Mechanistic Studies of a Novel Class of Trisubstituted Platinum(II) Antitumor Agents


ABSTRACT

Chemical and biological studies are presented for a new series of platinum(II) antitumor agents that violate the classical structure-activity relationships established for platinum complexes. These new agents, which have demonstrated activity against murine and human tumor systems, are cis-[Pt(NH$_3$)$_2$(Am)Cl]$^2^+$ cations, in which Am is a derivative of pyridine, pyrimidine, purine, or aniline. Members from this series block simian virus 40 DNA replication in vitro and inhibit the action of DNA polymerases at individual guanine residues in replication mapping experiments. Monoclonal antibodies that bind selectively to cisplatin lesions on calf thymus DNA were used in a competitive enzyme-linked immunosorbent assay study to show that the platinum-triamine complexes do not produce the type of intrastrand cross-links on DNA that are characteristic of cisplatin and analogues. These results indicate that cis-[Pt(NH$_3$)$_2$(Am)Cl]$^2^+$ cations form monofunctional adducts on DNA rather than eliminate NH$_3$ or Am to afford bifunctional lesions.

INTRODUCTION

Cisplatin (cis-DDP$^3$) is an effective anticancer drug presently approved for the treatment of testicular, ovarian, and bladder carcinomas (1–5). The drug is also widely used in combination with other agents, such as VP16, doxorubicin, and bleomycin, in the treatment of small cell lung carcinoma and head and neck cancers (6–9). While cisplatin is one of the most successful antitumor compounds to be developed in recent years, it displays limited activity against some of the most common and deadly forms of the disease, such as colon and breast cancers (10). In addition, a variety of adverse effects, such as nephrotoxicity, myelosuppression, neurotoxicity, and severe emesis, is observed in patients receiving cisplatin chemotherapy (11–15). These limitations reduce the efficacy of the compound and have inspired efforts to develop new agents that will display improved therapeutic properties.

One approach to this problem is to identify new classes of active platinum complexes having structural features that differ from those of the existing cisplatin analogues. The structure-activity relationships that define this class of compounds were first summarized by Cleare and Hoeschele in 1973 (21). In general, the majority of analogues that display antitumor activity are neutral platinum(II) complexes of the form cis-[Pt$_2$A$_2$X$_4$], in which A is an amine ligand and X is an anionic leaving group (22). Since the corresponding trans-[Pt$_2$A$_2$X$_4$] complexes exhibit no antitumor activity, numerous studies have focused on the correlation between the cis geometry of the leaving groups (X) and the observed antitumor response (23–26). The evidence from these investigations suggests that the antitumor activity stems from the interaction of these complexes with DNA. Cisplatin and related analogues produce bifunctional lesions on DNA that are capable of disrupting replication and transcription (27). The most common adduct is an intrastrand cross-link between adjacent guanine bases that has been shown to inhibit DNA synthesis (27, 28).

Aside from complexes belonging to the cis-[Pt$_2$A$_2$X$_4$] structural class, relatively few platinum compounds have demonstrated activity in preclinical tumor screens (29). Exploring new structural classes of platinum antitumor drugs may allow the discovery of agents that possess chemical and biological properties quite different from those of cisplatin. The recent discovery of one such class of antitumor agents may provide the basis for expanding the spectrum of activity of platinum-based drugs (30). These compounds are platinum-triamine cations of the form shown below, in which Am is an amine ligand derived from pyridine, pyrimidine, purine, piperidine, or aniline.
Members of this series of compounds show activity against a number of murine tumors, including S180a, P388, and L1210, and human tumor cell lines. Since these platinum-triamine complexes are cationic species that contain three nitrogen donors and only one leaving group, they cannot be considered as classical cisplatin analogues. Without loss of NH₃ or Am from complexes are cationic species that contain three nitrogen donors, but inactive, platinum-triamine complexes such as [Pt(dien)Cl]+ (31, 32). Monofunctionally binding complexes have not proved to have antitumor activity (21). In order to address the binding mode of the new platinum-triamine complexes, as well as other fundamental questions about their mechanism of action, the studies described in the present article were carried out.

MATERIALS AND METHODS

Chemical Studies

Compound Preparation

Platinum-triamine complexes were prepared as described previously (30). Two additional compounds investigated here were obtained in the following manner.

trans-[Pt(NH₃)₂(4-Br-py)Cl]Cl. Method a: cis-[Pt(NH₃)₂(4-Br-py)Cl]Cl (88 mg) was dissolved in 3.2 ml of PBS (pH 7.2; Sigma Chemical Co.) and the resulting solution was stored at 37°C for 14 days. Well-formed crystals, suitable for X-ray diffraction studies, were collected by filtration, washed with water, and air dried (yield, 45 mg). Method b: cis-[Pt(NH₃)₂(4-Br-py)Cl]Cl (1.0 g) was refluxed in 0.1 M HCl (50 ml) for 1 h. The resulting yellow precipitate was collected by filtration, washed with water, and dried under vacuum (yield, 0.53 g). cis-[Pt(NH₃)₂(4-CH₃-py)Cl]Cl. A solution of [K(Pt(NH₃)Cl)] (1.95 g) and 4-methylpyridine (0.509 g) in 25 ml of water was stirred at 50°C for 1 h. The resulting yellow precipitate was collected by filtration, washed with water, and dried under vacuum (yield, 0.53 g) (analysis: C, H, N).

