Apoptosis Induction and DNA Interstrand Cross-Link Formation by Cytotoxic *trans*-[$PtCl_2(NH(CH_3)_2)(NHCH(CH_3)_2)$]: Cross-Linking between d(G) and Complementary d(C) within Oligonucleotide Duplexes**

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In memory of Marc Leng

We have investigated the cytotoxic activity, the induction of apoptosis, and the interstrand cross-linking efficiency in the A2780cisR ovarian tumor cell line, after replacement of the two NH_3 nonleaving groups in trans-*[$PtCl_2(NH_3)_2$] (*trans-*DDP) by dimethylamine and isopropylamine. The data show that* trans-*[$PtCl_2(NH(CH_3)_2)(NHCH(CH_3)_2)$] is able to circumvent resistance to* cis-*[$PtCl_2(NH_3)_2$] (*cis-*DDP, cisplatin) in A2780cisR cells. In fact,* trans-*[$PtCl_2(NH(CH_3)_2)(NHCH(CH_3)_2)$] shows a cytotoxic potency higher than that of* cis-*DDP and* trans-*DDP, with the mean IC_{50} values being 11, 58, and 300 μM, respectively. In addition, at equitoxic doses (concentrations of the platinum drugs equal to their IC_{50} values) and after 24 hours of drug treatment, the level of induction of apoptosis by* trans-*[$PtCl_2(NH(CH_3)_2)(NHCH(CH_3)_2)$] is twice that produced by* cis-*DDP. Under the same experimental conditions,* trans-*DDP does not induce significant levels of apoptosis in A2780cisR cells. After 24 hours of incubation of A2780cisR cells at concentrations equal to the IC_{50} value of the platinum drugs, the level of DNA interstrand cross-links (ICLs) induced by* trans-*[$PtCl_2(NH(CH_3)_2)(NHCH(CH_3)_2)$] is two and three times higher, respectively, than those induced by* cis-*DDP and* trans-*DDP. We also found that* trans-*[$PtCl_2(NH(CH_3)_2)(NHCH(CH_3)_2)$] formed DNA ICLs between guanine and complementary cytosine. We propose that, in A2780cisR cells, the induction of apoptosis by* trans-*[$PtCl_2(NH(CH_3)_2)(NHCH(CH_3)_2)$] is related to its greater ability (relative to* cis-*DDP and* trans-*DDP) to form DNA ICLs.*

KEYWORDS:

apoptosis · bioinorganic chemistry · DNA damage · platinum drugs

Introduction

cis-Diamminedichloroplatinum(II) (*cis*-DDP, cisplatin) is an antitumor drug widely used for the treatment of many malignancies, including testicular, ovarian, bladder, cervical, head and neck, and small-cell and non-small-cell lung cancers. The *trans* isomer of *cis*-DDP, *trans*-diamminedichloroplatinum(II) (*trans*-DDP), is devoid of antitumor activity.[1] Thus, it is generally accepted that a *cis* configuration of the two leaving groups is the main prerequisite for Pt^{II} antitumor complexes. However, it has been reported that several classes of platinum complexes with *trans* configurations have antitumor activity and that some of them are able to circumvent resistance to *cis*-DDP.[2] In view of these findings, we recently synthesized new *trans*-$PtCl_2$ complexes, each with an asymmetric set of aliphatic amines as nonleaving groups.[3] The compounds were characterized by ^1H, ^{13}C, and ^{195}Pt NMR spectroscopy, elemental analyses, mass spectroscopy, and X-ray diffraction analyses. The cytotoxic activity was assayed. The results showed that the compound *trans*-[$PtCl_2(NH(CH_3)_2)(NHCH(CH_3)_2)$] (tdmaipa; Scheme 1) has higher cytotoxic activity than *cis*-DDP in cell lines (Jurkat, HeLa, and Vero) sensitive to this drug. In addition, this novel *trans*-$PtCl_2$ compound circumvents *cis*-DDP resistance in tumor cells that

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[**] *A list of abbreviations can be found at the end of the Experimental Section.*

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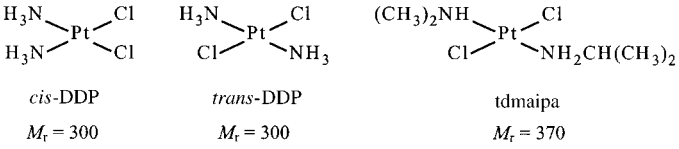
d(TGTC): 5'TC-CTC- TCC-**TGT**- **C**TC- TTC- TCT
d(ACAG): AG-GAG-AGG-**ACA**-**G**AG-AAG-AGA5'

d(TGCT): 5'TC-CTC- TCC- **TGC**-**T**TC- CTC- TCT
d(ACGA): AG-GAG-AGG-**ACG**-**A**AG-GAG-AGA5'



Scheme 1. *Top: Sequences of the oligonucleotides used in this study. Bottom: Structures of* cis-*DDP,* trans-*DDP, and* trans-*[$PtCl_2(NH(CH_3)_2)(NHCH(CH_3)_2)$] (tdmaipa).*

overexpress *ras* oncogenes (HL-60 and Pam 212-*ras*). Moreover, tdmaipa kills Pam 212-*ras* cells that are resistant to *cis*-DDP through apoptosis induction.[4]

