The new antitumor trinuclear platinum compound \[\{\text{trans-PtCl(NH}_3\text{)}_2\text{Cl}\}_{\text{trans-}}\text{Pt(NH}_3\text{)}_2\text{H}_2\text{N(CH}_2\text{)}_6\text{NH}_2\text{]}_{\text{4}}\text{\} is} \text{ currently} \text{ in} \text{ phase} \text{ II} \text{ clinical} \text{ trials.} \text{ DNA is generally considered the major pharmacological target of platinum drugs. As such it is of considerable interest to understand the patterns of DNA damage. The bifunctional DNA binding of BBR3464 is characterized by the rapid formation of long range intra- and interstrand cross-links. We examined how the structures of the various types of the intrastrand cross-links of BBR3464 affect conformational properties of DNA, and how these adducts are recognized by high mobility group 1 protein and removed from DNA during \textit{in vitro} nucleotide excision repair reactions. The results have revealed that intrastrand cross-links of BBR3464 create a local conformational distortion, but none of these cross-links results in a stable curvature. In addition, we have observed no recognition of these cross-links by high mobility group 1 proteins, but we have observed effective removal of these adducts from DNA by nucleotide excision repair. These results suggest that the processing of the intrastrand cross-links of BBR3464 in tumor cells sensitive to this drug may not be relevant to its antitumor effects. Hence, polynuclear platinum compounds apparently represent a novel class of platinum anticancer drugs acting by a different mechanism than cisplatin and its analogues.

The trinuclear compound \[\{\text{trans-PtCl(NH}_3\text{)}_2\text{Cl}\}_{\text{trans-}}\text{Pt(NH}_3\text{)}_2\text{H}_2\text{N(CH}_2\text{)}_6\text{NH}_2\text{]}_{\text{4}}\text{\} (Fig. 1) is currently in phase II clinical trials. The compound, designated as BBR3464, is the lead representative of an entirely new structural class of DNA-modifying anticancer agents based on the poly(di,tri)nuclear platinum structural motif (1–3). In phase I trials, objective par-
binding, cellular uptake, and antitumor activity within the polynuclear platinum family itself (1, 17, 18).

The high charge on BBR3464 facilitates rapid binding to DNA, which is significantly faster than that of the neutral cisplatin. This feature is also manifested in rapid binding to single-stranded DNA (19). Bifunctional binding to duplex DNA preferentially involves guanine (G) residues. Quantitation of interstrand DNA cross-linking in natural and linear DNA indicated ~20% of the DNA to be interstrand cross-linked. This value is significantly higher than that for cisplatin; on the other hand, an intriguing aspect of BBR3464 is that long range delocalized CLs in which the platinated sites are separated by one or more base pairs are equally or even more probable than interstrand adducts.

The (platinum,platinum) intrastrand CLs of BBR3464 are thus analogues of the major adducts of cisplatin, which forms on DNA ~90% bifunctional intrastrand adducts between neighboring purine residues, affording an unwound duplex with a directional fixed kink and a widened, shallow minor groove (20). The structure of these adducts determined by phasing assay based on gel electrophoresis and by chemical probes of DNA conformation has revealed (21–24) that these adducts induce the overall helix bend of 32–34° toward major groove, DNA unwinding of 13°, severe perturbation of hydrogen bonding within the 5'-coordinated GC bp, and distortion extended over at least 4–5 bp at the site of the CL. Similarly, the minor 1,3-intrastrand CL of cisplatin also bends the helix axis toward the major groove by ~3° and locally unwinds DNA by ~23° (24, 25). Another important feature of the conformational alteration induced by this lesion is that DNA is locally denatured and flexible at the site of the adduct (23, 26), in contrast to the case of the 1,2-intrastrand adduct. Given the recent advances in our understanding of the structural basis for the conformational alteration caused by intrastrand CLs of cisplatin, it is of considerable interest to examine how the structures of the various types of the intrastrand CLs of BBR3464 affect conformational properties of DNA.

