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Replacement of an NH_3 by an Iminoether in Transplatin Makes an Antitumor Drug from an Inactive Compound

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ABSTRACT

To investigate the modifications of antitumor activity and DNA binding mode of transplatin after replacement of one nonleaving group NH_3 by an iminoether group, *trans*-[$PtCl_2${*Z*-HN=C(OMe)Me}(NH_3)] and *trans*-[$PtCl_2${*E*-HN=C(OMe)Me}(NH_3)] complexes (differing in the *Z* or *E* configuration of iminoether, and abbreviated mixed *Z* and mixed *E*, respectively), have been synthesized. In a panel of human tumor cell lines, both mixed *Z* and mixed *E* show a cytotoxic potency higher than that of transplatin, the mean IC_{50} values being 103, 37, and 215 μM, respectively. In vivo mixed *Z* is more active and less toxic than mixed *E* in murine P388 leukemia and retains its efficacy against SK-OV-3 human cancer cell xenograft in nude mice. In the reaction with naked DNA, mixed *Z* forms monofunctional adducts that do not evolve into intrastrand cross-links but close slowly into interstrand cross-links between complementary guanine and cytosine residues. The monofunctional mixed *Z* adducts are removed by thiourea and glutathione. The interstrand cross-links behave as hinge joints, increasing the flexibility of DNA double helix. The mixed *Z*, transplatin, and cisplatin interstrand cross-links, as well as mixed *Z* monofunctional adducts are not specifically recognized by HMG1 protein, which was confirmed to be able to specifically recognize cisplatin d(GpG) intrastrand cross-links. These data demonstrate that the DNA interaction properties of the antitumor-active mixed *Z* are very similar to those of transplatin, thus suggesting that clinical inactivity of transplatin could not depend upon its peculiar DNA binding mode.

Cisplatin, *cis*-diamminedichloroplatinum(II), is one of the most largely used drugs in cancer chemotherapy. It displays significant activity against several types of cancers with an exceptional efficacy against testicular cancers. Despite this success, all the patients treated with cisplatin suffer from severe side effects, and quite often tumors become resistant to cisplatin (O'Dwyer et al.,1999). In order to overcome these drawbacks and to get more efficient drugs, many platinum(II) complexes have been synthesized. Most of them have the *cis* geometry because transplatin, *trans*-diamminedichloroplatinum(II), the stereoisomer of cisplatin, is clinically inefficient. A mechanistic explanation of transplatin inactivity has been based on the type of DNA adducts formed by this isomer, as well as on its chemical reactivity that could render the complex susceptible to deactivation before its delivery to the tumor site (Jamieson and Lippard, 1999, and references therein). However, several recent exceptions to the empiric rule that the presence of two leaving groups in *cis* position of platinum complexes is a necessary condition for their antitumor activity, have been reported. *trans*-Platinum(II) complexes with planar ligands (Farrell, 1996, and references therein), with iminoether (Coluccia et al., 1993; Natile and Coluccia, 1999) or asymmetric aliphatic amine ligands (Montero et al., 1999), and *trans*-ammine(amine)platinum(IV) complexes (Kelland et al., 1995), gave promising results in in vitro and in vivo assays.

Several *trans*-[$PtCl_2$(iminoether)$_2$] complexes differing by the nature of the iminoether groups have been studied, and an overview of their main properties has been recently reported (Natile and Coluccia, 1999). Briefly, they show in vivo antitumor activity toward both lymphoproliferative and solid metastasizing murine tumors. Their cytotoxicity toward several human tumor cell lines is comparable to that of cisplatin.

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† Marc Leng worked to the last of his days on this paper, giving the most important contribution to its realization. Unfortunately, Marc died of cancer in May 2000, but neither our long collaboration nor our friendship are interrupted, because we will always remember the joy of staying together.

ABBREVIATIONS: cisplatin and transplatin, *cis*- and *trans*-diamminedichloroplatinum(II); mixed *E* and mixed *Z*, *trans*-dichloro(ammine)(*E*-iminoether)platinum(II) and *trans*-dichloro(ammine)(*Z*-iminoether)platinum(II); ICL, interstrand cross-link; bp, base pairs; r_i, input molar ratio of drug over nucleotide residues; dsDNA, double-stranded DNA; HMG1, high mobility group 1; %T/C, mean survival time (%) of treated animals versus controls; %TWI, percentage of tumor weight inhibition of treated mice versus controls; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

The cytotoxic effect of *trans*-[$PtCl_2$(iminoether)$_2$] complexes appears to be a consequence of DNA adduct formation, as suggested by the greater sensitivity of cells deficient in nucleotide excision repair system. In the reaction with DNA, they form monofunctional adducts that are very slowly converted into bifunctional adducts.

To go further in the study of iminoether complexes with *trans* geometry, we have investigated some properties of complexes that contain only one iminoether group. These complexes are *trans*-[$PtCl_2(NH_3)$\{*Z*-HN=C(OMe)Me\}] and *trans*-[$PtCl_2(NH_3)$\{*E*-HN=C(OMe)Me\}] (mixed *Z* and mixed *E*, respectively). They differ in the configuration of the iminoether ligands (*Z* or *E*, depending upon the *cis*- or *trans*-position of the alkoxy group and the metal atom with respect to the C=N double bond). In this paper we show that both mixed *Z* and mixed *E* present a cytotoxic potency toward human tumor cells higher than that of transplatin and, in particular mixed *Z*, a promising in vivo antitumor activity. We show that the DNA interaction properties of mixed *Z* are very similar to those of transplatin. This similarity, which contrasts with the markedly different in vivo activity, is discussed in relation to the antitumor properties of mixed *Z*.

Experimental Procedures

Materials

The oligodeoxyribonucleotides from Eurogentec (Seraing, Belgium) were purified by strong anion exchange chromatography on a Mono Q HR5/5 column from Amersham Pharmacia Biotech (Orsay, France) as previously described (Boudvillain et al., 1995). The sequences of the oligonucleotides are given in Fig. 1. Plasmid pSPKB (Lemaire et al., 1991) was a derivative of plasmid pSP73 from Promega (Charbonnieres, France). The enzymes were from Promega or Biolabs (Hitchin, England). The radioactive products were from Amersham Pharmacia Biotech, and the chemicals were from Merck (Nogent-sur-Marne, France).

Synthesis of Mixed *E* and Mixed *Z*

The synthesis of the *trans*-[$PtCl_2(NH_3)$\{HN=C(OMe)Me\}] complex was accomplished starting from *cis*-[$PtI_2(NH_3)_2$], which was first converted to *cis*-[$Pt(NH_3)_2(NCMe)_2](NO_3)_2$, then to *trans*-[$PtI_2(NH_3)$(NCMe)], and finally to *trans*-[$PtCl_2(NH_3)$(NCMe)]. Addition of methanol to the coordinated nitrile in the last complex afforded the desired iminoether species.

Synthesis of *trans*-[$PtCl_2(NH_3)$(NCMe)]

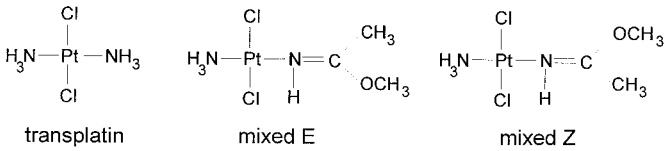
cis-[$PtI_2(NH_3)_2$] (1.4 g, 3 mmol) was suspended in H_2O (30 ml), and the temperature was increased to 70°C. Ten minutes after addition of $AgNO_3$ (1.0 g, 6 mmol), the solution was filtered through celite to remove AgI and then was treated with MeCN (25 ml); the solvent was evaporated under reduced pressure while keeping the reaction vessel at 70°C. The solid residue was dissolved in 50 ml of H_2O/CH_3CN (1:1, v/v), and the solvent was evaporated again. The solid residue of *cis*-[$Pt(NH_3)_2(NCMe)_2](NO_3)_2$ was dissolved in 100 ml of H_2O and treated with KI (1.66 g, 10 mmol), the solution was warmed to 70°C, and the solvent was evaporated under reduced pressure. A second volume of water (100 ml) was added to the solid residue, and the solvent was evaporated again under reduced pressure, always keeping the reaction flask at 70°C. The solid residue, suspended in a small volume of cold water, was transferred on a sintered glass filter, washed carefully with a small portion of cold water, and dried in a stream of dry air. The yield of *trans*-[$PtI_2(NH_3)$(NCMe)] was ca. 65% based on platinum. The iodo species (1 g, ca. 2 mmol) was suspended in acetone (50 ml), and the slurry was warmed to 60°C and treated with $AgNO_3$ (0.65 g, 3.8 mmol) dissolved in a minimum volume of water (ca. 1 ml). After 10 min the solution was filtered, the solvent was evaporated, and the residue was dissolved in water (100 ml). The water solution was treated with KCl (1.2 g, 16 mmol), and the solvent was evaporated under reduced pressure, keeping the reaction vessel in a water bath at 70°C. The solid residue was suspended in cold water, transferred on a glass filter, washed with cold water, and dried in the air. The yield of *trans*-[$PtCl_2(NH_3)$(NCMe)] was ca. 70%. Analysis calculated for $C_2H_6Cl_2Pt$: C, 7.4; H, 1.9; N, 8.6%. Found: C, 7.2; H, 1.8; N, 8.5%.

