DNA Modifications by Antitumor \(\text{trans-}[\text{PtCl}_2(E\text{-Iminoether})_2]\)

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SUMMARY

Recent findings that an analogue of clinically ineffective transplatin, \(\text{trans-}[\text{PtCl}_2(E\text{-Iminoether})_2]\), exhibits antitumor activity has helped reevaluation of the empirical structure-antitumor activity relationship generally accepted for platinum(II) complexes. According to this relationship, only the cis geometry of leaving ligands in the bifunctional platinum(II) complexes, should be therapeutically active. Global modifications of natural DNAs in cell-free media by \(\text{trans-}[\text{PtCl}_2(E\text{-Iminoether})_2]\) were studied through various molecular biological methods and compared with modifications by \(\text{cis-}[\text{PtCl}_2(E\text{-Iminoether})_2]\), transplatin, cisplatin, and monofunctional chlorodiethylenetriamineplatinum(II) chloride. Thus, the results of this study have extended our recent finding, indicating that the prevalent lesion occurring in double-helical DNA on its modification by \(\text{trans-}[\text{PtCl}_2(E\text{-Iminoether})_2]\) is a monofunctional adduct at guanine residues. The modification by \(\text{trans-}[\text{PtCl}_2(E\text{-Iminoether})_2]\) has been found to induce local distortions in DNA, which have a character differing fundamentally from those induced by both clinically ineffective or antitumor platinum complexes tested in this study. The different character of alterations induced in DNA by the adducts of \(\text{trans-}[\text{PtCl}_2(E\text{-Iminoether})_2]\) and transplatin has been suggested to be relevant to the unexpected observation that the new complex with leaving chlorine groups in trans position exhibits antitumor efficacy. In addition, the results support the idea that platinum drugs that bind to DNA in a manner fundamentally different from that of cisplatin can exhibit altered biological properties, including differing spectra and intensities of antitumor activity.

Cisplatin (Fig. 1) is a highly effective antitumor agent used, in particular, to treat genitourinary and head and neck cancers (1–4). Despite its remarkable biological activity, cisplatin displays a relatively limited spectrum of activity with associated toxicity, and some cancer cells develop resistance to the drug. Considerable modifications within the basic \(\text{cis-}[\text{PtX}_2(\text{ammine})_2]\) structure have been made in the attempt to overcome these clinical limitations. This modification has allowed reduced toxicity and altered modes of delivery, but none of these analogues of cisplatin has been shown to be likely to surpass the parent drug in efficacy. Considerable information has been accumulated regarding the mode of action. It is now firmly established that DNA is the principal target molecule in cells and the formation of kinetically sta-

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ABBREVIATIONS: cisplatin, cis-diaminedichloroplatinum(II) (cis-\([\text{PtCl}_2(\text{NH}_3)_2]\)); transplatin, trans-diaminedichloroplatinum(II) (trans-\([\text{PtCl}_2(\text{NH}_3)_2]\)); Me, methyl; cis-EE, cis-\([\text{PtCl}_2(E\text{-Iminoether})_2]\); E, iminoether = \(\text{HN} \equiv \text{C} \equiv \text{OMe} \equiv \text{Me}\); it can have either E or Z configuration depending on the relative position of OMe and N-bonded Pt with respect to the \(\text{C} \equiv \text{N}\) double bond, cis in the Z isomer and trans in the E isomer; trans-EE, trans-\([\text{PtCl}_2(E\text{-Iminoether})_2]\); dierenPt, chlorodiethylenetriamineplatinum(II) chloride ([\(\text{PtCl}_2(\text{H}_2\text{NCH}_2\text{CH}_2\text{NHC}(\text{Me})_2\text{N}_{\text{Me}})_2\text{Cl}\)]; \(t_m\), DNA melting temperature; DPP, differential pulse polarography; ELISA, enzyme-linked immunosorbent assay; ICL, interstrand cross-link or cross-linking; bp, base pair(s).

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We recently described some characteristics of DNA binding mode of antitumor trans-EE in a cell-free medium (14, 15). The results indicated that trans-EE preferentially forms monofunctional adducts at guanine residues in double-helical DNA that are kinetically stable (most of these monofunctional adducts at guanine residues in double-helical DNA are transformed to the relaxed form with an average time of 24 hr). It has been suggested that the different nature of the adducts formed on DNA by transplatin and cisplatin is relevant to their distinct clinical efficacy.