Some structures altered by platinum adducts, such as stable directional bending and unwinding, attract various damaged DNA-binding proteins such as those containing high mobility group (HMG) domain (27–29). This binding of these proteins has been postulated to mediate the antitumor properties of the platinum drugs (28, 29). In addition, several reports have demonstrated (30–32) that intrastrand CLs of cisplatin are removed from DNA during nucleotide excision repair (NER) reactions and that NER is also a major mechanism contributing to cisplatin resistance. Therefore, in addition to examining the structural alterations induced in DNA by the intrastrand CLs of BBR3464, we also investigated in the present work how these adducts are recognized by HMG1 protein and removed from DNA during in vitro NER reactions.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—BBR3464 (Fig. 1) was prepared by standard methods. Cisplatin was obtained from Sigma (Prague, Czech Republic). The stock solutions of platinum compounds were prepared at the concentration of 1 × 10⁻³ M in 10 mM NaClO₄ and stored at 4 °C in the dark. The synthetic oligodeoxyribonucleotides (Fig. 1) were synthesized and purified as described previously (33). HMG1 domain A (HMG1domA) and HMG1 domain B (HMG1domB) (residues 1–84 and 85–180, respectively) were prepared by M. Stros as described previously (34); their sequences (34) were derived from rat HMG1 cDNA. T4 DNA ligase, T4 polynucleotide kinase, and T4 DNA polymerase were purchased from New England Biolabs (Beverly, MA). Acrylamide, bis(acrylamide), urea, and NaCl were from Merck KGaA (Darmstadt, Germany). Dimethyl sulfate (DMS), KMnO₄, diethyl pyrocarbonate (DEPC), KBr, and KH₂SO₄ were from Sigma (Prague, Czech Republic). (γ²³⁵)P]ATP was from Amersham Pharmacia Biotech. ATP and deoxyribonucleoside triphosphates were from Roche Molecular Biochemicals (Mannheim, Germany).

**Platinations of Oligonucleotides**—The single-stranded oligonucleotides (the top strands of the duplexes in Fig. 1) were reacted in stoichiometric amounts with BBR3464. The platinated oligonucleotides were further purified by ion-exchange protein liquid chromatography (FPLC). It was verified by platinum flameless atomic absorption spectrophotometry and by the measurements of the optical density that the modified oligonucleotides contained three platinum atoms. It was also verified using DMS footprinting of platinum on DNA (35–37) that in the platinated top strands of all duplexes the N7 position of the two guanine (G) residues is not accessible for reaction with DMS. Briefly, platinated and nonmodified top strands (5' end-labeled with γ²³⁵P) were reacted with DMS. DMS methylates the N7 position of G residues in DNA, producing alkali-labile sites (38). However, if N7 is coordinated to platinum, it cannot be methylated. The oligonucleotides were then treated with hot piperidine and analyzed by denaturing 24% polyacrylamide gel electrophoresis. For the nonmodified oligonucleotides, shortened fragments due to the cleavage of the strand at one methylated G were observed in the gel. However, no such bands were detected for the oligonucleotides modified by BBR3464. These results indicate that one BBR3464 molecule was coordinated to both G residues in the top strands of all duplexes. If not stated otherwise, the platinated top strands were allowed to anneal with unplatinated complementary strands (bottom strands in Fig. 1) in 50 mM NaCl plus 10 mM Tris-HCl (pH 7.4) and used immediately in further experiments. This annealing procedure included a rapid heating of the mixture of the complementary oligonucleotides to 60 °C followed by the incubation at 25 °C for 2 h. It was verified that under these conditions the intrastrand CLs of BBR3464 were stable for at least 24 h. FPLC purification and flameless atomic absorption spectrophotometry measurements were carried out on an Amersham Pharmacia Biotech FPLC system with MonoQ HR 5/5 column and a Unicam 939 AA spectrometer equipped with a graphite furnace, respectively. Other details have been described previously (33, 35, 39).

**Chemical Modifications**—The modification by KMnO₄, DEPC, and KBr/KHSO₄ were performed as described previously (39–42). The strands of the duplexes (22 bp shown in Fig. 1B) were 5' end-labeled with γ²³⁵P]ATP. In the case of the platinated oligonucleotides, the platinum complex was removed after reaction of the DNA with the probe by incubation with 0.2 mM NaN₃ (pH 11) at 45 °C for 10 h in the dark.

**Ligation and Electrophoresis of Oligonucleotides**—Unplatinated 15- and 19–22-mer single strands (bottom strands in Fig. 1B) were 5' end-labeled with γ²³⁵P]ATP by using T4 polynucleotide kinase. Then they were annealed (see above) with their phosphorylated complementary single strands (unplatinated top strand containing intrastrand CL). Unplatinated and intrastrand CL-containing duplexes were allowed to react with T4 DNA ligase. The resulting samples along with ligated unplatinated duplexes were subsequently examined on 8% native polyacrylamide (monobis(acrylamide) ratio = 29:1) electrophoresis gels. Other details of these experiments were as described in previously published papers (23, 43).

