are serving as general bases, giving diphenylacetic acid product according to eq 7. Taking reciprocals of both sides of eq 7 leads to eq 8, which predicts a linear relationship between $1/R$ and

$$\frac{1}{R} = \frac{1-\omega}{\omega} + \frac{k_{H_2O}}{\omega k_B}\frac{1}{[B]} \quad (8)$$

$1/[B]$, with slope and intercept parameters from which ω and k_B may be obtained, the latter by making use of the known value of k_{H_2O} (=275 s^{-1}).[2b]

The experimental data for both bases conformed to this relationship well; this is illustrated for the case of morpholine in Figure 2. Least-squares analysis gave parameters which led to $\omega = 0.98 \pm 0.04$ and $k_B = (3.73 \pm 0.41) \times 10^4$ M^{-1} s^{-1} for ammonia and $\omega = 0.98 \pm 0.02$ and $k_B = (2.45 \pm 0.17) \times 10^5$ M^{-1} s^{-1} for morpholine. These rate constants agree well with the more precise values obtained from kinetic measurements, $k_B = (3.53 \pm 0.08) \times 10^4$ M^{-1} s^{-1} for ammonia and $k_B = (2.22 \pm 0.01) \times 10^5$ M^{-1} s^{-1} for morpholine, and both values of ω are, to a rather small statistical uncertainty, indistinguishable from unity. This analysis therefore shows that these two bases are functioning primarily as nucleophiles, with very little if any contribution from reaction as general base catalysts.

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Registry No. 1, 525-06-4; **2**, 3469-17-8; NH_3, 7664-41-7; $CNCH_2NH_2$, 540-61-4; $CF_3CH_2NH_2$, 753-90-2; NH_2OH, 7803-49-8; $CN(CH_2)_2NH_2$, 151-18-8; NH_2NH_2, 302-01-2; $(CH_2OH)_3CNH_2$, 77-86-1; $CH_3O(CH_2)_2NH_2$, 109-85-3; $CH_2HOCH_2NH_2$, 141-43-5; $CH_3O(CH_2)_3NH_2$, 5332-73-0; $CH_3(CH_2)_3NH_2$, 109-73-9; CH_3NH_2, 74-89-5; $(CF_3)_2C(OH)O^-$, 62394-21-2; $(CF_3)_2CHO^-$, 44870-01-1; $(CF_3)_2C(CH_3)O^-$, 130935-17-0; $CF_3CH_2O^-$, 24265-37-0; N_3^-, 14343-69-2; HPO_4^{2-}, 14066-19-4; $HO(CH_2)_2S^-$, 57966-62-8; morpholine, 110-91-8; thiomorpholine, 123-90-0; piperazine, 110-85-0; 2-methylpiperidine, 109-05-7; piperidine, 110-89-4; pyrrolidine, 123-75-1; *N*-methylmorpholine, 109-02-4; *N*-methylpiperidine, 626-67-5; 1,4-diazabicyclo[2.2.2]octane, 280-57-9; 1-azabicyclo[2.2.2]octene, 100-76-5; cyanide, 57-12-5.

DNA Binding Properties of *cis*-$[Pt(NH_3)(C_6H_{11}NH_2)Cl_2]$, a Metabolite of an Orally Active Platinum Anticancer Drug

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Abstract: The compound *cis,trans,cis*-$[Pt(NH_3)(C_6H_{11}NH_2)(OC(O)C_3H_7)_2Cl_2]$ (**1**) is the prototypical member of a new class of orally active platinum anticancer drugs. A major metabolite of this compound, formed after ingestion, is *cis*-$[Pt(NH_3)(C_6H_{11}NH_2)Cl_2]$ (**2**). We have used enzymatic digestion/HPLC analysis to investigate the spectrum of adducts formed by the reaction of this Pt(II) reduction product with calf thymus DNA. The major adduct (54%) formed is an intrastrand cross-link involving adjacent guanosine residues, followed in frequency by interstrand or long range intrastrand cross-links also involving guanosine nucleosides (18%). Unlike cisplatin, **2** forms d(ApG) intrastrand cross-links to only a minor extent (8%). The presence of the cyclohexylamine ligand gives rise to two orientational isomers of the platinated d(GpG) moiety, differing with respect to the positioning of the cyclohexyl group toward either the 3′ or the 5′ direction of the phosphodiester linkage. These isomers were observed for platination of calf thymus DNA (2:1 ratio) as well as shorter oligonucleotides. The coordination sites of platinum in the adducts were identified by studies of the reaction products of **2** with d(GpG). ^1H and ^{195}Pt NMR spectroscopy revealed that platinum is bonded to the N7 positions of the two guanine bases. The individual d(GpG)-**2** orientational isomers were synthesized and isolated from reagents in which both the ammine ligand and the N7 position of the 3′-guanine base were labeled with ^{15}N. Comparison with the ^{15}N–^{15}N coupling constants in the $^{15}N\{^1H\}$ NMR spectrum of the two isomers allowed for determination of the stereochemistry at the platinum metal center. From this information the orientational isomer having the cyclohexyl group directed toward the 3′ end of the platinated strand was identified as the more abundant of the two d(GpG)-**2** isomers formed in the reaction of **2** with calf thymus DNA. This isomer is less disruptive to the hydrogen bonding between the NH_3 ligand and the 5′-phosphate group, a structural feature previously identified as being important in the major cisplatin adducts with DNA. Two orientational isomers were also formed upon reaction of **2** with the dodecanucleotide d(TCTAGGCCTTCT), which contains a single d(GpG) platination site. Separation and purification of the two platinated dodecanucleotide orientational isomers allowed for construction of modified M13 genomes containing a single isomer of each of the two d(GpG)-**2** adducts. Each purified isomer was incorporated into a gapped heteroduplex, providing the two corresponding isomers of the site-specifically platinated M13 genomes. Studies of the replication of these platinated genomes with T7 DNA polymerase revealed differences in the position within the genome at which DNA synthesis is inhibited. The *cis*-$[Pt(NH_3)(C_6H_{11}NH_2)\{d(GpG)\text{-}N7(1), \text{-}N7(2)\}]$ orientational isomers inhibited DNA replication less efficiently than the parent cisplatin complex, allowing more (10–15% versus 8% for cisplatin) translesion synthesis.

Introduction

Two rational approaches to the development of new therapies for cancer are to investigate the molecular and cellular biological events that lead to tumorogenesis[1] and to study the mechanism of action of known chemotherapeutic agents. Platinum complexes are used in the treatment of ovarian and bladder cancers and have become indispensable for testicular cancer.[2,3] A long term objective in our laboratory is to elucidate the molecular basis for the anticancer activity of these platinum complexes. This research has focused on DNA as the primary target of the major platinum anticancer drug, *cis*-$[Pt(NH_3)_2Cl_2]$, (*cis*-DDP or cisplatin),[4] and

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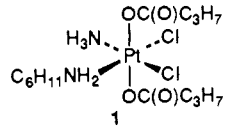
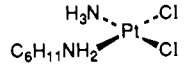
has led to considerable information concerning the structures of the DNA adducts and the binding of cellular proteins to cisplatin-modified DNA.[5-9]

Cisplatin treatments are expensive, in part due to the cost of required hospital visits for intravenous administration of the drug. Side effects such as renal toxicity, neurotoxicity, and severe emesis are often dose limiting. These problems have led to a search for platinum complexes that can be administered orally, a treatment that would potentially reduce the cost of chemotherapy and might also lead to reduced side effects compared to those experienced from intravenous injections. Nearly 1 year ago, platinum complexes were reported that could be administered orally while retaining excellent anticancer activity in animal screens; one such compound is scheduled to enter clinical trials shortly.[10] A prototypical member of this class is *cis,trans,cis*-[Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$)(OC(O)C$_3$H$_7$)$_2$Cl$_2$] (**1**), which contains a platinum(IV) metal center. Platinum(IV) complexes undergo ligand substitution reactions that are slow relative to their platinum(II) analogues.[11] The antitumor activity of Pt(IV) complexes is therefore likely to require in vivo reduction to the kinetically more labile, and therefore reactive, Pt(II) derivatives. Indeed, previous studies have shown that platinum(IV) metal centers are readily reduced by cellular components such as glutathione and ascorbic acid[12-15] to form the platinum(II) analogues that bind more rapidly to DNA. Thus, the orally ingested *cis,trans,cis*-[Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$)(OC(O)C$_3$H$_7$)$_2$Cl$_2$] compound probably serves as a pro-drug for the chemotherapeutically active platinum(II) analogue *cis*-[Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$)Cl$_2$] (**2**), which is presumably the complex that binds to DNA.[10]



1



2

cis-Diamminedichloroplatinum(II) forms covalent adducts with nitrogen donors of the nucleobases in DNA.[16-19] The predominant adduct is an intrastrand cross-link between the *N*7 positions of two adjacent guanosines, which accounts for 55–65% of the adducts. The second most abundant adduct is an *N*7–*N*7 d(ApG) intrastrand cross-link, accounting for 25–35% of the adducts. Interstrand and 1,3 intrastrand adducts comprise 10% or fewer of the adducts. Many studies have shown that the lesions caused by platinum binding to DNA inhibit DNA synthesis,[20-24] and it is commonly believed that the inability of tumor cells to replicate DNA accounts, at least in part, for the cytotoxicity of the platinum complexes.

