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Instability of the monofunctional adducts in *cis*-[Pt(NH$_3$)$_2$(*N7-N*-methyl-2-diazapyrenium)Cl]$^{2+}$-modified DNA: rates of cross-linking reactions in *cis*-platinum-modified DNA

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ABSTRACT

Single- and double-stranded oligonucleotides containing a single monofunctional *cis*-[Pt(NH$_3$)$_2$(dG)(*N7-N*-methyl-2-diazapyrenium)]$^{3+}$ adduct have been studied at two NaCl concentrations. In 50 mM and 1 M NaCl, the adducts within the single-stranded oligonucleotides are stable. In contrast, they are unstable within the corresponding double-stranded oligonucleotides. In 50 mM NaCl, the bonds between platinum and guanine or *N*-methyl-2,7-diazapyrenium residues are cleaved and subsequently, intra- or interstrand cross-links are formed as in the reaction between DNA and *cis*-DDP. In 1 M NaCl, the main reaction is the replacement of *N*-methyl-2,7-diazapyrenium residues by chloride which generates double-stranded oligonucleotides containing a single monofunctional *cis*-[Pt(NH$_3$)$_2$(dG)Cl]$^+$ adduct. The rates of closure of these monofunctional adducts to bifunctional cross-links have been studied in 60 mM NaClO$_4$. Within d(TG·CT/AGCA), d(CG·CT/AGCG) and d(AG·CT/AGCT) (the symbol · indicates the location of the adducts in the central sequences of oligonucleotides), the half-lifes ($t_{1/2}$) of the *cis*-[Pt(NH$_3$)$_2$(dG)Cl]$^+$ adducts are respectively 12, 6 and 2.8 hr and the cross-linking reactions occur between guanine residues on the opposite strands. Within d(AG·TC/GACT), d(CG·AT/ATCG) and d(TGTG·/CACA) or d(TG·TG/CACA) $t_{1/2}$ are respectively 1.6, 8 and larger than 20 hr and the intrastrand cross-links are formed at the d(AG), d(GA) and d(GTG) sites, respectively. The conclusion is that the rates of conversion of *cis*-platinum – DNA monofunctional adducts to minor bifunctional cross-links are dependent on base sequence. The potential use of the instability of *cis*-[Pt(NH$_3$)$_2$(dG)(*N7-N*-methyl-2-diazapyrenium)]$^{3+}$ adducts is discussed in the context of the antisense strategy.

INTRODUCTION

It is generally accepted that the cytotoxic activity of the antitumor drug *cis*-diamminedichloroplatinum (II) (*cis*-DDP) is related to its ability to react with cellular DNA (for general reviews see 1–4). Lesions produced in DNA have been characterized as bifunctional adducts including mainly intrastrand and interstrand cross-links (5–6). Numerous studies suggest that DNA modification by *cis*-DDP is controlled kinetically and that the adducts are formed in two solvent assisted reactions in sequence (for general reviews 7–8). The exchange of the chloro groups of *cis*-DDP is rate-limiting in both the initial attack of DNA and the closure of monofunctional adducts to bifunctional cross-links. The preferred site of initial binding of *cis*-DDP to DNA is the N7 atom of G residues. Subsequently the monofunctional adducts react with the neighboring bases to form intrastrand or interstrand cross-links. Most of the kinetic studies deal with the major lesions or do not differentiate among the minor lesions (7–10).

Whether one kind or several kinds of adducts contribute to the activity of *cis*-DDP is under discussion. It is also accepted that the monofunctional adducts of *cis*-DDP have no biological activity. However, Hollis *et al.* (11–12) showed that platinum–triamine cations of the form *cis*-[Pt(NH$_3$)$_2$(Am)Cl]$^+$ in which Am is an amine ligand derived from pyridine, pyrimidine, purine, piperidine, or aniline are active against murine and human tumor systems. These compounds form covalent monofunctional adducts on DNA and thus do not follow the classical structure–activity relationship established for platinum complexes (13). Similar monofunctional adducts are also formed when the *in vitro* reaction between DNA and *cis*-DDP is done in the presence of some intercalators such as proflavine, ethidium bromide or methyl-2,7-diazapyrenium (MDAP) (14–16).

The stability of these monofunctional adducts appears to depend upon DNA conformation. At low NaClO$_4$ concentration, the monofunctional *cis*-Pt[(NH$_3$)$_2$(*N7*-dG)(*N7-N*-methyl-2-diazapyrenium)]$^{3+}$ (dG-*cis*Pt-MDAP) adduct is stable within a single-stranded (ss) oligonucleotide and unstable within the corresponding double-stranded (ds) oligonucleotide (17). In the latter case, two reactions occur (cleavage of the bonds between Pt and MDAP or G residues) as summarized in Figure 1.