cis-[Pt(NH₃)₂(4-CH₃-py)]Cl. A solution of [K(Pt(NH₃)Cl)] (1.95 g) and 4-methylpyridine (0.509 g) in 25 ml of water was stirred at 50°C for 12 h. The solution was cooled to 25°C and the resulting yellow precipitate was collected by filtration, washed with water, and dried (yield, 1.2 g) (analysis: C, H, N).

Physical Studies

NMR Spectroscopy. ¹H NMR spectra were obtained with a Varian XL-300 spectrometer. ¹⁹⁵Pt and ¹⁷¹N spectra were recorded in dimethyl formamide with a Varian XL-200 spectrometer by using previously described methods (33). ¹⁹⁵Pt chemical shifts are reported relative to ¹⁹⁵Pt in H₂O at 8 ppm. Well-formed crystals suitable for X-ray diffraction studies were collected by filtration, washed with water, and dried (yield, 0.53 g) (analysis: C, H, N). cis-[Pt(NH₃)₂(4-CH₃-py)]Cl. A solution of [K(Pt(NH₃)Cl)] (1.95 g) and 4-methylpyridine (0.509 g) in 25 ml of water was stirred at 50°C for 12 h. The solution was cooled to 25°C and the resulting yellow precipitate was collected by filtration, washed with water, and dried (yield, 1.2 g) (analysis: C, H, N).

In Vitro Replication Assays

Preparation of Cytosolic Cell Extracts, SV40 T-Antigen, and Plasmid DNAs. The human embryonic 293 cell cytoplasmic extract was prepared as described above (28). Precipitation of the DNA. pSV011 plasmid DNA was incubated at 37°C for 16 h in the presence of platinum compound at several formal (D/N) ratios in 3 mM NaCl, 1 mM Na₂HPO₄ (pH 7.4) or 10 mM Tris·Cl, 1 mM Na₂EDTA (pH 7.6). Amount of bound platinum was measured by ethanol precipitation of the DNA. The amount of platinum bound/nucleotide, (D/N)ₚ, was measured by atomic absorption spectroscopy. Replication Assays and Analysis. Reactions were carried out in 50-μl solutions at 37°C for 60 min as described above (28). Reactions were terminated by addition of Na₂EDTA to stop the reaction. The reaction mixture was then washed with ethanol and centrifuged to precipitate the DNA. The amount of DNA was determined by measurement of DNA concentration by absorption at 260 nm. Background density readings were subtracted from the density readings to obtain a corrected density for each sample. The amount of DNA bound was determined by measurement of DNA concentration by absorption at 260 nm. The amount of DNA bound was determined by measurement of DNA concentration by absorption at 260 nm. Biological Studies

TRISUBSTITUTED PLATINUM(II) ANTITUMOR AGENTS

* Data on file with the National Auxiliary Publication Service.
with ice-cold ethanol. Radioactivity was determined by liquid scintillation counting.

Replication Mapping of Platinum-Triamine Complexes

Preparation and Platination of Plasmid DNA. Single-stranded viral DNA was purified from bacteriophage M13mp18 by precipitation, followed by phenol/chloroform extraction, and separated from any double-stranded contaminants by hydroxylapatite chromatography. Replicative form M13mp18 DNA was isolated by the cleared lysate method. DNA, either single or double stranded, was allowed to react with the platinum complexes at a (D/N) ratio of 1.25 x 10⁻³ for 16 h at 37°C in 3 mM NaCl, 1 mM Na₂HPO₄, pH 7.4. Following ethanol precipitation and careful washing to remove the excess platinum complex, the DNA was quantitated by UV absorbance and the level of platinum was determined by atomic absorption spectroscopy.

Replication Mapping on Adducted Templates. The platinated DNA was primed with an oligonucleotide and used as a template for DNA replication. For replication experiments on single-stranded DNA, an equimolar amount of a 17-mer primer (Universal primer; New England Biolabs) was added and the template was primed by heating to 85°C and cooling to room temperature. Double-stranded M13mp18 DNA was first denatured with 2 N NaOH and a 2-fold excess of a 24-mer primer (New England Biolabs; 40 primer or reverse primer) was added just prior to neutralization with ammonium acetate and ethanol precipitation. The 24-mer primer was necessary to ensure adequate annealing to the platinated templates. Replication was initiated by addition of Sequenase enzyme (United States Biochemical) or the Klenow fragment to the platinated templates. Replication was stopped by the addition of 90% formamide, 5 mM EDTA-dye mixture.

