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Lability of monofunctional *cis*-platinum adducts: role of DNA double helix

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ABSTRACT

Recently, we have shown that the adduct formed in the reaction between the platinum-triamine complex *cis*-[Pt(NH_3)$_2$(N7-N-methyl-2-diazapyrenium)Cl]$^{2+}$ and one single-stranded oligonucleotide was stable but became labile as soon as the platinated oligonucleotide was paired with its complementary strand (Gaucheron et al. Proc. Natl. Acad. Sci. USA 88, 3516–3519 (1991)). To generalize this finding we have now studied large DNA fragments containing several adducts. The stability of the adducts within single-stranded DNA is demonstrated by absorption spectrophotometry and by replication mapping experiments. Several approaches are used to prove the lability of the adducts within double-stranded DNA. Replication mapping experiments reveal that an unmodified single-stranded DNA when mixed with double-stranded DNA modified by the platinum-triamine complex behaves as a single-stranded DNA modified by the triamine complex. After double-stranded DNA is modified by the platinum-triamine complex, intrastrand and interstrand cross-links are progressively formed during subsequent incubation as revealed by transcription mapping experiments and gel electrophoresis under denaturing conditions. Finally, replication mapping experiments show that the lability of the adducts within a double-stranded DNA depends upon the nature of the flanking nucleotide residues. All these results support the proposal that the DNA double helix acts as a catalyst in the reaction between DNA, *cis*-diamminedichloroplatinum(II) and N-methyl-2,7-diazapyrenium.

INTRODUCTION

Several drugs have cellular DNA as a target. They act by binding reversibly or irreversibly to sites located within the minor or major groove of the double helix or between base pairs (1). Quite often, two drugs which interact with DNA at different sites are used in combination. It is not yet completely understood how the binding of a drug can influence the binding of another drug. Among the studies devoted to the antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP) (2–6), *in vitro* experiments have shown alteration of bleomycin cleavage specificity in platined DNA (7–8). The presence of the intercalating drug ethidium bromide during platination of double-stranded DNA modulates *cis*-DDP binding to DNA (9–10). One reason for this modulation is the formation of a new kind of adduct (10–12). In the reaction between *cis*-DDP and DNA the major adducts arise from intrastrand cross-links between adjacent purine residues (2–6). If intercalating compounds (Am) such as ethidium bromide, proflavine, N-methyl-2,7-diazapyrenium are present during the platination reaction, the major adduct (dG-*cis*Pt-Am) arises from a cross-link between Am and guanine residue (10–13). In the same experimental conditions but without double-stranded DNA (or with single-stranded DNA), *cis*-DDP is hardly reactive with Am. Thus, double-stranded DNA promotes the binding of *cis*-DDP to Am by acting as a matrix achieving a favorable orientation of the reactants.

It is generally accepted that once *cis*-DDP or the monofunctional derivative chlorodiethylenetriamineplatinum(II) are bound to DNA, the covalent adducts are kinetically inert (2–6). A few examples of metal migration have been observed with mononucleotide and single-stranded oligonucleotides (14–16). On the other hand, the conformation of the nucleic acid can interfere with the stability of the adducts. Recently we found that (dG-*cis*Pt-Am) monofunctional adduct in which Am is N-methyl-2,7-diazapyrenium (MDAP) was inert within a single-stranded oligonucleotide. On the opposite, the adduct became labile as soon as the modified single-stranded oligonucleotide was hybridized with its complementary strand and the two products *cis*-[Pt(NH_3)$_2$(N7-N-methyl-2-diazapyrenium) H_2O]$^{3+}$ (*cis*Pt-MDAP) and MDAP were released (17). Thus, in the reaction among *cis*-DDP, MDAP and DNA, the DNA double helix favors in a first step the binding of *cis*-DDP to MDAP and in a second step the cleavage of Pt-G and Pt-MDAP bonds respectively. We have proposed that DNA acts as a catalyst in the general reaction summarized in fig. 1.

Up to now, the lability of (dG-*cis*Pt-MDAP) monofunctional adduct has been demonstrated in the case of one double-stranded oligonucleotide (17). The purpose of the present work was to generalize this finding. To this end, we have used large DNA fragments containing several (dG-*cis*Pt-MDAP) adducts. The release of MDAP and *cis*-Pt-MDAP according to reactions 1 and 2 (fig. 1) respectively has been mainly followed by replication and transcription mapping experiments.