Another observation is that dG-*cis*Pt-MDAP adducts within ds DNA fragments are progressively transformed during incubation at 37°C (18). It was proposed that the *cis*-[Pt(NH$_3$)$_2$(dG)H$_2$O]$^{2+}$ adducts generated by the release of MDAP

(reaction 2, Fig. 1) react further with the adjacent bases to form bifunctional cross-links. To prove this proposal, we have undertaken a study of several oligonucleotides containing a single dG-*cis*Pt-MDAP adduct, at low and high NaCl or $NaClO_4$ concentrations. In the present paper, we show that in 50 mM salt, dG-*cis*Pt-MDAP adducts within ds oligonucleotides are unstable, in agreement with Figure 1. Furthermore, subsequently to reaction 2 (Fig. 1), intrastrand or interstrand cross-links are formed, depending upon the nature of the bases adjacent to the adducts. In contrast, in 1 M NaCl, mainly replacement of MDAP by chloride occurs which generates the monofunctional *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adducts. Taking advantage of this finding, the kinetics of the cross-linking reaction in these *cis*-DDP-modified oligonucleotides have been studied. We show that the rates of closure of the monofunctional *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adducts to bifunctional cross-links are dependent on base sequence. Finally, we discuss the potential use of the instability of dG-*cis*Pt-MDAP adducts in the context of the antisense strategy.

MATERIALS AND METHODS

The oligodeoxynucleotides synthesized on an Applied Biosystems solid-phase synthesizer were purified by ion-exchange chromatography on a pharmacia FPLC system. The sequences of the oligonucleotides were

d(TGCT)	C T T C T C C T T **G C** T C T C C T T C T T C
d(AGCA)	A A G A G G A A **C G** A G A G G A A G A A G G
d(AGTC)	C T T C T C C T C **A G T C** T C C T T C T T C
d(GACT)	A A G A G G A G **T C A G** A G G A A G A A G G
d(AGCT)	T C T C T C C T C T **A G C T** C T C C T T C T
d(AGCT)	G A G A G G A G A T **C G A** G A G G A A G A A
d(CGAT)	C T C C T C T C T **C G A T** C T C C T C T
d(ATCG)	A G G A G A G A G **C T A** G A G G A G A G
d(CGCT)	T C T C T C C T C T **C G C T** C T C C T T C T
d(AGCG)	G A G A G G A G A G **C G A** G A G G A A G A A
d(TGTC)	C T T C T C C T C T **G T C** T C C T T C T T C
d(GACA)	A A G A G G A G A **C A G** A G G A A G A A G G
d(GTGT)	C T T C C T C T T C T **G T G** T C T T C T C T
d(CACA)	A A G G A G A A G A **C A C** A G A A G A G A G

The ss oligonucleotides will be referred by their central sequences as for example d(TGCT) for d(CTTCTCCT**TGCT**CTCCTTC-TTC) and d(TGCT/AGCA) for d(CTTCTCCT**TGCT**CTCCT-TCTTC).d(GGAAGAAGGAG**AGCA**AGGAGAA). Plasmid pSP73KB was prepared as previously described (19). T4 polynucleotide kinase, T4 DNA polymerase, endonuclease P1 and alkaline phosphatase were purchased from Boehringer-Mannheim. Electrophoresis grade acrylamide and *N-N'*-methyl-enebisacrylamide were from Merck, *cis*-DDP was from Johnson-Mattey.