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Preparation of Platinated DNA

Native calf thymus DNA (Sigma) was extracted with 24:1 chloroform/isoamyl alcohol (3 times), precipitated with ethanol (2 times), and dialyzed against low salt buffer (3 mM sodium chloride, 1 mM Na₂HPO₄, pH 7.4) prior to platination. DNA samples were subsequently incubated in low salt buffer with either cis-DDP, cis-[Pt(NH₃)₂(4-Cl-py)Cl]Cl, or cis-[Pt(NH₃)₂(N3-cytosine)Cl]Cl at a formal drug to nucleotide ratio of 0.05 (37°C, 24 or 168 h). Under these conditions, the binding of all four platinum complexes was essentially quantitative at both time points.

Platinum-modified DNA samples were precipitated from ethanol to stop the platination reactions and to remove any unbound platinum. The precipitated DNA was washed with 70% ethanol and redissolved in PBS buffer (0.15 mM sodium chloride, 10 mM sodium phosphate, pH 7.2). DNA nucleotide concentrations were determined by UV spectroscopy (assuming ε = 6600 M⁻¹ cm⁻¹) and bound platinum was measured by atomic absorption spectroscopy.

ELISA

ELISA experiments were performed as described previously (42). Briefly, cis-DDP-DNA, (D/N)₀ = 0.040, was bound to microtiter plates (Dynatech) coated with poly-L-lysine (Sigma) and the plates were subsequently blocked with poly-D-glutamate (Sigma) and 1% bovine serum albumin (Sigma). Unbound reagents were removed with successive washes of buffer (150 mM sodium chloride, 10 mM Tris-HCl, pH 7.2). The monoclonal antibody CPT2 was diluted 1:100 from culture supernatant and preincubated for 60 min with DNA competitors prior to addition to the microtiter wells. Unbound antibody was removed from the wells with 0.2% Tween 20/PBS washes (3 times) and the remaining bound antibody was detected using an alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin antibody (Zymed). The latter antibody was quantitated by measurement of absorbance at 405 nm, 60 min after addition of the alkaline phosphatase substrate p-nitrophenylphosphate (1 mg/ml in 50 mM sodium carbonate, 2 mM magnesium chloride, pH 9.5; 37°C incubation). The specificity and avidity of CPT2 have been described previously (42) and the antibody was used at a concentration that gave absorbances of 1–2 AU (405 nm) after 60 min in the absence of competitor.

Animal Studies

Antitumor Screening

The platinum complexes trans-[Pt(NH₃)₄(4-Br-py)Cl]Cl and cis-[Pt(NH₃)₄(4-CH₃-py)Cl]Cl were screened versus Sarcoma 180 ascites (i.p.) in female CFW mice by using previously described methods (30). Screening data for these compounds are presented as supplementary material (Table 6).

RESULTS AND DISCUSSION

Biochemical Studies of the cis-[Pt(NH₃)₄(Am)Cl]⁺ Complexes

Inhibition of DNA Replication. Since cisplatin is known to bind DNA and inhibit replication in vitro and in vivo (28, 43–45), studies were conducted to determine whether the platinum-triamine complexes behave in a similar fashion. An in vitro system described previously (28) was used to measure the inhibitory effects of two platinum-triamine complexes on SV40 DNA replication. This system utilizes cytosolic extracts from the human embryonic kidney cell line 293 in combination with SV40 origin-containing plasmid templates, SV40 large T-antigen, and nucleotide triphosphates to replicate the SV40 genome. The extracts contain all the necessary enzymes to support DNA synthesis with the exception of large T-antigen, a virally encoded protein supplied exogenously. The conditions closely mimic those required in eukaryotic cells for replication of nuclear DNA (41, 46).

In separate experiments, SV40 origin-containing plasmid DNAs were platinated at low drug/nucleotide levels [(D/N)₀ = 1.4–1.7 x 10⁻³] with the platinum-triamine complexes and two control compounds, cisplatin and [Pt(dien)Cl]Cl (28). The modified templates were subjected to the in vitro replication assay and DNA synthesis was monitored by following the incorporation of [α-³²P]dAMP into the newly synthesized DNA.

Table 1: Effect of platinum complexes on in vitro DNA replication from pSV011 templates in 293 cytosolic extracts at (D/N)₀ levels indicated

<table>
<thead>
<tr>
<th>Modification</th>
<th>(D/N)₀</th>
<th>pmol dAMP/h</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>0.0</td>
<td>56.8</td>
<td>100</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>1.7 x 10⁻³</td>
<td>2.6</td>
<td>46</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>9.0 x 10⁻³</td>
<td>2.8</td>
<td>4.9</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>4.0 x 10⁻³</td>
<td>10.4</td>
<td>18.3</td>
</tr>
<tr>
<td>[Pt(dien)Cl]⁺</td>
<td>3.4 x 10⁻³</td>
<td>35.0</td>
<td>61.6</td>
</tr>
<tr>
<td>[Pt(dien)Cl]⁺</td>
<td>0.9 x 10⁻³</td>
<td>45.6</td>
<td>80.3</td>
</tr>
<tr>
<td>cis-[Pt(NH₃)₄(4-Me-py)Cl]⁺</td>
<td>1.7 x 10⁻³</td>
<td>8.6</td>
<td>15.1</td>
</tr>
<tr>
<td>cis-[Pt(NH₃)₄(4-Br-py)Cl]⁺</td>
<td>1.4 x 10⁻³</td>
<td>8.5</td>
<td>15.0</td>
</tr>
</tbody>
</table>