**Mobility Shift Assay**—The 20-mer oligonucleotides 5'-d(AAGAAAGACCGAGAGAGG), 5'-d(AAGAAAGACCAAGAGAGG), or 5'-d(AAGAAAGAACACAGAGAGG) were 5' end-labeled and annealed (see above) to their complementary strands 5'-d(CTTCTCTCGTGTCTTCTTCTCT), 5'-d(CTTCTTC-TTCGTTCTTCT), or 5'-d(CCTCTCTC-TGTTCTTCTTC) respectively, where the asterisks represent a platinum CL. The duplexes (0.6 nM) were incubated with increasing concentrations of proteins in 20-μl sample volumes containing 10 μM HEPES (pH 7.5), 10 mM MgCl₂, 50 mM LiCl, 100 mM NaCl, 1 mM spermidine, 0.2 mM BSA, and 0.05% Nonidet P40. Samples were incubated on ice for 30 min and then made 7% in sucrose and 0.017% in xylene cyanol prior loading on prerun, precooled (4 °C) 6% native polyacrylamide gels (monobis(acrylamide) ratio = 29:1). Gels were electrophoresed for 3 h, visualized by using a Molecular Dynamics PhosphorImager (Storm 860 system), and the bands were quantitated with the ImageQuant software.

**Nucleotide Excision Assay**—The 20-mer oligonucleotides 5'-d(CCTCT-TCTTCTCTG*TGTCTCTTCT), 5'-d(CCTCTCTCCTG*TGTTCTTCT), and 5'-d(CCTCTCTCCTG*TGTTCTTCTC) respectively, where the asterisks represent a platinum CL, were used for the production of linearized DNA with two centrally located 1,2-, 1,3-, or 1,5-intrastrand CL of BBR3464 at nucleotides 75 and 76, 75 and 77, or 75 and 78, respectively. Uniquely modified 20-mers were end-labeled to introduce a radiolabel at the 11th phosphodiester bond 5' to the CL, annealed with a set of five complementary and partially overlapping oligonucleotides, and ligated with T4 DNA ligase. Full-length substrates were separated from unligated products in a 6% denaturing polyacrylamide gel, purified by
electroelution, renatured, and stored in annealing buffer (50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM MgCl₂, and 1 mM dithiothreitol) at -20 °C. Other details of the purification of DNA substrates for NER were the same as described previously (44, 45).

Oligonucleotide excision reactions were performed in cell-free extracts (CFEs) prepared from the HeLa S3 and CHO AA8 cell lines as described (31, 46). These extracts were kindly provided by J. T. Reardon and A. Sancar from the University of North Carolina (Chapel Hill, NC). In vitro repair of intrastrand CLs of BBR3464 was measured with excision assay using these CFEs and 148-bp linear DNA substrates (see above) in the same way as described previously (31) with small modifications. The reaction mixtures (25 μl) contained 10 fmol of radiolabeled DNA, 50 μg of CFE, 20 μg each of dATP, dTTP, dGTP, and TTP in the reaction buffer (23 mM HEPES (pH 7.9), 44 mM KCl, 4.8 mM MgCl₂, 0.16 mM EDTA, 0.52 mM dithiothreitol, 1.5 mM ATP, 5 μg of BSA, and 2.5% glycerol) and were incubated at 30 °C for 40 min. DNA was deproteinized and precipitated by ethanol. The excision products were separated on 10% denaturing polyacrylamide gels and visualized by using a Molecular Dynamics PhosphorImager (Storm 860 system), and the bands were quantitated with the ImageQuant software.

Mapping of incision sites was performed as described in a previous report (31) with small modifications. Briefly, the major excision product (gel-purified) was further incubated for 10 min at 30 °C with T4 DNA polymerase (0.1 units) in 20 μl of buffer composed of 50 mM Tris-HCl (pH 7.9), 15 mM (NH₄)₂SO₄, 7 mM MgCl₂, 0.1 mM dithiothreitol, 50 mM captoethanol, and 20 μg/ml BSA, supplemented with 0.5 μg of Small-digested pBluescript DNA, and visualized by autoradiography following resolution in 10% denaturing polyacrylamide gel. Similar analyses using radiolabeled, platinumated 20-mers (used in the nucleotide excision assays) were also used to identify the nucleotide(s) at which the excinuclease activity of T4 DNA polymerase is blocked 5' to the lesion. The location of the 5’ incision site made by the excinuclease was determined by comparison with the length of excision products observed in the absence of T4 DNA polymerase digestion.