Platination of oligonucleotides

The platinum−triamine complex *cis*-$[Pt(NH_3)_2(N7\text{-}N\text{-}$methyl-2-diazapyrenium)$Cl]^{2+}$ (*cis*Pt-MDAP) was synthesized in organic solvent as previously described (16). The reaction between the oligonucleotides (c ≃0.3 mM in nucleotide residues) containing a single guanine G residue and the platinum−triamine complex (input molar ratio platinum−triamine complex per G residue ≃1.2) were carried out in 0.1 mM $HClO_4$, pH 3.8 during 16 hours at 37°C. The platinated oligonucleotides were purified by ion-exchange chromatography. The nature of the adducts was verified by reverse phase HPLC analysis (on a C18 column attached to a Hitachi model 655 chromatograph) of the digests after enzymatic hydrolysis with the endonuclease P1 [12 μl (c = 2 mg/ml) in 25 mM sodium acetate pH 5.5, 10 mM $MgCl_2$, 1 mM $ZnSO_4$ and 30 mM NaCl, during 20 hours at 37°C] and then treatment with alkaline phosphatase [2 units in endonuclease P1 buffer supplemented with 20 mM Tris−HCl pH 8.8, during 30 minutes at 50°C] as described (5−6). The reaction occurred at the G residues and the adduct was dG-*cis*Pt-MDAP (16). The location of the adducts within the oligonucleotides was confirmed as follows. The modified oligonucleotides were labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P] ATP (20) and then treated with T4 DNA polymerase [3 units per 50 nmol of the oligonucleotide in 50 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT and 10 mM Tris−HCl pH 7.9, during 10 minutes at 37°C] conditions in which the enzyme had 3′→5′ exonuclease activity so that digestion stopped at the lesion. The digests were incubated in 0.2 M NaCN (basic pH) for 12 hours to remove the platinum and then were separated by gel electrophoresis (denaturing 24% polyacrylamide gel). For each oligonucleotide, the autoradiogram of the gel revealed the presence of only one product with the expected length. The same protocol was used for platinated d(TGTG). The autoradiogram of the gel revealed the presence of two bands of approximately equal intensity. The oligonucleotide contained one adduct but the two G residues were equally reactive.

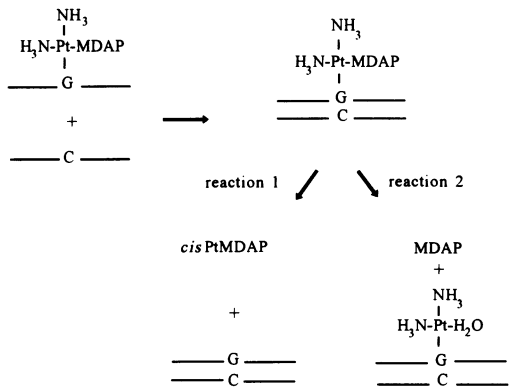

Figure 1. Scheme of the instability of dG-*cis*Pt-MDAP adducts within ds oligonucleotides.

The ss oligonucleotides d(AGTC) and d(GTGT) were reacted with *cis*-DDP as previously described (21). After purification, the oligonucleotides contained a single intrastrand cross-link at the d(AG) and d(GTG) sites, respectively.

Kinetics of conversion of dG-*cis*Pt-MDAP adducts

The *cis*Pt-MDAP-modified oligonucleotides were ^{32}P-end labeled and then paired with their respective complementary oligonucleotides. The hybrids (c ≃0.05 mM in nucleotide residues), in 50 mM $NaClO_4$, 5 mM Tris−HCl, pH 7.4 with 1 mM EDTA, were heated to 55°C for 3 min and subsequently cooled to 20°C (within 10 min) and then kept at 4°C for at least 3 hr. It was verified by the variation of the absorbance at 260 nm versus temperature that in these conditions, we were dealing with double helices (the melting temperatures (Tm) of the ds oligonucleotides containing a single dG-*cis*Pt-MDAP were lower by about 3°C than the Tm of the corresponding unplatinated samples). The ss and ds were incubated in various NaCl or $NaClO_4$ concentration, 5 mM Tris−HCl pH 7.5 and at 37°C. As a function of time, aliquots were withdrawn and analyzed by gel electrophoresis under denaturing conditions (17).

To identify the nature of the bases in the interstrand cross-links, the oligonucleotides were purified by gel electrophoresis, and then treated with T4 DNA polymerase (6 units per 50 nmol of the oligonucleotides in 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 10 mM Tris−HCl pH 7.9 during 2 hours at 37°C). The oligonucleotides were completely digested as verified by gel electrophoresis. After treatment with alkaline phosphatase, the digests were analyzed by reversed-phase HPLC with a gradient of acetonitrile in 0,1 M ammonium acetate (2−9% CH_3CN in 35 min gradient at 1 ml/min flow). The standard *cis*-$[Pt(NH_3)_2(dG)_2]^{2+}$ was prepared as described (22).

To identify the nature of the bases in the intrastrand cross-links, the ss oligonucleotides were treated with endonuclease P1 and then alkaline phosphatase as described (5−6).

Reaction with chemical probes

To confirm the formation and location of an intrastrand or interstrand cross-link after the conversion of dG-*cis*Pt-MDAP adducts within the ds oligonucleotides, chemical probes (osmium tetroxide, dimethylsulfate and hydroxylamine) were used as described (21,23).