* Except for the last two entries, the data in this table are reproduced from Ref. 11.
The results of these studies (Table 1) show that the two biologically active platinum-triamine complexes, cis-[Pt(NH₃)₂(4-CH₃-py)Cl]Cl and cis-[Pt(NH₃)₂(4-Br-py)Cl]Cl, are nearly as effective as cisplatin (85 versus 95% inhibition) in blocking DNA replication in vitro. In contrast, the related triamine complex, [Pt(dien)Cl]Cl, which displays no antitumor activity, does not effectively inhibit DNA synthesis under similar conditions.

Since cis-[Pt(NH₃)₂(Am)Cl]⁺ complexes and [Pt(dien)Cl]⁺ are both expected to bind to DNA in a monofunctional fashion, it is difficult to explain why these compounds differ in their ability to inhibit DNA replication. Previously, platinum complexes that produce monofunctional lesions on DNA were found to be inactive as antitumor agents (21). Since the cis-[Pt(NH₃)₂(Am)Cl]⁺ complexes display activity, it is important to understand the nature of their Pt-DNA adducts. In particular, is it the monofunctional DNA adducts produced by the platinum-triamine complexes that are ultimately responsible for the disruption of DNA synthesis, or do ligand exchange reactions occur leading to bifunctional cross-links having properties similar to those produced by cisplatin? This question is addressed by the experiments described below.

Sequence Specificity of Platinum-Triamine Complex Binding to DNA. Replication mapping studies were carried out to determine the DNA regiospecificity of the platinum-triamine complexes (45). DNA from bacteriophage M13, both single-stranded and double-stranded forms, was modified with cis-DDP, cis-[Pt(NH₃)₂(4-Br-py)Cl]⁺, cis-[Pt(NH₃)₂(4-CH₃-py)Cl]⁺, or [Pt(dien)Cl]⁺ to a level of approximately one adduct/1000 nucleotides, (D/N)b ~ 0.001. The adducted DNA was primed with an oligonucleotide and used as a template for second strand synthesis by either T7 DNA polymerase (Sequenase) or the Klenow fragment of DNA polymerase I. The stop sites resulting from the presence of the platinum-triamines were compared directly with those resulting from the presence of the active antitumor drug cis-DDP and also with the inactive platinum-triamine [Pt(dien)Cl]⁺.

The triamine complexes cis-[Pt(NH₃)₂(4-Br-py)Cl]⁺ and cis-[Pt(NH₃)₂(4-CH₃-py)Cl]⁺ both block replication, as evidenced by the strong bands observed when second strand synthesis products are run on sequencing gels (Fig. 1). The same results were obtained when double-stranded DNA was modified with the triamine complexes and replicated in a similar manner or when the Klenow fragment of DNA polymerase I was substituted for T7 DNA polymerase (data not shown). The inhibition of replication by the platinum-triamines in this experiment corroborates the results obtained for SV40-platinated templates replicated in the cell extracts, in which incorporation of dATP was inhibited by these complexes (vide supra).

It has previously been shown that cis-DDP inhibits replication by coordinating to multiple guanine sequences (45). The tendency of the modified T7 polymerase to stop at pairs of adjacent guanines on DNA modified by cis-DDP is clearly seen in Fig. 1, lane 2. The pattern of stop sites is quite different for DNA modified with cis-[Pt(NH₃)₂(4-Br-py)Cl]⁺ and cis-[Pt(NH₃)₂(4-CH₃-py)Cl]⁺ (Fig. 1, lanes 4 and 5). The principal stop sites on DNA modified with these triamines are at single guanine residues with no preference for the multiple guanine sites. This observation suggests that cis-[Pt(NH₃)₂(4-Br-py)Cl]⁺ and cis-[Pt(NH₃)₂(4-CH₃-py)Cl]⁺ bind monofunctionally to isolated guanine residues. It is clear from the differences between the mapping pattern produced by cis-DDP and those afforded by the cis-[Pt(NH₃)₂(Am)Cl]⁺ complexes that the platinum-triamine complexes do not bind to DNA in the same manner as cis-DDP, nor do they form bifunctional complexes containing the cis-[Pt(NH₃)₂]⁺² fragment through the loss of the pyridine ligand prior to binding to DNA.