Synthesis of *trans*-[$PtCl_2(NH_3)$\{*Z*-HN=C(OMe)Me\}]

trans-[$PtCl_2(NH_3)$(NCMe)] (0.43 g, 1.3 mmol) was suspended in methanol (5 ml), cooled to 0°C (ice bath) and treated with powdered KOH (50 mg). After stirring for 30 min, the solution was filtered, the solid residue was washed with cold methanol until complete removal of residual base (neutral pH of the filtered washing solvent) and dried in the air. The yield of *trans*-[$PtCl_2(NH_3)$\{*Z*-HN=C(OMe)Me\}] was ca. 80%. Analysis calculated for $C_3H_{10}N_2OCl_2Pt$: C, 10.1; H, 2.8; N, 7.9. Found: C, 10.2; H, 2.8; N, 7.8%.

Synthesis of *trans*-[$PtCl_2(NH_3)$\{*E*-HN=C(OMe)Me\}]

The *E* isomer was obtained by isomerization of the *Z* isomer. In a typical experiment, *trans*-[$PtCl_2(NH_3)$\{*Z*-HN=C(OMe)Me\}] (0.15 g, 0.42 mmol) was suspended in methanol (60 ml) and treated with 30 mg of powdered KOH. The slurry was left stirring overnight. After



TCCTCTCC**TGT**CTCTTCTCT
AGGAGAGGACAGAGAAGAGA

TCCTCTCC**TGC**TTCCTCTCT
AGGAGAGGACGAAGGAGAGA

20-mer TCCTCTCC**TGT**CTCTTCTCT
GAGAGGACAGAGAAGAGAAG

21-mer CTCCTCTCC**TGT**CTCTTCTCT
GGAGAGGACAGAGAAGAGAGA

22-mer CTCCTCTCC**TGT**CTCTTCTCTT
GGAGAGGACAGAGAAGAGAAGA

18-mer TCCTCTCA**GG**ATCTTCTC
AGGAGAGTCCTAGAAGAG

18-mer TCCTCTCA**GC**ATCTTCTC
AGGAGAGTCGTAGAAGAG

Fig. 1. Formula of platinum(II) complexes with *trans* geometry. Top, transplatin: *trans*-diamminedichloroplatinum(II); mixed *Z*: *trans*-[$PtCl_2$\{*Z*-HN=C(OMe)Me\}(NH_3)]; and mixed *E*: *trans*-[$PtCl_2$\{*E*-HN=C(OMe)Me\}(NH_3)]. Bottom, sequences of the oligonucleotides used in the present work. Central bases in bold characters indicate oligonucleotide abbreviations used throughout the text. The three duplexes, 20-, 21-, and 22-mers, with 5′ complementary overhangs were used to prepare multimers of the unplatinated or platinated duplexes.

neutralization with concentrated HCl, the solvent was evaporated under reduced pressure, and the solid residue was extracted with dichloromethane. The dichloromethane solution was concentrated to a small volume by evaporation of the solvent under reduced pressure and chromatographed on an open column of silica gel using dichloromethane containing increasing amounts of acetone (from 0.5 to 20%, v/v) as eluant. The first eluted product was the desired compound obtained in ca. 50% yield. Analysis found: C, 10.5; H, 2.9; N, 7.7%.

Tumor Cell Lines and In Vitro Cytotoxicity Assay

Tumor cell lines representative of ovarian (SK-OV-3, OVCAR-8), lung (A549/ATCC, NCI-H460), colon (KM12, COLO 205, HCT-116), and breast (T-47D, MCF7, MDA) cancers were obtained from the National Cancer Institute, Biological Testing Branch (Frederick, MD), and maintained in the logarithmic phase at 37°C in a 5% CO_2-humidified air in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 μM glutamine, penicillin, and streptomycin (100 U/ml and 0.1 mg/ml, respectively). A2780 ovarian cancer cells were kindly supplied by Dr. R. Ozols (Fox Chase Cancer Center, Philadelphia, PA) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 μM glutamine, 10 μg/ml insulin, penicillin, and streptomycin (100 U/ml and 0.1 mg/ml, respectively). LOVO colon cancer cells and CALU-3 lung cancer cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in Ham's F12K medium, 10% fetal bovine serum, and minimal essential medium Eagle, 2 mM glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10% fetal bovine serum, respectively. All culture media and reagents were from Euroclone (Paignton, UK).

The growth inhibitory effect of platinum complexes was evaluated by using the cell proliferation kit I (MTT) (Roche Molecular Biochemicals, Milano, Italy), following the supplier's protocol. Briefly, the cells grown in the culture flasks were trypsinized, and 100 μl of medium containing the appropriate cell number (A2780: 50,000; SK-OV-3, T-47D, MDA: 20,000; KM12, COLO, LOVO, CALU: 15,000; OVCAR8: 10,000; A549, H460: 7,500; HCT116, MCF7: 7,500) was inoculated into 96-well microplates. After 24 h from seeding, the cells were incubated with various concentrations of platinum complexes—freshly dissolved in the culture medium—for 72 h at 37°C, followed by an additional 4-h incubation with 10 μl/well tetrazolium salt solution (5 mg/ml). The cells were dissolved in 100 μl of 10% SDS solubilization solution, and the absorbance was measured at 570 nm. The drug concentration that inhibited cell growth by 50% (IC_{50}) was obtained from semilogarithmic dose-response plots.

Assessment of In Vivo Antitumor Activity

Murine P388 Leukemia. P388 leukemia cells were obtained from the National Cancer Institute (Bethesda, MD) and maintained in DBA/2J female mice (Charles River, Calco, Italy). For experimental purposes, female B6D2F1 mice weighing 18 to 20 g were used, and the antileukemic effect of platinum complexes was evaluated as previously described (Coluccia et al., 1999a).

SK-OV-3 Ovarian Carcinoma Cell Xenograft. SK-OV-3 tumor cell suspensions (2×10^6 cells/mouse) were injected s.c. into CD-1 female nude (nu/nu) mice (Charles River) on day 0. Treatment with cisplatin (Pharmacia and Upjohn, Milano, Italy) or mixed *Z* (freshly dissolved in saline) was performed i.p. on days 7, 14, and 21 from tumor implant (10 mice/group, 10 controls) at 2 and 4 mg/kg doses. Tumor mass was measured by caliper on days 9, 16, 23, and 31 from tumor implant, and the effect of treatment was expressed as %TWI, calculated according the following formula: %TWI = 100 − [(mean tumor weight of treated mice/mean tumor weight of controls) × 100]. Results of in vivo antitumor activity experiments were analyzed by the Mann-Whitney nonparametric test.

All procedures involving animals were in accord with institutional guidelines in compliance with national and international laws and policies (EEC Council Directive 86/109, OJL 358, December 1, 1987, and the National Institutes of Health, Guide for the Care and Use of Laboratory Animals, NIH publication 85-23, 1985).