In the present study, we have applied the methodology previously developed for cisplatin to investigate the reaction products of *cis*-[Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$)Cl$_2$] with DNA. Since this complex may be interesting from a clinical point of view, it is important to determine the extent to which it resembles the parent drug cisplatin in binding to its biological target. Moreover, since the platinum coordination sphere in **2** is unsymmetrical, the possibility exists that orientational isomers[25] might be formed upon binding to DNA. Understanding this stereochemical phenomenon is of fundamental interest to the developing field of metal-DNA chemistry.[7,26,27] In addition, the orientational isomers might interact differently with DNA processing enzymes, which could lead to altered chemotherapeutic properties. These issues have also been addressed in the experiments described here by using the powerful approach of site-specifically modified genomes.[28,29]

Experimental Section

Materials and Methods. Platinum complexes were synthesized as described in the literature.[30] *cis*-DDP was obtained as a gift from Johnson-Matthey. The compounds d(GpG), 2′-deoxyguanosine, 2-*N*-isobutyryl-5′-dimethoxytrityl-2′-deoxyguanosine 2-chlorophenyl diester, 2-*N*-isobutyryl-5′-dimethoxytrityl-2′-deoxyadenosine 2-chlorophenyl diester, and 2-*N*-isobutyryl-3′-benzoyl-2′-deoxyguanosine were purchased from Sigma. [^{15}N]2′-Deoxyguanosine, labeled at the N7 position, was obtained from the Stable Isotopes Laboratory at Los Alamos National Laboratories. ^{15}N-labeled 2-*N*-isobutyryl-2′-deoxyguanosine,[31] 2-*N*-isobutyryl-5′-dimethoxytrityl-2′-deoxyguanosine,[32] and 2-*N*-isobutyryl-3′-benzoyl-2′-deoxyguanosine[33,34] were prepared by procedures analogous to those reported for unlabeled material. d(ApG) and ^{15}N-labeled d(GpG) were prepared according to published procedures.[35,36] The oligonucleotide d(TCTAGGCCTTCT), abbreviated Stu-12T,[4] was prepared and purified by reversed phase HPLC, as described previously.[28,29] Preparative and analytical HPLC employed C$_{18}$ reversed phase columns (Waters μ-Bondapak). Linear gradients of acetonitrile in 0.1 M ammonium acetate (pH 6.0) were used on a Perkin-Elmer Series 4 liquid chromatograph linked to an LC terminal, an LC 95 UV–vis spectrophotometer detector set at 260 nm, and an LCI integrator. Calf thymus DNA was purchased from Sigma and purified by phenol/chloroform extraction and precipitation before use. Cloned, modified T7 polymerase was obtained as part of the Sequenase 2.0 system purchased from United States Biolabs. Restriction enzymes, polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs. DNase I (from bovine pancreas) was obtained from Sigma. P1 nuclease (from *Penicillium citrinum*) and alkaline phosphatase (from calf intestine) were purchased

(4) Abbreviations: bp, base pair; cisplatin or *cis*-DDP, *cis*-diamminedichloroplatinum(II); NaOAc, sodium acetate; RF, replicative form; RP-HPLC, reversed phase HPLC; TE, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; d(TCTAGGCCTTCT) and d(AGAAGGCCTAGA) are abbreviated Stu-12-T and Stu-12-A; M13-Stu-12-A is the product of insertion of Stu-12-A into the plus strand of M13mp18 at the *Hind* II site.

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from Boehringer Mannheim or Sigma. Platinum analyses were performed by flameless atomic absorption spectroscopy on a Varian AA1475 instrument equipped with a GTA95 graphite furnace. ^{1}H NMR spectra were obtained on a Bruker AC 250-MHz spectrometer; chemical shifts were referenced relative to internal DSS, which was calibrated against an external standard of tetramethylammonium chloride set to δ 3.18. ^{195}Pt NMR spectra were obtained on a Varian VXR 500-MHz spectrometer; chemical shifts were referenced relative to Na_2PtCl_6, with an external standard of K_2PtCl_4 set to δ −1624. ^{15}N NMR spectra were obtained on a Varian Unity 300-MHz spectrometer. FAB (MS/MS) mass spectral analyses were obtained according to published procedures.[37,38]

Platination Reactions. Because of the low solubility of [Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$)Cl$_2$] (**2**) in water, all DNA platination reactions were carried out with the diaqua derivative [Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$)(OH$_2$)$_2$](NO$_3$)$_2$. This species was obtained by stirring a heterogeneous aqueous solution containing 2–5 mmol of **2** and 1.98 equiv of silver nitrate for 8–16 h. The precipitated silver chloride was removed by centrifugation and the supernatant was employed in the reactions, assuming quantitative conversion to the diaqua derivative. Atomic absorption analysis of the supernatant demonstrated that all of the starting platinum material was present in the supernatant.

Platination of calf thymus DNA was performed for 12–16 h at 37 °C with 2–10 OD of DNA at r_f (formal drug-to-nucleotide ratio) values ranging from 0.01 and 0.10. Traces of unbound platinum were removed by precipitating the DNA and washing with 70% ethanol. Bound drug-to-nucleotide ratios (r_b) were determined by measuring DNA concentrations by UV spectroscopy and platinum concentrations by flameless atomic absorption spectroscopy. Platinations of 2′-deoxyguanosine, d(GpG), and oligonucleotides on a preparative scale were conducted at a platinum-to-strand ratio of 1.5 for 1–2 h. The products were isolated by preparative RP-HPLC.

pH-Dependent NMR Spectroscopy. Platinum binding sites were determined by pH-dependent NMR spectroscopy as described previously.[24,39,40] Samples containing 50–100 OD of [Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$){d(GpG)}] or [Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$){d(G)}$_2$] were dissolved in 0.5 mL of 99.9 atom % D_2O and lyophilized three times before being dissolved in 0.5 mL of 99.9 atom % D_2O for data collection. The pH of the solutions was adjusted by addition of 1% and 10% DCl or NaOD solutions and was measured in the NMR tube by using a 3 × 180 mm Ingold pH probe. pH measurements were corrected for the deuterium isotope effect by subtracting 0.4 from the observed values. The pH, determined before and after NMR data collection, proved to be reproducible.

Enzymatic Degradation of Calf Thymus DNA and Oligonucleotides. Digestions to yield mononucleosides and platinated dinucleoside monophosphates for subsequent RP-HPLC analysis were carried out by using DNase I, P1 nuclease, and alkaline phosphatase according to procedures reported previously.[18,24,41] Platinated, precipitated calf thymus DNA or lyophilized oligonucleotide Stu-12T was dissolved in 100 μL of NaOAc at pH 5.5, 1 mM $MgCl_2$ per 2 OD of DNA. Subsequently, DNase I (20 units per 2 OD) and P1 nuclease (0.02 mg per 2 OD) were added and the sample was incubated at 37 °C for 16–24 h. The pH was then raised by addition of Tris-HCl at pH 9.0 to a final concentration of 100 mM, calf intestinal phosphatase (5 units per 2 OD) was added, and the sample was incubated for an additional 4 h at 37 °C. Samples were then analyzed by RP-HPLC, and the product ratios were determined by relative peak areas, correcting for extinction coefficients of the nucleobases.