The aromatic amine ligand appears to be essential for the antitumor activity of the platinum-triamine complexes. The related triamine complexes, [Pt(dien)Cl]⁺ and [Pt(NH₃)₂Cl]⁺, also form monofunctional lesions on DNA yet are completely inactive as antineoplastic agents. The monofunctional adducts formed when [Pt(dien)Cl]⁺ is used to modify DNA do not block replication either in the mammalian cell extract system (Table 1) or in the replication mapping assay (Fig. 1, lane 3). These observations indicate the requirement for the aromatic amine ligand for replication inhibition by these complexes and suggest...
that disruption of DNA synthesis might be an important component of the antitumor activity of this class of platinum complexes.

Immunological Studies. The foregoing results indicate that platinum-triamine complexes do not form bifunctional adducts on DNA through loss of an amine ligand. This conclusion was further supported by using antibodies raised against the double-stranded DNA adducts of cis-DDP. These antibodies have been found to be quite useful for probing the structures of DNA adducts formed by a number of different platinum complexes (47–50). One such monoclonal antibody, CPT2, binds selectively to cis-bidentate platinum cross-links of adjacent purine nucleotides, e.g., cis-[Pt(NH3)2(N7-,N7-GpG)] and cis-[Pt(NH3)2(N7-,N7-ApG)], but not to DNA adducts formed by trans-DDP or the monofunctional complex [Pt(dien)Cl]2. This antibody is relatively insensitive to the chemical nature of the inert platinum amine ligands, however, and therefore binds to DNA adducts formed by complexes such as [Pt(en)Cl2] and [Pt(dach)Cl2] in addition to cis-DDP itself (47). We have used these properties of CPT2 specificity to investigate whether platinum-triamine complexes form cis-bidentate cross-links with adjacent purine nucleotides in DNA.

The initial DNA binding of platinum-triamine complexes such as cis-[Pt(NH3)2(4-CH3-py)Cl]2 is expected to occur via substitution of the relatively labile chloride ligand to yield monofunctional adducts (31). Closure of these monofunctional platinum adducts could then, in principal, occur to form two different types of cis-bidentate adducts depending upon which of the ligands is replaced (NH3 or 4-CH3-py). In either case, however, the resulting platinumated DNA should be recognized by the CPT2 antibody since the two types of adducts would differ if cis-[Pt(NH3)2(4-CH3-py)Cl]2 were to have lost a ligand upon DNA binding. As shown in Fig. 2, cis-DDP-DNA adducts ([D/N]b = 0.049) competitively inhibited the binding of CPT2 to immobilized cis-DDP-DNA by 50% at a concentration of 4 nM (IC50 = 4 nM). Similarly, cis-[Pt(NH3)2(4-CH3-py)Cl2]-DNA adducts ([D/N]b = 0.051) also competitively inhibited antibody binding, although a slightly higher concentration of these adducts was required for equivalent inhibition (IC50 = 7 nM).

In order to test the possibility that the monofunctional DNA adducts formed by platinum-triamine complexes close very slowly to form bifunctional cis adducts, DNA samples were incubated for 7 days at 37°C with cis-[Pt(NH3)2(4-CH3-py)Cl]2 or cis-[Pt(NH3)2(N3-cytosine)Cl]2 and again tested for antibody recognition in a competitive ELISA. In spite of the long incubation period, however, there was no increase in antibody recognition of DNA adducts formed by either complex ([D/N]b = 0.055 and 0.050, respectively). In control experiments, DNA adducts formed after 7 days incubations with cis-DDP ([D/N]b = 0.046) and cis-[Pt(NH3)2(4-CH3-py)Cl2] ([D/N]b = 0.051) were again efficiently recognized by the antibody (IC50 = 3 nM and 6 nM, respectively).

These experiments demonstrate that, in spite of their significant biological activity, cis-[Pt(NH3)2(4-CH3-py)Cl]2 and cis-[Pt(NH3)2(N3-cytosine)Cl]2 do not predominantly form cis bifunctional adducts on DNA in vitro. By analogy to the chemistry of [Pt(dien)Cl]2 and [Pt(NH3)2Cl2], we expect that platinum-triamine complexes should instead bind to DNA in a monodentate fashion, with a preference for the nucleophilic N7 position of guanine. The results of our immunological studies are consistent with this binding mode since monofunctional adducts are not recognized by the monoclonal antibody CPT2 (42). This lack of recognition does not prove the presence of monofunctional adducts, however, since other nonrecognizable adducts are also possible (e.g., trans-[Pt(NH3)(Am)(purine)]2).