Platination of the Oligonucleotides

The oligonucleotides containing a single mixed *Z* monofunctional adduct were obtained by reacting the pyrimidine-rich d(TGT) or d(TGC) oligonucleotides, at 30 μM concentration, with mixed *Z* at a platinum to oligonucleotide molar ratio of 6 in 10 mM $NaClO_4$, 5 mM acetate buffer, pH 3.6, for 40 min at 37°C, as previously described (Bernal-Mendez et al., 1997). The platinated oligonucleotides were purified by strong anion exchange chromatography (Mono Q HR5/5 column) with a 2 mM NaOH, 0.2 to 0.8 NaCl gradient. The fractions were neutralized with HCl and desalted on Sep-Pak C_{18} cartridges (Waters, St Quentin en Yvelines, France). The platinated oligonucleotides, in 100 mM NaCl, were kept at −20°C. It has been verified that the oligonucleotides contained one platinum residue that was bound at the single guanine residue by reaction with dimethyl sulfate as previously described (Lemaire et al., 1991; Boudvillain et al., 1995).

Interstrand Cross-Links in Double-Stranded Oligonucleotides

To follow the formation of interstrand cross-links, the single-stranded oligonucleotides containing a single monofunctional adduct were hybridized with their complementary strands and then incubated at 37°C in 50 or 150 mM $NaClO_4$, 3 mM phosphate buffer, pH 7.5, 0.5 mM EDTA. Aliquots were withdrawn at various time intervals and analyzed by gel electrophoresis under denaturing conditions as described (Brabec and Leng, 1993). Quantitation of the gel bands was done on a Molecular Dynamics PhosphorImager, using ImageQuant software version 3.3 (Molecular Dynamics, Sunnyvale, CA) for data processing.

Interstrand Cross-Links in DNA Restriction Fragments

Plasmid pSPKB was digested by *Hpa*I and *Ssp*I endonucleases into two fragments (1871 and 588 bp, respectively). The two restriction fragments were reacted with transplatin or mixed *Z* at a molar ratio of platinum/nucleotide residue equal to 0.001 or 0.005. The samples were incubated in 10 mM $NaClO_4$, at 37°C for 24 h. After addition of NaCl (100 mM), DNA fragments were ^{32}P-phosphorylated at the 3′-end with the Klenow fragment of DNA polymerase I and [α-^{32}P]ATP. The 588-bp fragments with and without interstrand cross-links were separated by electrophoresis on denaturing 1% agarose (Brabec and Leng, 1993). The number of interstrand cross-links per bound platinum residue was determined from the amounts of DNA in the two corresponding bands and assuming a Poisson distribution of ICLs (Farrell et al., 1990).

RNA Mapping Experiments

The unplatinated transcription template was synthesized by polymerase chain reaction amplification of a 135-bp fragment from plasmid pSPKB. This fragment contained T7 RNA polymerase promoter 10 bases away from the 3′-end of the upper strand. The primers were 5′-d(CGATTTAGGTGACACTATAG) and 5′-d(CGAAATTAATACGACTCACTATAGGG). The DNA fragments were reacted with transplatin or mixed *Z*, in 10 mM $NaClO_4$, for 24 h at 37°C. The molar ratios of platinum over DNA were chosen to get about 50% of the DNA molecules containing at least one interstrand cross-link. The samples were treated with thiourea (10 mM) for 40 min at 37°C to remove the monofunctional adducts. The two types of molecules, those containing or not containing interstrand cross-links, were purified by agarose (1.5%) gel electrophoresis under denaturing conditions. The gel was neutralized, and the two types of fragments were eluted. After one heating/reannealing cycle, the fragments were used as templates for RNA synthesis by T7 RNA polymerase as described (Lemaire et al., 1991).

Hydroxyl Radical Footprinting

The protocol for the footprinting experiments was essentially that previously described (Churchill et al., 1990). The reaction mixtures contained 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, and the platinated (or unplatinated) duplexes (about 6×10^{-9} M) with either the pyrimidine-rich or the purine-rich strand ^{32}P-labeled at the 5′-end. Cleavage of the phosphodiester backbones was done by introducing iron (40 μM), EDTA (80 μM), 0.03% H_2O_2, and sodium ascorbate (2 mM) for 5 min at 20°C. The reaction was stopped by adding thiourea (15 mM), EDTA (3 mM), sodium acetate (0.3 M), and tRNA (0.3 mg/ml). After precipitation, the samples were loaded onto a 24% denaturing polyacrylamide gel. Maxam-Gilbert sequencing reactions were run in parallel.

Ligation and Electrophoresis

Duplexes of various lengths (from 20- to 22-mer, see Fig. 1), unplatinated or containing a single ICL, were ^{32}P 5′-end phosphorylated with polynucleotide kinase and [α-^{32}P]ATP. The duplexes were incubated with T4 DNA ligase in 10 mM $MgCl_2$, 1 mM ATP, 10 mM Tris-HCl, pH 7.5, at 16°C for 1 h. The migration of the ligated products was examined on 8% native polyacrylamide electrophoresis gels as described (Koo et al., 1986; Boudvillain et al., 1995).

Interaction between HMG1 and Platinated DNAs

Rat HMG1 and the corresponding domain A were expressed in *Escherichia coli* from previously reported plasmids (pT7-RNHMG1 and K82Z, respectively). The purification of the proteins was done following the protocols already described (Bianchi, 1991; Locker et al., 1995).

The determination of the dissociation constants was done as described (Dunham and Lippard, 1997) with minor modifications. The reaction mixtures containing 6% Ficoll, 1 mM EDTA, 10 mM HEPES, pH 7.5, 0.2 mg/ml bovine serum albumin, 80 mM NaCl, 10 mM $MgCl_2$, 2000 cpm platinated DNA (about 10^{-10} M), and varying concentrations of HMG1 (or domains) in 10 μl were kept in ice for 20 min prior to loading. The reaction mixtures were analyzed by gel electrophoresis in prerun and precooled 45 mM Tris-borate, 1 mM EDTA, pH 8.3, 8% native polyacrylamide gels [29:1 acrylamide/*N,N*′-methylenebis(acrylamide), 0.5% ammonium persulfate]. The gels were run at 200 V. Quantitation of the gel bands was done on a Molecular Dynamics Phosphorimager. In the competition experiments to determine the relative affinities of HMG1 (or domain A) for platinated or unplatinated duplexes, the reactions were done as just described, but first the protein and the competitor were mixed and incubated in ice for 10 min, and then the probe (^{32}P 5′-end-labeled duplex containing a single cisplatin intrastrand cross-link) was added. After 20 min in ice, the reaction mixtures were analyzed by gel electrophoresis. The apparent dissociation constants of the competitors were calculated as described (Dunham and Lippard, 1997). The results were also plotted as inhibition (percentage) of binding of ^{32}P-labeled duplex as a function of the logarithm of the competitor concentration.

Results

In Vitro Cytotoxicity. The effect of mixed *Z*, mixed *E*, and transplatin was evaluated in a panel of human tumor cell lines containing examples of ovary (A2780, SK-OV-3, OVCAR-8), lung (A549/ATCC, NCI-H460, CALU), colon (KM12, LOVO, COLO 205, HCT-116), and breast (T-47D, MCF7, MDA) cancer (Fig. 2). Both mixed *Z* and mixed *E* showed a cytotoxic potency higher than that of transplatin, the mean IC_{50} values in the tumor cell panel being 103, 37, and 215 μM, respectively. Interestingly, the platinum-iminoether complexes showed major efficacy against ovarian and colon tumor cells, whereas lung cancer cells appeared to be more resistant.

In Vivo Antitumor Activity. The antitumor activity of platinum-iminoether complexes was investigated in the i.p.-i.p. murine P388 leukemia system (Table 1). Both mixed *Z* and mixed *E* showed in vivo activity (%T/C > 125). Mixed *Z* was definitely more active (and less toxic) than mixed *E*. The treatment with mixed *Z* at 15 and 20 mg/kg determined %T/C values of 173 and 190, along with one of six and two of six cures, respectively. On the contrary, the best %T/C for mixed *E* was obtained at 15 mg/kg (%T/C, 152), and a severe reduction of body weight along with two of six toxic deaths were observed at the 20-mg/kg dose.

The antitumor activity of mixed *Z* was also compared with that of cisplatin against SK-OV-3 tumor cells grown as s.c. xenografts in nude mice. Cisplatin or mixed *Z* was administered at the same dose levels (2 and 4 mg/kg). The treatment with mixed *Z* led to a significant reduction of tumor mass, not statistically different from that obtained by cisplatin (Table 2).