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MATERIALS AND METHODS

The 17-mer M13 sequencing primer d(GTAAAACGACGGCCAGT) was from Biolabs. The 22-mer d(TCACAATTCCACACAACATACG), synthesized on a Applied Biosystems solid-phase synthesizer was purified by ion-exchange chromatography on a Pharmacia FPLC system.

Restriction enzymes, T4 polynucleotide kinase, DNA polymerase I large fragment from *E.coli* (Klenow fragment) were from Boerhinger Mannheim. Enzyme buffers were those recommended by the suppliers. T7 RNA polymerase, ribonucleoside triphosphates and RNasin ribonuclease inhibitor were bought from Promega. 3'-deoxynucleoside triphosphates and ultrapure agarose were bought from Bethesda Research Laboratories. The radioactive products were from Amersham. Electrophoresis-grade acrylamide, N-N'-methylenebisacrylamide were from Merck. *cis*-DDP was from Johnson Matthey. The platinum-triamine complex *cis*-$[Pt(NH_3)_2$(N7-N-methyl-2-diazapyrenium)$Cl]^{2+}$ was synthesized as previously described (13). Single-stranded DNA from bacteriophage M13mp19 and double-stranded pSPKB DNA, a derivative of pSP73 DNA (18), were purified as described (19).

Platination of DNA

Reaction between *cis*-DDP and single-stranded or double-stranded DNA was performed in 10 mM $NaClO_4$ at 37°C and during 24 hours (18). For the reaction between DNA and the platinum-triamine complex *cis*-$[Pt(NH_3)_2$(N7-N-methyl-2-diazapyrenium)$Cl]^{2+}$, the triamine complex dissolved in water was first incubated overnight and at 37°C, then DNA was added ($c \approx 0.5$ mM) and $NaClO_4$ concentration adjusted to 10 mM. The solution was incubated for 1 hour at 37°C (13). Then, DNA was precipited three times with ethanol. In these reactions, more than 90% of the added triamine complex or *cis*-DDP were bound to DNA as verified by atomic absorption spectroscopy.

Replication mapping experiments

The procedure was that described (20) with minor modifications. The 17-mer M13 sequencing primer labeled at the 5' end with [gamma-^{32}P]ATP by means of T4 polynucleotide kinase was mixed with DNA in 60 µl of sequencing buffer (50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol). After heating to 70°C and cooling at room temperature, 170 µM each of dGTP, dCTP, dTTP and dATP and 2.5 units of Klenow fragment were added. The solution was incubated for 15 min at 42°C. The reaction was stopped by addition of 90% formamide, 5 mM EDTA-dye mixture. The samples were denatured by heating to 85°C for 3 min and then analysed by electrophoresis on 8% polyacrylamide, 7 M urea.

Transcription mapping experiments

Transcription with T7 RNA polymerase was performed according to the protocol recommended by Promega. For nucleotide sequence analysis, the reaction mixtures were supplemented with 3'-deoxynucleoside triphosphates according to the protocol of Axelrod and Kramer (21). The electrophoretic analysis of transcripts was done as (21).

Absorption

Spectra were recorded on a Kontron Uvikon 810 spectrophotometer.

RESULTS AND DISCUSSION

Stability of dG-*cis*Pt-MDAP adducts within a single-stranded DNA

To show that dG-*cis*Pt-MDAP adducts surrounded by different base residues within a single-stranded (ss) DNA were stable, we have studied by absorption spectrophotometry and by replication experiments ss DNA which has been reacted with the platinum-triamine complex *cis*-$[Pt(NH_3)_2(MDAP)H_2O]^{3+}$. Previous works have shown that the triamine complex reacts with ss and ds DNA and that the main adduct is dG-*cis*Pt-MDAP (13,17).

In the first set of experiments, spectra were recorded in the 300−500 nm range where absorption is only due to MDAP residues (13). The ss DNA from bacteriophage M13 was reacted with *cis*-$[Pt(NH_3)_2(MDAP)H_2O]^{3+}$ at $r_b = 0.03$ (r_b is the molar ratio bound drug per nucleotide residue). The modified DNA was then dialyzed against 0.2 M $NaClO_4$ during 24h and at 37°C. The absorption spectra before and after dialysis were the same (not shown). One can conclude that no loss of MDAP or *cis*-Pt-MDAP occurred during the dialysis.