Chemical Studies of the cis-[Pt(NH3)2(Am)Cl]+ Complexes

Nucleoside Binding Properties of cis-[Pt(NH3)2(N3-cytosine)]Cl. Since the monoclonal antibody and replication mapping studies reveal no evidence for the formation of bifunctional DNA adducts, several chemical studies were conducted to examine the nature of the products obtained in reactions with model nucleobases. The reaction of d(GpG) with cis-[Pt(NH3)2(N3-cytosine)]Cl was investigated, since the products are easily identifiable as monofunctional adducts. The major species formed in this reaction, A, was isolated by reversed phase HPLC and studied by NMR spectroscopy and enzymatic digestion. Other, less abundant, species were not further investigated. Product A eluted with a retention time distinct from that of free d(GpG) or cis-[Pt(NH3)2(d(GpG))]+, immediately eliminating the possible loss of cytosine from the starting triamine to form the same adduct as cis-DDP. Product A was found to contain one bound platinum atom/d(GpG) molecule. The pH-dependent behavior of the H8 'H NMR resonances of compound A was examined in order to assign the
site of platination. As demonstrated previously (52, 53), coordination of a platinum atom to the N7 position of the guanine ring lowers the $p_K_a$ of the H1 proton. Titration of this proton causes a shift in the H8 proton resonance, an effect which is readily monitored. Platinum complexes of the (d(GpG)) ligand are expected to display two H8 resonances, one for the 5'- and one for the 3'-guanosine. Interestingly, the 1H NMR spectrum of product A contains four H8 resonances of equal intensity, which titrate in two groups of two, as shown in Fig. 3. The titration behavior of two of these resonances, denoted 3 and 4, is consistent with unplatinated guanosines. Resonances 1 and 2 display a completely different titration behavior, titrating at pH ~8. This lowered $p_K_a$ is characteristic of platinum binding to the N7 position of guanine (52, 53). From this behavior it can be concluded that product A is a monofunctional adduct.

Further insight into the nature of the platinum adduct in product A was provided by enzymatic digestion analyses (38). The platinumated (d(GpG)) was first treated with DNase I and P1 nuclease to cleave the DNA phosphodiester backbone leaving nucleotides with 5'-phosphates. Alkaline phosphatase was then added to remove the 5'-phosphate to yield the corresponding nucleosides. An HPLC analysis of the products of this enzymatic digestion procedure is shown in Fig. 4a. Peak 1 is free deoxyguanosine nucleoside, assigned by coinjection with a sample of pure dG. The cis-[Pt(NH3)2(N3-cytosine)(dG)]2+ cation, prepared by reaction of cis-[Pt(NH3)2(N3-cytosine)Cl]Cl with dG, was used to assign peak 2 by coinjection. Since these are the only products observed in the enzymatic digestion analysis, it can be concluded that product A is a monofunctional adduct.

The assignment of product A as a single monofunctional adduct does not explain the presence of four H8 resonances in its 1H NMR spectrum, however. The occurrence of two sets of resonances suggests either that product A is a mixture of two chromatographically inseparable compounds or that it is a single compound which can exist in either of two slowly interconverting conformations. Thus, product A might be a 50:50 mixture of compounds in which platinum bound either at the 3'- or the 5'-guanosine of (d(GpG)). Alternatively, it could be a chemically pure compound with platinum bound to only one guanosine, present in two slowly interconverting forms. The former of these two possibilities was ruled out by repeating the enzymatic digestion but omitting the alkaline phosphatase step. This "modified digest" leaves the 5'-phosphate attached to the 3'-guanosine, in effect labeling it and allowing it to be differentiated from the 5'-guanosine, which will not have such a phosphate group. If platinum were bound only to the 5'-guanosine, one would expect to see two products appear in the modified digestion, namely, cis-[Pt(NH3)2(N3-cytosine)(dG)]2+ and d(GpG). If platinum were coordinated only to the 3'-nucleoside, the modified digestion would produce two completely different products, free dG and cis-[Pt(NH3)2(N3-cytosine)(dGpG)]+. The modified digest of a mixture of platinum bound to both the 3' and 5' positions would yield all four of these products.

Fig. 4b displays an HPLC trace of the modified enzymatic digestion of product A. Peak 2 is the same as found in the complete enzymatic digestion procedure, cis-[Pt(NH3)2(N3-cytosine)(dG)]2+. Peak 1 is identified as d(GpG) by coinjection with authentic material. The small amount of peak 3 arises from dephosphorylation of d(GpG) under the reaction conditions, as demonstrated by a control experiment in which this nucleotide was incubated under the digestion protocol but in the absence of alkaline phosphatase. These results can be nicely accounted for if platinum coordinates only to the 5'-guanosine of d(GpG). If platinum were bound to the 3'-guanosine, one would expect to observe cis-[Pt(NH3)2(N3-cytosine)d(GpG)]2+ produced from the modified digestion of A. This compound was synthesized independently by allowing cis-[Pt(NH3)2(N3-cytosine)Cl]Cl to react with d(GpG). The reaction proceeded cleanly to a single product and HPLC analysis revealed a peak shape and retention time completely distinct from peaks 1, 2, or 3 in Fig. 4 (data not shown). Thus, the digestion analyses allow product A to be identified as a single species, cis-[Pt(NH3)2(N3-cytosine)(dGpG)-N7(1)].