RESULTS

Chemical Probes of DNA Conformation—We demonstrated in our previous paper (14) that preferential G binding of BBR3464 results in various types of adducts including long range intrastrand and interstrand CLs. Quantitation of cross-linking revealed that intrastrand CLs are equally or even more probable than interstrand adducts. Considering these facts we have designed a series of synthetic oligodeoxyribonucleotide duplexes, TGTT, TGTTG, and TGTTTGTG, whose sequences are shown in Fig. 1. The pyrimidine-rich top strands of these duplexes only contained two G residues in the sequences TGTT, TGTTG, and TGTTTGTG in the center (Fig. 1, A). All T residues were strongly reactive with unpaired and distorted adenine (A) residues in a previous report (31) with small modifications. The reaction mixtures (25 μl) contained 10 fmol of radiolabeled DNA, 50 μg of CFE, 20 μg each of dATP, dTTP, dGTP, and TTP in the reaction buffer (23 mM HEPES (pH 7.9), 44 mM KCl, 4.8 mM MgCl₂, 0.16 mM EDTA, 0.52 mM dithiothreitol, 1.5 mM ATP, 5 μg of BSA, and 2.5% glycerol) and were incubated at 30 °C for 40 min. DNA was deproteinized and precipitated by ethanol. The excision products were separated on 10% denaturing polyacrylamide gels and visualized by using a Molecular Dynamics PhosphorImager (Storm 860 system), and the bands were quantitated with the ImageQuant software.

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The oligonucleotide duplexes containing a site-specific 1,2-, 1,3-, or 1,5-intrastrand CL between G residues were further analyzed by chemical probes of DNA conformation. The intrastrand cross-linked duplexes (22-bp, shown in Fig. 1B) were treated with several chemical agents that are used as tools for monitoring the existence of conformations other than canonical B-DNA. These agents include KMnO₄, DEPC, and bromine. They react preferentially with single-stranded DNA and distorted double-stranded DNA (39–42, 48). The results of the analysis by chemical probes of the TGTT(22), TGTTG(22), or TGTTTGTG(22) duplexes containing intrastrand CLs of BBR3464 are summarized in Fig. 2B.

KMnO₄ is hyperreactive with thymine (T) residues in single-stranded nucleic acids and in distorted DNA as compared with B-DNA (40, 42, 49, 50). KMnO₄ reacted with no residue within the unplatinated duplexes (shown for the TGTT(22) duplex in Fig. 2A (left side, lane ds)). All T residues were strongly reactive in the unplatinated single-stranded top oligonucleotide (shown for TGTT duplex in Fig. 2A (left side, lane IAC)). The intrastrand cross-linked duplexes showed strong reactivity of the 5’ T residue adjacent to the adduct (shown for the TGTT(22) duplex in Fig. 2A (left side, lane IAC)). A strong or somewhat weaker reactivity was also observed for the second 5’ T adjacent to the 1,3- or 1,2-CL, respectively. Similarly, a weaker reactivity was observed for the T residues between the platinated G residues in the TGTT or TGTTG cross-linked duplexes.

DEPC carbethoxylates purines at the N7 position. It is hyper-reactive with unpaired and distorted adenine (A) residues in DNA and with left-handed Z-DNA (40, 42, 51, 52). A and G residues within the unplatinated single-stranded oligonucleotide (top and bottom) readily reacted with DEPC (shown for the bottom strand of the 1,2 duplex in Fig. 2A (center, lane ss)). No reactivity of A and G residues was observed within the unplatinated duplex (shown for the bottom strand of the TGTT(22)
DNA Cross-links of Antitumor Trinuclear Platinum Drug

Among the alterations of secondary and tertiary structure of DNA to which it may be subject, the role of intrinsic bending of DNA is increasingly recognized as of potential importance in regulating replication, transcription and repair functions through specific DNA-protein interactions. For DNA adducts of cisplatin, the structural details responsible for bending and subsequent protein recognition have recently been elucidated (28, 29). Given the recent advances in our understanding of the structural basis for the bending of DNA caused by cisplatin CEs, it is of considerable interest to examine how intrastrand DNA adducts of BBR3464 affect conformational properties of DNA such as bending. In this work we performed further studies on the bending induced by single, site-specific 1,2-, 1,3-, and 1,5-intrastrand CL of BBR3464 formed in the oligodeoxyribonucleotide duplexes using electrophoretic retardation as a quantitative measure of the extent of planar curvature.