To explain the cytotoxicity of trans-EE in several tumor cell lines and its antitumor efficacy, it is necessary to examine in detail how this platinum complex modifies DNA conformation and to place these results in context with those previously elucidated for diaminedichloroplatinum(II) iso-
mers. We report the results of a comparison between the DNA binding in a cell-free medium of trans-EE with its cis isomer, cisplatin; transplatin; and monofunctional dienPt with respect to DNA stability, conformational changes, DNA ICLs, and sequence specificity.

Experimental Procedures

Starting materials. Cisplatin, transplatin, and dienPt were synthesized and characterized at Lachema (Brno, Czech Republic). cis-EE, trans-EE, and its mononitrate analogue trans-[Pt(NO)₂Cl(E-iminoether)₂] were prepared and characterized as previously described (15, 17). Calf thymus DNA (42% G + C; mean molecular mass, ~2 × 10⁷) was also prepared and characterized as described previously (18). Plasmid pSP73 (2464 bp) was isolated according to standard procedures and band was twice in CsCl/EtBr equilibrium density gradients. Restriction endonucleases were purchased from New England Biolabs (Beverley, MA). T4 polynucleotide kinase and the Klenow fragment of DNA polymerase I were from Boehringer-Mannheim Biochemicals (Mannheim, Germany). Riboprobe Gemini System II for transcription mapping containing T7 RNA polymerase was purchased from Promega (Madison, WI). The radioactive products were from Amersham (Arlington Heights, IL).

Platination reactions. DNAs were modified by platinum complexes in 10 mM NaClO₄ at 37°C in the dark for 48 hr if not otherwise stated in detail how this platinum complex modifies DNA conformation and to place these results in context with those previously elucidated for diaminedichloroplatinum(II) iso-

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**Platination reactions.** DNAs were modified by platinum complexes in 10 mM NaClO₄ at 37°C in the dark for 48 hr if not otherwise stated. In these samples, the *r*ᵣ (number of molecules of the platinum complex fixed per nucleotide residue) values were determined by flameless atomic absorption spectrophotometry or DPP (19).

**DNA melting.** The melting curves of DNAs were recorded by measuring the absorbance at 260 nm using a Beckman DU-8 spectrophotometer (Beckman Instruments, Columbia, MD). If not stated otherwise, the melting curves were recorded in media containing various concentrations of NaCl and 1 mM Tris-HCl with 0.1 mM EDTA, pH 7.4. The value of the *t*ₐ was determined as the temperature corresponding to a maximum on the first-derivation profile of the melting curves. The *t*ₐ values could be thus determined with an accuracy of ± 0.3°C.

**Immunological analysis.** Polyclonal antibodies were elicited against double-helical calf-thymus DNA modified by cisplatin or transplatin, respectively, at an *r*ᵣ value of 0.08 in 10 mM NaClO₄ for 48 hr at 37°C. They were purified and characterized as previously described (20–22). The procedures for their immunoenzymatic analysis and ELISA have also been previously described (20–22).

**Unwinding of negatively supercoiled DNA.** Unwinding of closed circular supercoiled pSP73 plasmid DNA was determined with an agarose gel mobility shift assay (23). The unwinding angle Φ, induced per platinum-DNA adduct, was calculated on determination of the *r*ᵣ value at which the complete transformation of the supercoiled form to the relaxed form of the plasmid was attained. Samples of pSP73 plasmid were incubated with *trans-EE* or *cis-EE* at 37°C in the dark for 24 hr. All samples were precipitated by ethanol and redissolved in Tris-borate/EDTA buffer. An aliquot of the precipitated sample was subjected to electrophoresis on 1% agarose gel at 30 V. The gels were then stained with EtBr, followed by photography with Polaroid 667 film and transilluminator. The other aliquot was used for the determination of *r*ᵣ values by flameless atomic absorption spectrophotometry.

**ICL assay.** If not otherwise stated, *trans-EE* or *cis-EE* at varying concentrations was incubated with 2 μg of pSP73 DNA linearized by EcoRI. The platinated samples were precipitated by ethanol and analyzed for DNA ICLs as recently described (24–26). The linear duplexes were first 3’-end labeled with the Klenow fragment of DNA polymerase I and [α-³²P]dATP. The samples were deproteinized by phenol and precipitated by ethanol, and the pellet was dissolved in 18 μl of a solution containing 30 mM NaOH, 1 mM EDTA, 6.6% sucrose, and 0.04% bromphenol blue. The number of ICLs was determined by elec-
trophoresis under denaturing conditions on alkaline agarose gel (1%). After the electrophoresis was completed, the bands corresponding to single strands of DNA and IC L duplex were cut off, and radioactivity was quantified on an LKB Wallac 1410 Beta spectrometer (Wallac Oy, Turku, Finland). As shown below, some platinated DNA samples were also analyzed for ICLs using milder conditions; the DNA samples were treated with formamide or dimethylsulfoxide at 40 or 55° and then analyzed in native agarose gel. The details of these milder assays have been previously reported (27, 28).