The presence of four H8 resonances in the 1H NMR spectrum of cis-[Pt(NH3)2(N3-cytosine)(dGpG)-N7(1)] arises from the presence of two slowly interconverting rotational isomers. In
order to evaluate further the stereochemical origin of this barrier to rotation, cis-[Pt(NH$_3$)$_2$(N3-cytosine)(dG)]$^{2+}$ and cis-[Pt(NH$_3$)$_2$(4-CH$_3$-py)(dG)]$^{2+}$ were synthesized by allowing deoxyguanosine to react with cis-[Pt(NH$_3$)$_2$(N3-cytosine)Cl]$^+$/cis-[Pt(NH$_3$)$_2$(4-CH$_3$-py)Cl]$^+$. A similar reaction was observed when cis-[Pt(NH$_3$)$_2$(4-Br-py)Cl]$^+$ was reacted with deoxyguanosine to form cis-[Pt(NH$_3$)$_2$(4-Br-py)(dG)]$^{2+}$, respectively. If the rotational barrier in product A was due to steric interactions between substituents on the 5'-guanosine base and either the carbonyl or exocyclic amino groups of the coordinated cytosine, then a ligand lacking these groups, such as pyridine, should not exhibit such a barrier to rotation. The H8 resonance in the $^1$H NMR spectrum of cis-[Pt(NH$_3$)$_2$(N3-cytosine)(dG)]$^{2+}$ is split, owing to the presence of two conformational isomers. When this sample was heated to 70°C, the resonances broadened, but the sample temperature could not be raised high enough to cause them to coalesce.

While this work was in progress, the $^1$H NMR spectrum of cis-[Pt(NH$_3$)$_2$(4-CH$_3$-py)(dG)]$^{2+}$ was observed by single crystal X-ray diffraction studies (Fig. 5). Ammonia release thus occurs at the monocationic metal ion (NH$_4^+$) which was detected in solution by $^1$H NMR spectroscopy. The product of this reaction is trans-[Pt(NH$_3$)$_2$(N3-cytosine)(dG)]$^{2+}$, a monomeric ion with a square planar configuration, and not the previously reported cis-[Pt(NH$_3$)$_2$(N3-cytosine)(dG)]$^{2+}$, which is a dimeric ion with a tetrahedral configuration. The H8 resonance of this species is not split, owing to the absence of rotational isomers. In contrast, the cis-[Pt(NH$_3$)$_2$(4-CH$_3$-py)(dG)]$^{2+}$ and cis-[Pt(NH$_3$)$_2$(4-Br-py)(dG)]$^{2+}$ were prepared to evaluate the relative donor properties of several triamine ligands. The results of an NMR study of a series of eight complexes of the form cis-[Pt($^{15}$NH$_3$)$_2$(Am)Cl]$^+$ are summarized in Table 2. From the coupling constant data, it is apparent that chloride is the weakest donor ligand in each case, as the $^{195}$Pt-$^{15}$N-coupling constant for an ammine trans to chloride being 24-50 Hz greater than for the corresponding coupling constant trans to Am. The relative donor strength of each Am ligand can also be estimated from these data. Based on the $^{195}$Pt chemical shifts, the Am donor increased in the following order: $^{15}$NH$_3$ > py > dC > dG > trans to py, 4-CH$_3$-py, 4-Br-py. In contrast, the $^{195}$Pt-$^{15}$N-coupling constant data suggest a similar but somewhat different ordering: $^{15}$NH$_3$ > py, 4-CH$_3$-py > 4-Br-py > 1-CH$_3$-cytosine, dC, dG. Although the donor properties of the heterocyclic Am ligands appear somewhat different, as judged by these parameters, both series indicate that these ligands are slightly weaker donors than NH$_3$.

Although the NMR studies of these complexes reveal that NH$_3$ is a stronger donor than Cl$^-$, it has similar donor properties to the heterocyclic Am ligands. Consequently, labilization of ammonia at the position trans to the Am ligand should not be highly favored in cis-[Pt(NH$_3$)$_2$(Am)Cl]$^+$ complexes in the absence of Cl$^-$ substitution. The results suggest that the first step in the reaction of the Am complexes with DNA is formation of monofunctional cis-[Pt(NH$_3$)$_2$(Am)(DNA-base)]$^{2+}$ adducts. Based on the $^{195}$Pt-$^{15}$N-coupling constant data obtained on the dG complex cis-[Pt($^{15}$NH$_3$)$_2$(4-Br-py)(dG)]$^{2+}$, the 4-Br-py ligand is a slightly better donor than deoxyguanosine. Since each of the amine groups in this species has similar donor strengths, it is difficult to predict from these data alone which ligand would be the leaving group if substitution by a second DNA base were to occur. Kinetic and steric effects would play an important role in such ligand exchange reactions.