In the second set of experiments, the stability of the adducts was confirmed by replication mapping studies . The ss DNA modified by *cis*-$[Pt(NH_3)_2(MDAP)H_2O]^{3+}$ at $r_b = 0.004$ was used as a template for second strand synthesis by the Klenow fragment of *E.coli* DNA polymerase I. The primer was a 17-mer oligonucleotide labeled at 5' end. As shown in fig. 2, DNA synthesis by the enzyme generates a population of DNA

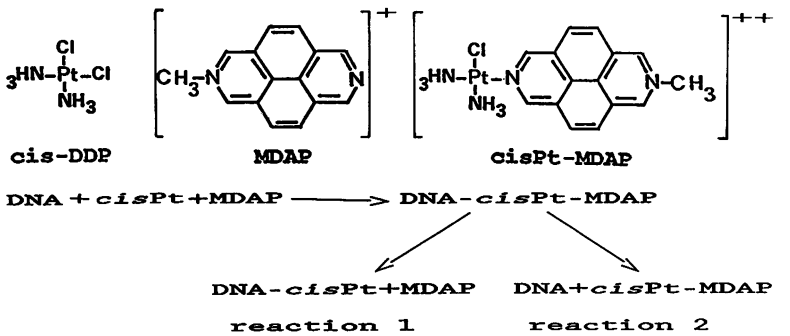

Figure 1. Reaction between DNA, *cis*-DDP and MDAP.

fragments of defined sizes. Sequence analysis of the fragments indicates that the main bands appear at the guanine residues. However, the intensities of the bands are not uniform and there are less bands than guanine residues suggesting that the reactivity of guanine residues with *cis*-$[Pt(NH_3)_2(MDAP)H_2O]^{3+}$ is influenced by neighboring bases. (In first approximation, a similar spectrum of modification was obtained with double-stranded (ds) DNA from bacteriophage M13 which had been reacted with the platinum-triamine complex, not shown). The modified ss DNA was dialyzed against 0.2 M $NaClO_4$ during 24 h at 37°C and then used as a template for the Klenow fragment. The patterns of stop sites were identical before and after dialysis of the sample(not shown).

For comparison, ss DNA modified by *cis*-DDP at $r_b = 0.004$ was replicated in a similar manner. As shown in fig. 2 the patterns for DNA modified by either *cis*-DDP (lane 2) or the triamine complex (lane 3) are different.

From these two sets of experiments, one can conclude that dG-*cis*Pt-MDAP adducts are stable within ss DNA. The reactivity of guanine residues with the platinum-triamine complex is modulated by the adjacent bases. By means of the Klenow fragment, one can differentiate between *cis*-DDP- and triamine complex-modified DNAs. This is in agreement with a recent work by Hollis et al. (22). They conclude from replication experiments that the triamine complexes *cis*-$[Pt(NH_3)_2(4\text{-methylpyridine})Cl]^+$ and *cis*-$[Pt(NH_3)_2(4\text{-bromopyridine})Cl]^+$ have no preference for multiple guanine sites and that the patterns of stop sites for DNA modified by these triamine complexes and *cis*-DDP are different. It could be argued that some translesion synthesis occurs and that all the adducts are not detected (23). This is not a crucial point in our work since we will compare samples modified either by *cis*-DDP or the triamine complex.

Lability of dG-*cis*Pt-MDAP adducts within a double-stranded DNA

To show the lability of dG-*cis*Pt-MDAP adducts within ds DNA, several experiments have been done.

In the first set of experiments we wanted to reveal the release of *cis*Pt-MDAP according to reaction 2 (fig. 1). We reasoned that once *cis*Pt-MDAP is released in solution, it can be trapped by any guanine residue within ss DNA. Since the adducts within ss DNA are stable, their presence can be subsequently detected by replication mapping studies.