**Sequence specificity of DNA adducts of trans-EE.** Transcription of the (NdeI/HpaI) restriction fragment of pSP73 DNA with T7 RNA polymerase and electrophoretic analysis of transcripts were performed according to the protocols recommended by Promega and previously described in detail (25, 26, 29, 30).

### Results and Discussion

**DNA binding.** Solutions of calf thymus DNA at a concentration of 0.32 mg/ml were incubated with trans-EE or cis-EE at r_m (molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA) values of 0.01 in 10 mM NaClO_4 at 37°. At various time intervals, an aliquot of the reaction mixture was withdrawn and assayed by DPP for platinum not bound to DNA (19). The amount of platinum bound to DNA (r_m) was calculated by subtracting the amount of free (unbound) platinum from the total amount of platinum present in the reaction. After ~48 hr, both iminoether compounds were bound quantitatively. The \( t_{1/2} \) values of these binding reactions were 294 and 176 min for trans-EE and cis-EE, respectively; \( t_{1/2} \) values of the reactions of transplatin and cisplatin with DNA under identical conditions were 141 and 42 min, respectively. This comparison indicates that the replacement of the amonia groups in diaminodichloroplatinum(II) complexes by iminoether groups significantly decreases the rate of the binding of platinum(II) complexes to DNA.

**DNA melting.** The stability of DNA double helix may be affected by the interaction with low-molecular-mass compounds. Numerous studies have shown that \( t_m \) characterizes the stability of the double helix. The modification of DNA by platinum(II) complexes results in a decrease in \( t_m \) if DNA melting is measured in media containing sufficiently high salt concentrations (31). This effect is reversed at lower salt concentrations, and the ionic strength at which this reversal occurs can differ markedly for individual platinum(II) complexes. It has been suggested (31, 32) that at least three major factors affect the thermal stability of DNA modified by platinum(II) complexes: stabilizing effects of the positive charge on the platinum(II) moiety and of DNA ICLs and a destabilizing effect of conformational distortions induced in DNA by platinum coordination. Calf thymus DNA was modified by trans-EE or cis-EE to various \( r_m \) values (0–0.1) [in 10 mM NaClO_4 at 37° for 48 hr so no free molecules of the platinum complexes (not coordinated to DNA) remained in the solution]. The effect of these modifications on \( t_m \) was measured in samples taken from the equilibrated reaction mixtures (in 10 mM NaClO_4 at 37° after 48 hr), in which the concentration of Na^+ was adjusted with NaCl to values in the range of 0.01–0.2 M. The modifications of DNA by trans-EE at \( r_m \) values in the range of 0.001–0.1 resulted in a slight increase in \( t_m \) at a low concentration of Na^+ (0.01 M) (Fig. 2B). This enhancement was reduced with increasing NaCl concentration. At a salt concentration of 0.2 M, the modification of DNA by trans-EE resulted in a decrease in \( t_m \) which was increasingly pronounced with increasing \( r_m \) value. For comparison, the effect of cis-EE on the melting behavior of DNA at the two extreme concentrations of salt in the medium (0.01 and 0.2 M) is also shown in Fig. 2A.

The observation that the \( t_m \) values of DNA modified by trans-EE are considerably decreased at higher \( r_m \) values (>0.05) at the higher salt concentration (0.2 M) (Fig. 2B) is consistent with the occurrence of conformational alterations induced in DNA by trans-EE that destabilize the duplex (31). At higher salt concentrations (0.2 M), the negative charges of DNA phosphate groups are already efficiently neutralized by the cations present in the medium, so an electrostatic contribution of the positive charge on the coordinated platinum complex to the stability of DNA is less expressed than in a medium of a lower salt concentration. In other words, at higher salt concentrations, the destabilization of DNA resulting from conformational distortions induced by trans-EE overcompensates stabilizing electrostatic effects arising from the positive charges located on the platinum atoms and from DNA ICLs (as will be shown, trans-EE forms a small amount of ICLs in DNA). In contrast, in the medium of a low salt concentration, the electrostatic stabilizing effects are apparently more effective (because the negative charges of DNA phosphate groups are no more sufficiently neutralized by the Na^+ counterions) and, along with the stabilization effects of ICLs, apparently predominate over the destabilization effects of conformational distortions induced in DNA by platinum coordination. Thus, at the low salt concentration (10 mM), no reduction in \( t_m \) is observed, even at relatively high levels of modification of DNA by trans-EE (\( r_m = 0.1 \)) (Fig. 2B).

