DNA adducts of antitumor trans-[PtCl₂ (E-imino ether)₂]

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Received September 1, 1995; Revised and Accepted November 29, 1995

ABSTRACT

It has been shown recently that some analogues of clinically ineffective trans-diaminedichloroplatinum(II) (transplatin) exhibit antitumor activity. This finding has inverted the empirical structure-antitumor activity relationships delineated for platinum(II) complexes, according to which only the cis geometry of leaving ligands in the bifunctional platinum complexes is therapeutically active. As a result, interactions of trans platinum compounds with DNA, which is the main pharmacological target of platinum anticancer drugs, are of great interest. The present paper describes the DNA binding of antitumor trans-[PtCl₂(E-imino ether)₂] complex (trans-EE) in a cell-free medium, which has been investigated using three experimental approaches. They involve thiourea as a probe of monofunctional DNA adducts of platinum(II) complexes with two leaving ligands in the trans configuration, ethidium bromide as a probe for distinguishing between monofunctional and bifunctional DNA adducts of platinum complexes and HPLC analysis of the platinated DNA enzymatically digested to nucleosides. The results show that bifunctional trans-EE preferentially forms monofunctional adducts at guanine residues in double-helical DNA even when DNA is incubated with the platinum complex for a relatively long time (48 h at 37°C in 10 mM NaClO₄). It implies that antitumor trans-EE modifies DNA in a different way than clinically ineffective transplatin, which forms prevalent amount of bifunctional DNA adducts after 48 h. This result has been interpreted to mean that the major adduct of trans-EE, occurring in DNA even after long reaction times, is a monofunctional adduct in which the reactivity of the second leaving group is markedly reduced. It has been suggested that the different properties of the adducts formed on DNA by transplatin and trans-EE are relevant to their distinct clinical efficacy.

INTRODUCTION

The standard cis/trans structure-activity relationship of platinum antitumor complexes, exemplified by cis-diaminedichloroplatinum(II) (cisplatin), is that only cis geometry is therapeutically active. The observed inactivity of the trans isomer, trans-diaminedichloroplatinum(II) (transplatin), has had a major influence on both the synthesis of new platinum antitumor agents and the mechanistic interpretation of the antitumor activity of cisplatin. It is generally accepted that cisplatin manifests its biological activity through coordination to DNA (1–3). Possible explanations for the different biological activity of the cis and trans isomers are that cis compounds form platinum–DNA adducts which inhibit DNA replication or transcription to a greater extent than those formed by transplatin (5,6), and alternatively, that DNA adducts formed by trans complexes may be repaired more efficiently (7).

The nature of the DNA adducts formed by cisplatin and its trans isomer has also been explored to explain the differences in biological activity between the two isomers. Cisplatin produces a range of adducts on DNA including bidentate intrastrand cross-links such as 1,2-GG or AG and 1,3-GNG (1–3,8,9) and interstrand cross-links at the GC sequence (10). These lesions result in conformational alterations in DNA and represent blocks to DNA and RNA polymerases (10–13).

Transplatin shows a distinct sequence preference upon binding to DNA, different from that of cisplatin (11,14). Transplatin also produces bidentate intrastrand cross-links, such as 1,3-GNG and interstrand cross-links between complementary guanine and cytosine residues (8,14–16). These lesions of transplatin, however, induce in DNA different conformational alterations than cisplatin (1–4,17,18).

It has been reported in recent contributions that trans-[PtCl₂(E-imino ether)₂] complex (trans-EE) (imino ether=HN=C(OCH₃)-CH₃; it can have either E or Z configuration depending on the relative position of OCH₃ and N-bonded Pt with respect to the C=N double bond, cis in the Z isomer and trans in the E isomer) is not only more cytotoxic than its congeners cis-[PtCl₂(E-imino ether)₂] (cis-EE), but is also endowed with significant antitumor...
activity (19, 20). These results strongly imply a new mechanism of action for trans-EE. By analogy with the diaminedichloroplatinum(II) isomers, the inhibition of DNA synthesis by cis-EE and trans-EE (19) implies a role for DNA binding in the mechanism of action. The presence of the imino ether group may result in altered hydrogen bonding and steric effects affecting the kinetics of DNA binding, the structures and/or stability of the adducts formed and resulting local conformational alterations in DNA.