Kinetics of conversion of *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adducts

To generate *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adducts, the ds oligonucleotides (c ≈0.05 mM) containing a single dG-*cis*Pt-MDAP adduct were incubated during 24 hours in 1 M NaCl, 5 mM Tris−HCl pH 7.5 and at 37°C. Then the oligonucleotides were precipitated with ethanol, dissolved in 0.3 M $NaClO_4$, and precipitated again (2 times). After dissolution (c ≈0.05 mM) in 60 mM $NaClO_4$, 5 mM Tris−HCl pH 7.5, they were incubated at 37°C. At various times, aliquots were withdrawn and analyzed by gel electrophoresis under denaturing conditions (17).

The platinum contents of the samples were measured with an atomic absorption spectrophotometer by Dr J.L.Butour (Toulouse). A Camag microdensitometer was used to collect the data from sequencing gels. Absorption spectra were recorded on a Kontron Uvikon 810 spectrophotometer.

RESULTS

Instability of dG-*cis*Pt-MDAP adducts versus salt concentration

The following 20- or 22-mer oligonucleotides [referred by their central sequences d(AGTC), d(CGAT), d(AGCT), d(TGCT), d(TGTC), d(CGCT) and d(TGTG)] which contain a single G residue (except d(TGTG) which contains two G residues), were reacted with the platinum−triamine complex *cis*Pt-MDAP. After purification, the oligonucleotides contained a single dG-*cis*Pt-MDAP adduct. The *cis*Pt-MDAP-modified ss and ds oligonucleotides were incubated at 37°C, in low salt conditions (50 mM NaCl, 50 mM $NaClO_4$) or in high salt conditions (1 M NaCl or 1 M $NaClO_4$). At various times, aliquots were withdrawn and analyzed by gel electrophoresis under denaturing conditions (17). A similar behavior was observed for all the samples and only the results relative to two samples are presented.

d(TG*CT/AGCA)

Low salt conditions. We consider first the results relative to d(TG*CT/AGCA) (the symbol * denotes the location of the adduct) incubated in 50 mM NaCl (Fig. 2A, left). At time t = 0 of incubation, only one band (a) is present. It migrates more slowly than the band (b) corresponding to unplatinated d(TGCT).

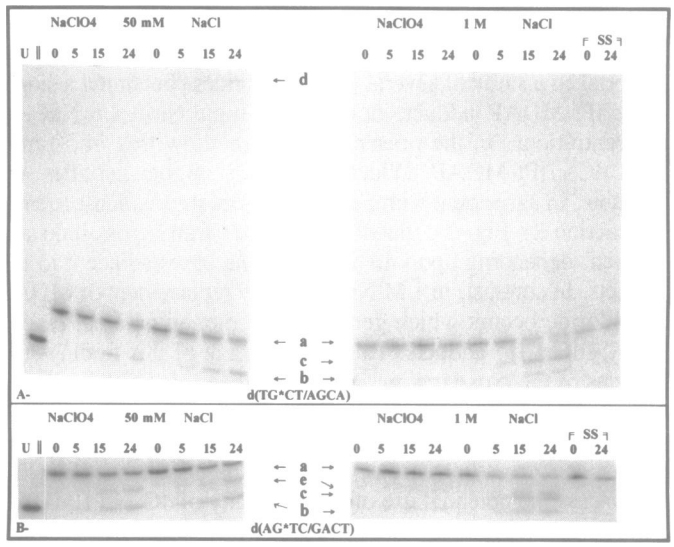

Figure 2. Instability of dG-*cis*Pt-MDAP adducts within ss or ds oligonucleotides. Autoradiogram of a denaturing 24% polyacrylamide gel of d(TG*CT/AGCA) (**A**) or d(AG*TC/GACT) (**B**) incubated at 37°C and in 50 mM NaCl or $NaClO_4$ (left) or in 1 M $NaClO_4$ or NaCl (right) (the symbol * indicates the location of the adducts). The incubation times (in hours) of the samples are indicated above the lanes. The concentrations of the samples were about 0.05 mM. Lanes (ss) correspond respectively to single-stranded d(TG*CT) and d(AG*TC) incubated in 1 M NaCl. Lanes (U) correspond to unplatinated d(TGCT) and d(AGCT), respectively. The letters (a, b, c, d and e) correspond respectively to the cisPt-MDAP-modified oligonucleotides (a), to the unplatinated oligonucleotides (b), to the oligonucleotides containing a *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct (c), to the oligonucleotides containing an interstrand cross-link (d) and to the oligonucleotides containing an intrastrand cross-link (e)

The adduct interferes with the electrophoretic mobility of the oligonucleotide by its mass and its three positive charges. After longer incubation times, the intensity of the band (a) decreases whereas two bands (b and d) appear. The presence of d(TGCT) (band b) suggests the release of *cis*Pt-MDAP from d(TG*CT). The band (d) migrates more slowly than the band (a), which suggests that the oligonucleotide contains an interstrand cross-link. The same results are obtained in 50 mM $NaClO_4$ (Fig. 2A, left). In both salts, the results are independent of d(TG*CT/AGCA) concentration.