The effect of cis-EE on melting properties of DNA (Fig. 2A) is similar to that of cisplatin (31), suggesting that the replacement of NH_3 groups in cisplatin by iminoether ligands has no pronounced effect on its capability to affect DNA properties. On the other hand, the modification of DNA by trans-EE destabilizes DNA (Fig. 2B) more efficiently than transplatin or monofunctional dienPt (31), even when less efficient than cisplatin or cis-EE. These results are consistent with the view that a global modification by trans-EE induces

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in DNA conformational distortions different from those induced by other platinum complexes that we tested.

**Immunological analysis.** We prepared two types of the antibodies: one that binds specifically to DNA modified by cisplatin and its analogues (Abcis) and one that binds specifically to DNA modified by transplatin (Abtrans). Abcis recognized two neighboring purine residues of the same strand of DNA cis coordinated to the platinum atom of cis-[Pt(amine)₂]²⁺ moiety (20, 22). On the other hand, Abtrans recognized specifically a short single-stranded segment in double-stranded DNA containing the platinated site; this platinated site was either an intrastrand cross-link between two nonadjacent base residues in DNA trans coordinated to the platinum atom of trans-[Pt(amine)₂]²⁺ moiety or the platinum(II) atom coordinated in a monodentate manner to a base residue (21).

With the use of competitive ELISA, we measured the inhibition of the binding of Abcis or Abtrans to their immunogens (double-stranded calf thymus DNA modified by cisplatin or transplatin at an r₀ value of 0.08 for 48 hr, respectively) by double-stranded DNA modified by trans-EE at various r₀ values in the range of 0.005–0.1. Double-stranded DNA modified by trans-EE did not inhibit the binding of the Abcis or Abtrans (shown for r₀ = 0.02 in Fig. 3, A and B). Importantly, double-stranded DNA modified by cis-EE inhibited the binding of Abcis (Fig. 3A) but not that of Abtrans (Fig. 3B).

Abcis exhibit equally good specificity for DNA modified by several analogues of cisplatin with varied nonleaving amine groups, (20, 22). Consistent with this conclusion is the observation that double-stranded DNA modified by cis-EE is recognized by Abcis (Fig. 3B).

Interestingly, double-stranded DNA modified by trans-EE and subsequently thermally denatured (10 min at 100°C followed by quick cooling on an ice bath) or DNA that was thermally denatured before modification by trans-EE inhibited the binding of the Abtrans to the immunogen with a similar efficiency as DNA modified by transplatin under identical conditions (shown for r₀ = 0.02 in Fig. 3C). Abtrans specifically recognized nonpaired nucleotide residues or a short segment of single-stranded DNA that is formed in double-stranded DNA due to the monofunctional platination or intrastrand cross-link between nonadjacent base residues (21). We speculated that Abtrans also recognize DNA adducts formed by analogues of transplatin with varied nonleaving groups, similar to the recognition by Abcis of such analogues of cisplatin (including cis-EE). However, the recognition by Abtrans would only take place if the DNA adducts of trans compounds were accessible for interaction with the antibodies. In this way, some structural element or elements of DNA adducts of transplatin and trans-EE might be similar and recognizable by the Abtrans. It is possible that the adducts of trans-EE remain buried in the double helix, where they are not accessible for the antibodies, whereas the adducts of transplatin are more accessible because their formation is accompanied by denaturational changes in DNA (32, 35). Consistent with this suggestion is the observation that trans-EE adducts formed in double-helical DNA inhibit Abtrans only after the modified DNA is denatured (Fig. 3C). In other words, the results of our immunochemical analysis could also be interpreted to mean that modification of double-helical DNA by trans-EE does not result in denaturation alterations in DNA. The latter conclusion is consistent with the results of DPP analysis (Fig. 4). This behavior of trans-EE would be in contrast to the modification of DNA by clinically ineffective transplatin or monofunctional dienPt, both of which induce in DNA denaturation conformational alterations (29, 32, 33). In this respect, trans-EE would resemble antitumor cisplatin, which also induces local conformational changes of the nondenaturational character in DNA (5, 32, 35).