The breaking of the paradigm for structure–activity relationships of platinum antitumor complexes poses new fundamental questions on the mechanism of antitumor activity of platinum complexes. To explain the cytotoxicity of imino ether derivatives, it could be important to examine in detail the DNA binding of the new complexes and compare these results with those previously obtained for other diaminedichloroplatinum isomers. Thus, these results could lead to the development of new strategies for the systematic design of platinum antitumor complexes acting by mechanisms different from the presently used agents, and eventually having a different clinical profile of antitumor activity.

This paper describes the results of studies of DNA binding of trans-EE with respect to characterization of preferential binding sites in double-helical DNA and the types of the resulting adducts.

MATERIALS AND METHODS

Starting materials

Cisplatin, transplatin and chlorodiethylenetriamineplatinum(II) chloride, [Pt(dien)Cl]Cl, were from Lachema (Brno, Czech Republic). cis-EE and trans-EE were synthesized as previously described (21). Calf thymus DNA (the content of guanine + cytosine is 42%) was purchased from Sigma and used without further purification. Deoxyriboguanosine (dGuo), DNase I from bovine pancreas, nuclease P1 from Penicillium citrinum and alkaline phosphatase from calf intestine were also from Sigma. Thiourea and ethidium bromide (EtBr) were from Merck. Thiourea ($^3$H) was from Amersham.

Preparation of HPLC standards

The standards were prepared by reaction of dGuo with the mononitratomonochloro and dinitrato platinum-iminoether species. Mononitrato platinum-iminoether derivative, trans-[PtCl(NO$_3$)$_2$(E-imino ether)$_2$] was prepared by dissolving 169 mg (0.41 mmol) of trans-EE in 40 ml aceton and treating it with an equivalent amount of AgNO$_3$ (70 mg, 0.41 mmol). After stirring in the dark for 1 h, the reaction mixture was filtered through celite, the solution was dried, the solid residue extracted with ethyl ether and the solution again filtered. By evaporation of the solvent a solid of the desired compound was obtained in ~60% yield. Anal. Calcd for C$_{6}$H$_{14}$N$_{4}$O$_8$Pt: C, 15.5; H, 3.0; N, 12.0%. Found: C, 15.4; H, 3.0; N, 12.0%.

Preparation of trans-[PtCl(dGuo)(E-imino ether)$_2$](NO$_3$)$_2$ was done by dissolving 0.043 g (0.1 mmol) of trans-[PtCl(NO$_3$)$_2$(E-imino ether)$_2$] in 50 ml of water to which a stoichiometric amount of dGuo (26 mg, 0.1 mmol) was added. The solution was stirred at room temperature in the dark for 2 days. After this time the solvent was evaporated under a reduced pressure and trans-[PtCl(dGuo)(E-imino ether)$_2$](NO$_3$)$_2$ was recovered quantitatively. Anal. Calcd for C$_{6}$H$_{12}$N$_{8}$O$_{18}$Pt.2H$_2$O: C, 25.9; H, 3.9; N, 15.1%. Found: C, 25.9; H, 3.8; N, 14.6%. trans-[Pt(dGuo)$_2$(E-imino ether)$_2$](NO$_3$)$_2$ was prepared by suspending 0.015 g (0.032 mmol) of trans-[Pt(NO$_3$)$_2$(E-imino ether)$_2$] in 20 ml of water to which 2 eq of dGuo (17 mg, 0.064 mmol) were added. The suspension was stirred at room temperature in the dark for 2 days. After this time a clear solution was obtained, the solvent was evaporated under a reduced pressure and trans-[Pt(dGuo)$_2$(E-imino ether)$_2$](NO$_3$)$_2$ was recovered quantitatively. Anal. Calcd for C$_{26}$H$_{40}$N$_{14}$O$_{18}$Pt.2H$_2$O: C, 29.2; H, 4.1; N, 18.4%. Found: C, 29.8; H, 4.2; N, 18.5%.