Several complementary experiments were carried out to confirm the formation of an interstrand cross-link and the release of MDAP and *cis*Pt-MDAP.

A large quantity (≈50 μg) of d(TG*CT/AGCA) dissolved in 50 mM $NaClO_4$ was mixed with plasmid DNA (200 μg) and incubated during 24 hours and at 37°C (the plasmid DNA was added to trap the released *cis*Pt-MDAP; without the plasmid DNA, the released *cis*Pt-MDAP could react with any G residue of the oligonucleotide (17)). Then the $NaClO_4$ concentration was adjusted to 0.3 M and after precipitation of the sample by ethanol, the supernatant was analyzed by HPLC. MDAP (and not *cis*Pt-MDAP) was found in the supernatant approximately in the quantity expected from the ratio of intensity of the band (d) over the sum of the intensities of the three bands (a, b, d) present in the autoradiogram (Fig. 2A left).

The oligonucleotides in the precipitate were separated by gel electrophoresis as in Fig. 2. After elution from the band (d) (the slowest migrating band), the oligonucleotide was studied by means of chemical probes (dimethylsulfate, osmium tetroxide,

Table 1. Half-lives ($t_{1/2}$) in hours (precision 10%) of dG-*cis*Pt-MDAP adducts within ds oligonucleotides

	$t_{1/2}$ (hr)
d(AG*TC/GACT)	55
d(AG*CT/AGCT)	15
d(TG*CT/AGCA)	35
d(CG*AT/ATCG)	15
d(TG*TC/GACA)	24
d(TG*TG/CACA)	14
d(CG*CT/AGCG)	24

Solvent 50 mM $NaClO_4$, 5 mM Tris−HCl pH 7.5. Temperature 37°C.

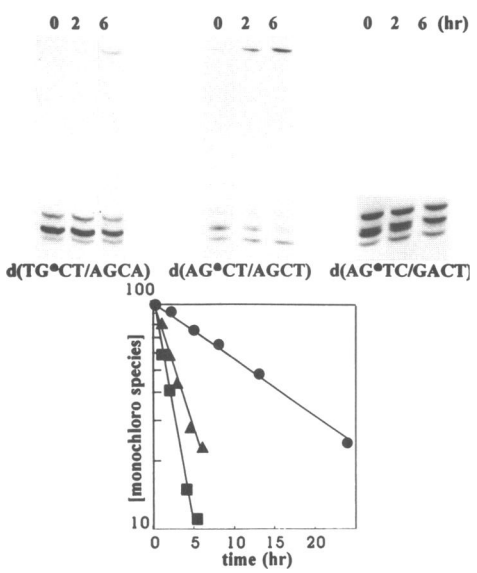

Figure 3. Reaction of closure of *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adducts to bifunctional cross-links within ds oligonucleotides. Top: autoradiogram of a denaturing 24% polyacrylamide gel of platinated d(TG*CT/AGCA), d(AG*CT/AGCT) and d(AG*TC/GACT) (the symbol * indicates the location of the adducts) incubated at 37°C and in 60 mM $NaClO_4$ for various times. Bottom: percentage of the initial monochloro species concentration (logarithmic scale) versus time, (■) d(AG*TC/GACT), (▲) d(AG*CT/AGCT) and (●) d(TG*CT/AGCA).

hydroxylamine) and HPLC after hydrolysis of the oligonucleotide by T4 DNA polymerase. The results confirm the presence of the interstrand cross-link at the d(GC/GC) site (19,21).

High salt conditions. In 1 M $NaClO_4$, the adducts could be considered as stable. Even after 24 hours of incubation, only one new band of weak intensity is detected (Fig. 2A, right). This band migrates as the unplatinated d(TGCT).

In contrast the adduct was unstable in 1 M NaCl. As a function of the incubation time, the intensity of the band (a) corresponding to d(TG*CT) decreases whereas two new bands (b and c) appear (Fig. 2A, right). The more rapidly migrating band (b), of weak intensity, corresponds to unplatinated d(TGCT). The band (c) of intermediate mobility corresponds to d(TGCT) containing *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct as deduced from the following experiment.