**DPP.** DPP analysis readily and with a great sensitivity distinguishes between nondenaturational and denaturational conformational alterations induced in DNA by various physical or chemical agents (34). This analysis is based on the observation that intact double-helical DNA is polaro-

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**Fig. 3.** Competitive inhibition in an ELISA of the binding of the antibodies elicited against double-helical DNA modified by cisplatin (Abcis) (A) or transplatin (Abtrans) (B and C) to the competitor, which was double-helical or denatured DNA platinated at an r₀ value of 0.02. A. The competitor was double-helical DNA modified by cisplatin (x), cis-EE (○), trans-EE (●), or transplatin (+). B. The competitor was double-helical DNA modified by cisplatin (+), cis-EE (○), trans-EE (●), or cisplatin (x). C. The competitor was either double-helical DNA modified by transplatin (+) or DNA thermally denatured and subsequently modified by transplatin (▲) or trans-EE (□). The inhibition expresses the antibody binding as percentage of binding in the absence of any competitor. Data represent mean (±2%) values from triplicate measurements.

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**Fig. 4.** DPP analysis of calf thymus DNA (modified by platinum complexes) at a concentration of 0.21 mg/ml in 0.3 M ammonium chloride with 0.01 M Tris-HCl buffer, pH 7.0. A. DPP curves; DNA modified by trans-EE at r₀ = 0 (control), curve 1; r₀ = 0.01, curve 2; r₀ = 0.03, curve 3; and r₀ = 0.05, curve 4. Arrows II and III, potentials at which native or denatured DNA samples yielded DPP peaks II or III, respectively (see the text). Curve 1, x indicates the height of DPP peaks. B. Dependence of the relative height of the DPP peak II (R) yielded by DNA modified by the platinum complexes on r₀, x, cisplatin; ○, cis-EE; and ●, trans-EE. The relative height of the DPP peak II was calculated as the ratio of the peak height yielded by the modified DNA over the peak height yielded by the control (nonmodified) DNA.
graphically inactive because its reduction sites are involved in hydrogen bonds and unable to make contact with the working electrode in a manner suitable for electron transfer. Electroreduction in adenine or cytosine residues present in distorted but still double-stranded (non-denatured) regions of DNA is responsible for the appearance of the small DPP peak II (Fig. 4A, curve 1). Base residues in these distorted regions become more accessible for electroreduction at the mercury electrode and can yield a small polarographic current. On the other hand, the appearance of a more negative peak III on DPP curves of DNA indicates the presence of single-stranded, denatured regions in the DNA molecule, in which hydrogen bonds between complementary bases have been broken (32, 35). Differences in the adsorption properties of double-helical and denatured DNA at the mercury electrode have been suggested to give rise to different reduction potentials that are observed for the two DNA conformations. Importantly, <1% denatured material in the excess of double-helical DNA can be determined by DPP (36).

DPP has been already used to analyze DNA modified by various physical or chemical agents, including platinum compounds, with different clinical efficacies. It has been found that DNA globally modified by antitumor cisplatin or its analogues at $r_b$ values of $\leq 0.05$ yields the DPP peak II, indicating that these antitumor drugs induce non-denaturational conformational changes in DNA (32, 35). In contrast, the more negative DPP peak III is noticed on DPP curves of DNA globally modified by clinically ineffective transplatin or monofunctional dienPt and other inactive platinum(II) complexes, indicating that the clinically ineffective platinum complexes induce denaturational conformational alterations in DNA (32, 35).

DPP analysis also sheds considerable light on the conformational basis for DNA binding of trans-EE (Fig. 4A). The modification of calf thymus DNA by trans-EE at an $r_b$ value of 0.01–0.05 resulted in an increase in the DPP peak II with growing level of the modification. The more negative peak III has not been detected even on the DPP curves recorded for DNA modified at the highest $r_b$ value used in our experiments (0.05) (Fig. 4A, curve 4). It could be argued that the absence of peak III on the DPP curves recorded for the samples of DNA modified at relatively high $r_b$ values (0.05) could be due to an increase in the slope of the part of the DPP curve corresponding to the background electrolyte discharge (Fig. 4A, curve 4). The fact that the peak III was not buried under the background electrolyte discharge curve was verified using the sample of DNA modified by trans-EE at an $r_b$ value of 0.05 (Fig. 4A, curve 4), to which 0.8% thermally denatured calf thymus DNA was added. This sample yielded a small, more negative peak III on the DPP curve (recorded under conditions specified for curve 4 in Fig. 4A), which was clearly observed (not shown). Thus, the absence of the peak III on the DPP curves of DNA modified by trans-EE (Fig. 4A) suggests that trans-EE, similar to antitumor cisplatin and other antitumor analogues of this drug, induces non-denaturational conformational distortions in DNA at relatively low levels of the global modification ($r_b < 0.05$). The DNA binding mode of trans-EE is also in sharp contrast to the modification of DNA by clinically ineffective transplatin or dienPt (32, 35). Importantly, the DPP behavior of DNA modified by cis-EE was similar to that of DNA modified by cisplatin (Fig. 4B); this result indicates that there is no substantial difference in the modification of DNA by cisplatin or cis-EE. On the other hand, the relative increase of the peak II due to the global modification by trans-EE was considerably smaller at the same level of the DNA platination ($r_b$) (Fig. 4B) than the increase of the peak II due to the modification by cisplatin (32). This finding supports the view that non-denaturational distortions of DNA due to the global binding of antitumor cisplatin or transplatin are not identical.