Numerous studies suggest than the cytotoxic action of *cis*-DDP is related to its ability to react with cellular DNA.[5, 6] *trans*-DDP also binds to DNA, although it is clinically ineffective. Both isomers bind to DNA in a two-step process, initially forming monofunctional adducts that may subsequently evolve into bifunctional lesions.[7] However, while the DNA interstrand cross-links (ICLs) of *cis*-DDP are preferentially formed between guanine residues at d(GC/GC) sites, *trans*-DDP preferentially forms lesions between complementary guanine and cytosine residues.[8, 9] Thus, the natures and the kinetics of formation of the bifunctional lesions produced by each derivative are thought to be related to their differential biological activities.[10, 11] Although it is not yet firmly established that DNA is the biological target of the novel *trans*-platinum complexes, their antitumor activity might originate from interaction with DNA.[2] It is therefore important to identify and quantify the adducts arising from the binding of these *trans*-$PtCl_2$ compounds to DNA and to compare them with those produced with *trans*-DDP. We have previously reported that *trans*-[$PtCl_2(NH(CH_3)_2)(NHCH(CH_3)_2)$] (tdmaipa) has an enhanced capacity—relative to that of *cis*-DDP—for DNA interstrand cross-linking in linear pBR322 plasmid DNA.[12] In this paper we report the induction of apoptosis and the formation of DNA ICLs by tdmaipa in A2780cisR cells. We also report that tdmaipa preferentially forms DNA ICLs between complementary d(G) and d(C) residues within oligonucleotides.

Results

Cytotoxic activity of the platinum drugs in A2780cisR cells

We tested the cytotoxic activity of tdmaipa, *cis*-DDP, and *trans*-DDP against A2780cisR cells over 24 hours of drug treatment. Table 1 shows that the cytotoxic activity of tdmaipa was 2.6 times higher than that of *cis*-DDP and more than 13.6 times higher than that of *trans*-DDP (IC_{50} values of 22, 58, and 300 μM, respectively). Interestingly, after 24 hours of treatment at concentrations of the platinum drugs equal to their IC_{50} values, a greater degree of cell detachment from the surface of the culture plates was shown by A2780cisR cells treated with tdmaipa than in the other treatments, as found by phase-contrast microscopy (data not shown). Cell detachment has previously been reported as an indication of apoptosis induction.[4, 13] Both detached and attached cells were mixed and assayed by a flow cytometric annexin V binding assay.[14] Annexin V binds phosphatidyl serine residues, which are asymmetrically distributed toward the inner plasma membrane but move to the outer plasma membrane during apoptosis. Figure 1 shows that treatment of A2780cisR cells with tdmaipa induced a greater increase in the annexin V positive/propidium iodide (PI) negative cell population (bottom right quadrant) than treatment with *cis*-DDP or *trans*-DDP did (Figure 1 B, C, and D, respectively). The annexin V positive/PI negative cell population constitutes the fraction of apoptotic cells.[14] Table 1 shows the percentage of apoptotic cells induced by the platinum compounds in A2780cisR cells as calculated from the scattergrams of Figure 1. It can be seen that tdmaipa induced apoptosis in 35% of the cells. Moreover, the percentage of apoptotic A2780cisR cells induced tdmaipa was approximately twice that induced by *cis*-DDP (15%). Interestingly, treatment of A2780cisR cells with *trans*-DDP only induced 1% of apoptotic cells.

Table 1. *IC_{50} mean values and percentage mean values of apoptotic cells obtained for* tdmaipa, cis-*DDP, and* trans-*DDP against A2780cisR cell lines for a drug treatment period of 24 hours.*

	IC_{50} value [μM]	% Apoptotic cells
tdmaipa	22 ± 3	35 ± 2
cis-DDP	58 ± 4	15 ± 1
trans-DDP	300 ± 12	1 ± 0.2

Formation of DNA ICLs in A2780cisR cells

We had previously reported that tdmaipa induces a greater quantity of ICLs in linear pBR322 plasmid DNA than *cis*-DDP does.[12] The next step was to analyze whether tdmaipa also produces a greater quantity of DNA ICLs than *cis*-DDP in culture cells. Thus, A2780cisR cells were exposed to equitoxic doses (concentrations of the platinum drugs equal to their IC_{50} values) of tdmaipa, *cis*-DDP, and *trans*-DDP for a drug treatment period of 24 hours, and the cross-link indexes (see Experimental Section) of these drugs were determined after various incubation periods. Figure 2 shows that tdmaipa, *cis*-DDP, and *trans*-DDP produced increasing levels of ICLs with increasing duration of drug treatment. Moreover, tdmaipa induced a quantity of ICLs higher than those produced either by *cis*-DDP or by *trans*-DDP at all the periods of incubation tested. Interestingly, after 24 hours of drug treatment the cross-link index of tdmaipa was two and three times as high, respectively, as those of *cis*-DDP and *trans*-DDP.