Preparation of Double-^{15}N-Labeled Complexes. ^{15}N7-labeled 2-*N*-isobutyryl-3′-benzoyl-2′-deoxyguanosine[33,34] and unlabeled 2-*N*-isobutyryl-5′-dimethoxytrityl-2′-deoxyguanosine 2-chlorophenyl diester were employed in a solution phase synthesis of 3′-^{15}N7-d(GpG).[35,36] The labeled complex *cis*-[Pt(^{15}NH$_3$)(C$_6$H$_{11}$NH$_2$)Cl$_2$] (^{15}N-**2**) was prepared[30] from *cis*-[Pt(^{15}NH$_3$)$_2$Cl$_2$].[42] Platination and purification of the two products, d(GpG)-**2a** and d(GpG)-**2b**, of 3′-^{15}N7-d(GpG) platination with ^{15}N-**2** were carried as described for unlabeled material.

Preparation of Heteroduplex DNA with a (−) Strand Gap. M13 genomes containing one isomer of a single [Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$){d(GpG)-*N*7-G(1),*N*7-G(2)}] adduct were synthesized according to the strategies previously employed for the preparation of an M13 genome containing single *cis*-DDP adducts.[28,29] Briefly, M13-Stu-12A clones were prepared in 2-L growths from the progeny phage containing the Stu-12A oligonucleotide (complementary to Stu-12T), inserted by blunt end ligation into the *Hin*d II recognition site in M13mp18. Single-stranded DNA phage were isolated by polyethylene glycol precipitation. M13mp18 RF DNA was isolated from 2-L growths and was purified by two CsCl/ethidium bromide density gradient centrifugation steps. The single-stranded phage DNA containing the Stu-12A insert was mixed with a 20-fold molar excess of *Hin*d II linearized M13mp18 RF DNA. The 20:1 mixture was dialyzed against 95% formamide to denature the linearized M13mp18 RF strands and then was dialyzed against decreasing amounts of formamide to allow renaturation of the (−) strand to the excess circular viral (+) strands. These gapped heteroduplexes were separated from the remaining closed and linear single strands by hydroxylapatite chromatography. Double-stranded cloned M13-Stu-12A was also isolated for use in constructing a molecular weight/sequence ladder for replication inhibition experiments.

Insertion of Platinated and Unplatinated Dodecanucleotides into the (−) Strand of Gapped Duplex Genomes. Unplatinated Stu-12T and the two isomerically pure Stu-12T-**2** platinated oligonucleotides (see Results) were phosphorylated by using polynucleotide kinase (PNK) and [γ-^{32}P]ATP for genome characterization by restriction digest inhibition, and with PNK and cold ATP for use in replication inhibition, experiments. Ligation reactions were performed with freshly phosphorylated oligomers at a 300–1000:1 molar ratio of oligonucleotide to gapped duplex. After deactivating the PNK by heating to 65 °C for 20 min, fresh ATP was added, along with gapped duplexes and T4 DNA ligase. Ligation reactions were performed at 16 °C for 12–16 h. Both the kinase and ligase reactions were conducted in solutions containing Tris-HCl (50 mmol, pH 7.6), $MgCl_2$ (10 mM), and dithiothreitol (20 mM). Radioactive samples were purified by Sepharose CL-4B size exclusion chromatography, eluting with 50 mM NaCl in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Large-scale preparative ligations were used for replication inhibition studies after phenol/chloroform extraction to remove enzyme and precipitation to remove salts and unincorporated ATP.

Characterization of Platinated Genomes by Restriction Digest Inhibition. ^{32}P-labeled M13 genomes prepared by ligation of platinated or unplatinated, ^{32}P-labeled Stu-12T into gapped heteroduplexes were treated with *Stu* I restriction enzyme at 37 °C for 2 h in the recommended buffers. Carrier M13-Stu-12A DNA was added to each restriction digest to assist in recovery of precipitated samples. A portion of each sample was treated with one-third volume of a 1 M NaCN solution, providing a final cyanide concentration of 0.25 M, to remove the bound platinum prior to *Stu* I restriction digests. The NaCN solutions were freshly prepared in 100 mM Tris-HCl (pH 7.5) with the pH adjusted to 8.5 by the addition of HCl in a fume hood. The samples were exposed to NaCN for 5 h at 37 °C before precipitation of the samples. The cyanide reversed samples were treated with *Stu* I restriction enzyme at the same time as the platinated samples. The final samples (200–300 cpm) were analyzed on a 0.8% agarose gel containing 0.5 μg/mL of ethidium bromide. Visualization of the samples was achieved by autoradiography after drying the gel at 60 °C for 4 h in a vacuum gel dryer.

Preparation of Samples for Replication Inhibition Experiments. The site-specifically modified genomes (3–5 μg) were linearized with 20 units of *Hin*d III in a 200 μL volume containing the recommended buffer. Complete linearization was verified by electrophoresis through an agarose gel containing ethidium bromide. The samples were extracted with phenol/chloroform, divided into two equal portions, precipitated with sodium acetate and ethanol, and washed with 70% ethanol. The samples were then reconstituted in the appropriate buffer, primer was added, and the volume was adjusted to 10 μL.

Replication Inhibition Experiments. The linearized samples were denatured by boiling for 3 min, and a 1–5-fold excess of the primer d(AGCGGATAACAATTTCACACAGGA) obtained from New England Biolabs (primer No. 1233) was annealed by rapid cooling on ice. The procedure for DNA replication experiments was adapted from directions supplied with the Sequenase sequencing kit. For experiments incorporating [α-^{35}S]dATP, 4.5 pmol of dGTP, dCTP, dTTP and 5 pmol of [α-^{35}S]dATP were added to 0.2–2 μg of the linearized, site-specifically modified genomes with annealed primer and to 2 μg of the denatured, primer-annealed M13-Stu-12T RF samples for molecular weight/sequencing ladders. All samples contained 25 mM Tris-HCl (pH 8), 6.5 mM dithiothreitol, 12 mM $MgCl_2$, and 30 mM NaCl. After addition of 1.5 units of T7 polymerase, the samples were incubated for 5 min at room temperature. At this point, the concentrations of all four dNTPs were raised to 10–45 mM, which allowed for completion of DNA synthesis. In separate reactions, ddNTP/dNTP samples were added to portions of the M13-Stu-12A RF sample in order to produce sequence ladders. Reactions were terminated after 15 min at 37 °C by the addition of stop solution containing 95% formamide, 20 mM EDTA, and xylene cylanol and bromophenol blue tracking dyes. Stopped reactions were

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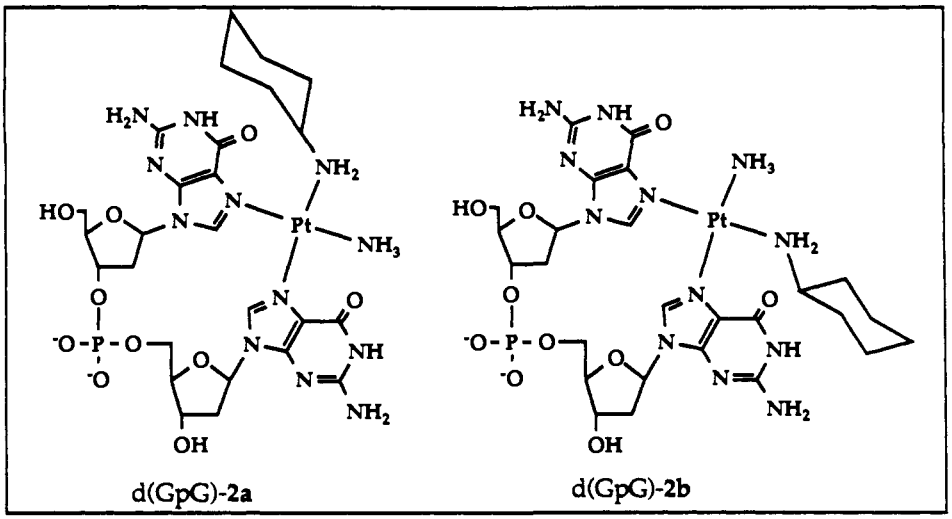
(42) Nee, M.; Roberts, J. D. *Biochemistry* **1982**, *21*, 4920.