**Unwinding induced in DNA by trans-EE binding.**

Electrophoresis in native agarose gel was used to determine the unwinding induced in pSP73 plasmid by trans-EE and its cis isomer by monitoring the degree of supercoiling (23) (Fig. 5). A compound that unminds the DNA duplex reduces the number of supercoils so the superhelical density of closed circular DNA decreases. This decrease on binding of unwinding agents causes a decrease in the rate of migration through agarose gel, which makes it possible for the unwinding to be observed and quantified. Fig. 5 shows electrophoresis gels in which increasing amounts of trans-EE or cis-EE have been bound to a mixture of relaxed and supercoiled pSP73 DNA. The unwinding angle is given by $\Phi = 18 \pi r_b(c)$, where $\pi$ is the superhelical density and $r_b(c)$ is the value of $r_b$ at which the supercoiled and relaxed forms comigrate (23). Under the present experimental conditions, $\pi$ was calculated to be $-0.063$ on the basis of the data of cisplatin for which the $r_b(c)$ was determined in this study and $\Phi = 13^\circ$ was assumed (23, 37). Unwinding angles for the trans-EE and its cis isomer calculated in this way were 6$^\circ$ and 12$^\circ$, respectively. The unwinding angles for cisplatin, transplatin, and dienPt taken from the literature (23, 37, 38) are 13$^\circ$, 9$^\circ$, and 6$^\circ$, respectively. Thus, the order of the DNA unwinding ability is cisplatin = cis-EE > transplatin > dienPt = trans-EE.

Although the molecular details of the interactions of [PtCl$_2$(E-iminoether)$_2$] complexes with DNA described in this section are not fully understood, the information obtained from the unwinding experiments is valuable. The values of unwinding angles are affected by the nature of the ligands in the coordination sphere of platinum and the stereochemistry at the platinum center. A previous systematic work (23) revealed that the platinum(II) compounds fall into different classes according to their DNA binding modes. It has been shown that platinum(II) compounds with the smallest unwinding angles (6$^\circ$) are those that can bind DNA only mono-

![Fig. 5. Unwinding of supercoiled pSP73 plasmid DNA by trans-EE or cis-EE. Top bands, forms of nicked plasmid. Bottom bands, closed, negatively supercoiled plasmid. A, Plasmid was incubated with trans-EE with the $r_b$ values of 0 (control) (lanes 1 and 16) and 0.1, 0.12, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.20, 0.21, 0.23, 0.25, 0.27, and 0.29 (lanes 2–15, respectively). B, cis-EE was studied in the same way with $r_b$ values of 0 (control) (lanes 1 and 12) and 0.005, 0.02, 0.04, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, and 0.13 (lanes 2–11, respectively).](image)
functionally [dienPt or [Pt(NH₃)₃]Cl][Cl]. The observation that the complex trans-EE, which unwinds DNA by 6°, can be grouped with other monofunctional platinum(II) compounds is readily understood in terms of an adduct structure in which trans-EE is preferentially coordinated to DNA in a monodentate manner. This is in agreement with our recent finding (15) that trans-EE preferentially forms kinetically stable monofunctional adducts in double-helical DNA. In this configuration, the iminoether ligands in trans-EE have apparently little or no opportunity to interact with the double helix in the way that they would contribute to the unwinding induced by the compound.