ICL formation in model oligonucleotide duplexes

Further investigations were aimed at studying ICL formation induced by tdmaipa in double-stranded oligonucleotides. Experiments were performed on double-stranded oligonucleotides with the central sequences d(TGTC) and d(TGCT), with each

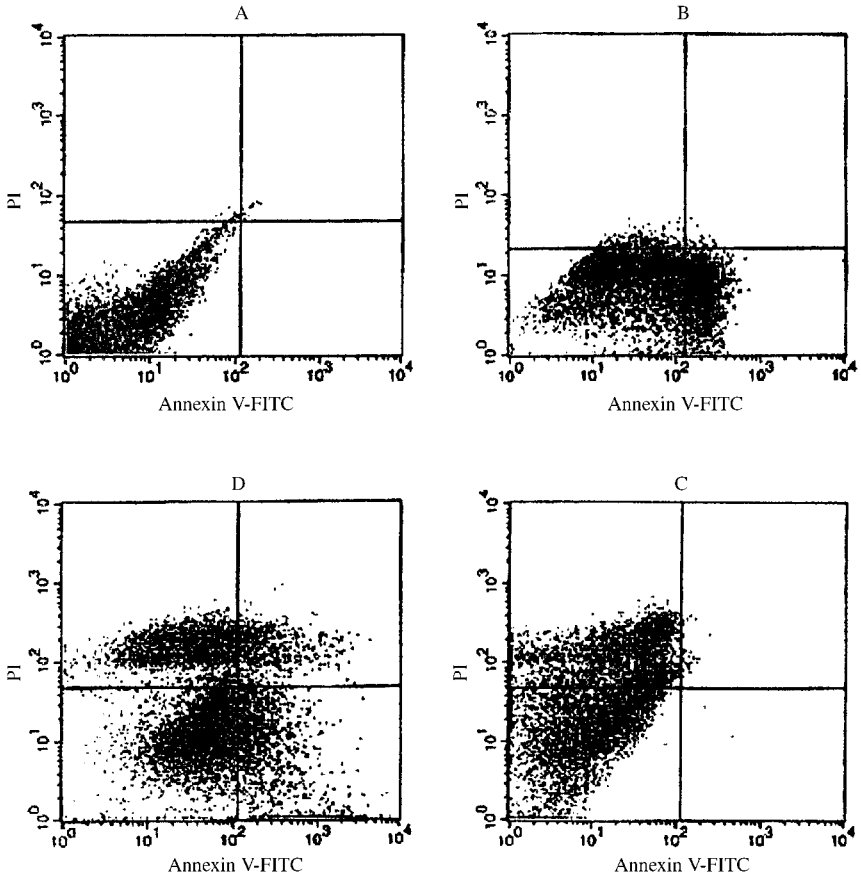


Figure 1. *Quantification of apoptosis after 24 hours exposure to concentrations equal to the IC_{50} values of the platinum drugs. Representative annexin V/PI fluorescence scattergrams showing A2780cisR cells: A) Control, B) tdmaipa treatment, C)* cis-*DDP treatment, and D)* trans-*DDP treatment.*

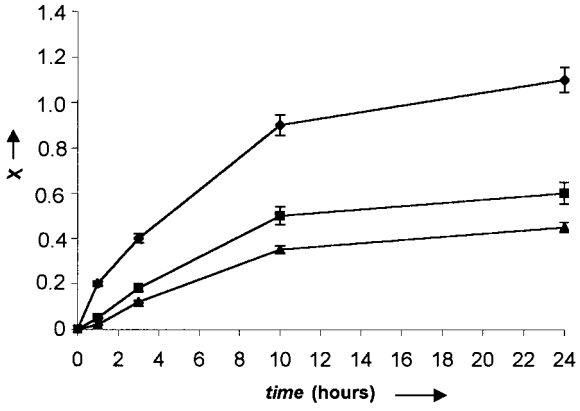


Figure 2. *Alkaline elution plots showing the time course for formation of DNA ICLs in A2780cisR cells by equitoxic doses of tdmaipa (◆),* cis-*DDP (■), and* trans-*DDP (▲). X = ICL index.*

containing a single *trans*-$[Pt(NH(CH_3)_2)(NH_2CH(CH_3)_2)(d(G))Cl]^+$ monofunctional adduct (see Scheme 1 for the complete sequence of oligonucleotides). For the sake of clarity, we will present only results with oligonucleotides of the central sequence d(TGCT), since data obtained with oligonucleotides containing the central sequence d(TGTC) are similar. The single-stranded d(TGCT) oligonucleotide containing a *trans*-$[Pt(NH(CH_3)_2)(NH_2CH(CH_3)_2)(d(G))Cl]^+$ monofunctional adduct was first ^{32}P labeled at the 5′ end and then mixed with its complementary strand. The duplexes were incubated at 37 °C in an aqueous solution of $NaClO_4$ (100 mM), phosphate buffer (pH 7.5; 3 mM), and ethylenediaminetetraacetate (EDTA; 0.5 mM). Aliquots were withdrawn at various time intervals and analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (24% polyacrylamide/7 M urea). The results are shown in Figure 3 (lanes 1 – 5). At time 0 (lane 2), a single band migrating slightly more slowly than the control single-stranded nonplatinated oligonucleotide band (lane 1) was present. The band of lane 2 corresponds to the single-stranded oligonucleotide containing a monofunctional tdmaipa adduct, as later confirmed by treatment with thiourea.[15] The band corresponding to the initial product disappeared as a function of incubation time (at 37 °C), to yield three new, slowly migrating bands (lanes 3 – 5). The presence of three bands of lower mobility indicates that at least three types of ICLs had been formed.[15–17] As shown in Figure 3 (top), we can observe that one of these bands has a much higher intensity than the other two, which suggests that the cross-linking reaction yields mainly one type of ICL (80%).