Chart I



stored at −80 °C for subsequent analysis. Experiments involving ^{32}P-end-labeled primer were conducted by adding 1–5 units of enzyme to 1.5–2.5 μg of samples in solutions containing 25 mM Tris-HCl at pH 8, 6.5 mM dithiothreitol, 12 mM $MgCl_2$, 30 mM NaCl, and 10–45 μM of each dNTP. The samples were incubated for 15 min at 37 °C and stopped as for ^{35}S-labeled samples.

Electrophoresis. The products of replication and sequencing reactions were resolved on 8% polyacrylamide/7 M urea denaturing gels and were fixed (10% methanol/10% acetic acid), dried, visualized by autoradiography, and analyzed by densitometry. Analytical experiments were conducted by using 0.4 mm thick sequencing gels. Samples (2–6 μL) were heated at 75–80 °C for 3 min immediately before loading, and the gels were run for 2.5–3 h at a constant voltage of 1500–1800 V.

Results

Characterization of Adducts Formed by *cis*-[Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$)Cl$_2$] with Calf Thymus DNA. The spectrum of adducts formed upon global platination of double-stranded DNA with cisplatin and [Pt(en)Cl$_2$] (en = 1,2-diaminoethane) has been previously established by enzymatic degradation of the platinated DNA to single nucleosides and platinated dinucleosides, followed by chromatographic analysis.[17,18,41,43] In order to determine the adduct profile of *cis*-[Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$)Cl$_2$] (**2**), and also to ascertain whether the cyclohexylamine ligand leads to separable 3′- and 5′-orientational isomers of the platinated dinucleosides obtained following digestion of DNA,[25] the binding of **2** to calf thymus DNA was analyzed. The products were identified by coinjection of digested DNA with independently prepared platinated dinucleosides or dinucleoside monophosphates onto a reversed phase C$_{18}$ HPLC column.

Figure 1 shows a typical HPLC trace for a sample of digested calf thymus DNA platinated with hydrolyzed **2** at a r_b of 0.057. As previously reported,[18] in HPLC analyses of the digestion products of DNA containing platinum adducts the platinated fragments were difficult to separate from unplatinated mononucleosides. In the present study, the hydrophobic cyclohexyl group afforded significantly different retention times for the platinated dinucleosides and platinated dinucleoside monophosphates compared to those of the unplatinated mononucleosides. Product ratios were determined from the relative peak areas (see Experimental Section). Table I summarizes the spectrum of adducts formed in appreciable quantities for **2** and, for comparison purposes, cisplatin. As is characteristic of cisplatin, the most abundant adducts of **2** with calf thymus DNA are intrastrand d(GpG) cross-links, which account for 54% of the total bound platinum. Moreover, two orientational isomers form in the reaction of **2** with DNA,[25] leading to the generation of the corresponding isomers (Chart I) of d(GpG)-**2** in a 2:1 ratio. The second most abundant digestion product is the bis(guanosine) complex, which could result from the formation of a 1,3-intrastrand cross-link, a longer range intrastrand cross-link, or an interstrand cross-link. In this case, the absence of the phosphodiester linkages eliminates the asymmetry of the DNA fragment and only one isomer is observed following enzymatic digestion. Unlike cisplatin, complex **2** forms d(ApG) adducts to only a minor extent. These adducts account for 8% of the digestion products, appearing as a single peak in the HPLC trace of the digestion mixture. This result most likely indicates that only one d(ApG)-**2** isomer forms in appreciable quantities, although we have not been able to rule out the possibility that two isomers are present that coelute under the HPLC conditions employed (vide infra).

Table I. Quantities of Various Adducts Formed upon Global Platination of Calf Thymus DNA with Cisplatin or the Hydrolysis Product of *cis*-[Pt(NH$_3$)(C$_6$H$_{11}$NH$_2$)Cl$_2$] (**2**)[a]

adduct	cisplatin[b]	**2**	ratio of isomers for **2**
d(GpG)	55–65	54 (4)	35 (2), 19 (2)
d(ApG)	25–35	8 (2)	
(Guo)$_2$	5–10	18 (6)	

[a] Numbers in parentheses are estimated errors. [b] Data taken from refs 17 and 18.

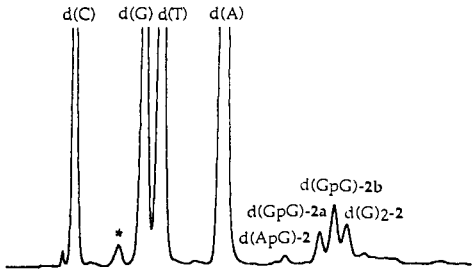


Figure 1. HPLC trace of enzymatically degraded, platinated (r_b = 0.057) calf thymus DNA in the region containing platinum-modified nucleosides. The small peak marked with an asterisk is present in unplatinated control digests.

The adduct peaks in the HPLC trace of the digested, platinated calf thymus DNA were identified by coinjection of independently prepared samples of the two orientational isomers of d(GpG)-**2** and d(G)$_2$-**2**. Platination of d(GpG) with the diaqua derivative of **2** at a platinum-to-strand ratio of 1.5 provided the two isomers in a ratio of 3:2, with the more rapidly eluting isomer formed in greater quantities. This preference is in contrast to the results obtained for platination of duplex DNA by **2** where the major product found after nuclease and phosphatase digestion elutes more slowly from the column. The isomers were separated by reversed phase C$_{18}$ HPLC chromatography. The d(G)$_2$ adduct was similarly prepared by addition of 2 equiv of 2′-deoxyguanosine to an aqueous solution of the diaqua derivative of **2**. This bis(guanosine) adduct was purified by RP-HPLC. All three complexes were characterized as N7–N7 adducts by pH-dependent ^{1}H NMR

(43) Eastman, A. *Biochemistry* **1982**, *21*, 6732.

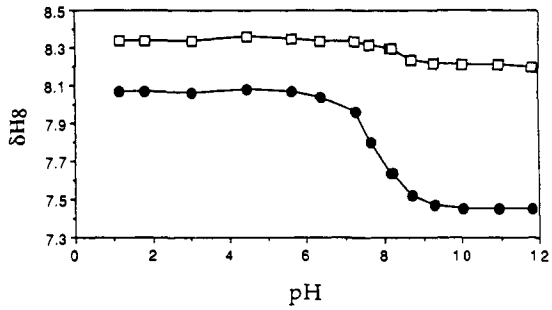


Figure 2. Dependence of the guanosine H8 chemical shifts on pH for one isomer of d(GpG)-**2**.

titrations and by ^{195}Pt NMR spectroscopy at neutral pH. The effect of pH on the chemical shift of the two nonexchangeable H8 guanine resonances for one of the isomers of GpG-**2** is shown in Figure 2. Nearly identical titration curves were observed for the other two complexes (data not shown). The three complexes all exhibited the characteristic inflection point near pH 8.5, reflecting the increased acidity of the proton attached to N1 upon platination of the guanine base.[39,44,45] The absence of chemical shift changes near pH 2, observed for unplatinated d(GpG) as a result of protonation of N7, confirms that platination occurs at the most nucleophilic N7 position of both guanine bases. The ^{195}Pt NMR chemical shifts of δ −2485, −2495, and −2467 for the two d(GpG) adducts and the $d(G)_2$ adduct, respectively, are consistent with coordination of a diamineplatinum(II) fragment to two additional nitrogen donor ligands.[46]

Initial studies to determine the relative orientations of the cyclohexyl group in the two d(GpG) isomers were carried out by employing two-dimensional NOESY and ROESY NMR experiments. No crosspeaks between the H8 protons of the guanine bases and any of the cyclohexylamine protons were observed, however. Instead, the geometry at platinum for the two orientational isomers of d(GpG)-**2** was assigned by a double-label experiment, in which ^{15}N was incorporated both into the ammine ligand and into the N7 position of the 3′-guanine base. Although cis and trans geometric isomers have not been typically distinguished by measuring the coupling between two chemically distinct ^{15}N-labeled atoms coordinated to a transition metal center, analogous stereochemical determinations are common for phosphine complexes and have been especially useful in determining the structures of compounds containing Pt–P bonds.[47] Similar stereochemical determinations should also be possible with ^{15}N-labeled complexes. Several ^{15}N NMR spectroscopic studies of square planar and octahedral platinum ammine complexes have been conducted. Although $J_{^{15}N-^{15}N}$ between ^{15}N-labeled ligands in mutually cis positions has not been observed, coupling constants of 4–8 Hz have been reported for complexes with an ammine positioned trans to ^{15}N-labeled glycine or its derivatives,[48-50] $^{15}NO_2^-$,[51,52] or ^{15}N-labeled imidazole.[53]

