affects DNA conformation, its recognition by cellular proteins and repair distinctly different from the monofunctional adduct formed by chlorodiethylenetriamineplatinum(II) chloride ([PtCl-(dien)]Cl) (Figure 1A) or [$PtCl(NH_3)_3$]Cl [the monodentate complexes (*15*)]. The monofunctional adduct of *trans*-PtTz creates a local conformational distortion very similar to that produced in DNA by the major 1,2-GG intrastrand CL of antitumor cisplatin. In addition, these monofunctional adducts are recognized by HMGB1 domain proteins (HMG = high mobility group) and removed by the nucleotide excision repair (NER) system similarly as the 1,2-GG intrastrand CL of antitumor cisplatin. As the monofunctional lesions represent a significant fraction of stable adducts formed in DNA by bifunctional antitumor *trans*-platinum compounds with planar ligands, the results of the present work may contribute to understanding the molecular mechanism that underlies antitumor activity of this class of platinum drugs.

MATERIALS AND METHODS

Chemicals. *trans*-PtTz (Figure 1A) was prepared by standard methods (*16*). Cisplatin (Figure 1A) was obtained from Sigma-Aldrich sro (Prague, Czech Republic). 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 (Figure 1B−E) were synthesized and purified as described previously (*17*). Expression and purification of domains A (residues 1−84) and B (residues 85−180) of recombinant rat HMGB1 protein (HMGB1a and HMGB1b, respectively) were carried out as described (*18*, *19*). T4 DNA ligase, T4 polynucleotide kinase, and the Klenow fragment of *Escherichia coli* DNA polymerase I deficient in 3′→5′ proofreading

exonuclease activity (KF) were purchased from New England Biolabs (Beverly, MA). Acrylamide, bis(acrylamide), urea, and NaCN were from Merck KgaA (Darmstadt, Germany). Dimethyl sulfate (DMS), $KMnO_4$, diethyl pyrocarbonate (DEPC), KBr, $KHSO_5$, DNase I from bovine pancreas, nuclease P1 from *Penicillium citrinum*, and alkaline phosphatase from calf intestine were from Sigma-Aldrich sro (Prague, Czech Republic). [γ-^{32}P]ATP was from Amersham (Arlington Heights, IL). ATP was from Boehringer (Mannheim, Germany). Deoxyribonucleoside 5′-triphosphates were purchased from Pharmacia.

Platinations of Oligonucleotides. The single-stranded oligonucleotides [the top strands of the duplexes in Figure 1B−E except duplexes TGGT, TGGT(KF), and TGGT(NER)] were reacted in stoichiometric amounts with the monoaqua derivative of *trans*-PtTz generated by allowing this complex to react with 0.9 molar equiv of $AgNO_3$. The platinated oligonucleotides were repurified by ion-exchange fast protein liquid chromatography (FPLC). It was verified by platinum flameless atomic absorption spectrophotometry (FAAS) and by the measurements of the optical density that the modified oligonucleotides contained one platinum atom. It was also verified using DMS footprinting of platinum on DNA (*12*) that one *trans*-PtTz molecule was coordinated to a single G at the N7 position in the top strands of all duplexes. FPLC purification and FAAS measurements were carried out on a Pharmacia Biotech FPLC System with a MonoQ HR 5/5 column and a Unicam 939 AA spectrometer equipped with a graphite furnace, respectively. The duplexes TGGT, TGGT(KF), and TGGT(NER) (Figure 1B−D) containing the single, 1,2-GG intrastrand CL of cisplatin in the top strand were prepared as described (*12*). The unmodified or platinated duplexes used in the studies of recognition by HMGB1 domain proteins were still purified by electrophoresis on native 15% polyacrylamide (PAA) gel [monoacrylamide:bis(acrylamide) ratio = 29:1]. Other details have been described previously (*12*, *17*, *20*).

High-Pressure Liquid Chromatographic (HPLC) Analyses. These analyses were performed using a Hitachi Series 4 liquid chromatograph equipped with a LCI-100 computing integrator and a Waters μBondapak C18 column. The products were separated by reversed-phase HPLC (RP-HPLC). The products of the enzymatic digestion were analyzed using isocratic elution with 0.1 M ammonium acetate, pH 5.5, in 3.9% CH_3CN at a 1 mL/min flow rate. The following enzymatic digestion protocol was used to characterize the platinated deoxyribooligonucleotide duplexes. The samples (50 μg of the duplex) were incubated with 72 units of DNase I at 37 °C. After 4 h nuclease P1 (40 μg) was added, and the reaction was allowed to continue at 37 °C for 18 h. Finally, alkaline phosphatase (39 units) was added and the incubation continued for an additional 4 h at 37 °C. The digested samples containing constituent nucleosides were then heated for 2 min at 80 °C and centrifuged, and the supernatant was analyzed by RP-HPLC. In the experiments in which dissociation of thiazole from the monofunctional adduct of *trans*-PtTz was examined, the concentration of the duplex was 1 mM (related to the monomer content) and the concentration of thiazole and *trans*-PtTz was 0.1 mM. In these experiments, the products were separated using isocratic elution with 3.9% CH_3CN in water at a 1 mL/min flow rate. Other details are in the text below.