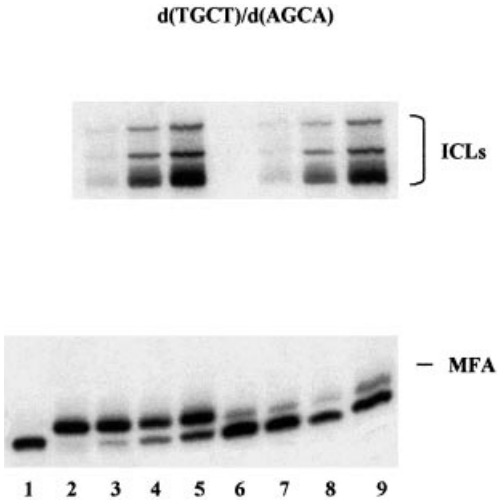


Figure 3. *ICL formation in the duplex formed by mixing the single-stranded pyrimidine-rich oligonucleotide, of central sequence d(TGCT) and containing the monofunctional adduct* trans-*$[Pt(NH(CH_3)_2(NHCH(CH_3)_2)(dG)Cl]^+$, with the single-stranded oligonucleotide of central sequence d(AGCA). Autoradiogram of a 24% polyacrylamide gel/7 M urea denaturing gel. Lane 1: control nonplatinated single-stranded oligonucleotide. Lanes 2 – 5 refer to incubation times of 0, 24, 48, and 72 h, respectively, at 37 °C and in 100 mM $NaClO_4$/3 mM phosphate buffer (pH 7.5) with 0.5 mM EDTA. Lanes 6 – 9 refer to treatment of the products with thiourea for 30 min at 37 °C after 0, 24, 48, and 72 h of incubation, respectively. MFA = monofunctional adduct.*

Removal of *trans*-Pt monofunctional adducts from duplexes with thiourea

Single-stranded oligonucleotides of central sequence d(TGCT), labeled at the 5′ end and each containing a single *trans*-$[Pt(NH(CH_3)_2)(NH_2CH(CH_3)_2)(d(G))Cl]^+$ monofunctional adduct, were mixed with their complementary strands as before. At various times, aliquots were withdrawn, treated with thiourea (10 mM) for 30 min at 37 °C, and analyzed by PAGE under denaturing conditions. As expected, the monofunctional adducts were removed by treatment with thiourea.[15] The band corresponding to the starting oligonucleotide modified with a monofunctional adduct disappeared, to yield a band that comigrated with the nonplatinated control strand (Figure 3, lanes 6 – 9). This result strongly suggests that, under our experimental conditions, the cross-linking reaction yields ICLs but no intrastrand cross-links.[15] The same results were obtained with a duplex oligonucleotide of central sequence d(TGTC)/d(GACA) (data not shown).

Kinetics of ICL formation within oligonucleotide duplexes

Figure 4 shows the percentage of ICL adducts versus reaction time for a duplex oligonucleotide of central sequence d(TGCT)/d(AGCA). The percentage of interstrand cross-linked duplexes was calculated from the ratio between the intensities of the bands corresponding to the fragments containing ICLs (upper bands in Figure 3) and the sum of the intensities of the bands corresponding to the fragments which were not cross-linked (lower bands in Figure 3) and the bands corresponding to the fragments containing ICLs (upper bands in Figure 3). The rate of appearance of the ICL adducts was deduced from the linear plots of the logarithm of the ICL percentages (calculated as above) versus reaction time (data not shown). The half-reaction time ($t_{1/2}$) of the interstrand cross-linking reaction was 48 hours. (The same value was also obtained for the other central sequence.)

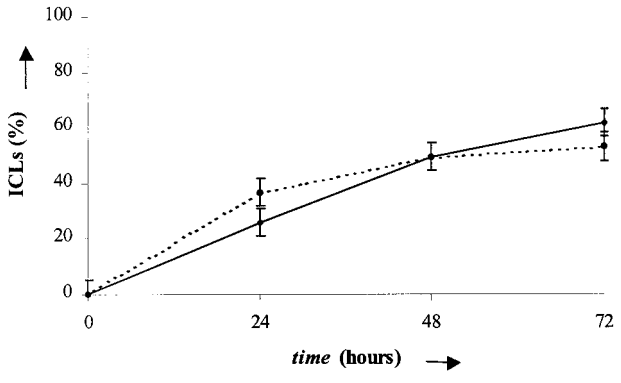


Figure 4. *Plots showing the kinetics of ICL formation in d(TGCT) (- - - -) and d(TGTC) (——) duplexes. The percentage of interstrand cross-linked duplexes (% ICLs) was calculated from the ratio of the intensities of the bands corresponding to the fragments containing the ICLs (three upper bands in Figure 3) to the sum of the intensities of the bands corresponding to the fragments which are not cross-linked (two bottom bands in Figure 3) and the bands corresponding to the fragments containing the ICLs (three upper bands in Figure 3).*