Analytical and physico-chemical methods

Ultraviolet spectra were collected on a Beckmann DU-8 spectrophotometer. Fluorescence measurements of DNA modified by platinum in the presence of EtBr were performed using a Perkin-Elmer LS 5B spectrophotometer. The excitation wavelength was 546 nm and the emitted fluorescence was measured at 590 nm. The fluorescence was measured at 25°C in 0.4 M NaCl to avoid the second fixation site of EtBr to DNA (22). The concentrations were 0.01 mg/ml for DNA and 0.04 mg/ml for EtBr, which corresponded to the saturation of all intercalation sites of EtBr in DNA (22, 23). Flameless atomic absorption spectroscopy (FAAS) measurements were carried out on a Perkin-Elmer 560 instrument with a graphite furnace. For FAAS analysis, DNA was precipitated with ethanol and dissolved in 0.1 M HNO$_3$. High-pressure liquid chromatographic (HPLC) analyses were performed by using a Perkin-Elmer Series 4 liquid chromatograph equipped with a LCI-100 computing integrator and a Waters µBondapak C$_{18}$ column. Gradient was 0–60% methanol in 0.02 M ammonium acetate, pH 5.5; flow rate was 1 ml/min. Spectra ($^3$H NMR) were obtained with a Bruker AM 300 spectrometer. pH measurements were performed with a CRISON microPH 2002 apparatus.

Modification of DNA by platinum complexes

If not stated otherwise, the platination reactions were performed in 10 mM NaClO$_4$ at 37°C in the dark. If required, the reactions were terminated by adding thiourea to 10 mM and incubating at 37°C for 10 min. The ratio of platinum atoms fixed per nucleotide residue ($n_t$) was determined by FAAS. Enzymatic digestions of DNA modified by platinum were carried out by using DNase I, nuclease P1, and alkaline phosphatase (8). In a typical experiment, samples (45 µg of DNA) were allowed to react with 72 U of DNase I at 37°C. After 4 h nuclease P1 (40 µg) was added, and the reaction was allowed to continue at 37°C for 18 h. Finally, alkaline phosphatase (39 U) was added and the incubation continued for additional 4 h at 37°C. The samples were then heated for 2 min at 80°C, centrifuged and the supernatant analyzed by HPLC.
Figure 1. Kinetics of reaction of transplatin (A,C) and trans-EE (B,D) with double-stranded DNA (A,B) and thermally denatured DNA (C,D) at r_i = 0.05 in 10 mM NaClO_4 at 37°C. DNA concentration was 0.15 mg/ml. Reactions were stopped with (□) or without (▲) 10 mM thiourea (10 min), and Pt associated with DNA was assessed by FAAS.

Tritiated thiourea binding assay

Calf thymus DNA (double-stranded or thermally denatured) at the concentration of 0.16 mg/ml was incubated with trans-EE at various r_i values (r_i is defined as the molar ratio of free metal complex to nucleotide-phosphates at the onset of incubation). At each concentration of trans-EE, the aliquots were withdrawn at 10 min, 2, 24 and 48 h time intervals. After the withdrawal, the unbound platinum was immediately removed by a centrifugation (1500 r.p.m., 30 s) through a column of Sephadex G25 (coarse). The r_i values were chosen in the way that the values of r_i, determined after this separation step by FAAS were 0.01, 0.05 and 0.2 for each incubation time. Of these solutions, 0.15 ml was added to 0.15 ml of 0.9 mM 3H labeled thiourea, prepared as described previously (24), having specific radioactivity of 77 MBq/mmol. After 10 min incubation at 37°C, 0.8 ml of 0.15 M NaCl, pH 7.0 was added and 1.0 ml of the resulting solution was layered on a nitrocellulose filter having pores of 0.4 mm in diameter (Synpor, VCHZ Syntezia, Pardubice). In order to remove the unreacted thiourea the filter was washed with 15 ml of 5% trichloroacetic acid. The filters were dried under an infrared lamp, transferred to glass tubes to which 5 ml of toluene scintillator was added. The radioactivity was measured on a LKB Wallac1410 Betaspectrometer (Finland).