Another group of platinum compounds are those that bind to DNA in a bifunctional manner. The principal compound of this category is cisplatin, which unwinds DNA by 13° (37, 38). The complex cis-EE unwinds DNA by 12°, which is a value very similar to that determined for DNA unwinding by cisplatin. This result can be interpreted to mean that the platinum atom in cis-EE has two available coordination sites in cis positions and is therefore expected to mimic cisplatin with respect to its coordination to DNA. It is therefore likely that in prevalent DNA adducts of cis-EE, its iminoether ligands have little opportunity for interaction with DNA that would result in additional unwinding.

**ICL.** The amount of ICLs formed by trans-EE or cis-EE in linear DNA was measured in pSP73 plasmid (2464 bp) that was first linearized by EcoRI (EcoRI cuts only once within pSP73 plasmid) and subsequently modified by trans-EE or cis-EE at various rₚ values. The samples were analyzed for ICLs by agarose gel electrophoresis under denaturing conditions.

An electrophoretic method for precise and quantitative determination of ICL by platinum complexes in DNA was previously described (24–26, 39). On electrophoresis under denaturing conditions, 3’-end-labeled strands of linearized pSP73 plasmid containing no ICLs migrate as a 2464-base single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species. The bands corresponding to more slowly migrating ICL fragments were noticed if trans-EE or cis-EE was used to modify linearized DNA at an rₚ value as low as 1 × 10⁻⁶ (shown for trans-EE in Fig. 6A). The intensity of the more slowly migrating band increased with the growing level of the modification. The radioactivity associated with the individual bands in each lane was measured to obtain estimates of the fraction of non-cross-linked or cross-linked DNA under each condition. The frequency of ICLs (amount of ICLs/one mole of drug) was then calculated using the Poisson distribution from the fraction of non-cross-linked DNA under each condition. The ratio of ICLs to total platinum bound was calculated as previously described (24); % ICL/Pt was then calculated by multiplying this ratio by 100.

**Fig. 6.** The formation of ICLs by trans-EE in linearized pSP73 plasmid (2464 bp). A, Autoradiograms of a denaturing 1% agarose gel electrophoresis of labeled pSP73 plasmid containing no ICLs (lanes 1 and 6) and 0.0002, 0.0005, 0.001, and 0.002 (lanes 2–5, respectively). B, Dependence on rₚ of the number of ICLs per adduct (% ICL/Pt) formed after 48 hr by trans-EE (○) or cis-EE (●) in linearized DNA. The ratio of ICLs to total platinum bound was calculated as previously described (24); % ICL/Pt was then calculated by multiplying this ratio by 100. C, Kinetics of the ICL by trans-EE in linearized DNA at an rₚ value of 0.002. The ICL reaction was stopped by adjusting the NaOH concentration to 30 mM and cooling the samples to −70°.

Agarose gel electrophoresis under denaturing conditions was also used to investigate the kinetics of ICL in linearized DNA by trans-EE or cis-EE at 37°. DNA was mixed with mononitrate derivative trans-[Pt(NO₃)₂Cl(E-iminoether)]_2 or cis-[Pt(NO₃)₂Cl(E-iminoether)]₂ at an rₚ value of 0.001 in 10 mM NaClO₄. The samples were withdrawn from the reaction mixture at various time intervals and immediately cooled at −70°. The rate of ICL formation was estimated from gel electrophoresis experiments as described above. The platinum-binding studies confirmed that monoaquamonochloro derivatives of trans-EE or cis-EE were bound to linearized DNA Modifications by trans-[PtCl₂(E-iminoether)]
DNA within <60 min. As shown in Fig. 6C, the DNA ICL by trans-EE in linear DNA was not complete after 48 hr of the ICL reaction. The $t_{1/2}$ of the ICL was $>18$ hr. In contrast, the DNA ICL by cis-EE was similar to that by cisplatin (i.e., $t_{1/2} \approx 4$ hr), and the ICL reaction was complete after 48 hr. Thus, the DNA ICLs of trans-EE are formed noticeably more slowly than the same lesions of cisplatin or cis-EE and transplatin ($t_{1/2} \approx 4$ or 11 hr for ICL by cisplatin or transplatin, respectively (26)).

Transcription inhibition experiments. Recent work has shown that the in vitro RNA synthesis by RNA polymerases on DNA templates containing several types of bidentate adducts of platinum complexes can be prematurely terminated at the level or in the proximity of adducts (25, 26, 29, 30, 40). Importantly, monofunctional DNA adducts of several platinum(II) complexes are unable to terminate RNA synthesis (25, 26).