Identification of the interstrand cross-linked bases in oligonucleotide duplexes

The nature of the bases involved in the cross-linking reaction was determined by the Maxam – Gilbert sequencing method.[18] Dimethyl sulfate (DMS) would not be expected to react with guanine when the N7 position in this base was platinated, because this atom would no longer be accessible.[8, 18] Samples of the two duplexes containing a single ICL, purified from the gel, were ^{32}P labeled at the 5′ ends and treated with DMS followed by piperidine. Figure 5 shows the piperidine-induced strand cleavage at DMS-modified bases in the pyrimidine-rich and purine-rich strands of the major interstrand cross-linked product

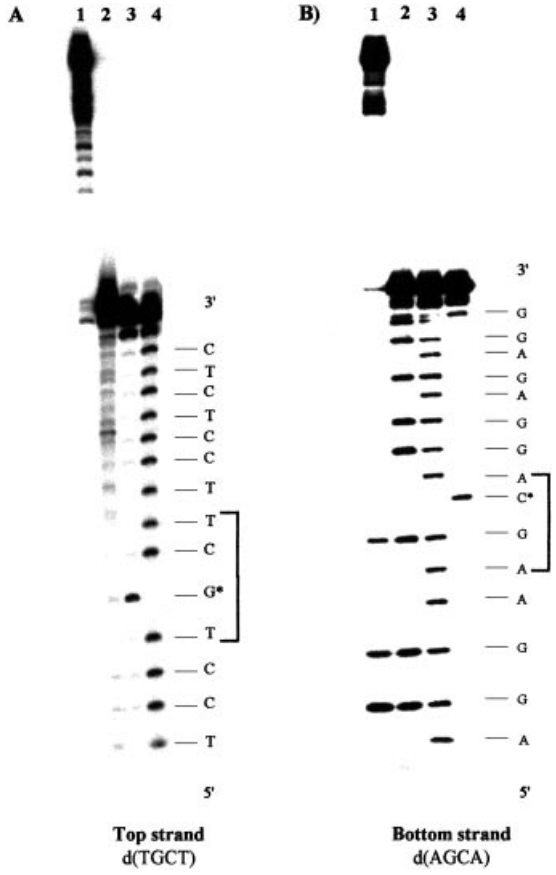


Figure 5. *Autoradiogram of a 24% polyacrylamide gel/7 M urea denaturing gel of the products resulting from piperidine-induced specific strand cleavage at DMS-modified bases in the major gel-purified cross-linked duplex. The cross-linking reaction occurred within duplexes formed by mixing the top strand d(TGCT), which incorporates a single* tdmaipa *moiety at the dG, with an unplatinated d(AGCA) bottom strand. A) Results obtained for the top strand (pyrimidine-rich strand; only this strand was labeled at the 5′ end); lane 1: interstrand cross-linked sample; lane 2: double-stranded oligonucleotide incorporating a monofunctional adduct; lane 3: unplatinated duplex (G reaction); and lane 4: unplatinated duplex treated with hydrazine (C+T reaction). B) Results obtained for the bottom strand (purine-rich strand; only this strand was labeled at the 5′ end); lane 1: interstrand cross-linked sample; lane 2: unplatinated oligonucleotide treated with DMS (G reaction); lane 3: unplatinated oligonucleotide treated with formic acid (G+A reaction); and lane 4: unplatinated oligonucleotide treated with hydrazine (C+T reaction). The base sequences of the top and bottom strands are shown on the right-hand side of each panel. The star designates the d(C) moiety opposite to the platinated d(G) in the top strand.*

obtained from the cross-linking reaction in the monofunctionally modified duplex with the central sequence d(TGCT)/d(AGCA). As can be seen in Figure 5 A, the guanine in the pyrimidine-rich strand is resistant to DMS attack (lanes 1 and 2), which shows that the monofunctional tdmaipa adduct is indeed bound at the N7 position in the single guanine residue in the pyrimidine-rich strand. Figure 5 B shows that, after subsequent treatment of the purine-rich strand with piperidine, all the bands detected correspond to cleavage at d(G) residues on the 5′ side of the d(C*) residue (d(C*) indicates the d(C) residue complementary to the platinated d(G) residue, lanes 1 and 2). In contrast, no bands corresponding to fragments formed by cleavage at d(G) residues on the 3′ side of the d(C*) were detected. The reason for this is that the fragments generated by cleavage at the d(G) residues on the 3′ side of the cross-linked base in the purine-rich strand are still cross-linked to the top strand and, thus, migrate more slowly (Figure 5 B, lane 1). This result strongly suggests that the platinated base residue within the purine-rich strand is d(C). Thus, in duplexes with d(TGCT)/d(ACGA) and d(TGTC)/d(GACA) central sequences, tdmaipa forms ICLs mainly between d(G) and complementary d(C) residues.