RESULTS AND DISCUSSION

Characterization of DNA adducts of trans-EE by thiourea

Cisplatin, transplatin and analogous bifunctional platinum compounds bind to DNA in a two-step process, forming first monofunctional adducts, preferentially at guanine residues, which subsequently close to bifunctional lesions (1–4,8,9). Thus, monofunctional adducts are formed in DNA at an early stage of the reaction. Thiourea is successfully used to labilize monofunctional transplatin coordination to DNA (25). The displacement of transplatin takes place via coordination of thiourea trans to the base residue. Because of the strong trans effect of sulphur, the nitrogen–platinum bond is weakened so that it becomes susceptible to substitution. Importantly, whereas thiourea effectively trans labilizes transplatin in monofunctional DNA adducts, bifunctional adducts of this platinum complex are resistant to the thiourea treatment (25).

The initial experiment aimed at characterization of DNA adducts of trans-EE was conducted employing thiourea as a probe of DNA monofunctional adducts of trans-dichloroplatinum(II) complexes. Double-stranded and thermally denatured DNAs were incubated with transplatin or trans-EE at a formal drug to nucleotide ratio r_i = 0.05. At various times the reaction was stopped by ethanol precipitation of the DNA. In parallel tubes, the reactions were stopped by addition of thiourea to 10 mM. These samples were incubated for 10 min at 37°C and then precipitated by ethanol. The DNA was redissolved, and the platinum content was determined by FAAS (Fig. 1).

The modification of double-helical or thermally denatured DNA by transplatin was maximal after ~8 h whereas the modification by trans-EE was completed in ~10 h (Fig. 1B and D). In accordance with the previous results (25), thiourea displaced ~90% transplatin from double-helical DNA at early time intervals (1–5 h) (Fig. 1A). At longer incubation times (24–48 h), thiourea was less efficient in removing transplatin from DNA since at these time intervals a large fraction of monofunctional adducts of transplatin had closed to a bifunctional lesion resistant to the treatment with thiourea (25). Thus, after 48 h of the reaction only ~35% transplatin was displaced from double-stranded DNA, which indicates that ~65% monofunctional adducts had evolved to
bifunctional lesions. In a sharp contrast to the behaviour of transplatin, thiourea displaced only 10–20% of DNA-bound trans-EE independently of the length of the platination reaction (Fig. 1B). These observations indicate that as compared with transplatin, trans-EE forms on double-helical DNA the adducts, which are resistant to the thiourea treatment already at early time intervals. Interestingly, if denatured instead of double-helical DNA was modified by transplatin, ~10–50% adducts were removed by thiourea at early time intervals of the platination reaction (Fig. 1C). If denatured DNA was modified by trans-EE, 10–20% platinum was only displaced by thiourea and independently of the time of incubation of this platinum complex with DNA (Fig. 1D). Thus, thiourea was only slightly effective in displacing trans-EE adducts from DNA independently of its secondary structure. It was verified that 5–60 min incubations with 10 mM thiourea gave the same results as are those shown in Figure 1.

It is reasonable to suggest that the resistance of DNA adducts of trans-EE to the treatment with thiourea is either due to the formation of an extensive amount of bifunctional adducts already at early time intervals or, alternatively, that trans-EE forms on DNA monofunctional adducts different from those formed by transplatin. This new type of monofunctional adduct either would not bind readily thiourea or, although binding thiourea, would be resistant to the trans labilizing effect of the sulphur donor ligand. The latter eventuality was tested with the titrated thiourea binding assay. No radioactivity associated with double-stranded or denatured DNAs, both modified by trans-EE ($n_0 = 0.01–0.2$), was detected after their treatment with titrated thiourea. Thus, these results are consistent with the idea that trans-EE forms on DNA preferentially either monofunctional adducts which, in contrast to the same type of DNA adducts of transplatin, do not readily bind thiourea, or forms bifunctional adducts already at early times of the reaction of trans-EE with DNA. Interestingly, independent $^1$H NMR experiments performed on trans-[PtCl(dGMP)(E-imino ether)$_2$] and trans-[Pt(dGMP)$_2$(E-imino ether)$_2$] complexes have shown that thiourea (10 mM) reacts with the former complex replacing chloride ion and dGMP (the reaction is complete in <10 min at 30°C) while, under similar reaction conditions, it leaves the latter complex unaltered.