In the case of the platinum d(GpG) adducts, we observed coupling between the two ^{15}N labels with only one orientational

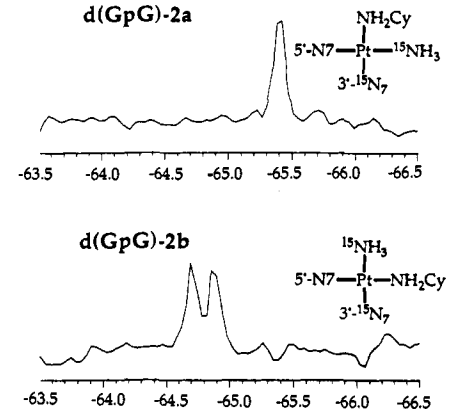


Figure 3. $^{15}N\{^1H\}$ NMR spectra of the two linkage isomers of d(GpG)-**2**. Chemical shifts are reported in ppm downfield of 5 M $^{15}NH_4$ as $^{15}NH_4{}^{15}NO_3$ in 2 M $D^{15}NO_3/D_2O$.[52]

isomer and attribute this isomer to that containing the labeled ammine ligand trans to the labeled 3′ base. This ^{15}N–^{15}N coupling was determined from the $^{15}N\{^1H\}$ NMR spectra of the ammine resonances, presented in Figure 3, of the two orientational isomers. The ^{15}N resonance of the 3′-deoxyguanosine ligand was not observable in these spectra owing to the limited quantities of material available from the synthesis and the lack of an NOE enhancement similar to that afforded to the ammine ^{15}N resonance by proton decoupling in the protiated H_2O solvent. The low signal-to-noise ratio also prevented unambiguous observation of ^{195}Pt satellites for the ammine resonance. A 6-Hz coupling between the two ^{15}N nuclei was observed for d(GpG)-**2b**, whereas no $J_{^{15}N,^{15}N}$ coupling was observed for the doubly ^{15}N labeled isomer d(GpG)-**2a**. These results indicate that the cyclohexylamine ligand is located cis to the 5′ nucleoside in the more rapidly eluting isomer, d(GpG)-**2a**, and cis to the 3′ nucleoside in d(GpG)-**2b** (Chart I).

We were unable to identify the adduct formed in the reaction of the hydrolysis products of **2** with d(ApG) sites in calf thymus DNA by independent synthesis. Attempts to prepare samples of d(ApG) platinated with *cis*-$[Pt(NH_3)(C_6H_{11}NH_2)(H_2O)_2]^{2+}$ in acceptable yields were unsuccessful at any platinum concentration (1–10 equiv) or pH (3.3 to 8) employed, precluding NMR spectroscopic analysis of this adduct. Quantities of the d(ApG) adduct sufficient for mass spectral analysis, however, were obtained by preparative HPLC of digested, platinated calf thymus DNA. By using FAB (MS/MS) techniques it was possible to establish the presence of a product with the stoichiometry expected for a *cis*-$[Pt(NH_3)(C_6H_{11}NH_2)\{d(ApG)\}]$ adduct; spectra are supplied in Figure S1 (supplementary material). In particular, the molecular ion at *m/e* 889 (for the ^{194}Pt component) appeared as an envelope of peaks corresponding to the different platinum isotopes. MS/MS analysis of this ion showed fragmentation patterns arising from loss of the ammine (*m/e* 872) or cyclohexylamine (*m/e* 790) ligand, or the deoxyribose sugar (*m/e* 774). In addition, fragments of *m/e* 152 and 137 were observed, corresponding to adenine and guanine bases, at *m/e* 479, arising from platinum bound to adenine and guanine, and 496, corresponding to platinum bound to adenine, guanine, and one ammine. Mass spectral analysis cannot distinguish between a *cis*-$[Pt(NH_3)(C_6H_{11}NH_2)\{d(ApG)\}]$ and a *cis*-$[Pt(NH_3)(C_6H_{11}NH_2)\{d(GpA)\}]$ adduct. Since cisplatin d(GpA) adducts have not been observed in the platination of duplex DNA, however, we tentatively assign the spectrum of a *cis*-$[Pt(NH_3)(C_6H_{11}NH_2)(dApG)]$ adduct. Moreover, the presence of orientational isomers cannot be determined by mass spectral techniques, and therefore, it is unknown whether only one d(ApG) adduct is formed in measurable quantities or whether there are two platinated d(ApG) isomers that coelute under the HPLC conditions employed.

Platination and Enzymatic Characterization of d(TCTAGGCCTTCT). Reaction of *cis*-$[Pt(NH_3)(C_6H_{11}NH_2)(H_2O)_2]^{2+}$ with the dodecanucleotide d(TCTAGGCCTTCT) (Stu-12T), containing a unique d(GpG) site within a single strand of the *Stu* I restriction site, resulted in the formation of two major

(44) Chottard, J. C.; Girault, J. P.; Chottard, G.; Lallemand, J.-Y.; Mansuy, D. *J. Am. Chem. Soc.* **1980**, *102*, 607.
(45) Marcellis, A. T. M.; den Hartog, J. H. J.; Reedijk, J. *J. Am. Chem. Soc.* **1982**, *104*, 2664.
(46) A list of ^{195}Pt chemical shifts is provided in: Bancroft, D. P.; Lepre, C. A.; Lippard, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 6860, and references therein.
(47) Verkade, J. G.; Quin, L. D. *Phosphorus-31 NMR Spectroscopy in Stereochemical Analysis*; VCH Publishers: New York, 1987.
(48) Appleton, T. G.; Hall, J. R.; Ralph, S. F. *Aust. J. Chem.* **1986**, *39*, 1347.
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(50) Appleton, T. G.; Hall, J. R.; Ralph, S. F. *Inorg. Chem.* **1985**, *24*, 673.
(51) Appleton, T. G.; Hall, J. R.; Ralph, S. F. *Inorg. Chem.* **1988**, *27*, 4435.
(52) Appleton, T. G.; Hall, J. R.; Ralph, S. F. *Inorg. Chem.* **1985**, *24*, 4685.
(53) Alei, M. J.; Vergamini, P. J.; Wageman, W. E. *J. Am. Chem. Soc.* **1979**, *101*, 5415.

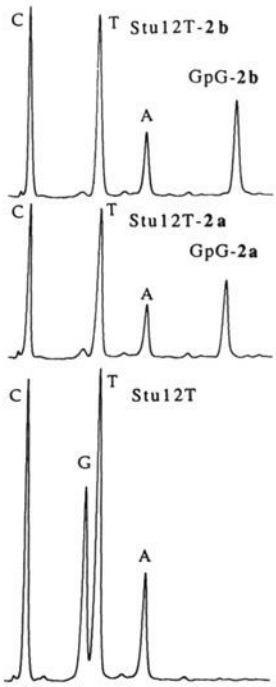


Figure 4. Enzymatic digestion of unplatinated Stu-12T and the two Stu-12T-**2** isomers. The peaks labeled d(GpG)-**2** correspond to the two independently synthesized, purified, and NMR spectroscopically characterized GpG adducts.

adducts in a ratio of 3:4. These two adducts were separated by HPLC. A complete NMR spectroscopic characterization of the analogous *cis*-DDP platinated dodecanucleotide Stu-12T, reported previously, demonstrated that platinum binds to the N7 positions of the adjacent guanosine residues.[28,29] Enzymatic digestion of the single nucleotides dA, dC, and dT and to the intrastrand cross-link *cis*-[Pt(NH_3)$_2$\{d(GpG)-*N*7(1), -*N*7(2)\}] confirmed this assignment.[24] Enzymatic digestion of the reaction product of hydrolyzed **2** with Stu-12T also demonstrates the formation of the d(GpG, *N*7–*N*7) intrastrand cross-link. As shown in Figure 4, digestion of the individually purified isomers of the platinated dodecanucleotide gave rise to the appropriate ratio of single nucleosides dA, dC, dT and to two different isomers of *cis*-[Pt(NH_3)($C_6H_{11}NH_2$)\{d(GpG)\}]. The more rapidly eluting *cis*-[Pt(NH_3)($C_6H_{11}NH_2$)\{d(GpG)\}] isomer, known from the work described above to contain the cyclohexylamine ligand positioned cis to the 5′-deoxyguanosine, corresponds to the more rapidly eluting isomer of platinated Stu-12T. We therefore conclude that, in this isomer, Stu-12T-**2a**, the cyclohexylamine ligand is oriented toward the 5′ end of the dodecanucleotide. From the digestion analysis it was also clear that the more slowly eluting isomer, Stu-12T-**2b**, has its cyclohexylamine ligand positioned toward the 3′ end of the dodecanucleotide.