Linear ds DNA pSPKB was reacted with *cis*-$[Pt(NH_3)_2(MDAP)H_2O]^{3+}$ at $r_b = 0.1$. After three precipitations to completely remove the unreacted triamine complex, the modified ds DNA was mixed with an equal amount of unmodified ss DNA from bacteriophage M13 and then incubated in 10 mM $NaClO_4$, 5 mM Tris-HCl pH 7.5, at 37°C. At various times, aliquots were withdrawn. The ss DNA was primed with the 17-mer oligonucleotide and replicated as in fig. 2. The results are presented in fig. 3. At time=0, long DNA fragments are synthesized. At time=0.5, 1, 2 h...bands corresponding to shorter DNA fragments appear. The intensities of the bands increase as a function of time but their locations do not change. The stop pattern is similar to that relative to DNA modified directly by the triamine complex. Thus, incubation of *cis*Pt-MDAP-modified ds DNA with unmodified ss DNA leads to a modified ss DNA which in

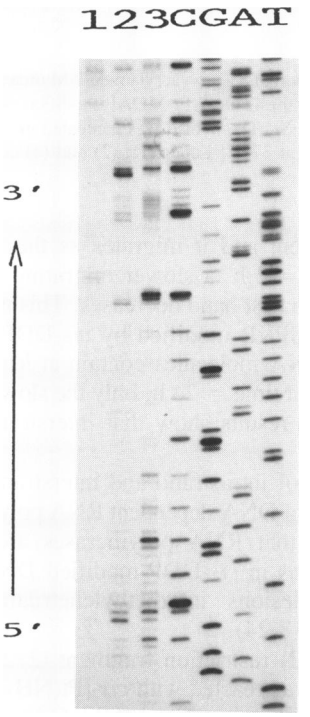

Figure 2. Autoradiogram of 8% polyacrylamide/7 M urea sequencing gel showing inhibition of DNA synthesis on *cis*-DDP- and *cis*Pt-MDAP-modified ss DNA. The direction of DNA synthesis by the Klenow fragment of *E.coli* DNA polymerase I is from 5′ (bottom) to 3′ (top). Unplatinated DNA (lane 1); *cis*-DDP-modified ss DNA at $r_b = 0.004$ (lane 2); *cis*Pt-MDAP-modified ss DNA at $r_b = 0.004$ (lane 3). Lanes C, G, A and T correspond to the sequence of neosynthesized strand.

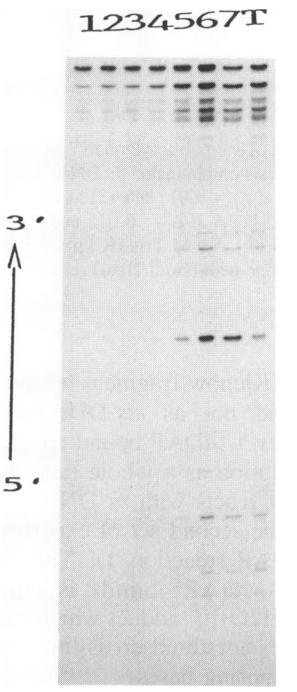

Figure 3. Autoradiogram of 8% polyacrylamide/7 M urea sequencing gel showing inhibition of DNA synthesis on ss DNA incubated in the presence of a *cis*Pt-MDAP-modified ds DNA at $r_b = 0.1$. Lanes (1−7) correspond to times of incubation equal to 0, 0.5, 1, 2, 5, 10 and 20 h. Lane T coresponds to the same ss DNA modified directly by the platinum-triamine complex at $r_b = 0.004$.

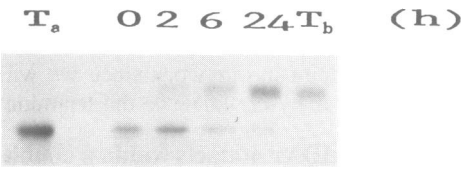

Figure 4. Autoradiogram of a denaturing 1.5% agarose gel showing the formation of interstrand cross-links in *cis*Pt-MDAP-modified ds DNA. Times of incubation of the modified samples are indicated above the lanes. T_a and T_b correspond to the unplatinated DNA and to the same DNA modified by *cis*-DDP at r_b = 0.01, respectively.

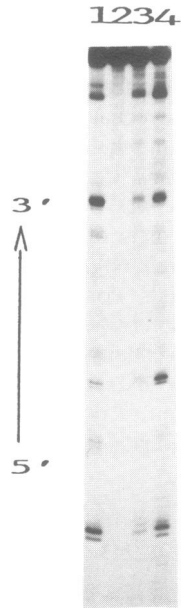

Figure 5. Autoradiogram of 8% polyacrylamide/7 M urea sequencing gel showing inhibition of RNA synthesis on platinated ds DNA. Lane (1) corresponds to *cis*-DDP-modified ds DNA at r_b = 0.01, lane (2) to unplatinated ds DNA, lane (3) to *cis*Pt-MDAP-modified DNA at r_b = 0.015 and lane (4) to the same sample incubated in 10 mM $NaClO_4$, 5 mM Tris-HCl pH 7.5 during 24 h and at 37°C. The experiments were done with (NdeI/HpaI) restriction fragment from pSPKB DNA.