Characterization of DNA adducts of cis- and trans-EE by EtBr fluorescence

In order to differentiate between the latter two eventualities we have employed EtBr as a fluorescent probe. This probe can be used to distinguish between perturbations induced in DNA by monofunctional and bifunctional adducts of platinum compounds (22,23). Binding of EtBr to DNA by intercalation is blocked in a stoichiometric manner by formation of the bifunctional adducts of a series of platinum complexes including cisplatin and transplatin, which results in a loss of fluorescence intensity (22). On the other hand, modification of DNA by monodentate platinum complexes (having only one leaving ligand) results in only a slight decrease of EtBr fluorescence intensity as compared with non-platinated DNA–EtBr complex.

Double-helical DNA was first modified by cisplatin, transplatin, cis-EE, trans-EE and by monodentate [Pt(dien)Cl]Cl for 48 h. The levels of the modification corresponded to the values of $n_0$ in the range between 0 and 0.1. Modification of DNA by all platinum complexes resulted in a decrease of EtBr fluorescence (Fig. 2). In accordance with the results published earlier (22,23), monodentate [Pt(dien)Cl]Cl decreased the fluorescence only to a small extent, whereas the decrease induced by the DNA adducts of cisplatin, transplatin and cis-EE was significantly more pronounced. The decrease induced by the adducts of trans-EE was markedly less pronounced than that induced by the DNA adducts of other bidentate complexes tested in this work. In fact, the fluorescence intensity was only slightly lower than the fluorescence intensity of DNA modified by the monodentate [Pt(dien)Cl]Cl. This result suggests that trans-EE complex forms the DNA adducts which resemble, from the viewpoint of their capability to inhibit EtBr fluorescence, those formed by monofunctional platinum complexes. Importantly, the DNA adducts of cis-EE inhibited EtBr fluorescence almost to the same extent as cisplatin. Taken together, the fluorescent analysis is consistent with the idea and supports the postulate that the major DNA adducts of trans-EE are monofunctional lesions even after long incubations of DNA with this platinum complex (48 h). On the other hand, under comparable conditions cis-EE forms on DNA mainly bifunctional adducts similar to those formed by cisplatin.

Characterization of DNA adducts of trans-EE by HPLC analysis of enzymatically digested DNA

To characterize further the coordination mode of trans-EE, DNA modified by this platinum complex at $n_0$ of 0.08 for 48 h was enzymatically digested to mononucleosides and analyzed by reversed-phase HPLC. Cisplatin or transplatin exhibit a strong preference for binding to guanine residues in DNA (they also bind in a much smaller extent to other base residues) (1–4,8,9). Therefore, we first characterized the products of the reactions of trans-EE with monomeric dGuo by NMR spectroscopy and then used these products as HPLC standards. The $^1$H NMR spectral data are shown in Table 1. In the case of both standards containing trans-EE coordinated to one or two dGuo molecules, trans-[PtCl(dGuo)(E-imino ether)$_2$]$^+$ or trans-[Pt(dGuo)$_2$(E-imino ether)$_2$]$^{2+}$, respectively, a major shift was noticed for the H8 protons (0.4–0.6 p.p.m. downfield), which is typical of the N7 coordinated guanines (26,27). The N7 coordination was also supported by the pH dependence of chemical shifts (Fig. 3) and platinum coupling constants of H8 proton (30 and 26 Hz for the mono- and bis-dGuo complexes, respectively).
Table 1. Proton chemical shifts (δ, downfield from trimethylsililpropionic acid, TSP) in D$_2$O (pH 7.4) of deoxyriboguanosine (dGuo), trans-[PtCl(NO$_3$)$_2$(E-imino ether)$_2$], trans-[Pt(NO$_3$)$_2$(E-imino ether)$_2$] and the complexes of these platinum compounds with dGuo