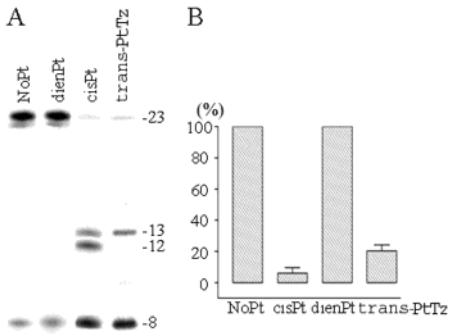

FIGURE 2: Primer extension activity of the exonuclease-deficient Klenow fragment of DNA polymerase I using the 8-mer/23-mer primer/template duplex. The experiments were conducted for 1 min using undamaged template (panel A, lane NoPt) or template containing the 1,2-GG intrastrand CL of cisplatin (panel A, lane cisPt), the monofunctional adduct of [PtCl(dien)]Cl (panel A, lane dienPt), or *trans*-PtTz (panel A, lane trans-PtTz). The graph (B) shows relative inhibition of DNA synthesis (%) on undamaged (control) template (NoPt) or on DNA containing the 1,2-GG intrastrand CL of cisplatin (cisPt), the monofunctional adduct of [PtCl(dien)]Cl (dienPt), or *trans*-PtTz (trans-PtTz). Data are the means (±standard error) from three different experiments with two independent template preparations.

DNA Synthesis. The 23-mer templates (for sequences, see Figure 1C) containing a single monofunctional adduct of *trans*-PtTz or [PtCl(dien)]Cl or the 1,2-GG intrastrand CL of cisplatin were prepared in the same way as described in the paragraph Platinations of Oligonucleotides (vide supra). The 8-mer DNA primer whose sequence is also shown in the Figure 2A was complementary to the 3′ termini of the 23-mer template. The DNA substrates were formed by annealing templates and 5′-end-labeled primers at a molar ratio of 3:1. All experiments using KF were performed at 25 °C in a volume of 50 μL in a buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 50 μg/mL BSA, 25 μM dATP, 25 μM dCTP, 25 μM dGTP, 25 μM TTP, and 0.5 unit (approximately 1 pmol) of KF. Reactions were terminated by the addition of EDTA so that its resulting concentration was 20 mM and heated at 100 °C for 30 s. Products were resolved by denaturing 24% PAA/8 M urea gel and then visualized and quantified by using a Molecular Dynamics PhosphorImager and ImageQuant software.

The relative inhibition of DNA synthesis on damaged templates was calculated as follows: % inhibition of DNA synthesis = (1 − Pt/NoPt) × 100, where Pt = full-length DNA synthesis on the platinated template/total primer termini and NoPt = full-length DNA synthesis on the control, unplatinated template/total primer termini. This calculation was made for that concentration and incubation time for which replication was incomplete (i.e., not all molecules of the primer had been fully elongated) on both the control and platinated templates. Thus, the inhibition of DNA synthesis relative to synthesis on unplatinated DNA templates was determined for 1 min incubation.

Gel Mobility Shift Assay. The 5′-end-labeled 20 base pair (bp) oligonucleotide duplexes (TGT or TGGT shown in Figure 1B) either unplatinated (controls) or containing the central platinum adduct in their top strands were used, and

their reaction with HMG-domain proteins was performed and analyzed as described previously (*21*). Apparent dissociation constants, $K_{D(app)}$, were estimated in the manner described in ref *21*. Each $K_{D(app)}$ is the average of at least two measurements.

Nucleotide Excision Assay. The 148 bp substrates containing the single, central monofunctional adduct of *trans*-PtTz, [PtCl(dien)]Cl, or the 1,2-GG intrastrand CL of cisplatin were assembled from three oligonucleotide duplexes as described previously (*22*, *23*).

Oligonucleotide excision reactions were performed in cell-free extracts (CFEs) prepared from the HeLa S3 and CHO AA8 cell lines as described (*24*, *25*). These extracts were kindly provided by J. T. Reardon and A. Sancar from the University of North Carolina (Chapel Hill, NC). In vitro repair was measured with excision assay using these CFEs and 148 bp linear DNA substrates (vide supra) in the same way as described previously (*25*).

Ligation, Electrophoresis of Oligonucleotides, and Chemical Modifications. Details of these experiments were as described in previously published papers (*26*−*28*).