Interaction between DNA and Mixed *Z*. Numerous studies support that the formation of adducts in the reaction between cisplatin or transplatin and DNA proceeds in two successive solvent-assisted reactions (Bancroft et al., 1990). Assuming a similar mechanism for mixed *Z*, we wanted to know whether the reaction of mixed *Z* with double-stranded DNA resulted in the formation of intrastrand and/or ICL and then to determine the rates of the cross-linking reactions. These studies were performed on large platinated DNA restriction fragments or on double-stranded oligonucleotides containing a single adduct, and we chose to investigate mixed *Z* because of its higher antitumor activity.

Reactivity of DNA Restriction Fragments with Mixed *Z*. The formation of ICLs can be easily revealed by gel electrophoresis under denaturing conditions, the migration of the DNA molecules with and without ICLs being very different because of the difference in molecular weight. The 588-bp DNA restriction fragments from plasmid pSPKB were reacted with either mixed *Z* or transplatin for 24 h, and then the molecules with and without ICLs were separated on an alkaline agarose gel (1%) (Fig. 3). The values of ICL/adduct were 0.13 for transplatin and 0.10 for mixed *Z*, respectively (precision 10%).

RNA Polymerase Mapping. It is known that bifunctional adducts in cisplatin- or transplatin-modified DNA can

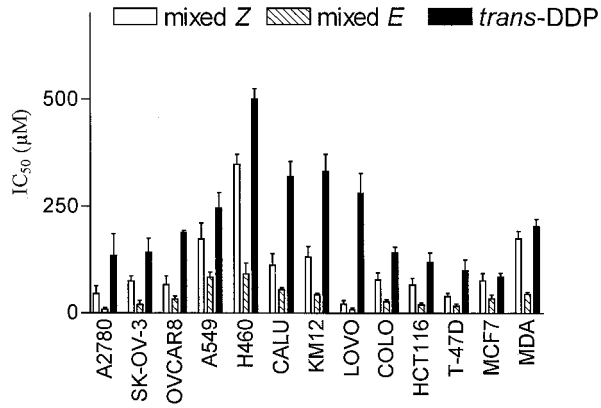


Fig. 2. Cytotoxicity of mixed *Z*, mixed *E*, and transplatin against human cancer cells (MTT assay; mean IC_{50} values; bars, ±S.D.; 72-h treatment; $n \geq 3$). DDP, diamminedichloroplatinum(II).

prematurely terminate in vitro RNA synthesis by T7 or SP6 RNA polymerases (Lemaire et al., 1991; Brabec and Leng, 1993). This approach has been used to characterize the preferred binding sites for mixed *Z*. After a 24-h reaction with mixed *Z* or transplatin, DNA fragments (135 bp) were treated with thiourea to remove the monofunctional adducts (vide infra). The fragments with and without ICLs were separated by agarose gel electrophoresis under denaturing conditions and then used as templates for RNA synthesis by T7 RNA polymerase. Sequence analysis of the fragments (Fig. 4A) did not reveal any stop during transcription of transplatin- or mixed *Z*-modified DNA that did not contain ICLs. We previously concluded that in double-stranded DNA modified by transplatin no intrastrand cross-links are formed (Boudvillain et al., 1995; Bernal-Mendez et al., 1997). The present results suggest that transplatin and mixed *Z* behave similarly as far as their inability to form intrastrand cross-links.

Sequence analysis of the fragments containing ICLs revealed several stops during transcription. Most of the stops are located at the level of G or C residues. This suggests that mixed *Z*, like transplatin (Brabec and Leng, 1993), cross-links complementary G and C residues.

The next step was to determine whether the monofunctional adducts could stop RNA polymerase. It has been already shown that the monofunctional adducts formed in the reaction between DNA and the monofunctional platinum derivative chlorodiethylenetriamineplatinum(II) do not arrest T7 or SP6 RNA polymerases (Lemaire et al., 1991; Brabec and Leng, 1993). On the other hand, the nature and/or the size of the nonleaving groups could interfere with the action of RNA polymerase, as in the case of the monofunctional adducts formed in the reaction between DNA and *trans*-[$PtCl_2$(*E*-iminoether)$_2$] (Zaludova et al., 1997).

The 135-bp DNA fragments were reacted with transplatin or mixed *Z* (r_i equal to 0.02, 10 mM $NaClO_4$, 37°C). After 3 h, the salt concentration was adjusted to 0. 3 M NaCl, and DNA fragments were precipitated twice with ethanol to remove completely the unbound complex. Each sample, dissolved in 10 mM $NaClO_4$, was divided into two aliquots. One was frozen after addition of NaCl (150 mM), whereas the other aliquot was further incubated for 24 h. All these modified fragments were used as templates for RNA synthesis. As shown in Fig. 4B, no stops were detected during transcription of the DNAs that were platinated for 3 h. After 3 h of reaction, mainly monofunctional adducts are formed and thus the monofunctional adducts do not stop T7 RNA polymerase. In the case of the samples incubated for 24 h, the patterns for the two complexes were essentially the same and were identical to the patterns in Fig. 4A. These experiments confirm that after 3 h of incubation with both complexes the DNAs were platinated and contained, at that step, only monofunctional adducts.

Closure of the Monofunctional Adducts within Double-Stranded Oligonucleotides. Further investigations were aimed at determining the rate of the interstrand cross-linking reaction and the nature of the cross-linked bases. Experiments were done on double-stranded d(TGC) and d(TGT) oligonucleotides containing a single mixed *Z* monofunctional adduct. For sake of simplicity, we present only the results relative to the d(TGC) sequence, because they are identical to those of the d(TGT) sequence.

The single-stranded oligonucleotide containing a single mixed *Z* monofunctional adduct was ^{32}P-labeled at the 5′-end and then hybridized with its complementary strand. The sample was incubated at 37°C in 50 or 150 mM $NaClO_4$. The reaction products were analyzed by polyacrylamide gel electrophoresis under denaturing conditions. This technique allows the separation of oligonucleotides containing intrastrand or interstrand cross-links (Bernal-Mendez et al., 1997, and references therein). The results are shown in Fig. 5. At time 0, a single band was present that corresponded to the single-stranded oligonucleotide containing a monofunctional adduct. Upon incubation, the band corresponding to the initial product disappeared at the expense of a much slower mobility species. This indicates the formation of an ICL within the duplex. The formation of intrastrand cross-links

TABLE 1
Antileukemic activity (murine P388 system) of mixed *E* and mixed *Z* complexes[a]

Complex	Dose	%T/C[b]	Body Weight Variation[c]
	mg/kg, qd1–7		%
Vehicle			+5.5
Mixed *E*	5	121	0
	10	128	−1.8
	15	152	−3.1
	20	132[d]	−6.2
Mixed *Z*	5	140	−1.5
	10	165	−2.4
	15	173[e]	−5
	20	190[f]	−5

[a] P388 leukemia cells (10^6/mouse) were implanted i.p. on day 0 in B6D2F1 female mice (18 g of body weight, six animals/group, eight controls. The treatment with platinum complexes freshly dissolved in saline was performed i.p. on days 1 to 7 (qd1–7).
[b] %T/C, mean survival time (%) of treated animals vs. controls.
[c] Body weight variation at the end of treatment with respect to day 0.
[d] Two of six toxic deaths.
[e] One of six cures.
[f] Two of six cures.