The cutting of pSP73 DNA by NdeI and HpaI restriction endonucleases yielded a 221-bp fragment containing T7 RNA polymerase promoter in the upper strand close to the 3’-end of the fragment (25, 26). The experiments were carried out using this linear DNA fragment, modified by cisplatin, transplatin, trans-EE, or cis-EE at an $r_b$ value of 0.005, for RNA synthesis by T7 RNA polymerase (shown for cisplatin, transplatin, or trans-EE in Fig. 7, lanes cisDDP, transDDP, and transEE, respectively). RNA synthesis on these templates yielded fragments of defined sizes, which indicates that RNA synthesis on DNA modified by the four platinum complexes was prematurely terminated. The major stop sites observed for cis-EE (not shown) were mainly at guanine residues and identical to those produced by cisplatin (Fig. 7, lane cisDDP) (25, 26). In contrast, RNA synthesis on the template globally modified by transplatin was terminated less regularly (lane transDDP) (26).

The most striking feature of these transcription experiments is that the adducts in DNA globally modified by trans-EE prematurely terminate RNA synthesis at similar sites and with an efficiency similar to that of major DNA adducts of cisplatin (Fig. 7). The prevalent lesions formed on DNA by trans-EE are monofunctional adducts at guanine residues (15), and it has been shown (25, 26, 29) that monofunctional DNA adducts of other platinum(II) complexes (e.g., dienPt, cisplatin, transplatin) do not terminate RNA synthesis. In addition, it is generally accepted that monofunctional DNA adducts of cisplatin or transplatin are not relevant to the cytostatic effects of these metal complexes. Thus, the results of this study are consistent with the view that monofunctional DNA adducts of trans-EE on one hand and those of other platinum complexes on the other modify DNA conformation in a fundamentally different manner. Unique properties of monofunctional DNA adducts of trans-EE revealed in the transcription mapping experiments and the fact that trans-EE coordination to DNA exhibits a sequence preference similar to that exhibited by antitumor cisplatin (compare lanes transEE and cisDDP in Fig. 7) might be of fundamental importance in explaining the anticancer activity of this platinum(II) complex with leaving ligands in trans positions.

Conclusions. The extensive biochemical and biophysical analyses of DNA interactions with trans-EE that we describe provide further experimental support for the view that the binding of this platinum complex modifies DNA in a way that is different from the modification by clinically ineffective transplatin. Because DNA is a main pharmacological target of platinum(II) complexes, the latter view is also consistent with the hypothesis that radically altered DNA binding mode of trans-EE (in comparison with transplatin) is a very important factor that is responsible for its unexpected biological activity [i.e., this new platinum(II) complex with the leaving groups in trans positions exhibits antitumor activity].

The current results indicate that trans-EE modifies DNA conformation in a way that also differs from the modification by cis-dichloroplatinum(II) complexes with various inert amine ligands. Importantly, similar antitumor activities have been reported for the latter cis compounds. This observation was explained on the basis of the results indicating that all of the cis-dichloroplatinum(II) complexes make stereochemically similar adducts on DNA that modify DNA conformation in a similar manner. The view that the modification of the inert ammonia groups in cisplatin does not alter its DNA binding mode in a cell-free medium and, con-

Fig. 7. Autoradiogram of 6% polyacrylamide/8 M urea sequencing gel showing inhibition of RNA synthesis by T7 RNA polymerase on the NdeI/HpaI fragment containing adducts of trans-EE, cisplatin, or transplatin. Control, nonplatinated template; transEE, DNA modified by trans-EE at an $r_b$ value of 0.005; transDDP, DNA modified by transplatin at an $r_b$ value of 0.005; cisDDP, DNA modified by cisplatin at an $r_b$ value of 0.005; and C, G, U, and A, chain-terminated marker RNAs. Numbers, nucleotide numbering in the sequence map of pSP73 plasmid.
seqently, its antitumor activity is also supported by the results of this study. The results show similar DNA binding modes of cisplatin and its analogue cis-EE (Figs. 2 and 4), and as has been shown previously, cisplatin and cis-EE exhibit similar antitumor activities. Importantly, the antitumor efficacy of the trans-EE complex is not identical to that exhibited by the cis compounds (9, 14). Thus, the results are also consistent with the hypothesis that platinum drugs that bind to DNA in a manner fundamentally different from that of cisplatin can exhibit altered biological properties that include the spectrum and intensity of antitumor activity. Further studies are warranted to reveal other details of the contribution of DNA by trans-EE, probably relevant to its therapeutic properties. These studies will undoubtedly contribute to reevaluation of the structure-pharmacological relationship used in the search for new platinum cytostatics that would have distinct or more efficient anticancer activity than platinum drugs currently in clinical use.

References