RESULTS

Stability of the Adduct. We demonstrated previously (*9*, *11*) that preferential G binding of *trans*-[$PtCl_2(NH_3)$(quinoline)] or *trans*-PtTz results in monofunctional adducts and CLs. Quantitation of monofunctional adducts revealed that these adducts are equally or even more probable than interstrand or intrastrand CLs. Considering these facts, we have designed a series of synthetic oligodeoxyribonucleotide duplexes, TGT, whose sequences are shown in Figure 1B−E. The pyrimidine-rich top strands of these duplexes only contained one G residue in the central sequence TGT (printed in bold in the Figure 1B−E). These top strands were modified by *trans*-PtTz so that they contained its single monofunctional adduct at the G residue at the central sequence TGT. The platinated top strands were hybridized with their complementary strands. The samples of the platinated duplexes in which either the upper or bottom strand was only 5′-end-labeled with ^{32}P were reacted with DMS, which does not react with platinated G because the N7 position is no longer accessible (*12*). The adducts were removed by NaCN, and then the samples were treated with piperidine. In the unplatinated duplexes, the central G residue in the top strands or all G residues in the bottom strands were reactive with DMS (not shown). The single G residue in the top strands was no longer reactive in the platinated duplexes whereas reactivity of all G residues in the complementary bottom strands remained unchanged. This observation confirms that the single G residue in the upper strands remained platinated even after the duplex was formed whereas no G residue in the bottom strands of these duplexes became platinated. The stability of the central monofunctional adducts in the top strands of these duplexes was also confirmed by electrophoretic analysis in denaturing PAA gel (*12*, *28*), demonstrating that no interstrand CL was formed (not shown).

The platinated site in the 20 bp duplex TGT (for the nucleotide sequence, see Figure 1B) modified by *trans*-PtTz in the way described above was also identified using enzymatic digestion analysis. The platinated or nonmodified duplexes TGT were treated with DNase I, P1 nucleases, and finally alkaline phosphatase to yield corresponding deoxyribonucleosides. An RP-HPLC analysis of the products of this enzymatic digestion procedure revealed well-separated peaks A, C, G, and T, which corresponded to nonmodified deoxyriboadenosine, deoxyribocytidine, deoxyriboguanosine, and thymidine, respectively, assigned by co-injection with samples of pure deoxyribonucleosides (*29*). The area under peak G yielded by the platinated duplex was only reduced, and this reduction was exactly eight-ninths of that found for the nonmodified, control duplex (containing nine guanine residues) (not shown). The only reasonable explanation of this result is consistent with only one platinated guanine residue in the platinated duplex. We have found conditions for RP-HPLC analysis which allowed detecting quantitatively free thiazole in the presence of *trans*-PtTz also free (not bound to DNA) in the solution. The lower limit of this determination was 0.5% thiazole in the presence of *trans*-PtTz. The 20 bp duplex TGT (Figure 1B) containing the single monofunctional adduct at the central G residue in the top strand was dissolved in 0.01 and 1.0 M $NaClO_4$ or NaCl and incubated in the dark at 37 °C for 1 week. No peak in the RP-HPLC profile corresponding to free thiazole or *trans*-PtTz was observed, indicating that no thiazole spontaneously dissociated from the monofunctional adduct of *trans*-PtTz in any of the solutions tested and also confirming the stability of this monofunctional lesion.

DNA Synthesis. It is generally accepted that antitumor effects of platinum drugs, such as cisplatin, are associated with the capability of DNA adducts of these compounds to inhibit replication and/or transcription and induce programmed cell death (*30*). Inhibition of transcription of DNA globally modified by a transplatin analogue with a planar amine ligand in in vitro transcription mapping experiments has been already demonstrated (*9*). On the other hand, replication or transcription of the adducts of "classical" monofunctional platinum(II) compounds, such as [PtCl(dien)]Cl or $[PtCl(NH_3)_3]Cl$, is not inhibited or is inhibited much less effectively than replication or transcription of platinum CLs (*3*). It is, therefore, of fundamental importance to know whether the monofunctional adducts of *trans*-PtTz can inhibit replication.

In the present work we constructed the 8-mer/23-mer primer/template duplexes TGT(KF) and TGGT(KF) (Figure 1C) unplatinated or containing either the monofunctional adduct of *trans*-PtTz or [PtCl(dien)]Cl at the G residue of the template strand in the central TGT sequence or the 1,2-GG intrastrand CL of cisplatin in the central TGGT sequence. The first eight nucleotides on the 3′ terminus of the 23-mer template strand were complementary to the nucleotides of the 8-mer primer and single monodentately modified guanine, or two adjacent guanines cross-linked by cisplatin on the template strand were located at its 13th or 13th and 14th position, respectively, from the 3′ terminus (Figure 1C). After annealing a eight nucleotide primer to the 3′ terminus of the unplatinated 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 monofunctional adduct or the 1,2-intrastrand CL on the template by KF in the presence of all four deoxyribonucleoside 5′-triphosphates. The reaction was stopped at 1 min, and the products were analyzed using a sequencing

gel (Figure 2A). Polymerization using the 23-mer 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 Figure 2A, lane cisPt). There was almost no 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 Figure 2A, lane NoPt). The full-length products were also noticed with the 23-mer template containing the CL of cisplatin, but only in a relatively very low amount. This result is in agreement with previously published work (*31*) in which HIV-1 reverse transcriptase and T7 DNA polymerase were used and confirms that the 1,2-GG intrastrand CL of cisplatin strongly inhibits DNA synthesis (*32*). In contrast, under the same experimental conditions no products shorter than the full-length product were seen with the 23-mer template containing a single monofunctional adduct of [PtCl(dien)]Cl, and the full-length product was only formed (shown in Figure 2A, lane dienPt). This result confirms that monofunctional adducts of [PtCl(dien)]Cl inhibit DNA synthesis much less efficiently or negligibly in comparison with the 1,2-GG intrastrand CL of cisplatin.