The oligodeoxyribonucleotide duplexes TGGT(15,19–22), TGGT(15,19–22), and TGGTTTGT(15,19–22) (15 and 19–22 bp long, for their sequences see Fig. 1B) were used for the bending studies of the present work. All sequences were designed to leave a one nucleotide overhang at their 5’ ends in double-stranded form. These overhangs facilitate polymerization of the monomeric oligonucleotide duplexes by T4 DNA ligase in only one orientation and maintain a constant interadduct distance throughout the resulting multimer. Autoradiograms of electrophoresis gels revealing resolution of the ligation products of unplatinated TGGT(15,19–22) duplexes or containing a unique 1,2 GG intrastrand CL of BBR3464 are shown in Fig. 3A. A significant retardation was observed for the multimers of all unplatinated duplexes. Decreased gel electrophoretic mobility may result from a decrease in the DNA end-to-end distance (53). Various platinum(II) complexes have been shown to form DNA adducts, which decrease gel mobility of DNA fragments due to either stable curvature of the helix axis or increased isotropic flexibility (23, 39, 54–56). DNA multimers of identical length and number of stable bend units, but with differently phased bends, have different end-to-end distances. The DNA bends of a multimer must be, therefore, spaced evenly and phased with the DNA helical repeat in order to add constructively. Such constructively phased bends add in plane, yielding short end-to-end distances and the most retarded gel migration. In other words, gel electrophoresis of multimers of oligonucleotide duplexes that only differ in length and contain a stable curvature induced by the same platinum adduct should exhibit a phase effect, i.e. the maximum retardation should be observed for the multimers having the bends in phase with the helix screw. In contrast, the normal electrophoretic mobility should be observed for the multimers having the bends separated by a half-integral number of DNA turns. Importantly, a gel mobility retardation of multimers due to the platinum adducts introducing isotropic flexibility rather than stable curvature is not expected to display a phase dependence (55). The K factor is defined as the ratio of calculated to actual length. The calculated length is based on a multimer’s mobility and is obtained from a calibration curve constructed from the mobilities of unplatinated multimers. The variation of the K factor versus sequence length obtained for multimers of the TGGT 15,19–22 bp long and containing the unique 1,2-GG intra-

Fig. 2. Chemical probes of DNA conformation. Piperidine-induced specific strand cleavage at KMnO₄-modified (A, left), DEPC-modified (A, center), and KBr/KHSO₄-modified (A, right) bases in the duplex TGGT(22) unplatinated or containing single, 1,2-di(GpG) intrastrand CL of BBR3464. The oligomers were 5’ end-labeled at their top or bottom strands. Lanes in panel A, left (KMnO₄, only top strand end-labeled); ss, the unplatinated top strand; ds, the unplatinated duplex; IAC, the duplex intrastrand cross-linked by BBR3464; G, a Maxam-Gilbert specific reaction for the unplatinated duplex. Lanes in panel A, center (DEPC, only bottom strand end-labeled); ss, the unplatinated bottom strand; ds, the unplatinated duplex; IAC, the duplex intrastrand cross-linked by BBR3464; G, a Maxam-Gilbert specific reaction for the unplatinated duplex. Lanes in panel A, right (KBr/ KHSO₄, only bottom strand end-labeled), ss, the unplatinated bottom strand; ds, the unplatinated duplex; IAC, the duplex intrastrand cross-linked by BBR3464; G, a Maxam-Gilbert specific reaction for the unplatinated duplex. A summary of the reactivity of chemical probes in the duplexes TGGT(22), TGGT(22), and TGGTTTGT(22) containing single, 1,2-, 1,3-, and 1,5-intrastrand CL of BBR3464, respectively. Closed and open circles designate strong or weak reactivity, respectively.

duplex in Fig. 2A (center, lane ds)). Within the double-stranded oligonucleotides containing either intrastrand CL, other A residues in the bottom strand became reactive (shown for the TGGT(22) duplex in Fig. 2A (center, lane IAC)). These are readily identified as the A residues complementary to the reactive T residues of the top strand. Importantly, A residues complementary to strongly reactive T residues also reacted with DEPC strongly whereas those A residues complementary to more weakly reactive T residues also reacted with DEPC only more weakly.