TABLE 2
Antitumor activity of mixed *Z* and cisplatin against SK-OV-3 tumor implanted in nude mice[a]

Complex	Treatment Schedule[b]	Dose	Total Dose	%TWI[c] (after the First Treatment)	%TWI[c] (after the Second Treatment)	%TWI[c] (after the Third Treatment)	%TWI[c] (on Day 31 from Tumor Implant)
		mg/kg/day	*mg/kg*				
Cisplatin	q7d × 3	2	6	18	45	43	40[d]
		4	12	41	40	48	52[e]
Mixed Z	q7d × 3	2	6	6	30	22	50[e]
		4	12	17	49	44	42[d]

[a] Three groups of 10 CD-1 female nude (nu/nu) mice were injected s.c. with a SK-OV-3 cell suspension (2×10^6/cells/mouse) into the right flank. On day 7 after tumor implant, when treatment started, a tumor was evident—although not measurable by caliper—in all mice.
[b] Treatment with cisplatin or mixed *Z* (freshly dissolved in sterile saline) was performed by three i.p. administrations on days 7, 14, and 21 after tumor implant (q7d × 3).
[c] %TWI, percentage of tumor weight inhibition of treated mice vs. controls on days 9, 16, 23, and 31 (nadir of cisplatin effect) from tumor implant.
[d,e] Statistical analysis by Mann-Whitney nonparametric test on TWIs calculated 10 days after the last treatment; [d] $P < .05$; [e] $P < .01$.

seems unlikely because no bands with a migration slightly slower than that of the starting material were detected.

The rate of disappearance of mixed *Z* monofunctional adduct was deduced from the roughly linear plots of the logarithm of the monofunctional adduct percentages (calculated from the ratio of the intensity of the corresponding band to the sum of the intensities of the two bands) versus reaction time (not shown). In 50 mM $NaClO_4$, the values of $t_{1/2}$ within d(TGT) and d(TGC) duplexes were 62 and 38 h, respectively. In the same experimental conditions and in the d(TGT) duplex, the value of $t_{1/2}$ for monofunctional transplatin adduct was 27 h (not shown). The closure of the monofunctional mixed *Z* adducts was slowed down as the salt concentration of the medium was increased to 150 mM $NaClO_4$ ($t_{1/2}$ values were equal to 85 and 60 h within d(TGT) and d(TGC) duplexes, respectively).

Hydroxyl Footprinting. The hydroxyl radicals generated by reaction of the EDTA complex of iron(II) with hydrogen peroxide initiate cleavage of the DNA phosphodiester backbone by abstracting a hydrogen atom from a deoxyribose. The hydroxyl radicals cleave mixed-sequence DNA nearly equally at each backbone position (Draganescu and Tullius, 1996). We have taken advantage of this reaction to identify the nature of the bases in the mixed *Z* ICLs.

The double-stranded d(TGC) oligonucleotide containing a single interstrand cross-link was ^{32}P-labeled at the 5′-end of either the pyrimidine-rich strand or the purine-rich strand. The fragments generated by hydroxyl radicals were separated on a polyacrylamide gel under denaturing conditions. The cleavage patterns for the platinated and unplatinated oligonucleotides, as well as the Maxam-Gilbert sequencing patterns for the unplatinated oligonucleotide, are shown in Fig. 6. For the two strands of the unplatinated duplex a uniform cleavage was obtained. For the platinated duplex all the bases from the 5′-end up to the ICL were detected. All the other bases were not detected because the generated fragments were cross-linked and consequently migrated with a slower mobility than that of each unplatinated strand. In our experimental conditions these cross-linked fragments were poorly separated.

Similar results (not shown) were obtained with the d(TGT) duplex containing a single ICL. In both duplexes, the two cross-linked residues were complementary G and C residues.

Distortions of DNA Induced by the ICLs. Several studies have shown that some structural features of cisplatin- or transplatin-modified oligonucleotides, such as bending of the double helix longitudinal axis and variation from the canonical twist of B-DNA, can be determined from electrophoretic mobility of multimers of these oligonucleotides on polyacrylamide gels (Bellon and Lippard, 1990). We have applied this technique to characterize the conformational changes of the

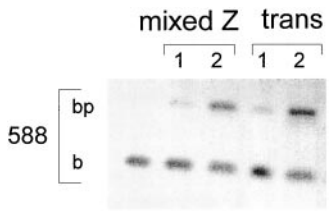


Fig. 3. Formation of ICLs by mixed *Z* or transplatin in the 588-bp DNA restriction fragment from pSPKB plasmid. Autoradiogram of a denaturing agarose (1.5%) gel of the platinated and unplatinated DNA fragments. The first lane corresponds to the untreated DNA fragment. Drug over nucleotide input molar ratios (r_i) were 0.001 and 0.005 (lanes 1 and 2, respectively).

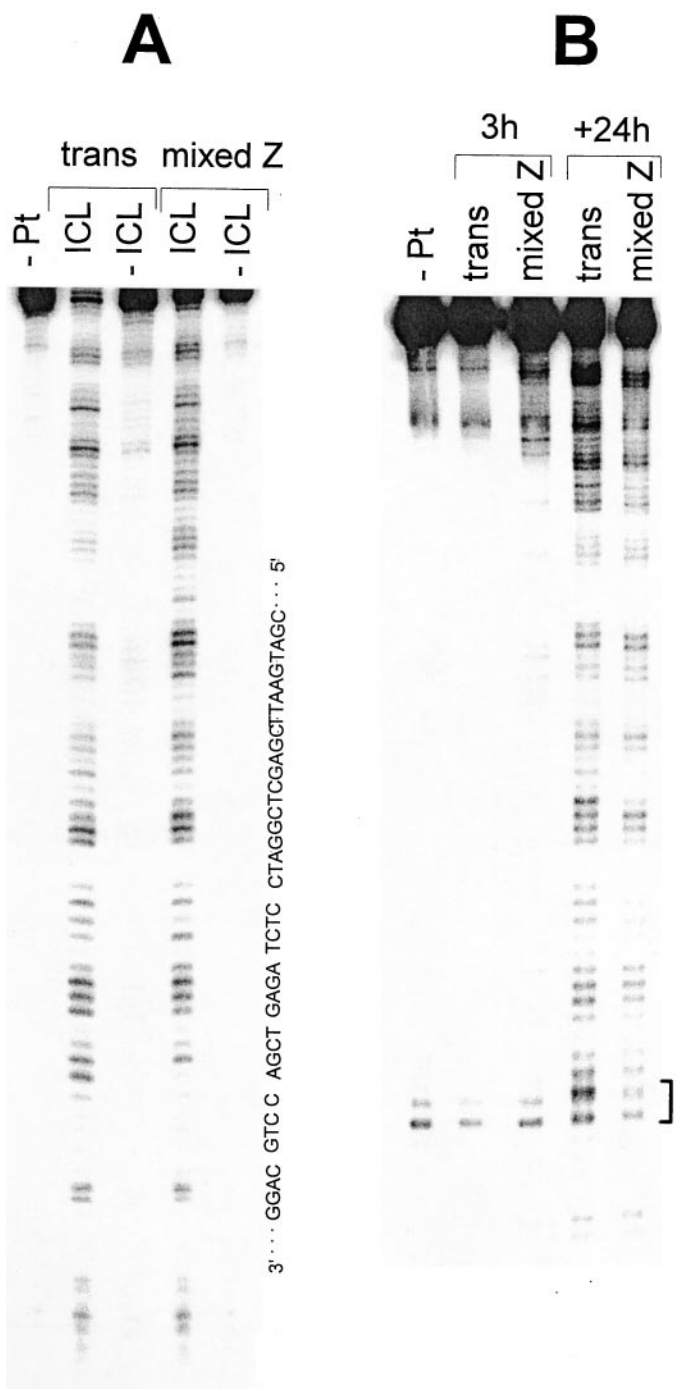


Fig. 4. Inhibition of RNA synthesis on transplatin- or mixed *Z*-modified DNA fragments. Autoradiograms of 6% polyacrylamide/7 M urea sequencing gel showing RNA fragments synthesized on unplatinated or platinated DNA. A, the DNA fragments (135 bp) were reacted with transplatin or mixed *Z* in conditions to have about half the DNA molecules containing at least one ICL. The fragments with and without (−) ICLs were purified by gel electrophoresis and then used as templates for RNA synthesis by T7 RNA polymerase. B, the DNA fragments were reacted with transplatin or mixed *Z* (r_i = 0.02) for 3 h and then were precipitated to remove unbound complex. Aliquots were further incubated for 24 h. The 3- and +24-h samples were used as templates for RNA synthesis by T7 RNA polymerase. The bracket indicates that the bands were due to an artifact.

DNA double helix induced by mixed *Z* ICLs within the d(TGT) duplex.