DNA polymerization by KF was also examined under identical conditions as in the previous experiment using the template containing the monofunctional adducts of *trans*-PtTz. Polymerization using this 23-mer template proceeded up to the nucleotide opposite the platinated G (Figure 2A, lane trans-PtTz). There was almost no accumulation of shorter and larger DNA intermediates. A small amount of the full-length products accumulated also with the 23-mer template containing the monofunctional adduct of *trans*-PtTz. This result indicates that the monofunctional adducts of *trans*-PtTz are very efficient inhibitors of DNA synthesis in contrast to the monofunctional adducts of [PtCl(dien)]Cl, but similarly as major 1,2-intrastrand CLs of cisplatin (Figure 2B) (the inhibition of DNA synthesis after 1 min incubation due to the 1,2-GG intrastrand CL of cisplatin and the monofunctional adduct of *trans*-PtTz was 94% and 80%, respectively). Hence, from the viewpoint of inhibition of replication, the monofunctional adducts of *trans*-PtTz resemble the 1,2-GG intrastrand CLs of cisplatin (although some features are different) and not the adducts of the clinically ineffective monofunctional platinum(II) compounds.

Recognition by HMGB1 Proteins. DNA modified by the *trans*-platinum compounds containing the planar ligand is recognized by cisplatin-specific antibodies, which suggests that these transplatin analogues behave in some respects like cisplatin (*9*). An important feature of the mechanism that underlies the antitumor activity of cisplatin is that the major adducts of this platinum drug (1,2-GG intrastrand CLs) are recognized by proteins containing HMG domains (*2*, *3*). Importantly, DNA modified by transplatin or monodentate platinum(II) compounds, such as [PtCl(dien)]Cl or [PtCl-$(NH_3)_3$]Cl, is not recognized by these cellular proteins. It has been also shown (*2*, *3*) that the binding of these proteins to DNA modified by cisplatin mediates antitumor effects of this platinum drug. Therefore, we tested in the present work whether the monofunctional adducts which are frequent lesions of *trans*-PtTz are also recognized by HMGB1 box proteins similarly as the 1,2-GG intrastrand CLs of cisplatin.

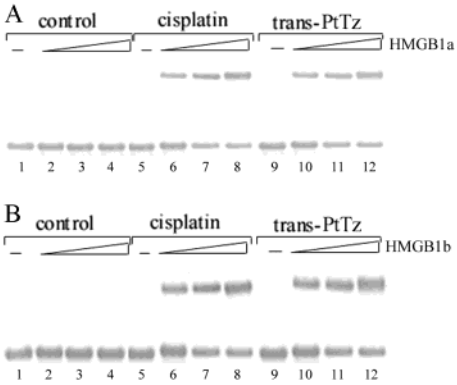

FIGURE 3: Analysis of the binding affinity of 20 bp DNA containing the single, site-specific monofunctional adduct of *trans*-PtTz or the 1,2-GG intrastrand CL of cisplatin to the HMGB1 domain A (A) and the HMGB1 domain B (B) proteins in a 6% PAA gel. The duplexes were at the concentration of 20 nM. Key: unplatinated duplex (lanes 1−4); the duplex containing the intrastrand CL of cisplatin (lanes 5−8); the duplex containing the monofunctional adduct of *trans*-PtTz (lanes 9−12). Lanes 1, 5, and 9: no protein added. Lanes 2, 6, and 10 in panels A and B: 19 nM HMGB1a and 109 nM HMGB1b added, respectively. Lanes 3, 7, and 11 in panels A and B: 38 nM HMGB1a and 273 nM HMGB1b added, respectively. Lanes 4, 8, and 12 in panels A and B: 72 nM HMGB1a and 546 nM HMGB1b added, respectively.

The interactions of the rat HMGB1 domain A (HMGB1a) and HMGB1 domain B (HMGB1b) with the monofunctional adduct of *trans*-PtTz were investigated. In these experiments, the 20 bp duplex TGT (Figure 1B) was modified so that it contained a single, site-specific monofunctional adduct of *trans*-PtTz at the G residue. For comparative purposes we also prepared the 20 bp duplex TGGT (Figure 1B), which was modified so that it contained a single, site-specific 1,2-GG intrastrand CL of cisplatin. The binding of the HMGB1a and HMGB1b to these DNA probes was detected by retardation of the migration of the radiolabeled 20 bp probes through the gel (*21*, *33*, *34*) (Figure 3).