<table>
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<tr>
<th>Compound</th>
<th>H8</th>
<th>C1'H</th>
<th>C2'H$_2$</th>
<th>C3'H$_2$</th>
<th>C4'H$_2$</th>
<th>C5'H$_2$</th>
<th>O-CH$_3$</th>
<th>C-CH$_3$</th>
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<tr>
<td>dGuo</td>
<td>7.98</td>
<td>6.30</td>
<td>2.79</td>
<td>4.62</td>
<td>4.13</td>
<td>3.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>trans-<a href="NO$_3$">Pt(dGuo)$_2$(E-imino ether)$_2$</a>$_2$</td>
<td>8.57</td>
<td>6.35</td>
<td>2.78</td>
<td>4.64</td>
<td>4.16</td>
<td>3.81</td>
<td>3.75</td>
<td>2.13</td>
</tr>
<tr>
<td>trans-<a href="NO$_3$">PtCl(dGuo)(E-imino ether)$_2$</a></td>
<td>8.39</td>
<td>6.32</td>
<td>2.75</td>
<td>4.63</td>
<td>4.15</td>
<td>3.82</td>
<td>3.77</td>
<td>2.44</td>
</tr>
<tr>
<td>trans-[Pt(NO$_3$)$_2$(E-imino ether)$_2$]$^a$</td>
<td>3.87</td>
<td></td>
<td>2.66</td>
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<td></td>
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<tr>
<td>trans-[PtCl(NO$_3$)(E-imino ether)$_2$]$^a$</td>
<td>3.84</td>
<td></td>
<td>2.63</td>
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$^a$In aqueous solution a rapid displacement of coordinated nitrate ions by solvent molecules takes place.

CONCLUSIONS

The purpose of this study was to examine the effect of substitution of imino ether group for NH$_3$ in dichloroplatinum(II) complexes on their DNA-binding properties. The results presented here show that imino ether ligands do not alter radically the first step of the binding of bifunctional platinum complexes to DNA, i.e., the formation of monofunctional adducts at N7 position of guanine residues. Importantly, the imino ether substitution, especially in the trans geometry, results in a greatly reduced rate of closure of the monofunctional platinum lesions in bifunctional adducts. This result implies an important difference in the nature and frequency of the DNA adducts of trans-EE and clinically ineffective transplatin.

Recently, some trans-[PtCl$_2$(amine)$_2$] complexes containing sterically hindered planar ligands instead of simple NH$_3$ groups were reported (28,29). These complexes exhibit greatly enhanced cytotoxicity in tumor cells in comparison with transplatin and in several cases their cytotoxicity was equivalent to that of the clinically used cisplatin. These results along with those obtained for the imino ether derivatives invert the standard cis/trans...
structure–pharmacological activity relationships observed previously for [PtCl₂(NH₃)₂] complexes. The presence of bulky planar amine ligands has been found to enhance strongly DNA interstrand cross-linking capability of the complexes with trans geometry. It has been suggested that this enhanced interstrand cross-linking efficiency of trans-[PtCl₂(amine)₂] complexes along with specific conformational changes in DNA could be relevant to their enhanced cytotoxicity in tumor cells.

The trans-EE complex also exhibits cytotoxicity in tumor cells which was much more pronounced than that of its cis congener. However, in contrast with the trans complexes of planar amine ligands and transplatin, trans-EE appears to form markedly lower amount of bifunctional DNA adducts. Thus, an important feature for biological activity of trans-EE is its capability to form in DNA relatively stable monofunctional adducts. Further investigations of conformational alterations induced in DNA by trans-EE are warranted to unravel the origin of antitumor activity of platinum complexes with leaving ligands in the trans configuration.

ACKNOWLEDGEMENTS

This work was supported in part by the Internal Grant Agency of the Academy of Sciences of the Czech Republic (grant nos 504406 and 404101), Grant Agency of the Czech Republic (grant no. 203/93/0092) and the Internal Grant Agency of the Ministry of Health of the Czech Republic (grant no. 1893-3). This joint research is also a part of the European Cooperation in the field of Scientific and Technical Research (COST) network (COST projects D1/0002/92 and D1/0001/95). One of the authors (V.B.) is grateful to the Consiglio Nazionale delle Ricerche in Italy for the NATO Guest Fellowship.

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