An autoradiogram of 8% native electrophoresis gel corresponding to the ligation products of 20- to 22-bp platinated and unplatinated duplexes is presented in Fig. 7 (top). In the three cases, the platinated multimers migrated slower than the corresponding unplatinated ones. This means that ICLs induce either bends or hinge joints in the DNA double helix. Retardation in mobility of the platinated duplexes depended slightly on the length of the oligonucleotides (20–22 bp), as shown by the variation of the K factor (apparent length relative to sequence length) as a function of the multimer length (Fig. 7, bottom). This does not argue in favor of induced bends. The end-to-end distance of the multimers is very sensitive to the position of the bends relative to the helix repeat of DNA, with the shortest end-to-end distance when the bends and the helix repeat are in phase (Koo et al., 1986; Crothers et al., 1990). The results suggest that the ICLs behave as hinge joints, increasing the flexibility of the double helix. This is also supported by the presence of sharp bands for the 21- and 22-mers at the top of the gel indicating the formation of circles.

Resistance of the Monofunctional Adducts to Thiourea. It is well known that transplatin monofunctional adducts are rapidly removed by thiourea (Eastman and Barry, 1987), a sulfur-containing molecule, whereas *trans*-[$PtCl_2$(E-iminoether)$_2$] monofunctional adducts are more resistant (Brabec et al., 1996; Boccarelli et al., 1999). We wanted to determine the effect of thiourea on mixed Z monofunctional adducts.

The single-stranded d(TGT) or d(TGC) oligonucleotides containing a single mixed *Z* monofunctional adduct at the G residue were ^{32}P-labeled at the 5′-end and then hybridized with their complementary strands. The samples were incubated at 20°C in the presence of thiourea (10 mM). Aliquots were withdrawn at various time intervals, and the samples were analyzed by gel electrophoresis under denaturing conditions. As shown in Fig. 8, the intensity of the band corresponding to the platinated sample decreased as a function of time. A new band with a slightly faster migration (corresponding to the unplatinated oligonucleotide) appeared and its intensity increased as a function of time. The rate of disappearance of the monofunctional adduct was deduced from the roughly linear plots of the logarithm of the monofunctional adduct percentage (calculated from the ratio of the intensity of the upper band to the sum of the intensities of the two bands) versus reaction time (not shown). The values of $t_{1/2}$ of mixed *Z* monofunctional adducts within d(TGT) and d(TGC) duplexes were equal to 12 and 21 min, respectively. In the same conditions, $t_{1/2}$ of transplatin monofunctional adducts within the d(TGT) duplex was 10 min. The conclusion is that the modification of one nonleaving group of transplatin by an iminoether group does not significantly change the stability of the monofunctional adducts in the presence of thiourea.

Similar experiments (not shown) have been done in the presence of glutathione, a cysteine-containing tripeptide, which is the predominant intracellular thiol. Glutathione is

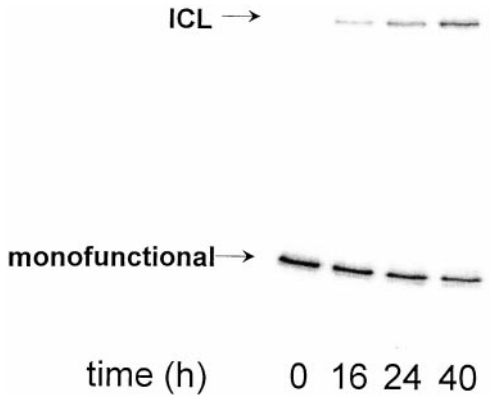


Fig. 5. Closure of the monofunctional adduct into ICL. Autoradiogram of a denaturing 24% polyacrylamide/7 M urea gel showing the conversion of the mixed *Z* monofunctional adducts into ICLs within the d(TGC) duplex. The platinated duplex was incubated at 37°C in 50 or 150 mM $NaClO_4$, 3 mM phosphate buffer, pH 7.3, 0.5 mM EDTA. At various time intervals (indicated in hours below the lanes), aliquots were withdrawn and electrophoresed.

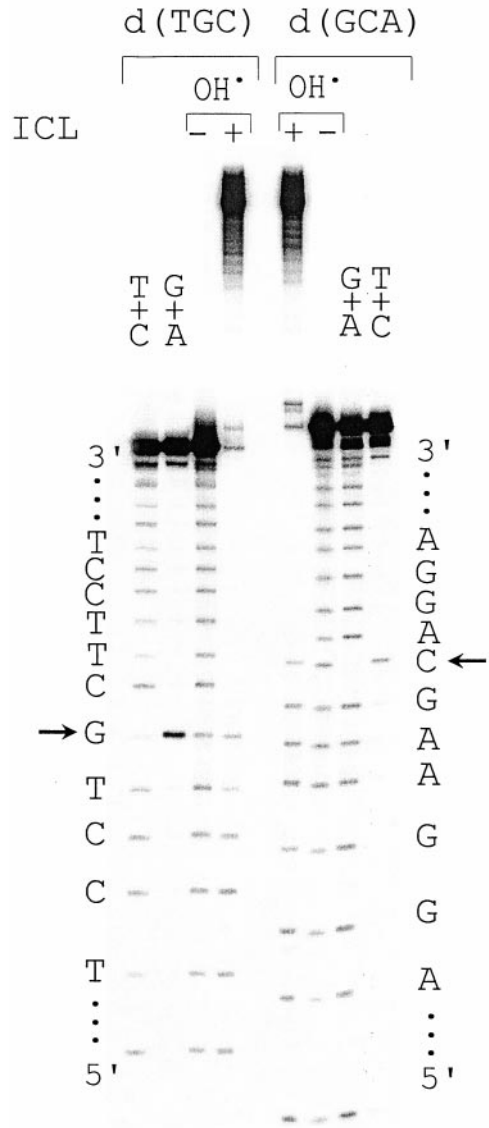


Fig. 6. Identification of the cross-linked bases in mixed *Z* ICLs. Autoradiogram of the products of cleavage by hydroxyl radicals in the d(TGC) duplex containing (+) or not containing (−) a single mixed *Z* ICL. Maxam-Gilbert (T + C) and (G + A) sequencing reactions were run in parallel on the unplatinated duplex. Arrows indicate the cross-linked bases.

known to react with transplatin monofunctional adducts (Eastman and Barry, 1987; Bancroft et al., 1990). In our experimental conditions (10 mM glutathione, 37°C) the values of $t_{1/2}$ for transplatin or mixed *Z* monofunctional adducts are about the same (~350 min).

Interaction between HMG1 and Platinated DNAs. We were interested to determine whether HMG domains were able to bind specifically to DNAs modified by mixed *Z*. The results relative to domain A from rat HMG1 are summarized in Fig. 9. In agreement with published data (Dunham and Lippard, 1997) domain A recognized the 18-bp d(AG*G*)duplex containing a single cisplatin intrastrand cross-link at the d(GpG) site of the pyrimidine-rich strand (Fig. 1), as indicated by the presence of a shifted band that increased with increasing protein concentration (Fig. 9, 1). The dissociation constant K_d for domain A-d(AG*G*) complex is about 5 nM. This value differs slightly from that previously reported (Dunham and Lippard, 1997), reflecting different experimental conditions. Competition experiments in which labeled d(AG*G*) was displaced from domain A by unlabeled d(AG*G*) (Fig. 9, 2) gave a K_d of about 10 nM. The affinities for domain A of several other duplexes were tested by competition experiments. None of the following duplexes, the unplatinated d(AGG) duplex, the unplatinated 18-bp d(AGC) duplex, the 18-bp d(AGC) duplex containing either a cisplatin ICL or a transplatin ICL, or a mixed *Z* ICL, or a mixed *Z* monofunctional adduct, was specifically recognized. They all behaved similarly, and the K_d for these duplexes-domain A complexes were in the 5 μM range.

The experiments were repeated with the whole protein HMG1 (not shown). The dissociation constants were different, but the results were qualitatively the same. All the competitors, except the one containing a single cisplatin intrastrand cross-link, behaved similarly, being not specifically recognized by HMG1.