HMGB1a and HMGB1b exhibited negligible binding to the nonmodified 20 bp duplexes. As indicated by the presence of a shifted band whose intensity increases with increasing protein concentration, both HMGB1a and HMGB1b recognize the duplex containing the monofunctional adduct of *trans*-PtTz (Figure 3). Since only a single-shifted band forms following incubation of the TGT duplex containing the monofunctional adduct of *trans*-PtTz with either HMGB1a or HMGB1b, detailed titration studies were possible (only the results of some typical analyses of these detailed titration studies are shown in Figure 3). Evaluations of these titration data afforded $K_{D(app)}$ values reported in Table 1. These titration data indicate that HMGB1a binds the probe containing the monofunctional adduct of *trans*-PtTz with a relatively high affinity, which was similar to that of HMGB1a to the analogous probe but containing the 1,2-GG intrastrand CL of cisplatin.

The titration of the 20 bp duplex containing the monofunctional adduct of *trans*-PtTz with HMGB1b has revealed (Figure 3B) that this protein also binds to the monofunctional adduct of *trans*-PtTz with an affinity similar to that of the probe containing the 1,2-GG intrastrand CL of cisplatin. The affinity of HMGB1b to these platinum lesions was, however, considerably smaller (∼50 times) than that of HMGB1a.

One Pt−Cl bond of *trans*-PtTz in its DNA monofunctional adduct remains available for coordination. Hence, a ternary

Table 1: Summary and Comparison of Basic Characteristics of the Monofunctional Adduct of *trans*-PtTz with the 1,2-GG Intrastrand CL of Cisplatin and the Monofunctional Adduct of [PtCl(dien)]Cl[a]

	monofunctional adduct of *trans*-PtTz	1,2-GG intrastrand CL of cisplatin	monofunctional adduct of [PtCl(dien)]Cl
termination of DNA synthesis	yes	yes	no
DNA bending[b]	34°	32−34° [c]	no[d]
DNA unwinding[b]	12°	13° [c]	no[d]
$K_{D(app)}$ (HMGB1a recognition)	38.5 nM	30.8 nM	no[e]
$K_{D(app)}$ (HMGB1b recognition)	2.05 μM	1.85 μM	no[e]
NER by eukaryotic excinuclease	yes	yes	no

[a] If not stated otherwise, the data are from this work. [b] Determined by gel electrophoresis. [c] Bellon and Lippard (*27*), Bellon et al. (*69*), and Stehlikova et al. (*54*). [d] Marrot and Leng (*55*) and Brabec et al. (*17*). [e] Donahue et al. (*64*) and Pil and Lippard (*65*).

DNA−Pt−protein complex could be formed if HMGB1a or HMGB1b is bound to the duplex containing the monofunctional adduct of *trans*-PtTz (Figure 3B). This eventuality was tested in the following way. The 20 bp duplex TGT (Figure 1B) containing the monofunctional adduct of *trans*-PtTz was incubated with HMGB1a or HMGB1b proteins in the same way as in the experiments shown in Figure 3 (for 1 h at 0 °C). The reactions were further incubated at 25 °C for 16 h, then divided in two, and analyzed by native PAA and 5%/10% SDS/PAA gel electrophoresis (not shown). Whereas analysis performed on native gels clearly revealed more slowly migrating bands due to formation of the complex between the platinated duplex and the protein (the results were the same as those shown in Figure 3), no such bands were noticed if the same samples were analyzed on SDS/PAA gel. This result indicates that HMGB1a and HMGB1b proteins were able to bind the 20 bp DNA duplex containing the monofunctional adducts of *trans*-PtTz but were unable to be cross-linked to this platinated DNA.

Nucleotide Excision Repair. NER is a pathway used by human cells for the removal of damaged nucleotides from DNA (*35*, *36*). In mammalian cells, this repair pathway is an important mechanism for the removal of bulky, helix-distorting DNA adducts, such as those generated by various chemotherapeutics including cisplatin (*37*). Efficient repair of the 1,2-GG intrastrand CL of cisplatin has been reported by various NER systems including human and rodent excinucleases (*25*, *38*−*42*). The result presented in Figure 4A, lane 8, is consistent with these reports. The major excision fragment contains 28 nucleotides, and other primary excision fragments are 23−27 nucleotides in length, although 22−31 nucleotide long fragments are also observed (*43*). This range of product sizes reflects variability at both the 3′ and 5′ incision sites (*25*, *44*); smaller excision products are due to degradation of the primary excision products by exonucleases present in the extracts (*25*). Importantly, the monofunctional adduct of *trans*-PtTz was also repaired with a similar efficiency by both human and rodent excinucleases (shown in Figure 4A, lane 6, and in Figure 4B for the adduct repaired by rodent excinuclease). We have also examined whether the adduct of [PtCl(dien)]Cl is a substrate for mammalian excinucleases because it has been shown (*45*) that this monofunctional adduct is even a better substrate