Bromination of cytosine (C) residues and formation of piperidine-labile sites are observed when two simple salts, KBr and KHSO₄, are allowed to react with single-stranded or distorted double-stranded oligonucleotides (41). All C residues within the unplatinated single-stranded top or bottom strands of the TGGT(22), TGGT(22), or TGGTTTGT(22) duplexes were strongly reactive (shown for the bottom strand of the TGGT(22) duplex in Fig. 2A (right side, lane ss)). No reactivity of these residues was observed within the unplatinated duplexes (shown for the bottom strand of the TGGT duplex in Fig. 2A (right side, lane ds)). Within the double-stranded duplexes containing the intrastrand CL, no C residue in the top strand was reactive (data not shown). In contrast, the only two C residues in the bottom strand of the platinated TGGT(22) duplex (complementary to the platinated G residues in the top strand of the TGGT(22) duplex (Fig. 2A, right side, lane IAC) and only one C residue in the bottom strands of the platinated TGGT(22) or TGGTTTGT(22) duplexes (complementary to the platinated 5’ G residue in the top strands of these duplexes) were reactive (data not shown).
The binding of the HMG1domA and HMG1domB to these DNA probes was detected by retardation of the migration of the radiolabeled 20-bp probes through the gel (28, 58, 59) (Fig. 4). There is no binding of the HMG1domA and HMG1domB to the DNA probe containing the 1,2-, 1,3-, or 1,5-intrastrand CL of BBR3464 (shown in Fig. 4 (lanes 6–8 and 11–13) for the 1,3-intrastrand CL of BBR3464) that would be evidenced by the presence of slower migrating band. Importantly, this more slowly migrating band was clearly seen for the probe containing 1,2-intrastrand CL of cisplatin analyzed in the presence of the HMG1domA and HMG1domB, even at concentrations 5–6 times lower than was their maximum concentration used in the experiments with the probes containing the CLs of BBR3464 (Fig. 4, lanes 4 and 10). Also importantly, no binding of the proteins occurred under identical experimental conditions in the cases where the same 20-bp DNA probes were not platinumated (shown in Fig. 4 (lane 1) for the duplex containing in its top strand the central TGTT sequence). From these results it is clear that in contrast to 1,2-d(GpG) intrastrand adducts of cisplatin the intrastrand CLs of BBR3464 are not recognized by HMG1 domain proteins.

Nucleotide Excision Repair—NER is a major pathway used by human cells for the removal of damaged nucleotides from DNA (60–62). In mammalian cells, this repair pathway is the only known mechanism for the removal of bulky, helix-distorting DNA adducts, such as those generated by various chemotherapeutics including cisplatin (63). Efficient repair of 1,2-d(GpG) and 1,3-d(GTG) intrastrand CLs of cisplatin has been reported by various NER systems including human and rodent excinuclease (30–32, 64–66). The results presented in Fig. 5A (lanes 4 and 8) confirm these reports. Importantly, 1,2-, 1,3-, and 1,5-intrastrand CLs of BBR3464 were also repaired with a similar efficiency as cisplatin 1,3-intrastrand adduct, but with a considerably higher efficiency than 1,2-intrastrand CL of cisplatin by both human and rodent excinuclease (shown in Fig. 5 (A and B) for the CLs repaired by rodent excinuclease).

The excision repair assay detects radiolabeled fragments resulting from dual incisions both 5’ and 3’ to the lesion. The mobility of these fragments is, however, considerably affected by the 4+ charge of the platinum complex moiety (Fig. 5C), which complicates determination of their length by comparing their migration in the gel with that of the unplatinated marker oligonucleotides. The decreased mobility of the excised platinumated fragments was, therefore, reversed by NaCN treatment (0.2 M, pH 10–11, 45 °C overnight), which removes platinum from DNA. Thus, for the substrate containing intrastrand adducts of BBR3464, the excised fragments were primarily 23–28 nucleotides in length, although 22–31-nucleotide-long fragments were also observed (Fig. 5, A (lane 6) and C (lane 2)). This range of product sizes reflects variability at both the 3’ and 5’ incision sites (31, 67); smaller excision products are due to degradation of the primary excision products by exonuclease present in the extracts (31).

Incubation of the 20-mer containing single 1,3-intrastrand CL of BBR3464 (used in the nucleotide excision assay) with T4 DNA polymerase in the absence of deoxyribonucleotide triphosphates (i.e. exploiting 3’ → 5’ exonuclease activity of T4 DNA polymerase) was used to determine the nucleotide at which the exonuclease activity is inhibited. After incubation (10 min) with T4 DNA polymerase under these conditions, the resulting products migrated as a species 5 nucleotides shorter than the starting material (Fig. 6A), indicating that the exonuclease...
activity is blocked at the second nucleotide 3' to the intrastrand CL. When the 25-mer excision product, generated by repair of the intrastrand CL of BBR3464, was treated in the same manner, it was shortened by 6 nucleotides (Fig. 6B). Because the data with the 20-mer show that T4 DNA polymerase 3'–5' exonuclease activity is stopped two nucleotides 3' to the CL, it implies that the 3' incision site is 8 nucleotides (or at the 9th phosphodiester bond) 3' to the CL. This in turn places the other incision site at the 15th phosphodiester bond 5' to the 1,3-intrastrand CL of BBR3464.