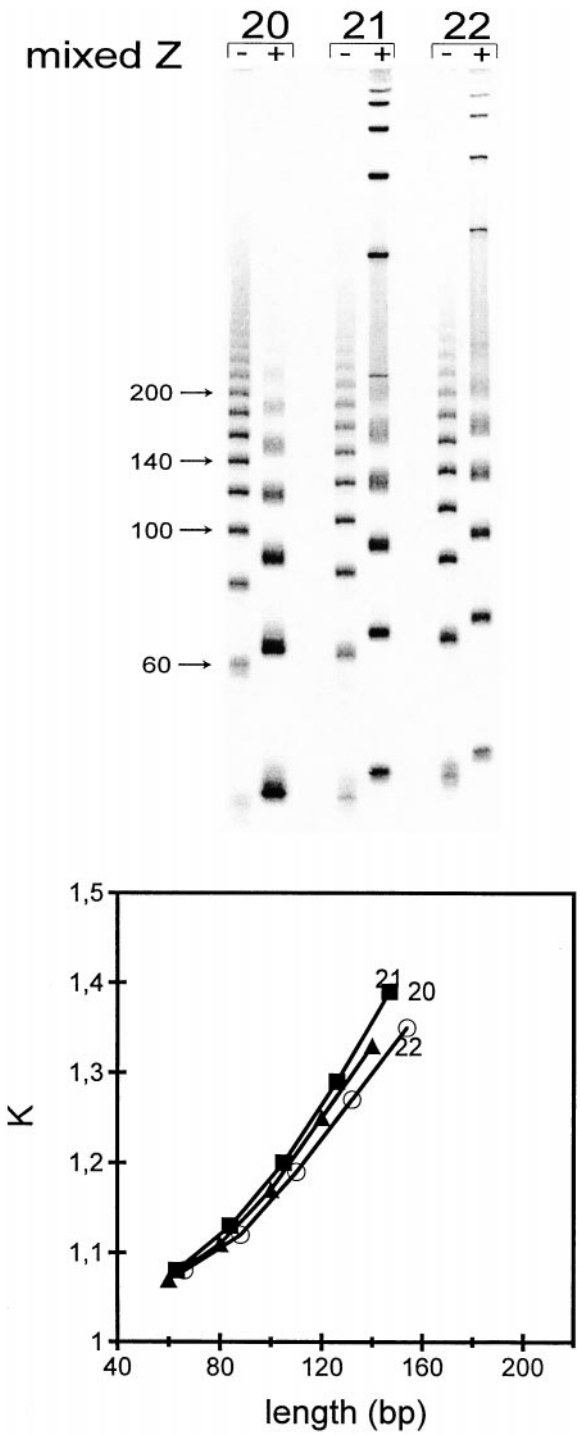


Fig. 7. Distortions induced by ICLs. Top, autoradiograms of the ligation products of the 20- to 22-mer double-stranded d(TGT) oligonucleotides containing a single mixed *Z* ICL separated on a 8% polyacrylamide gel. The numbers on the left represent the sizes of the ligation products in base pairs. Bottom, plot showing the relative mobility *K* versus sequence length for the 20- to 22-mers denoted 20, 21, and 22.

Discussion

The main purpose of this work was to determine the effect on antitumor activity and DNA interaction properties of transplatin after replacement of one NH_3 by an iminoether group.

The major biological effect has been observed with *trans*-[$PtCl_2${*Z*-HN=C(OMe)Me(NH_3)] (mixed *Z*). It is only 2-fold more cytotoxic than transplatin toward tumor cells in vitro, but, unlike transplatin, it is endowed with a selective antitumor activity in in vivo models. To our knowledge, this is the first time that a platinum complex with *trans* geometry has shown the ability to cure tumor-bearing animals (P388 system). Moreover, the antitumor efficacy of mixed *Z* was confirmed by using an established s.c. tumor (SK-OV-3 xenograft) and drug administration distant (i.p.) from the tumor site.

The next step was to characterize the interaction of mixed *Z*-DNA. No intrastrand cross-links are formed, as deduced from transcription footprinting on a mixed *Z*-modified DNA

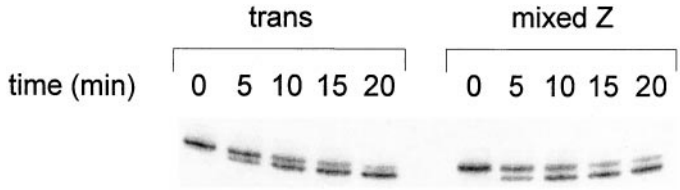


Fig. 8. Thiourea reaction on transplatin or mixed *Z* monofunctional adducts within duplexes. Autoradiogram of a denaturing 24% polyacrylamide/7 M urea gel. The d(TGT) duplexes containing a monofunctional adduct were incubated at 20°C in 100 mM NaCl, 3 mM phosphate buffer, pH 7.5, 10 mM thiourea. At various time intervals (indicated in minutes below the lanes), aliquots were withdrawn and electrophoresed.

fragment and from the electrophoretic pattern of double-stranded oligonucleotides containing a single monofunctional adduct. It has been previously reported that transplatin does not form intrastrand cross-links within dsDNA (Boudvillain et al., 1995; Bernal-Mendez et al., 1997), and thus, transplatin and mixed *Z* behave similarly as far as their inability to cross-link nucleotide residues on the same strand is concerned.

Both transplatin and mixed *Z* form ICLs between complementary G and C residues in the reaction with dsDNA, as deduced from RNA polymerase and hydroxyl radical footprinting experiments. The RNA polymerase footprinting reveals that potential (G·C) binding sites are unequally modified, as shown by the different intensities of the bands. This reflects a sequence effect that modulates the reactivity of G residues with mixed *Z* in the first step of reaction and/or the following closure of the monofunctional adducts. The sequence effect on formation of monofunctional adducts in the reaction between DNA and cisplatin is well documented (Davies et al., 1998; Legendre et al., 1998; Guo and Sadler, 1999; Jamieson and Lippard, 1999). The negative electrostatic potential at the N7 position of G residues [the preferred binding sites of platinum(II) complexes] depends upon the nature of the flanking residues (Pullman and Pullman, 1981). As far as the second step is concerned, we find a difference in the rate of closure of mixed *Z* monofunctional adducts within double-stranded d(TGT) and d(TGC) oligonucleotides. All these results support a sequence effect on the two steps of the interstrand cross-linking reaction. Finally, the rate of closure decreases as the salt concentration increases, suggesting that factors destabilizing the double helix favor the formation of the ICLs (Bernal-Mendez et al., 1997; Zaludova et al., 1997).

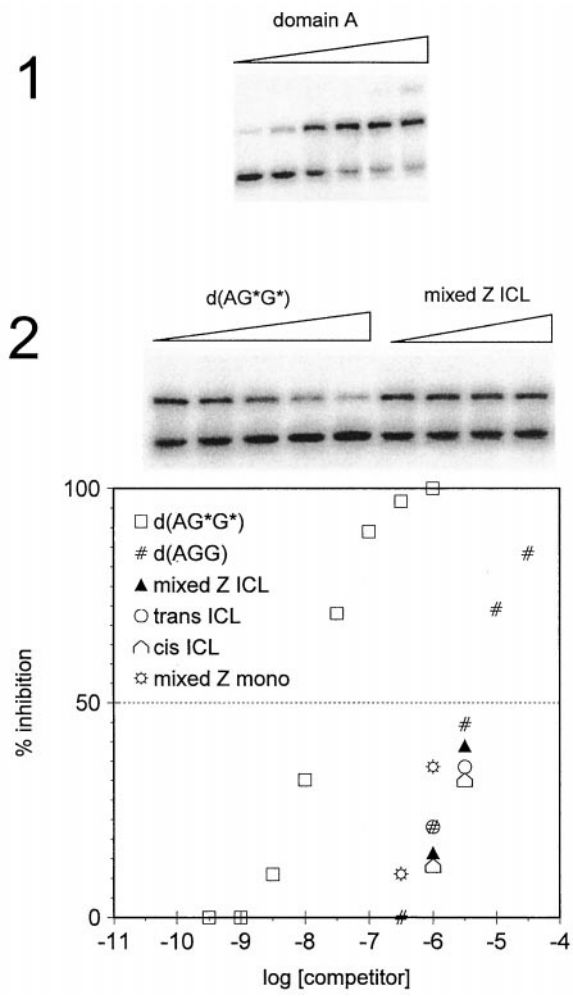


Fig. 9. Interaction between domain A from HMG1 and platinated DNAs. 1, gel retardation assay showing binding of domain A to an 18-bp d(AGG) duplex containing a single cisplatin intrastrand cross-link at the d(GpG) site. Duplex concentration, ~2 nM; domain A concentration, 1 to 100 nM. 2, competition experiments. Top, gel retardation assays showing displacement of the labeled cisplatin-modified d(AGG) duplex by the unlabeled cisplatin-modified d(AGG) duplex or by an 18-bp d(AGC) duplex containing a single mixed *Z* ICL. Bottom, inhibition of domain A binding to the 18-bp cisplatin-modified d(AGG) duplex by various duplexes. d(AG*G*), stands for the unlabeled 18-bp cisplatin-modified d(AGG) duplex; d(AGG), corresponding unplatinated duplex; mixed *Z* ICL, *trans*-ICL, or *cis*-ICL, the 18-bp d(AGC) duplex containing a single ICL formed by mixed *Z*, transplatin, or cisplatin, respectively); mixed *Z* mono, the 18-bp d(AGC) duplex containing a single mixed *Z* monofunctional adduct.