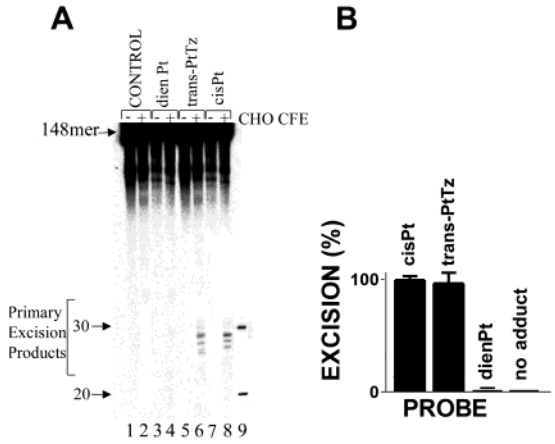

FIGURE 4: Excision of the monofunctional adduct of *trans*-PtTz or [PtCl(dien)]Cl and the 1,2-GG intrastrand CL of cisplatin by rodent excinuclease. (A) The substrates were incubated with CHO AA8 CFE for 40 min at 30 °C and subsequently treated overnight with NaCN prior to analysis in 10% PAA/8 M urea denaturing gel. Lanes: 1 and 2, control, unplatinated substrate; 3 and 4, the substrate containing the monofunctional adduct of [PtCl(dien)]Cl; 5 and 6, the substrate containing the monofunctional adduct of *trans*-PtTz; 7 and 8, the substrate containing the 1,2-GG intrastrand CL of cisplatin; 1, 3, 5, and 7, no extract added; 2, 4, 6, and 8, the substrates were incubated with CHO AA8 CFE for 40 min at 30 °C; all substrates were subsequently treated overnight with NaCN prior to analysis in 10% PAA/8 M urea denaturing gel. Lane 9: 20 and 30 nt markers. (B) Quantitative analysis of removal of the adducts. The columns marked cisPt, dienPt, and trans-PtTz are for the 1,2-d(GpG) intrastrand CL of cisplatin, the monofunctional adduct of [PtCl(dien)]Cl, and *trans*-PtTz, respectively. Data are the average of two independent experiments done under the same conditions; bars indicate range of excision.

than the 1,2-GG intrastrand CL of cisplatin for prokaryotic *E. coli* ABC excinuclease. Consistetly with previous reports (*46*, *47*) no excision fragments were noticed if the monofunctional adduct of [PtCl(dien)]Cl was used as a substrate for human and rodent excinucleases (shown in Figure 4A, lane 4, for rodent excinuclease).

Bending, Unwinding, and Chemical Probes of DNA Conformation. Important structural motifs that attract HMG-domain and NER proteins to 1,2-intrastrand CLs of cisplatin are the bending and unwinding of the helix axis (*2*, *3*, *48*, *49*). For DNA adducts of cisplatin, the structural details responsible for bending and unwinding and subsequent protein recognition have recently been elucidated (*33*, *50*). Given the recent advances in our understanding of the structural basis for the bending of DNA caused by cisplatin CLs, it is of considerable interest to examine how frequent monofunctional adducts of *trans*-PtTz affect conformational properties of DNA such as bending and unwinding. In this work we further performed studies on the bending and unwinding induced by a single, site-specific monofunctional adduct of *trans*-PtTz using electrophoretic retardation as a quantitative measure of the extent of planar curvature. Molecular modeling had previously indicated that such bending might be possible for the trans planar compounds (*45*).

The oligodeoxyribonucleotide duplexes TGT (*16*, *20*−*23*) (16 and 20−23 bp long shown in Figure 1E) were used for the bending and unwinding studies of the present work. Experimental details of these studies are given in our recent reports (*51*−*53*) and are also described in detail in the

Supporting Information. The DNA unwinding due to one monofunctional adduct of *trans*-PtTz has been found to be $12 \pm 2°$.

Moreover, the monofunctional adduct of *trans*-PtTz bends by about 34° DNA toward the major groove. The bending and local unwinding induced by the monofunctional adduct of *trans*-PtTz are very similar to those afforded by the 1,2-GG interstrand CL of cisplatin using the same experimental procedure [32−34° and 13°, respectively (*27*, *54*)], but in a distinct contrast to the monofunctional adduct of [PtCl(dien)]-Cl which does not bend DNA and unwinds it only negligibly (*17*, *55*). Further studies of the present work were also focused on analysis of the distortion induced by the monofunctional adduct of *trans*-PtTz by chemical probes of DNA conformation using the duplex containing the single, site-specific adduct. The platinated duplex TGT (Figure 1B) was treated with several chemical agents that are used as tools for monitoring the existence of conformations other than canonical B-DNA. These agents include $KMnO_4$, DEPC, and bromine. They react preferentially with single-stranded DNA and distorted double-stranded DNA (*20*, *56*). We used for this analysis exactly the same methodology described in detail in our recent papers aimed at DNA adducts of various antitumor platinum drugs (*53*, *57*) so that these experiments are only described in more detail in the Supporting Information. The results indicate that this adduct induces in DNA the distortion that extends over at least 2 bp and is localized mainly at the platinated base pair and that on its 5′ side.