**DISCUSSION**

This paper describes the conformational distortions induced in duplex DNA containing the unique 1,2-, 1,3-, and 1,5-intrastrand CLs of BBR3464 (Fig. 1). The phasing assay (Fig. 3) has revealed that none of three intrastrand CLs of BBR3464 re-
trans such as 

intrastrand CL has been observed for the dinuclear compounds indeed, interstrand CLs possible. The formation of the 1,2-

unlikely to be favored over the longer range intrastrand and, 

formed by cisplatin. Because of the long distance between the 

formally the structural analog of the most prominent adduct 

character or nature of the distortion. The 1,2-intrastrand CL is 

ized and its extent, but they do not provide all details about the 

base pairs between the platinated G residues. The chemical 

probes and phasing assays suggest that the structural 

motif recognized by HMG domain proteins is bent or 

kinked duplex axis. Thus, it is clear from the results of the 

incision site of the 9th phosphodiester bond 3’ to the CL, and the second incision occurs at the 

15th bond on the 5’ side to generate a 25-mer excision product. For other details, see “Experimental Procedure.”

FIG. 6. Mapping of incision sites. A, time-course analysis with T4 DNA polymerase and 20-mer containing 1,3-intrastrand CL of BBR3464 (for its sequence, see “Nucleotide Excision Assay” or the sequence in panel A) was used to identify site of inhibition of T4 DNA polymerase exonuclease activity. At all time points, this exonuclease activity was primarily blocked at the second nucleotide 3’ to the intrastrand CL, resulting in migration of platinated 20-mers as platinated 15-mers. B, limited (10 min) T4 DNA polymerase digestion was used to identify the 3’ incision site of gel-purified oligomers released during the excision repair reaction (Fig. 5A, lane 6). The excised 25-mer (lane 1) migrated as a 19-mer (lane 2) after treatment with T4 DNA polymerase. Thus, one incision occurs at the 9th phosphodiester bond 3’ to the CL, and the second incision occurs at the 15th bond on the 5’ side to generate a 25-mer excision product. For other details, see “Experimental Procedure.”

sults in a stable curvature (directional bending). Lack of phase dependence of the retardation of gel mobility of DNA containing the intrastrand CLs of the trinuclear BBR3464 is consistent with the view that these adducts increase flexibility of the duplex. An increased flexibility introduced to the helix in this manner is sustained by the observation that these lesions create a local conformational distortion revealed by the chemical probes (Fig. 2). This distortion mainly occurs on the 5’ side of the CLs and in the case of the 1,3 and 1,5 adducts also on the base pairs between the platinated G residues. The chemical probes allow detection of the sites where the distortion is localized and its extent, but they do not provide all details about the character or nature of the distortion. The 1,2-intrastrand CL is formally the structural analog of the most prominent adduct formed by cisplatin. Because of the long distance between the two platinating centers of BBR3464, the formation of this CL is unlikely to be favored over the longer range intrastrand and, indeed, interstrand CLs possible. The formation of the 1,2-intrastrand CL has been observed for the dinuclear compounds such as (trans-)[PtCl(NH$_3$)$_2$H$_2$N(CH$_2$)$_2$NH$_2$]Cl$_2$, with the relatively short bridging butane- and hexanediamines. The chemical probes and phasing assays suggest that the structures of the 1,2-intrastrand adduct in the present case are similar to those formed by the dinuclear compound (68, 69). Thus, the principal feature introducing conformational flexibility is reasonably inferred to be the presence of the monofunctional coordination spheres with only one purine base bound. The greater length of the BBR3464 linker and its charge and hydrogen-bonding capacity may, respectively, produce a bulkier adduct and enhance initial DNA recognition but do not appear to affect greatly the major structural feature of conformational flexibility. It is noted that this fundamental feature of monofunctional coordination spheres also leads to considerable conformational flexibility in interstrand CL structure (70).

It has been suggested (28, 29) 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 (28, 29). The molecular basis for this recognition is still not entirely understood, although several structural details of the 1:1 complex formed between HMG domain proteins and the duplex containing 1,2 d(GpG) intrastrand CL were recently elucidated (28, 59). The details of how the binding of HMG domain proteins to cisplatin-modified DNA sensitize 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 titrated away from their transcriptional regulatory function have been suggested (29, 71–73) as clues for how these proteins are involved in the antitumor activity.