Discussion

DNA is generally accepted to be the main pharmacological target of platinum complexes.[5] Moreover, DNA ICLs have often been implicated in the cytotoxicity of Pt^{II} and Pt^{IV} complexes.[9, 19, 20] It is not yet firmly established whether DNA is the biological target of active *trans*-platinum complexes. However, it might be that the cytotoxic activity of some *trans*-platinum complexes in cells resistant to *cis*-DDP is related to their ability to form particular types of DNA adducts. We have previously reported that tdmaipa has an enhanced capacity (relative to that of *cis*-DDP) to form DNA interstrand cross-links in linear pBR322 DNA.[12] The next step was to test whether this enhanced capacity of interstrand cross-link formation by tdmaipa could also be observed in DNA from culture cells. In this paper we show that tdmaipa is able to form more DNA interstrand cross-links than *cis*-DDP and *trans*-DDP in A2780cisR cells resistant to *cis*-DDP. In fact, after 24 hours treatment with the drugs at equitoxic doses, the level of DNA interstrand cross-linking induced by tdmaipa in A2780cisR cells was two and three times higher than those induced by *cis*-DDP and by *trans*-DDP, respectively.

We also studied the interstrand cross-linking formation, as well as the nature of the bases on the opposite strands of DNA cross-linked by tdmaipa, in double-stranded oligonucleotides with d(TGTC)/d(ACAG) and d(TGCT)/d(AGCA) central sequences. The results reported here indicate that tdmaipa may form at least three kinds of ICL, although one of these predominates. Moreover, the kinetics of ICL formation in double-stranded d(TGTC) and d(TGCT) oligonucleotides show that the half-reaction time ($t_{1/2}$) of interstrand cross-linking by tdmaipa is approximately 48 hours. The results reported here, determined by Maxam – Gilbert experiments, indicate a preferential tdmaipa-based cross-linking reaction between complementary G and C residues within double-stranded d(TGTC) and d(TGCT) oligonucleotides. These results are in agreement with previously reported data indicating that the compound shows a binding affinity towards alternating purine – pyrimidine sequences and inhibits the B – Z transition of poly(dG – dC) · poly(dG – dC).[12] Interestingly, the main bifunctional lesions formed by *trans*-DDP in double-stranded oligonucleotides are also ICLs between G and C residues.[8, 21] In contrast, only a small percentage ($< 10\,\%$) of the bifunctional lesions formed by *cis*-DDP in double-stranded oligonucleotides are ICLs.[7] Moreover, the nature of the ICLs produced by *cis*-DDP is different from that of those produced both by tdmaipa and by *trans*-DDP, since the *cis*-DDP interstrand cross-links are formed between two G residues located on opposite DNA strands at d(GC/GC) sites.[9, 22] It has been reported that resistance of cells to *cis*-DDP may be associated with increased gene-specific repair efficiency of interstrand cross-links.[23, 24, 25] On the other hand, A2780cisR cells exhibit acquired resistance to *cis*-DDP through a combination of decreased uptake, enhanced DNA repair, and elevated GSH levels.[26] We propose that the higher efficiency of DNA interstrand cross-linking and the different nature of the DNA ICL lesion produced by tdmaipa in relation to that of *cis*-DDP may be in part responsible for the biological activity of the drug in A2780cisR cells.

Emerging evidence suggests that an important cause of *cis*-DDP resistance may be the inability of this drug to induce apoptosis in particular cell lines.[27, 28] tdmaipa is the first representative of a novel class of cytotoxic *trans*-$PtCl_2$ complexes incorporating mixed aliphatic amines that are capable of circumventing *cis*-DDP resistance in several tumor cell lines through induction of apoptosis.[2] We have shown in this paper that tdmaipa is able to circumvent *cis*-DDP resistance in the A2780cisR tumor cell line. In fact, after treatment of A2780cisR cells with the drugs for 24 hours, tdmaipa displayed an IC_{50} value 2.6 times lower than that of *cis*-DDP and its ability to induce apoptosis was 2.3 times that of *cis*-DDP. Thus, the results suggest that, in A2780cisR cells, there is a relationship between the cytotoxic potency of the platinum drugs and their ability to induce apoptosis. In other words, the lower the dose of drug needed to kill the A2780cisR cells, the higher the percentage of apoptosis induction. It is interesting to note that *trans*-DDP induced a very low percentage of apoptosis in A2780cisR cells at its IC_{50} value. This finding might be related to the biological inefficacy of *trans*-DDP.

It is generally accepted that DNA damage and subsequent induction of apoptosis may be the primary mechanism of cytotoxic activity of platinum antitumor drugs.[28] The results reported in this paper further support previous evidence[2, 3, 12] that indicated that the replacement of the two NH_3 groups in *trans*-DDP by different aliphatic amines enhances the interstrand cross-linking efficiency of the *trans*-$PtCl_2$ center and very probably also its apoptotic properties. Insights into these aspects may facilitate rational design of new *trans*-platinum derivatives possessing therapeutic properties.