DISCUSSION

Binding of *trans*-PtTz to DNA in a monofunctional fashion represents a binding mode previously expected not to result in antitumor activity. This feature of the classical structure−pharmacological activity relationship of platinum(II) compounds was mainly based on the observation that clinically ineffective monofunctional compounds, such as [PtCl(dien)]-Cl or $[PtCl(NH_3)_3]Cl$, do not inhibit replication and transcription. Chemical modification such as the presence of one planar ligand clearly modifies the structures of DNA adducts in such a way that new biological activity is seen. More recently, several platinum(II) compounds have been synthesized that form on DNA stable monofunctional adducts, inhibit replication (and/or transcription), and exhibit antitumor activity. For instance, monofunctional agents *cis*-$[Pt(Am)Cl(NH_3)_2]^+$, in which Am is a derivative of pyridine, pyrimidine, purine, or aniline, demonstrate activity against murine and human tumor systems and block DNA replication (*58*). In addition, a novel monofunctional compound, *cis*-$[PtCl(NH_3)_2(N7\text{-ACV})]^+$ (*59*), which contains in the coordination sphere of cisplatin antiviral acyclovir (ACV), exhibits activity against various herpes viruses and has been also found as effective as cisplatin when equitoxic doses are administered in vivo to P388 leukemia-bearing mice. Also interestingly, the antitumor *trans*-$[PtCl_2(E\text{-iminoether})_2]$ complex, which preferentially forms stable monofunctional adducts at guanine residues in DNA (90%) (*60*), is also endowed with significant antitumor activity (*61*), and its DNA adducts inhibit replication and transcription (*61*, *62*). In addition, the results of the present work indicate that the monofunctional adducts of antitumor *trans*-PtTz inhibit DNA synthesis in vitro (Figure 2). Thus, the cytotoxicity of some platinum(II) compounds in tumor cells may also arise from the formation of monofunctional adducts that inhibit replication although the exact details of the mechanism that underlies these antitumor effects remain to be clarified.

The studies described in the present paper were carried out to understand the role of DNA monofunctional adducts of *trans*-PtTz in the antitumor effects of this drug as these adducts represent a significant fraction (30−40%) of all adducts formed by this drug on DNA. There is a large body of experimental evidence indicating that antitumor effects of cisplatin are mediated by cellular proteins that bind specifically to DNA CLs of cisplatin (*2*, *63*) but not to its monofunctional adducts or to DNA adducts of clinically ineffective transplatin or [PtCl(dien)]Cl (*64*, *65*). An example of the cellular proteins, whose interactions with the major adduct of cisplatin (1,2-intrastrand CL) have been studied in detail, is a class of HMG-domain proteins (*2*, *63*). The results of the present work clearly demonstrate that HMGB1 proteins bind to the monofunctional adducts of *trans*-PtTz with the same affinity as to the 1,2-GG intrastrand CL of cisplatin (Figure 3). This observation is unique in the field of DNA interactions of antitumor platinum compounds because this is for the first time when it is shown that HMG-domain proteins bind to monofunctional adducts of platinum-(II) compounds or that these proteins bind to the adducts of a *trans*-platinum compound.

The structural motif recognized by HMG-domain proteins on DNA modified by cisplatin is a rigid bend directed toward the major groove (*33*). It has been shown (*17*, *55*, *66*) that the monofunctional adducts of [PtCl(dien)]Cl, which are used as models of the initial (monofunctional) step of the binding of cisplatin or transplatin to DNA (*67*), do not bend DNA and the character of conformational distortions induced by these adducts is distinctly different from those induced by 1,2-intrastrand CLs of cisplatin (recognized by HMG-domain proteins with a high affinity). It was, therefore, somewhat surprising that the monofunctional adducts of *trans*-PtTz attract and bind to HMGB1 proteins.

A working hypothesis that has been further tested in the present work is that the monofunctional adducts of *trans*-PtTz in contrast to the adducts of [PtCl(dien)]Cl distort DNA conformation in a similar way as 1,2-intrastrand CLs of cisplatin. As summarized in Table 1, this hypothesis has strong experimental support. An intriguing observation is that the monofunctional adduct of *trans*-PtTz bends and unwinds DNA in the same way and extent as the 1,2-intrastrand CL of cisplatin. The reasons why this type of distortion, so far only observed in the case of the bifunctional CLs of platinum compounds, is also induced by the monofunctional platinum adducts may be due to the secondary perturbations of the planar ligand resulting in a bend mimicking the structural distortion produced by the 1,2-intrastrand CL of cisplatin (*9*). This conclusion is substantiated by the fact that the planar ligand in the *trans*-PtTz complex is cis to the binding site, affording a situation that allows the planar ligand to interact or stack with DNA (*68*). Details of the mechanisms underlying antitumor activity of cisplatin or resistance to this drug have been already proposed (*3*, *50*). One hypothesis is based on the fact that a number of proteins that exhibit an enhanced affinity to DNA modified by cisplatin, such as transcription factors, have key roles in the cells. Cisplatin−DNA adducts may hijack these proteins away from their normal binding sites, thereby disrupting fundamental cellular processes. As

the monofunctional adducts of *trans*-PtTz attract the cellular proteins with a similar efficiency as the major adduct of cisplatin, it is reasonable to suggest that this hijacking mechanism of antitumor activity is also effective in the case of antitumor analogues of transplatin containing the planar ligand.