The two isomers of Stu-12T-**2** do not interconvert. Samples stored at 10–15 °C for several months were isomerically pure as judged by HPLC analysis. Moreover, during the enzymatic degradation to single deoxynucleosides and platinated dinucleoside monophosphates, the complexes were incubated at 37 °C for 24 h, yet only one *cis*-[Pt(NH_3)($C_6H_{11}NH_2$)\{d(GpG)\}] isomer was observed for each of the digested samples upon HPLC analysis. Control experiments indicated that, even boiling the samples of the individual isomers for 5 min, a procedure employed for denaturing and primer annealing (see Experimental Section), did not isomerize the compound. It is perhaps not surprising that the purified isomers are so stable. Rearrangement from one isomer to the other would require simultaneous dissociation of both guanine bases from the platinum center, and little evidence exists for dissociation of even a single such deoxyguanosine.[24,37]

Construction of M13 Genomes Containing Isomerically Pure, Site-Specific [Pt(NH_3)($C_6H_{11}NH_2$)\{d(GpG)\}] Adducts. The ability to separate the two orientational isomers formed upon platination

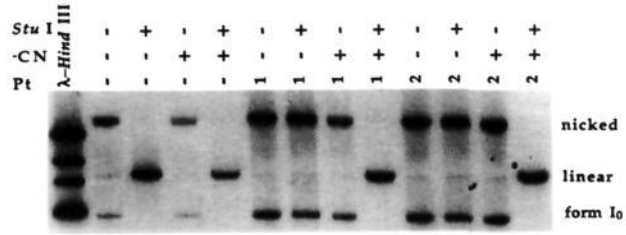


Figure 5. Characterization of single, isomerically pure d(GpG)-**2** adducts by *Stu* I restriction digest inhibition. Lane 1, λ-*Hind* III markers; lane 2, unplatinated ligated genomes; lane 3, *Stu* I digest of unplatinated ligated genomes; lane 4, unplatinated ligated genomes treated with NaCN; lane 5, *Stu* I digest of unplatinated ligated genomes after NaCN treatment; lane 6, Stu12T-**2a** ligated genomes; lane 7, *Stu* I digest of Stu12T-**2a** ligated genomes; lane 8, Stu12T-**2a** ligated genomes treated with NaCN; lane 9, *Stu* I digest of Stu12T-**2a** ligated genomes after NaCN treatment; lane 10, Stu12T-**2b** ligated genomes; lane 11, *Stu* I digest of Stu12T-**2b** ligated genomes; lane 12, Stu12T-**2b** ligated genomes treated with NaCN; lane 13 *Stu* I digest of Stu12T-**2b** ligated genomes after NaCN treatment.

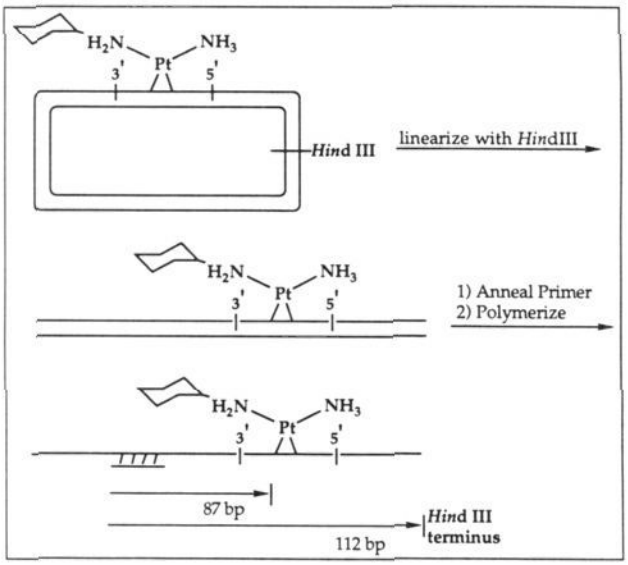
of Stu-12T with hydrolyzed **2** facilitated the construction of M13 genomes containing one isomer in a site-specifically placed d(GpG) site by using the strategy previously developed for cisplatin.[28,29] The resultant, site-specifically modified genome allows one to evaluate the effect of the cyclohexylamine ligand, and its orientation toward the 3′ or 5′ direction of the duplex, on processes such as DNA replication, mutagenesis, and repair. We have studied the ability of the two different isomers to inhibit DNA replication, a process believed to be central to the antitumor activity of the platinum complexes.[7] Moreover, it was expected that the processive 3′ to 5′ tracking of DNA polymerases on the template strand would reveal any differential or altered states of inhibition of DNA synthesis arising from the 3′ versus 5′ orientation of the cyclohexylamine.

The basic strategy for construction of the isomerically pure platinated genomes has been described previously.[28,29] Heteroduplexes containing a single-stranded 12-bp region complementary to the Stu-12T dodecamer are formed by denaturing *Hind* II linearized M13mp18 RF DNA and slowly renaturing in the presence of a large excess of the (+) strand of M13mp18 containing the Stu-12A dodecamer (complementary to Stu-12T) inserted into the single *Hind* II site by blunt end ligation. Phosphorylation of the purified, platinated Stu-12T oligomer with either [γ-^{32}P]ATP or cold ATP allows covalent insertion into the *gapped heteroduplex by T4 DNA ligase. The resulting material* contains covalently closed circular DNA (form I_0) and nicked (form II) circular DNA. The latter material contains nicks predominantly at the 5′ and 3′ ends of inserted oligomer owing to incomplete ligation of the inserted dodecamer (see below). A small quantity (~5-10%) of linear double-stranded material was also present. Presumably, this linear material is M13 RF DNA that was incompletely denatured or which self annealed during formation of the gapped heteroduplexes. These materials were either used directly for replication inhibition experiments or the material was enriched in form I_0 DNA by agarose gel electrophoresis purification.

The use of ^{32}P-end-labeled dodecamers facilitated the characterization of the genomes by gel electrophoresis following digestion by the restriction enzyme *Stu* I. This assay is particularly valuable since platinum complexes inhibit the activity of this restriction enzyme.[28] The formation of intrastrand cross-links between the two deoxyguanosines within the *Stu* I recognition site blocks the double-stranded cleavage, and removal of the platinum by treatment with NaCN[54] restores enzymatic activity. Lanes 3 and 5 of Figure 5 indicate that *Stu* I digestion of M13 genomes containing ligated, unplatinated Stu-12T affords linear double-stranded DNA from the mixture of forms I_0 and II either with or without NaCN treatment. These results confirmed that

(54) Bauer, W.; Gonias, S. L.; Kam, S. K.; Wu, K. C.; Lippard, S. J. *Biochemistry* **1978**, *17*, 1060.

Scheme I



the Stu-12T oligomer had been ligated into the gapped duplex by covalent linkage at either or both of the 3′ and 5′ ends. The formation of linear material would not be observed if only one strand of the *Stu* I recognition site were present in the genome. Lanes 6 and 10 of Figure 5, corresponding to material containing the individual Stu-12T-**2** isomers, show no increase in linear material upon *Stu* I digestion (lanes 7 and 11). Complete formation of linear, double-stranded material occurred, however, when the genomes were treated with NaCN prior to restriction digestion (lanes 9 and 13), demonstrating the presence of the ligated, platinum-modified Stu-12T dodecamer.