An important structural motif recognized by HMG domain proteins on DNA modified by cisplatin is a stable, directional bend of the helix axis (29, 57, 74). Therefore, it is not surprising that we have observed in the present work (Fig. 4) no recognition of DNA intrastrand CLs of BBR3464 by HMG1domA and HMG1domB consistent with the assumption that an important structural motif recognized by HMG domain proteins is bent or kinked duplex axis. Thus, it is clear from the results of the present work that the intrastrand DNA adducts of antitumor BBR3464 may present a block to DNA or RNA polymerases (14) but are not a substrate for recognition by HMG domain proteins. These results parallel our previous findings on the dinuclear compound (68). From these considerations we could conclude that the mechanism of antitumor activity of bifunctional polynuclear platinum BBR3464 does not involve recognition of its intrastrand CLs by HMG domain proteins as a crucial step, in contrast to the proposals for cisplatin and its direct analogues.

One possible role for binding of HMG domain proteins to DNA modified by cisplatin is that these proteins shield damaged DNA from intracellular excision repair (29, 30, 72, 73). The examinations of excision repair of DNA containing various intrastrand CLs of BBR3464 revealed that these adducts were readily removed from DNA by NER (Fig. 5). These results suggest that the processing of the intrastrand CLs of BBR3464 in tumor cells sensitive to this drug may not be relevant to its antitumor effects despite the fact that the trinuclear platinum compound forms on DNA intrastrand CLs with a relatively high frequency. An interesting feature of the repair assay is that a greater range of excised fragments is seen for the conformationally flexible adducts produced by BBR3464 than the stericly rigid adducts produced by cisplatin (Figs. 5 and 6). Indeed, the flexible 1,3-intrastrand adduct of cisplatin also appears to produce more variability in cutting site than the corresponding 1,2-adduct. It is tempting to speculate that de-localized lesions formed by long range adducts of polynuclear platinum compounds may still represent unique challenges for
recognition and excision by repair enzymes.

On the other hand, BBR3464 also forms on DNA interstrand CLs with a considerably higher frequency than cisplatin (14). In general, DNA interstrand CLs could be even more effective lesions than intrastrand adducts in terminating DNA or RNA synthesis in tumor cells and thus could be even more likely candidates for the genotoxic lesion relevant to antitumor effects of BBR3464. In addition, the interstrand CLs pose a special challenge to repair enzymes because they involve both strands of DNA and cannot be repaired using the information in the complementary strand for resynthesis. The fact that interstrand CLs cannot be removed so readily by excision repair as intrastrand lesions is also corroborated by the observation that excision repair of the interstrand CL formed by cisplatin was not detected under condition when intrastrand CLs of this drug were readily removed by a reconstituted system containing highly purified nucleotide excision repair factors (30). Hence, the interstrand CLs, which are rather frequent DNA adducts of BBR3464, would not even have to be shielded by damaged DNA recognition proteins to prevent their repair.

Cellular pharmacology studies in Li210 and osteosarcoma cells using alkaline elution show the persistence of interstrand CLs with time, consistent with a slower rate of repair (16, 18). Data on conformation, recognition by HMG domain proteins and NER of DNA interstrand CLs of BBR3464 will provide more insights into which DNA adduct of BBR3464 is a more likely lesion responsible for antitumor effects of this polynuclear platinum drug. The cytotoxic effects may also be due to a cumulative effect of the structurally heterogeneous adducts produced by polynuclear platinum drugs.

In conclusion, the results of the present work provide additional strong support for the hypothesis that platinum drugs that bind to DNA in a fundamentally different manner to that of cisplatin have altered pharmacological properties. Importantly, in contrast to cisplatin, the mediation of antitumor properties of bifunctional trinuclear platinum complex BBR3464 by shielding its intrastrand adducts by HMG domain proteins is unlikely so that polynuclear platinum compounds apparently represent a novel class of platinum antitumor drugs acting by a different mechanism than cisplatin and its analogues. A further understanding of how bifunctional polynuclear platinum compounds modify DNA and how these modifications are further processed in cells should provide a rational basis for the design of new platinum antitumor drugs and chemotherapeutic strategies.

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Conformation, Recognition by High Mobility Group Domain Proteins, and Nucleotide Excision Repair of DNA Intrastrand Cross-links of Novel Antitumor Trinuclear Platinum Complex BBR3464

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