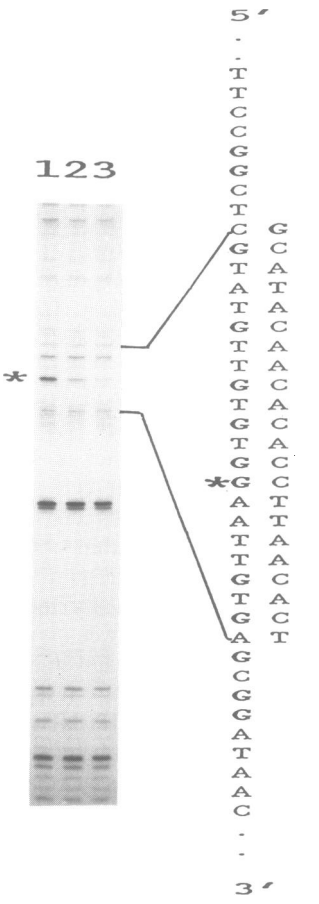

Figure 6. Autoradiogram of 8% polyacrylamide/7 M urea sequencing gel showing inhibition of DNA synthesis on *cis*Pt-MDAP-modified ss DNA hybridized with a 22-mer oligonucleotide. The hybrid was incubated in 40 mM $NaClO_4$, 5 mM Tris-HCl pH 7.5 and at 37°C. Lanes (1), (2) and (3) correspond to 0, 24 and 48 h of incubation.

the presence of the Klenow fragment behaves as *cis*Pt-MDAP-modified DNA and not as *cis*-DDP-modified DNA. The conclusion is that *cis*Pt-MDAP bound covalently to ds DNA is first released in solution as a whole (and not as *cis*-DDP and MDAP) and then it reacts with ss DNA.

The purpose of the second set of experiments was to reveal the release of MDAP (reaction 1). The consequence of the breakage of Pt-MDAP bond is the formation of *cis*-$[Pt(NH_3)_2(dG)(H2O)]^{2+}$ adduct which can further react and form intrastrand or interstrand cross-links depending upon the nature of the neighboring bases.

We have first studied the formation of interstrand cross-links. The linear plasmid DNA pSPKB was reacted with *cis*-$[Pt(NH_3)_2(MDAP)H_2O]^{3+}$ at r_b=0.025, precipitated three times and then incubated in 10 mM $NaClO_4$ at 37°C. At various times, aliquots were withdrawn and analysed by gel electrophoresis under denaturing conditions. At time=0, only one band is present and it migrates as the unmodified DNA (fig. 4). At time = 2 h, a slower migrating band appears and the intensity of the first band decreases. This new band migrates as linear DNA pSPKB modified by *cis*-DDP at r_b = 0.01 (at this r_b, all the DNA molecules contain at least one interstrand cross-link (18)). At time = 24 h, only the slower migrating band is present. These results show that interstrand cross-links are formed.

The formation of intrastrand and interstrand cross-links was studied by means of DNA-dependent RNA polymerase. Recently, we have shown that RNA polymerases are blocked by the bifunctional lesions in *cis*-DDP-modified DNA but not by the monofunctional lesions in diethylenetriamineplatinum (II)-modified DNA (18,24).

The (NdeI/HpaI) restriction fragment (212 base pairs) from plasmid pSPKB was reacted with *cis*-$[Pt(NH_3)_2(MDAP)H_2O]^{3+}$ at r_b = 0.015 and then incubated at 37°C for 24 h. The samples were used as a template for T7 RNA polymerase. As shown in fig. 5, only a few bands of weak intensity (lane 3) are detected with the modified DNA at incubation time = 0. The monofunctional adducts do not inhibit the enzyme. After 24 h of incubation of the sample, the bands are intense (lane 4) and are at the same position as those obtained with *cis*-DDP-modified DNA. This experiment is in agreement with reaction 1.