A large quantity of d(TG*CT/AGCA) (50 μg) was incubated in 1 M NaCl during 24 hours and at 37°C. After precipitation of the oligonucleotides with ethanol, the supernatant was analyzed by HPLC. MDAP and to a smaller extent *cis*Pt-MDAP were

Table 2. Half-lives ($t_{1/2}$) in hours (precision 10%) of *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adducts within ds oligonucleotides

	$t_{1/2}$ (hr)
d(AG*TC/GACT)	1.6
d(AG*CT/AGCT)	2.8
d(TG*TG/CACA)	>20
d(CG*CT/AGCG)	6
d(CG*AT/ATCG)	8
d(TG*CT/AGCA)	12

Solvent 60 mM $NaClO_4$, 5 mM Tris−HCl pH 7.5. Temperature 37°C.

detected. The oligonucleotides were separated by gel electrophoresis as in Fig. 2. Three experiments were carried out on the oligonucleotide eluted from the band (c). (1) The extent of platinum determined by atomic absorption spectrophotometry was 1 platinum per oligonucleotide. (2) the oligonucleotide was reacted with dimethylsulfate and then treated with piperidine. No cleavage of the chain was detected which suggests that the N7 position of the G residue is protected (19). (3) the oligonucleotide was incubated in 0.2 M NaCN (basic pH) during 12 hours at 37°C and then precipitated. No MDAP was detected in the supernatant by HPLC analysis. On the other hand the precipitated oligonucleotide migrated as d(TGCT).

In 1 M NaCl, dG-*cis*Pt-MDAP adduct within ss d(TG·CT) is stable (Fig. 2A, right). It was also verified by HPLC that in 1 M NaCl no degradation of the modified nucleoside, *cis*-$[Pt(NH_3)_2$(d-guanosine)(MDAP)$]^{3+}$, occurred after 24 h of incubation at 37°C.

d(AG*TC/GACT)

Low salt conditions. We consider first the results relative to d(AG*TC/GACT) incubated in 50 mM NaCl or $NaClO_4$ (Fig. 2B left). At time t = 0 of incubation, only one band (a) is present and it migrates more slowly than the band (b) corresponding to d(AGTC). Again the adduct interferes with the electrophoretic mobility of the oligonucleotide by its mass and its three positive charges. After longer incubation times, the intensity of the band (a) decreases with the concommitant appearance of two new bands (b and e). The band (b) migrates as d(AGTC) which suggests the loss of *cis*Pt-MDAP from d(AG*TC/GACT). The band (e) migrates slighty more slowly than the band (b) which suggests that the oligonucleotide contains an intrastrand cross-link at the d(AG) site (the platinum moiety carries 2 positive charges). In addition, it was found that d(AGTC) containing the d(AG) 1,2 intrastrand cross-link formed in the reaction between d(AGTC) and *cis*-DDP (23) migrated at the level of the band (e).

A large quantity (50 μg) of d(AG*TC/GACT) was mixed with a plasmid DNA (200 μg) and incubated in 50 mM $NaClO_4$ during 24 hours and at 37°C. Experiments similar to those described in the case of d(TG*CT/AGCA) were done. The results confirm the formation of an intrastrand cross-link at the d(AG) site and the release of MDAP and *cis*Pt-MDAP from d(AG*TC/GACT).

High salt conditions. In 1 M $NaClO_4$ the adduct can be considered as stable. Even after 24 hours of incubation, only one new band of very weak intensity is detected. This band corresponds to unplatinated d(AGTC).

In contrast, the adduct is unstable in 1 M NaCl (Fig. 2B right). As a function of time, the intensity of the band (a) decreases

whereas three new bands (b, c and e) appear. The most rapidly migrating band (b) corresponds to the unplatinated d(AGTC).

The band (e) migrates as d(AGTC) containing a d(AG) 1,2 intrastrand cross-link. Even 1 M NaCl does not prevent completely the cross-linking reaction to occur at this site. On the other hand, experiments similar to those described in the case of d(TG*CT/AGCA) were carried out with the oligonucleotide eluted from the band (c). All the results suggested that the oligonucleotide contained *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct.

d(TG*TC/GACA), d(AG*CT/AGCT), d(CG*CT/AGCG), d(CG*AT/ATCG) and d(TGTG*/CACA)

The instability of dG-cisPt-MDAP adducts within d(TG*TC/GACA), d(AG*CT/AGCT), d(CG*AT/ATCG), d(CG*CT/AGCA) and d(TGTG*/CACA) has been also studied and the results can be summarized as follows.