Experimental Section

Chemicals and oligonucleotides: *cis*- and *trans*-DDP were purchased from Sigma (Alcobendas, Spain). *trans*-[$PtCl_2(NH(CH_3)_2)$-

$(NHCH(CH_3)_2)]$ was synthesized as previously reported.[3] The oligodeoxyribonucleotides were supplied by Eurogentec (Seraing, Belgium) and were purified by strong anion-exchange chromatography on a Mono Q HR5/5 column as previously described.[21] The sequences of the oligonucleotides are given in Scheme 1. All enzymes were from Promega or Biolabs (Hitchin, UK). The radioactive products were from Amersham Pharmacia Biotech, and other chemicals were from Merck (Nogent-Sur-Marne, France).

Cell culture and platinum drug cytotoxicity: The *cis*-DDP-resistant human ovarian tumor cell line A2780cisR[20, 26] was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) together with glutamine (2 mM), penicillin (100 units mL^{-1}), and streptomycin (100 mg mL^{-1}) at 37 °C in an atmosphere of 95% air and 5% CO_2. Cell death was evaluated with a system based on 3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is reduced by living cells to yield a soluble formazan product that can be assayed colorimetrically.[29] Exponentially growing A2780cisR cells were plated at a density of 10^4 cells per well in 96-well sterile plates in DMEM (100 μL), and were incubated for 3 – 4 h. Stock solutions of the platinum compounds (1 mg mL^{-1}) in DMEM were added to the wells at final concentrations ranging from 0 to 350 μM, in a volume of 100 μL per well. After these had been incubated with the drugs for 24 h, a freshly diluted MTT solution (1:5 in culture medium, 50 μL) was added to each well to produce a final concentration of 1 mg mL^{-1}, and the plate was further incubated for 5 h. Cell survival was evaluated by measurement of the absorbance at 520 nm, with a Whittaker Microplate reader 2001. IC_{50} values were calculated from curves constructed by plotting cell survival (%) versus compound concentration (μM). The IC_{50} value is defined as the drug concentration that kills 50% of cells. All experiments were performed in quadruplicate.

Quantification of apoptosis by annexin V binding and flow cytometry: Exponentially growing A2780cisR cells were exposed to concentrations equal to the IC_{50} value of the platinum drugs for 24 hours. Subsequently, attached and detached cells were recovered, mixed, and resuspended in annexin V binding buffer (PharMingen). Propidium iodide (PI; 2.5 μL; Sigma) and annexin V – fluorescein isothiocyanate (1 μg mL^{-1}; PharMingen) were added, and the cells were left at room temperature prior to flow cytometric analysis in a FACScalibur Beckton – Dickinson apparatus. The percentage of apoptotic cells induced by each platinum drug (percentage of annexin V positive/PI negative cells) was calculated from annexin V/PI scattergrams.[14] Experiments were carried out in quadruplicate.

Kinetics of in vivo DNA ICL formation: DNA ICLs were determined by alkaline filter elution, with the A2780cisR cell line as described previously.[30] The DNA of the cells was labeled by seeding 10^6 cells in P100 tissue culture plates and growing for 24 h in the presence of [^{14}C]thymidine (0.03 μCi mL^{-1}; specific activity: 51 mCi $mmol^{-1}$; Amersham International). A plate of cells for use as an internal standard in the assay was labeled overnight with [methyl-^{3}H]thymidine (0.17 μCi; specific activity: 5 mCi $mmol^{-1}$) plus unlabeled thymidine (10^{-5} M). A2780cisR cells (labeled with ^{14}C) were treated with equitoxic doses (concentrations equal to their IC_{50} values) of tdmaipa, *cis*-DDP, and *trans*-DDP for various periods of time. In addition, an untreated control plate was included in all experiments. Immediately after drug treatment, the drug was washed off with ice-cold phosphate-buffered saline (PBS). Test (^{14}C-labeled) cells and internal standard (^{3}H-labeled) cells were then irradiated on ice with 5 and 1 Gy, respectively, of ^{60}Co γ rays from a 2000 Ci source (dose rate: 2 Gy min^{-1}). Approximately 10^6 cells of a mix (1:1) of test and internal standard cells were then added to duplicate polycarbonate filters (pore size: 2 μm; 25 mm; Millipore Co.) in ice-cold PBS (5 mL). Cells were then lysed by two additions of lysis buffer (2% sodium dodecyl sulfate in 0.1 M glycine and 0.02 M EDTA; pH 10; 10 mL). In the first quantity of buffer added, proteinase K (0.5 mg mL^{-1}; Sigma, Alcobendas, Spain) was added immediately prior to use. DNA was then eluted at pH 12, by use of tetrapropylammonium hydroxide (10 mL, 0.1 M) containing sodium dodecyl sulfate (0.1%) and EDTA (0.02 M). The elution rate was 0.010 mL min^{-1} (Pharmacia Biotech peristaltic pump), and fractions were collected at 90 min intervals over 24 hours. The ^{14}C and ^{3}H DNA radioactivity in each fraction and from the filters was then determined by liquid scintillation counting (Wallac 1209 Rackbeta). Results are expressed as the fraction of ^{14}C retained versus the fraction of ^{3}H (internal standard). DNA ICL units (Dalton$^{-1} \times 10^9$) were calculated by use of the expression:

$$\text{ICL index (X)} = [(B1 - r_o/1 - r)^{1/2} - 1] \times P_b \quad (1)$$

in which r and r_o are the fractions of ^{14}C-labeled DNA for treated versus control cells remaining on the filter when 60% of ^{3}H-labeled DNA is retained on the filter, and P_b is the radiation-induced break probability per Dalton. Control experiments were carried out to test for the presence of single-strand breaks induced by *cis*-DDP.