Adducts formed by cisplatin distort DNA conformation, inhibit replication and transcription, but are also bypassed by DNA or RNA polymerases (*3*, *70*). Another hypothesis of the mechanism that underlies antitumor effects of cisplatin is associated with the observation that cisplatin intrastrand CLs are removed from DNA mainly by NER. They are, however, also recognized by a number of proteins (*2*, *3*) which could block DNA adducts of cisplatin from damage recognition needed for repair. These adducts may thus persist, which would potentiate their cytotoxicity. Consistent with this hypothesis is the fact that the adducts of clinically ineffective transplatin are more efficiently repaired than those of cisplatin (*71*). Hence, this shielding mechanism might be also applicable for the mechanism of antitumor activity of *trans*-PtTz since the monofunctional adducts of this drug are removed by NER (Figure 4).

The observation that monofunctional adducts of *trans*-PtTz are removed by eukaryotic NER systems (Figure 4) deserves further discussion. The NER of monofunctional adducts of platinum compounds, such as those of [PtCl(dien)]Cl, has been already examined. Whereas the monofunctional adducts of [PtCl(dien)]Cl are removed by prokaryotic ABC excinuclease more efficiently than 1,2-intrastrand CLs of cisplatin (*45*), the opposite is observed, as shown in the present work, in the case of the NER by eukaryotic excinucleases (Figure 4). The monofunctional adduct of [PtCl(dien)]Cl is only excised negligibly whereas the 1,2-GG intrastrand CL of cisplatin is excised considerably more readily. This observation is consistent with the view that monofunctional lesions induced in DNA of eukaryotic cells treated with platinum compounds are tolerated by a mechanism much more effective in eukaryotic cells. However, the monofunctional adducts of *trans*-PtTz are removed from DNA by eukaryotic excinuclease considerably more efficiently than the adducts of [PtCl(dien)]Cl and it is particularly interesting that with almost identical efficiency as the 1,2-intrastrand CLs of cisplatin (Figure 4). Thus, it is necessary for a role of the monofunctional adducts of *trans*-PtTz in the mechanism of antitumor activity of this drug that the repair of these adducts is inhibited so that they will persist and inhibit replication (transcription) or trigger apoptosis. In the case of the major adducts of cisplatin, their repair is inhibited by shielding these CLs by cellular proteins, such as HMG-domain proteins. As the monofunctional adducts of *trans*-PtTz are also specifically recognized and bound to cellular proteins, such as HMG-domain proteins (Figure 3), they could be also protected from repair in the same way as 1,2-intrastrand CLs of cisplatin, which would potentiate antitumor effects of *trans*-PtTz.

The affinity of cellular proteins, such as HMG-domain proteins, to the monofunctional adducts of *trans*-PtTz (Figure 3) may have another consequences for their role in the mechanism of antitumor effects of this drug. The clinical ineffectivity of transplatin is also related to a relatively slow rate of the conversion of its monofunctional adducts into the interstrand CLs. It has been suggested that long-lived monofunctional adducts of transplatin may be trapped by sulfur-containing compounds (for instance, by glutathione), which may be a process leading to the inhibition of the biological effects of transplatin (*10*, *72*). The monofunctional adducts of *trans*-PtTz also persist, but in contrast to the adducts of transplatin they are shielded by cellular proteins, which may protect them also from trapping by sulfur-containing compounds. In addition, it has been shown (*73*, *74*) that binding of HMG-domain proteins to the major 1,2-GG intrastrand CL of cisplatin markedly reduces translesion synthesis by DNA polymerases across this adduct, which also may potentiate its cytostatic effects in the tumors sensitive to this drug. As HMG-domain proteins also bind to the monofunctional adducts of *trans*-PtTz with a similar affinity as to 1,2-intrastrand CLs of cisplatin (Figure 3), it is possible to expect that translesion synthesis by DNA polymerases will be also reduced across the monofunctional adduct of *trans*-PtTz, which may also contribute to the importance of this adduct for its antitumor effects.

The cytotoxic effects of *trans*-PtTz may most likely be due to a cumulative effect of the structurally heterogeneous adducts (bifunctional intrastrand and interstrand adducts as well as the monofunctional adducts discussed in the present work) produced by this drug, but the role of monofunctional adducts in the antitumor effects of *trans*-PtTz could be significant. This work demonstrates that transplatin analogues containing a planar amine ligand are unique from a mechanistic point of view and, therefore, worthy of further studies. These studies should reveal all details of the mechanism underlying antitumor effects of this new class of platinum anticancer drugs that would allow reevaluating the structure−pharmacological relationships of platinum compounds needed for the search and design of new, more effective platinum anticancer drugs. In conclusion, the results of the present work further support the view that the simple chemical modification of the structure of an inactive platinum compound alters its DNA binding mode into that of an active drug and that processing of the monofunctional DNA adducts of these *trans*-platinum analogues in tumor cells may be similar to that of the major bifunctional adducts of classical cisplatin.

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SUPPORTING INFORMATION AVAILABLE

Results describing in detail DNA bending, unwinding, and conformational distortion by chemical probes as a consequence of the formation of the monofunctional adduct of *trans*-PtTz. This material is available free of charge via the Internet at http://pubs.acs.org.

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