In 50 mM NaCl or $NaClO_4$ the adducts were unstable. MDAP and *cis*Pt-MDAP were released (*cis*Pt-MDAP was released more than MDAP) and subsequently two additional oligonucleotides were detected 1) the unplatinated oligonucleotide 2) the oligonucleotide containing either *cis*-$[Pt(NH_3)_2(dG)H_2O]^{2+}$adduct within d(TG*TC/GACA) or a bifunctional cross-link within the other oligonucleotides. The bifunctional cross-link was either an intrastrand cross-link located at the d(AG) site within d(AGTC/GACT), at the d(GA) site within d(CGAT/ATCG), at the d(GTG) site within d(TGTG/CACA) or an interstrand cross-link located at the d(GC/GC) site within d(TGCT/AGCA), d(CGCT/AGCG) and d(AGCT/AGCT). The half-lives of dG-*cis*Pt-MDAP adducts deduced from the plots of the dG-*cis*Pt-MDAP concentration (logarithmic scale) as a function of time (not shown) are given in Table 1. They are of the same order of magnitude.

In 1 M $NaClO_4$, dG-*cis*Pt-MDAP adducts could be considered as stable while in 1 M NaCl they were unstable. MDAP and *cis*Pt-MDAP were released. The main products of the reaction were oligonucleotides containing *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct.

At low and high salt concentrations, dG-*cis*Pt-MDAP adducts within all the ss oligonucleotides were stable. In the same conditions, the modified nucleoside cis-$Pt(NH_3)_2$(d-guanosine)$(MDAP)]^{3+}$ was also stable.

Conversion of monofunctional *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adducts to bifunctional cross-links

In order to generate *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adducts, the ds oligonucleotides containing a single dG-*cis*Pt-MDAP adduct were incubated during 24 hours in 1 M NaCl and at 37°C. Depending on the samples, about 50−80% of the MDAP residues were replaced by chloride. Then the oligonucleotides were incubated in 60 mM $NaClO_4$ and at 37°C. At various times, aliquots were withdrawn and analyzed by gel electrophoresis under denaturing conditions which allows to follow the conversion of monofunctional *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adducts to bifunctional cross-links. All the samples behave similarly and only the results relative to three oligonucleotides are shown in Fig. 3.

We consider first *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct within d(TG*CT/AGCA) (the symbol * indicates the location of the adduct). At time t = 0 of incubation, three bands are present (Fig. 3 top). The band of intermediate mobility is the most intense and corresponds to the oligonucleotide containing *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct. As a function of incubation time, the intensity of this band decreases whereas a new band of much slower mobility appears. This band corresponds to the oligonucleotide containing an interstrand cross-link. Even after 24 hours of incubation, no other band was detected.

Within d(AG*CT/AGCT), the monofunctional adduct reacts with the G of the opposite strand and forms an interstrand cross-link. No reaction between the adduct and the adjacent A on the same strand has been detected. Within d(AG*TC/GACT), the adduct cross-links the adjacent 5′ A residue on the same strand.

Within d(TG*TC/GACA), the adduct was stable and no intra or interstrand cross-link were detected. Within d(TGTG*/CACA) or d(TG*TG/CACA), d(GTG) 1,3 intrastrand cross-link was formed while within d(CG*CT/AGCG) an interstrand cross-link was formed (not shown).

For all the platinated oligonucleotides but d(TG*TC/GACA) the plots of the percentage of the monochloro species concentration (logarithmic scale) versus time were roughly linear. Three plots are shown in Fig. 3 (bottom). The half-lives of the monofunctional *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adducts are given in Table 2.

DISCUSSION

The first part of the study shows that the DNA conformation interferes with the stability of the monofunctional dG-*cis*Pt-MDAP adducts.

At low and high NaCl concentration, the adducts within ss oligonucleotides are stable at 37°C. This is not due to some interactions between the adducts and the neighboring nucleotide residues since even in 1 M NaCl the modified nucleoside, *cis*$[Pt(NH_3)_2$(d-guanosine)$(MDAP)]^{3+}$ is stable.

In contrast, in 50 mM NaCl or $NaClO_4$, dG-*cis*Pt-MDAP adducts within several ds oligonucleotides are unstable. Two independent reactions occur according to an intramolecular process (no effect of the platinated oligonucleotide concentration), releasing respectively *cis*Pt-MDAP and to a less extent MDAP. The similarity of the results in both salts excludes a displacement of the adducts by chloride ions. The half-lives of the adducts are comprised in the 15−50 hours range and thus, in the first approximation, are considered as independent of the nature of the bases surrounding the adducts. We tentatively exclude a key role played by a given base among those surrounding the adducts, in the cleavage of the bond between MDAP and Pt. We propose that the formation of the bifunctional adducts results from a two-step reaction. The first step is the cleavage of the Pt-MDAP bond which generates an aquated intermediate *cis*-$[Pt(NH_3)_2(dG)H_2O]^+$. Such an intermediate has been observed in the case of d(TG*TC/GACA). With the other oligonucleotides, the aquated intermediate reacts further and forms either intrastrand cross-links within d(AGTC/GACT), d(CGAT/ATCG), d(TGTG/CACA) or interstrand cross-links within d(TGCT/AGCA), d(CGCT/AGCG) and d(AGCT/AGCT).