Platination of the oligonucleotides: Single-stranded pyrimidine-rich oligonucleotides, with central sequences d(TGTC) or d(TGCT) and containing a monofunctional adduct of tdmaipa, were prepared by incubation of the oligonucleotides (concentration in oligonucleotide residues: 30 μM) with tdmaipa with an input platinum/strand molar ratio of 6 in 10 mM $NaClO_4$/5 mM acetate buffer (pH 3.6), over 90 min at 37 °C.[15] The platinated oligonucleotides were purified by strong anion-exchange chromatography on a Mono Q HR5/5 column (flow rate: 1mL min^{-1}) with a linear gradient of 25 to 750 mM NaCl and with 2 and 10 mM NaOH, respectively, for oligonucleotides of central sequences d(TGCT) and d(TGTC). The sample solutions were then neutralized with acetic acid, tris(hydroxymethyl)aminomethane/HCl (Tris-HCl; pH 7.5), and HCl. Both platinated oligonucleotides were desalted on Sep-Pack cartridges (Waters, St Quentin en Yvelines, France), lyophilized, and resuspended in NaCl (100 mM).

Removal of the monofunctional adducts from oligonucleotides with thiourea: Single-stranded oligonucleotides containing a monofunctional tdmaipa adduct were labeled at the 5′ end with [γ-^{32}P]ATP through use of T4 polynucleotide kinase.[31] We then checked that the platinated strands were modified at the guanine residues of the d(TGCT) or the d(TGTC) central sequence by treatment with DMS[8] as described in the legend of Figure 5. As previously reported,[15] thiourea has been used to remove *trans*-diammine platinum(II) monofunctional adducts from DNA under conditions in which bifunctional cross-links remain stable. The period of incubation with thiourea necessary for removal of the *trans*-$[Pt(NH(CH_3)_2)(NH_2CH(CH_3)_2)(d(G))Cl]^+$ monofunctional adduct from the oligonucleotides was determined. Single-stranded oligonucleotides labeled at the 5′ end and containing a single *trans*-$[Pt(NH(CH_3)_2)(NH_2CH(CH_3)_2)(d(G))Cl]^+$ monofunctional adduct were incubated with the respective complementary strand in NaCl (100 mM), phosphate buffer (pH 7.5; 3 mM), EDTA (0.5 mM), and thiourea (10 mM) at 37 °C. Aliquots were withdrawn every five minutes, and analyzed by PAGE under denaturing conditions (24% polyacrylamide/7 M urea; data not shown). This allowed us to determine that the incubation time necessary for removal of all the monofunctional adducts from the platinated strand was 30 min. The band intensities were quantified by use of a Molecular Dynamics PhosphorImager, with ImageQuant software, version 3.3 (Molecular Dynamics, Sunnyvale, CA), for data processing.

Kinetics of ICL formation and nature of the cross-linked bases: Single-stranded oligonucleotides, ^{32}P-labeled at the 5′ end and containing a monofunctional adduct, were incubated with their complementary strands (equimolar ratio of complementary strands) at 37 °C in 100 mM $NaClO_4$ (100 mM), phosphate buffer (pH 7.5; 3 mM), and EDTA (0.5 mM; final duplex concentration = 0.12 mM). Aliquots were withdrawn as a function of time, either treated or not treated with thiourea (10 mM) for 30 min at 37 °C, and analyzed by PAGE under denaturing conditions (24% polyacrylamide/7 M urea). Half-reaction times ($t_{1/2}$) for ICL formation were deduced by quantification of the band intensities as described before. To identify the nature of the bases in the ICLs, the cross-linked oligonucleotides (major band) were purified by gel electrophoresis. The location and nature of the ICL were determined by the chemical reactivity of the cross-linked duplexes with DMS and formic acid[8, 18] after labeling of either the pyrimidine-rich or the purine-rich strand.

Statistical analysis: Where appropriate, statistical significance was tested using a two-tailed Student's test. All values shown are mean values, with the corresponding standard deviation of the mean unless otherwise stated.

Abbreviations:

cis-DDP	*cis*-diamminedichloroplatinum(II)
DMEM	Dulbecco's modified Eagle's Medium
DMS	dimethyl sulfate
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
IC_{50}	drug concentration that induces 50% cell death
ICL	interstrand cross-link
MFA	monofunctional adduct
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PI	propidium iodide
tdmaipa	*trans*-[$PtCl_2(NH(CH_3)_2)(NHCH(CH_3)_2)$]
trans-DDP	*trans*-diamminedichloroplatinum(II)

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