T7 RNA polymerase is not stopped by transplatin or mixed *Z* monofunctional adducts. This result extends previous findings of DNA modified by the monofunctional platinum derivative chlorodiethylenetriamineplatinum(II) (Lemaire et al., 1991) or by more bulky compounds such as *cis*-$[Pt(NH_3)_2(N7\text{-}N\text{-methyl-2-diazapyrenium})Cl]^{2+}$ (Anin et al., 1992) or *trans*-$[PtCl_2(Z\text{-iminoether})_2]$ (M. Coluccia, A. Schwartz and M. Leng, unpublished results). On the other hand, *trans*-$[PtCl_2(E\text{-iminoether})_2]$ monofunctional adducts behave differently because they arrest T7 RNA polymerase (Zaludova et al., 1997).

The closure of mixed *Z* monofunctional adducts into ICLs within double-stranded oligonucleotides is about 2 times slower than that of transplatin, which is itself about 10 times slower than cisplatin (Malinge and Leng, 1999). An explanation for the slow closure of transplatin monofunctional adducts can be found in the three-dimensional structure of a duplex containing a single transplatin ICL. In this structure (Paquet et al., 1999), the platinated G residue is in the *syn* conformation and the adjacent base pairs are pushed away along the axis of the double helix by the platinum NH_3 ligands. Two events, rotation of the platinated G residue from *anti* to *syn* conformation and displacement of the adjacent base pairs, are necessary to locate the platinum residue near the complementary cytosine residue. These two independent events have to occur concomitantly, and this makes the interstrand cross-linking reaction unlikely.

The three-dimensional structure of a duplex containing a single mixed *Z* ICL has not yet been determined. Compared with transplatin ICL, we expect some structural changes due to the larger size of the iminoether group. In fact, the distortions induced by transplatin and mixed *Z* ICL are different, as judged by gel mobility shift of multimers of cross-linked duplexes relative to unplatinated controls. Transplatin ICL behaves as a directed bend and introduces some flexibility into the helix (Brabec et al., 1993). The mixed *Z* ICL introduces more flexibility and behaves as a hinge joint, as shown by the anomalous migration of the multimers of mixed *Z* duplexes that is almost independent from the length of the cross-linked duplexes. Despite these structural differences, both transplatin and mixed *Z* cross-link complementary G and C residues in dsDNA with about the same rate and yield.

It is known that transplatin monofunctional adducts are removed by thiourea, whereas the bifunctional adducts are

resistant (Eastman and Barry, 1987). The thiourea deplatination reaction rate on mixed *Z* monofunctional adducts within double-stranded oligonucleotides depends slightly upon the sequence ($t_{1/2}$ = 10 and 21 min for d/TGT) and d(TGC), respectively) and is in the same range of magnitude as that of transplatin monofunctional adducts ($t_{1/2}$ = 10 min). Both transplatin and mixed *Z* monofunctional adducts are also removed at the same rate by glutathione ($t_{1/2} \sim$ 350 min). Therefore, the accessibility of the platinum residue is not hampered by the iminoether group, and both adducts behave similarly with respect to small sulfur-containing molecules.

Several results support the implication of HMG1-like proteins in the mechanism of action of cisplatin (Jamieson and Lippard, 1999; Zamble and Lippard, 1999). They bend DNA and recognize specifically prebent DNA structures that they can further bend (Bianchi and Bertrame, 1998). HMG1 as well as its A and B domains interact specifically with DNA modified by cisplatin at d(GpG) (Jamieson and Lippard, 1999; Zamble and Lippard, 1999, and references therein), and recently the crystal structure of HMG1 domain bound to a 16-bp oligonucleotide containing a single (G1,G2)-intrastrand cross-link has been resolved (Ohndorf et al., 1999). We have compared the relative affinities of HMG1 (and domain A) to DNA containing a monofunctional mixed *Z* adduct, a transplatin ICL, or a mixed *Z* ICL. The proteins do not recognize preferentially the modified DNAs, which behave as the unplatinated sample. Unlike cisplatin, the recognition of a mixed *Z* monofunctional adduct or ICL by HMG1-like proteins does not seem to be implicated in the antitumor activity of the complex.

The results on the interaction between HMG1 and transplatin ICLs confirm previous findings (Kasparkova and Brabec, 1995; Locker et al., 1995). We have also studied the interaction between HMG1 (and domain A) and a duplex containing a cisplatin ICL. The structure of the cisplatin ICL has been resolved by NMR and X-ray crystallography (Malinge and Leng, 1999, and references therein). The double helix is largely unwound and its axis is bent toward the minor groove. The platinum residue is located in the minor groove of DNA and the cytosine residues are extruded from the double helix. The structures of the cisplatin (G1,G2)-intrastrand cross-link (Takahara et al., 1995; Ohndorf et al., 1999) and cisplatin ICL are quite different; therefore, the latter is not expected to be specifically recognized by HMG1. In fact, we do not observe any preferential binding. This result differs from the one recently reported (Kasparkova and Brabec, 1995).

The general conclusion of the in vitro experiments is that mixed *Z* resembles transplatin with respect to the interactions with DNA. On the other hand, mixed *Z* is an antitumor drug, but transplatin is not. The similar in vitro DNA interaction of mixed *Z* and transplatin, which contrasts with their in vivo behavior, might have relevant mechanistic implications, suggesting that the clinical inactivity of transplatin could not depend upon its peculiar DNA binding mode. A comparative investigation of the nature of adducts formed in the in vivo reaction between DNA and mixed *Z* or transplatin should shed light, in the near future, on this matter.

Replacement of a NH_3 by an iminoether very likely modifies some cellular pharmacological properties (increased accumulation and DNA binding) of the complex. Preliminary data support this hypothesis, which is also favored by the fact that the bis-imminoether complexes *trans*-[$PtCl_2${*E*-NH=C(OMe)Me}$_2$], *trans*-[$PtCl_2${*E*-NH=C(OEt)Me}$_2$], and *trans*-[$PtCl_2${*Z*-NH=C(OEt)Me}$_2$] enter cells much more efficiently than does cisplatin, and their DNA adduct formation is, at least, 20-fold higher than that of cisplatin (Coluccia et al., 1999a, b). Accordingly, it is tempting to speculate that both mixed *Z* monofunctional adducts and ICLs are involved in the pharmacological action of the complex. It is generally accepted that monofunctional adducts are not involved in the antitumor activity of cisplatin because platinum complexes that bind monofunctionally to DNA as $[Pt(NH_3)Cl]^+$ and chlorodiethylenetriammineplatinum(II)chloride are inactive (Jamieson and Lippard, 1999). However, the long-lived mixed *Z* monofunctional adducts could be important because of their potential to cross-link DNA to proteins. On the other hand, mixed *Z* ICLs could be important because they are expected to block DNA and RNA polymerases.

Importantly, the in vivo experiments show that the replacement of one NH_3 by an iminoether modifies the toxicity of transplatin and very likely also its pharmacokinetic properties, because mixed *Z* is able to reach, with therapeutic efficacy, the tumor site. Whether these promising preclinical investigations will lead to a new platinum drug for cancer patients will become clearer in the near future, after more detailed pharmacological and toxicological investigations.

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