In summary, the two sets of experiments show that *cis*-$[Pt(NH_3)_2(MDAP)(dG)]^{3+}$ adducts within ds DNA are labile. The formation of intra or interstrand cross-links suggest that first MDAP is released and then closure of *cis*-$[Pt(NH_3)_2(dG)(H_2O)]^{++}$ monofunctional adducts to bifunctional cross-links occur. We are aware that intra and interstrand cross-links can be formed if the ligands NH_3 were released. This seems to us unlikely taking into account our previous results on the ds oligonucleotide (18).

Sequence effect on the lability of *cis*-$[Pt(NH_3)_2(MDAP)(dG)]^{+++}$ adducts

The role of the adjacent base residues on the lability of the adducts within ds DNA has been studied by replication mapping experiments using as a template ss DNA containing a ds region obtained by pairing a 22-mer oligonucleotide.

The ss DNA from bacteriophage M13 was reacted with *cis*-$[Pt(NH_3)_2(MDAP)H_2O]^{3+}$ to $r_b = 0.004$, hybridized with the 22-mer d(TCACAATTCCACACAACATACG) and then incubated in 40 mM $NaClO_4$, 5 mM Tris-HCl pH 7.5 and at 37°C. At various times, aliquots were withdrawn. The ss DNA was primed with the 17-mer (as in fig. 2) and used as a template for second strand synthesis by Klenow fragment. The results are presented in fig. 6. At time = 0, the pattern of stop sites is the same as that presented in fig. 2. At times = 24 and 48 h, in the first approximation, the intensities of all bands but one located within the ds region are unchanged. The intensity of this band (marked by an asterisk in fig. 6) decreases as a function of incubation time which suggests release of *cis*Pt-MDAP. This experiment is in favor of a sequence effect on the lability of the adduct. A systematic study is in progress in order to determine the importance of the adjacent base residues on the rates of reactions 1 and 2.

CONCLUSION

In this work, we show that the adducts formed in the reaction between *cis*-$[Pt(NH_3)_2(MDAP)H_2O]^{3+}$ and DNA are kinetically inert within ss DNA and labile within ds DNA. All the results suggest that the two reactions 1 and 2 (fig. 1), first observed in the case of one double-stranded oligonucleotide containing a single adduct occur also in the case of large DNA fragments containing several adducts. A released *cis*Pt-MDAP can further react with any guanine residue present in the solution. If the solution contains no trapping agents other than the G residues within the double helix, the amount of *cis*Pt-MDAP covalently bound to DNA is constant as a function of time. However, a given *cis*Pt-MDAP residue does not necessarily remain bound to the same G residue. Due to the release of MDAP, *cis*-$[Pt(NH_3)_2(dG)H_20]^{2+}$ adducts are formed which can further react with the neighboring base residues within ds DNA and the consequence is the formation of intra and interstrand cross-links as in the reaction between *cis*-DDP and DNA. Since both reactions 1 and 2 occur within DNA modified by the triamine complex, the total amount of covalently bound MDAP residues decreases as a function of time while the total amount of covalently bound platinum residues remains constant.

It is presently unknown why the adducts are labile within double-stranded DNA and stable within single-stranded DNA. Since within double-stranded DNA the adducts surrounded by different nucleotide residues undergo reactions 1 and 2, it might be that a major destabilizing factor of the Pt-G and Pt-MDAP bonds originates from steric constraints imposed on the adducts by the DNA double helix itself (the lability of the adducts would be structure specific and sequence independent). According to such a view the neighboring bases of the adducts might modulate the constraints since the geometry of the double helix is locally sequence dependent. Thus quite generally, one expects the lability of the adducts to vary as a function of the DNA conformation (A-, B-, Z-form...) the chemical nature of Am and the chemical nature of the inert amine ligands bound to platinum residues.

Recently, it has been found that *cis*-$[Pt(NH_3)(Am)Cl]^{+}$ cations in which Am is an amine ligand derived from pyridine, pyrimidine, purine and piperidine are active against murine and human tumor systems (25). These cations are not supposed to form bifunctional cross-links in the reaction with DNA and thus they do not follow the structure-function relationship established for *cis*-platinum derivatives (2–6). Work is in progress to determine whether these agents which initially form monofunctional adducts on DNA (22,25) undergo reaction 1 and 2 as observed with *cis*Pt-MDAP.

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