The conclusion of this part of the work is that due to the instability of dG-*cis*Pt-MDAP adducts within ds DNA, intra- or interstrand cross-links are formed as in the reaction between DNA and *cis*-DDP. We find (unpublished results) that this conclusion holds for several other adducts in which MDAP is replaced by an heterocyclic amine. This might explain the antitumor activity of some platinum−triamine cations (11−12). These results might be also useful to design new platinum−triamine cations interacting specifically with given DNA sequences and subsequently forming bifunctionnal *cis*-DDP adducts within these sequences.

The second part of the present work deals with experiments in high salt concentration. Within ds oligonucleotides, dG-*cis*Pt-MDAP adducts are unstable in 1 M NaCl but stable in 1 M $NaClO_4$. Assuming that in first approximation, the conformation of the double-helices are the same in both salts, these results suggest that the main role of the double-helix is to block the adducts in a favorable orientation for the attack by chloride ions. (One can compare this reaction with the attack of *cis*-DDP-modified DNA at d(GpG) and d(ApG) sites by cyanide ions (24). The bound platinum was removed by cyanide ions much more rapidly within ds DNA than within ss DNA.) In 1 M NaCl, the main reaction generates *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct. Subsequent incubation in 60 mM $NaClO_4$ leads to conversion of the monofunctional adducts to bifunctional cross-links. The second part of our study deals with this conversion.

Within d(AGTC/GACT), the half-life ($t_{1/2}$) of *cis*-$[Pt(NH_3)_2(dG)Cl]^+$ adduct is about 1.5 hr which is in good agreement with recent results (9−10). As expected, the adduct reacts with the flanking 5′ A residue and forms the d(AG) 1,2 intrastrand cross-link.

Within d(CGAT/ATCG), $t_{1/2}$ of the adduct is about 8 hr and the closure of the adduct results in d(GA) 1,2 intrastrand cross-link. Although d(GA) 1,2 intrastrand cross-link is formed slower than d(AG) 1,2 intrastrand cross-link, this does not explain why after reaction between naked DNA and *cis*-DDP, one finds mainly the latter cross-link (5-6). It is likely that *cis*-DDP has more affinity for d(AG) sites than for d(GA) sites during the first step of the reaction (25).

Within d(TGTG/CACA), $t_{1/2}$ of the adduct is longer than 20 hr and closure of the adducts results in d(GTG) 1,3 intrastrand cross-link. The relative large stability of the monofunctional adduct suggests that in the reaction between *cis*-DDP and DNA, formation of d(GTG) 1,3 intrastrand cross-link is unlikely, in agreement with recent results (26).

Within d(TGCT/AGCA) and d(AGCT/AGCT), $t_{1/2}$ of the adduct are about 12 and 2.8 hr respectively and closure of the adducts results in interstrand cross-links at d(GC/GC) sites. Work is in progress to explain this difference in the life-times.

A striking result is that within d(AGCT/AGCT) the monofunctional adduct reacts mainly with the G residue on the opposite strand. No reaction was detected with the flanking A residue on the 5′ side of the adduct. This result confirms previous data showing that in *cis*-DDP modified DNA, intrastrand cross-links at the d(AG) sites within the sequences d(YGAGC)(Y is pyrimidine) are not formed (27).

To conclude, it seems worth noting the potential use of the stability of dG-*cis*Pt-MDAP adduct within ss DNA versus the instability of this adduct within ds DNA in the context of the antisense strategy. Numerous studies have demonstrated that antisense oligonucleotides can inhibit gene expression specifically (28−32). However, the binding of the oligonucleotides to their target sequences is reversible and therefore, it is difficult to achieve 100% inhibition. In order to make the process irreversible, chemically and photoactivatable reagents have been attached to the oligonucleotides. In the case of chemically-induced cross-links, non-sequence-specific reactions have often been observed. In the *in vivo* experiments, photochemical activation is not easy. The advantage of the oligonucleotides containing a dG-*cis*Pt-MDAP adduct is that reaction 2 (Fig. 1) and subsequently the cross-linking reaction occur only when the modified oligonucleotide binds to its target. The rate of reaction 2 is too slow to be useful in the antisense strategy. However, preliminary results show that the rate and the yield of reaction 2 can be improved by modifying the chemical nature of the non-leaving groups of *cis*-DDP and of the heterocyclic amine.

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