Replication Inhibition Studies. Replication inhibition studies were conducted by the procedure reported previously.[24] As illustrated in Scheme I for the genome containing the Stu-12T-**2b** insert, the ligated genomes were linearized at a single *Hin*d III restriction site located 25 bp downstream, on the platinated strand, from the platinum binding site. The resulting linear double-stranded DNA was denatured and the primer was annealed. A 24-bp primer was employed such that the 5′ end of the primer is positioned 87 bp upstream of the platinum binding site. In this manner, blockage of replication at the platinum site will yield a fragment 87 bp in length, whereas fragments 112 bp in length will result from replication past the adduct, termed translesion synthesis. Products were visualized by autoradiography either by employing [α-^{35}S]dATP during strand synthesis or by using ^{32}P-end-labeled primer. The stop sites were easily identified by comparison of the accompanying molecular weight sequencing ladder. The experiments used a cloned T7 DNA polymerase that contains a mutation destroying the 3′ to 5′ proofreading ability of the enzyme.

Figure 6 shows a typical autoradiogram for experiments employing ^{35}S-labeled dATP. The ladder at the right is labeled to identify the sequence of the template strand and therefore corresponds to the inserted Stu-12T fragment when read from top to bottom. The lane labeled UnPt corresponds to replication of unplatinated material and reveals a strong stop and a weak satellite stop at the *Hin*d III stie, as well as stops at the 3′ and 5′ ends of the inserted Stu-12T oligomer, reflecting a small amount of incompletely ligated material. The lane marked cis corresponds to fragments synthesized from material containing the Stu-12T fragment modified by *cis*-DDP. Lanes marked 3′ and 5′ correspond to fragments synthesized from templates containing the two orientational isomers of Stu-12T-**2**, with the cyclohexylamine directed toward the 3′- or 5′-deoxyguanosine residues of the platinated strand.

As reported earlier, cisplatin provides a strong but incomplete block to replication by T7 DNA polymerase.[24] Densitometric analysis of experiments involving [α-^{35}S]dATP or ^{32}P-end-labeled

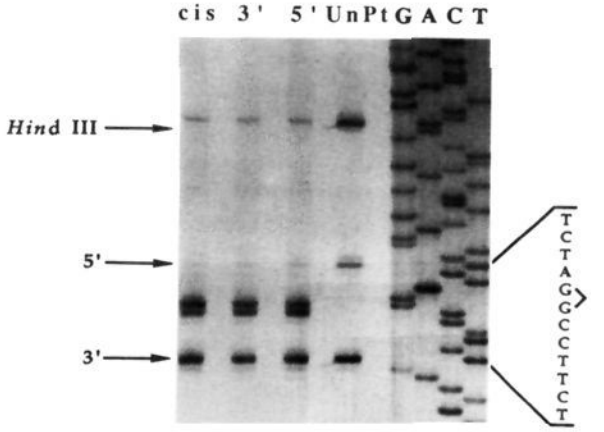


Figure 6. Results of a replication inhibition experiment incorporating α-[^{35}S]dATP into the synthesized strand. Lane 1, material synthesized from genome templates containing Stu12T-cisplatin insert; lane 2, material synthesized from genome templates containing the Stu12T-**2b** insert (cyclohexylamine oriented toward the 3′-guanosine); lane 3, material synthesized from genome templates containing the Stu12T-**2a** insert (cyclohexylamine oriented toward the 5′-guanosine); lane 4, material synthesized from genome templates containing unplatinated Stu12T insert; lanes 5–8, Sanger sequencing lanes, indicating the sequence of the template strand.

Table II. Percentage of Translesion Synthesis and Relative Intensities of Major Stop Sites (Normalized to the Full Length Fragments) for d(GpG)-Cisplatin and the Two d(GpG)-**2** Adducts

	translesion synthesis		
type of experiment	d(GpG)-*cis*	M13-Stu12T-**2a**	M13-Stu12T-**2b**
[α-^{35}S]dATP	7	15	12
	8	15	9
^{32}P-end-labeled primer	6	15	8
	relative intensities		
stop site	d(GpG)-*cis*	M13-Stu12T-**2a**	M13-Stu12T-**2b**
*Hin*d III	1	1	1
5′-ligation site	<0.1	0.29	0.34
3′-GpG-Pt	8.7	3.3	5.9
3′-cytosine	4.6	4.7	5.2

primer revealed that both M13-Stu12T-**2** isomers also act as strong inhibitors of DNA synthesis. Despite the increased size of the amine ligand, however, these isomers remain incomplete blocks to replication. The percentage of translesion synthesis measured in three experiments is given in Table II. The two experiments involving [α-^{35}S]dATP were conducted with modified M13 genomes obtained from two different preparations involving independently prepared stocks of platinated dodecanucleotides, demonstrating the reproducibility of the amount of translesion synthesis products formed. Although the differences were small, the M13-Stu12T-**2** adducts were less efficient at inhibiting DNA synthesis than cisplatin in all experiments. The orientational isomer with the cyclohexylamine at the 3′ site, d(GpG)-**2b**, provides a slightly stronger block to DNA synthesis than the isomer with a 5′-cyclohexylamine, d(GpG)-**2a**. The former isomer should more closely resemble the analogous cisplatin DNA adduct in which hydrogen bonding between the 5′-oriented NH_3 ligand and a phosphodiester oxygen atom has been identified to be an important structural feature.[55-58]

The use of ^{35}S-labeled material allows for clear identification of the nucleosides in the template strand at which synthesis is

(55) Sherman, S. E.; Gibson, D.; Wang, A. H.-J.; Lippard, S. J. *Science* **1985**, *230*, 412.
(56) Sherman, S. E.; Gibson, D.; Wang, A. H.-J.; Lippard, S. J. *J. Am. Chem. Soc.* **1988**, *110*, 7368.
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terminated. The intensities of the bands corresponding to major stop sites, normalized to the intensity for the full length fragments produced for each sample by translesion synthesis, are also displayed in Table II. All three adducts display two stop sites at identical locations in the region of the platinum adduct. The data reveal subtle differences, however, in the intensities of the two bands corresponding to the positions at which DNA synthesis is terminated near the platinum binding site. For cisplatin, a strong stop site is observed at the 3'-deoxyguanosine of the platinum binding site on the template strand, and a weaker stop appears at the cytosine located 3' to this platinated guanine, one base upstream. Two bands at the *Hin*d III terminus reflect translesion synthesis. Much weaker bands corresponding to the 5' position of the inserted oligomer are also observed, as in the control lane. These bands result from translesion synthesis which is terminated as a result of incomplete ligation of the inserted oligomer during synthesis of the platinated genomes. Control experiments have shown that these bands are not due to the presence of unplatinated material in the samples.[24]

Two stop sites of equal intensity are observed for the d(GpG)-**2b** isomer containing a 3'-oriented cyclohexylamine, one at the first (3') guanine position and one at the cytosine located 3' to this guanine. In contrast to fragments synthesized from *cis*-DDP modified templates, material obtained from templates containing the 5'-oriented cyclohexylamine, M13-Stu12T-**2a**, produces a stronger stop at the cytosine located 3' to the platinum binding site and a slightly weaker band reflecting replication inhibition at the first guanine. Thus, this isomer blocks DNA synthesis more efficiently at the base preceding the platinated site than does cisplatin.

Discussion

The present study reveals that the cyclohexyl substituent on the amine ligand in *cis*-[Pt(NH3)(C6H11NH2)Cl2] has a profound effect on the selectivity for DNA binding sites and the ability of the resulting lesions to alter the processing of the damaged DNA. The predominant binding site involves intrastrand cross-linking of two adjacent guanosines, as is the case with *cis*-DDP, but two orientational isomers are formed as a result of the asymmetry in the platinum coordination sphere. Structural studies have indicated that a hydrogen bonding interaction occurs between the 5'-phosphate and the ammine ligand of cisplatin.[55-58] The 2:1 ratio of d(GpG) orientational isomers formed upon platination of duplex DNA by **2** suggests that the difference in hydrogen bonding abilities of the cyclohexylamine and ammine ligands may play a role in determining the DNA binding preferences of *cis*-[Pt(NH3)(C6H11NH2)Cl2]. This conclusion is supported by the ^{15}N NMR studies which reveal that the more abundant isomer contains the ammine ligand oriented in the direction of the 5'-phosphate. In fact, the importance of the 5'-phosphate in determining this result is confirmed by the observation that the ratio of the orientational isomers in the platination of the dinucleoside monophosphate d(GpG), which contains no 5'-phosphate, shows the opposite selectivity to that observed for platination of duplex DNA. In the absence of a hydrogen bond between the 5'-phosphate and either amine ligand, the more abundantly formed d(GpG) orientational isomer contains the cyclohexylamine ligand located cis to the 5' nucleoside.