An example of the importance of the kinetic trans effect in the substitution reactions of platinum-triamine complexes can be seen when cis-[Pt($^{15}$NH$_3$)$_2$(4-Br-py)Cl]$^+$ is placed in chloride containing media. Dissolution of this complex in PBS followed by incubation at 37°C over a period of 14 days afforded ammonium ion ($^{15}$NH$_4^+$) which was detected in solution by $^{15}$N NMR spectroscopy. The product of this reaction is trans-[Pt(NH$_3$)$_2$(4-Br-py)Cl]$^+$, as revealed by single crystal X-ray diffraction studies (Fig. 5). Ammonia release thus occurs at the site trans to the chloride ligand. This reaction could also be conducted on a preparative scale by heating cis-[Pt(NH$_3$)$_2$(4-Br-py)Cl]$^+$ in 0.1 M HCl. A similar reaction occurs when cis-[Pt(NH$_3$)$_2$(1-CH$_3$-cytosine)Cl]$^+$ is heated in aqueous solution (67). While in principle such a reaction might occur in vivo before the platinum complex reaches DNA, it is unlikely that

Table 2 $^{195}$Pt and $^{15}$N chemical shifts (ppm) and coupling constants (Hz) for cis-[Pt($^{15}$NH$_3$)$_2$(Am)]$^+$ and related complexes*

<table>
<thead>
<tr>
<th>Complex</th>
<th>$^{195}$Pt (ppm)</th>
<th>$^{15}$N (ppm)</th>
<th>$^1$J($^{195}$Pt-$^{15}$N) (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-[Pt($^{15}$NH$_3$)$_2$(N3-cytosine)(dG)]$^{2+}$</td>
<td>-2357</td>
<td>-68.7</td>
<td>312</td>
</tr>
<tr>
<td>cis-[Pt($^{15}$NH$_3$)$_2$(4-CH$_3$-py)(dG)]$^{2+}$</td>
<td>-2777</td>
<td>-61.2</td>
<td>323</td>
</tr>
<tr>
<td>cis-[Pt($^{15}$NH$_3$)$_2$(4-Br-py)Cl]$^+$</td>
<td>-2345</td>
<td>-65.4</td>
<td>323</td>
</tr>
<tr>
<td>cis-[Pt($^{15}$NH$_3$)$_2$(1-CH$_3$-cytosine)(dG)]$^{2+}$</td>
<td>-2341</td>
<td>-65.7</td>
<td>324</td>
</tr>
<tr>
<td>cis-[Pt($^{15}$NH$_3$)$_2$(dG)]$^{2+}$</td>
<td>-3024</td>
<td>-64.1</td>
<td>317</td>
</tr>
<tr>
<td>cis-[Pt($^{15}$NH$_3$)$_2$(n-butyl-NH$_2$)]$^+$</td>
<td>-2410</td>
<td>-67.1</td>
<td>318</td>
</tr>
<tr>
<td>cis-[Pt($^{15}$NH$_3$)$_2$(Cl)]$^+$</td>
<td>-2086</td>
<td>-59.9</td>
<td>303</td>
</tr>
<tr>
<td>cis-[Pt($^{15}$NH$_3$)$_2$(4-Br-py)(dG)]$^{2+}$</td>
<td>-2086</td>
<td>-59.9</td>
<td>303</td>
</tr>
<tr>
<td>cis-[Pt($^{15}$NH$_3$)$_2$(4-Br-py(H$_2$O))$^{2+}$</td>
<td>-2433</td>
<td>-63.8</td>
<td>312</td>
</tr>
</tbody>
</table>

* All NMR spectra were obtained in dimethyl formamide, except where indicated.
anism of action will differ in some respects from that of cisplatin, which forms mainly intrastrand d(GpG) and d(ApG) cross-links. Recently, a cellular DRP that senses the structural distortions in DNA caused by cisplatin binding has been identified (69). This DRP does not bind to DNA damaged either by cis-[Pt(NH3)2(Am)Cl]Cl platinum-triamine complexes or by trans-DDP. One interesting hypothesis to account for the anticancer activity of cisplatin is that the DRP recognizes and binds cis-DDP lesions on DNA, protecting them from repair (69). Consistent with this hypothesis is the fact that trans-DDP adducts are more efficiently repaired than those of cisplatin (28). The inability of cisplatin-damaged DNA to be replicated or transcribed is presumably a major cause of tumor cell lethality. Perhaps the platinum-triamine complexes, although not recognized by the cisplatin DRP, are not efficiently repaired and, consequently, their DNA adducts are toxic to tumor cells. Whatever the detailed mechanism, however, these complexes are unique from a mechanistic point of view and, therefore, worthy of additional biological testing in the hope that they might also display a unique spectrum of activity. Further studies should reveal the extent to which these compounds hold promise as clinically useful antitumor drugs.

REFERENCES

TRISUBSTITUTED PLATINUM(II) ANTITUMOR AGENTS


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Mechanistic Studies of a Novel Class of Trisubstituted Platinum(II) Antitumor Agents

L. Steven Hollis, Wesley I. Sundquist, Judith N. Burstyn, et al.


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