Since platinum binding to DNA is irreversible under physiologically relevant conditions, the ratio of isomers reflects a kinetic selectivity, which can be attributed to the preferred hydrogen bonding interaction between the 5'-phosphate and the ammine ligand. The rate of platinum binding to short, single-stranded DNA fragments containing a 5'-phosphate has been shown to be faster than binding to fragments lacking a 5'-phosphate.[59-61] Our results with the mixed amine platinum complexes demonstrate that the 5'-phosphate–amine interaction may be important in the kinetic selectivity of platinum binding to duplex DNA.

The amount of **2** bound to d(ApG) sites is greatly reduced and the number of 1,3 adducts is increased relative to the corresponding adducts formed by *cis*-DDP. Two independent studies with site-specifically modified genomes containing *cis*-DDP, when viewed together, suggest that different adducts have altered abilities to cause mutations in vivo[62,63] and to inhibit DNA synthesis in vitro.[24] For cisplatin, the d(ApG) adduct appears to be more mutagenic than the d(GpG) cross-link.[63,64] If a similar situation were to obtain for compound **2**, the decreased number of d(ApG) adducts formed in comparison to those made by cisplatin might result in lower mutagenicity for the oral compound. Studies to determine directly the mutagenicity of complexes **1** and **2** are in progress.[65]

Replication inhibition experiments involving defined adducts afford an opportunity to determine whether substituents attached to the amine ligands, such as the cyclohexyl group employed in the present study, can affect the enzymatic processing of platinated DNA. One might expect bulkier DNA adducts to be more effective in blocking replication, and just such a trend has been observed with aminofluorene and acetylaminofluorene.[66,67] It is not clear, however, that the cyclohexyl group would provide enough steric bulk to add significantly to the distortion of DNA caused by a platinum lesion. In fact, antibodies elicited against *cis*-DDP modified DNA also recognize DNA modified with other *cis*-amine–platinum complexes such as the 1,2-diaminocyclohexane analogue,[68] and structural studies show similar duplex unwinding angles for a series of substituted *cis*-amine–platinum complexes.[69] Moreover, it appears that distortion of the normal B DNA structure upon platination does not necessarily lead to more efficient inhibition of DNA replication. A 1,3-d(GpCpG) intrastrand crosslinked *cis*-{Pt(NH3)2}2+ adduct displays markedly greater unwinding of duplex DNA and a similar bend as compared with 1,2-d(ApG) and 1,2-d(GpG) adducts,[8,70] whereas the amount of translesion synthesis can be significantly greater for the presumably more distorted 1,3 adduct.[24] These observations notwithstanding, the results of the present experiments indicate that the cyclohexylamine ligand does influence the recognition of platinated DNA, at least as reflected by the T7 DNA polymerase enzyme assay. DNA synthesis is inhibited at different positions with respect to the sites of platination compared to previous results for DNA site-specifically modified with cisplatin. In addition, replication inhibition occurs with different efficiencies depending on the 3' or 5' orientation of the cyclohexylamine ligand. It is possible that this latter finding arises from the different abilities of the two isomers to form hydrogen bonding interactions with the 5'-phosphate, but our results do not clearly identify this interaction as the important structural difference that leads to the altered results for the two isomers.

Summary and Conclusions. In broad terms, we have demonstrated that the DNA adduct profile, and most likely also the mechanism of antitumor activity, of the new class of orally active platinum complexes is similar to that of cisplatin. Some interesting aspects of the details of the DNA binding have been discovered, however, that reflect the substituents on the amine ligands and the stereochemistry at the platinum center. Although orientational isomers resulting from platination of r(ApG) have been previously

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identified,[71] the present study is the first demonstration of this phenomenon for platinated duplex DNA. This assignment was made by means of a careful analysis of platinum stereochemistry with the aid of ^{15}N NMR spectroscopy. In addition, modification of the amine ligands in the platinum coordination sphere has been shown to influence the selectivity for d(ApG) and d(GNG) intrastrand cross-link formation. The observed reduction in the number of d(ApG) cross-links suggests the exciting possibility that the mutagenic activity of compounds **1** and **2** may be decreased relative to cisplatin. The selective formation of the two d(GpG)-**2** orientational isomers, corresponding to the 3′ versus 5′ orientation of the cyclohexylamine ligand, has been shown to play a small but measurable role in the processing of DNA platinated with complex **2**. In particular, both isomers are slightly less efficient than cisplatin in blocking replication despite the increased bulk of the platinum lesion, and each terminates DNA synthesis to different degrees at the two observed sites. The availability of high-resolution X-ray structural studies of cisplatin and related complexes bound to duplex DNA will help in the understanding of the hydrogen bonding or steric factors that are responsible for these differences and should provide a basis for analyzing the relationship between the structures of specific platinum-DNA adducts and their processing by cellular components. Studies toward this end are in progress, from which may ultimately arise a rational basis for platinum antitumor drug design.

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Supplementary Material Available: Figure S1, displaying the FAB (MS/MS) spectrum of the d(ApG)-**2** adduct (1 page). Ordering information is given on any current masthead page.

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Dihydrogen Complexes of Metalloporphyrins: Characterization and Catalytic Hydrogen Oxidation Activity

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Abstract: A series of monometallic dihydrogen complexes of the type M(OEP)(L)(H_2) (M = Ru, Os; L = THF, *Im) was synthesized and characterized by ^1H NMR. The H–H bond length was found to increase when Os was replaced by Ru or when *Im was replaced by THF. The bond distances (as determined by T_1) range from 0.92 to 1.18 Å. The first example of a bimetallic bridging dihydrogen complex, Ru_2(DPB)(*Im)$_2$(H_2), was also prepared. The H_2 ligand is simultaneously bound to both Ru–metal centers. High-field ^1H NMR experiments (620 MHz) revealed a −7.37 Hz dipolar splitting of the H_2 ligand for this complex. Analysis of this splitting suggests that the H_2 ligand is bound with the H–H axis perpendicular to the Ru–Ru axis. These complexes were examined as possible catalysts for the oxidation of dihydrogen through prior heterolytic activation of H_2. Only Ru(OEP)(THF)(H_2) can be conveniently deprotonated. Ru(OEP)(THF)(H_2) is also implicated in the Ru(OEP)(THF)$_2$ catalyzed isotopic exchange between H_2 and D_2O in THF solution. Each step for this mechanism has been elucidated. We have also achieved catalytic dihydrogen oxidation using [Ru(OEP)]$_2$ adsorbed onto graphite. Two mechanisms for this ruthenium porphyrin catalyzed dihydrogen oxidation are presented and compared.

Though thermodynamically unstable in an oxygen atmosphere, dihydrogen is kinetically inert and is not reactive unless activated with a suitable catalyst. Such catalysis occurs naturally in certain microorganisms that have been known since the turn of the century to consume dihydrogen.[1] In 1931, Stephenson and Strickland[2] proposed the first description of this phenomenon in terms of enzymatic activation of hydrogen and named the relevant enzymes hydrogenases. The physiological role of hydrogenases is to mediate the production and consumption of dihydrogen in the presence of cofactors such as nicotinamide adenine dinucleotide, cytochrome c_3, and ferredoxin.[3,4] However, an important criterion for hydrogenase activity is the physiologically unimportant exchange of H_2 and D_2O first characterized by Farkas in 1934 (eq 1).[5,6]

$$H_2 + D_2O \rightleftharpoons D_2 + HD + HDO + H_2O \qquad (1)$$

Several authors[3,7] have suggested that this activity indicates

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