Journal of Inorganic Biochemistry 126 (2013) 46–54

Contents lists available at SciVerse ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Activation of trans geometry in bifunctional mononuclear platinum complexes by a non-bulky methylamine ligand

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ARTICLE INFO

Article history:
Received 19 February 2013
Received in revised form 23 May 2013
Accepted 23 May 2013
Available online 28 May 2013

Keywords:
Platinum(II)-based agents
Activation of trans geometry
Cytotoxicity
DNA adducts

ABSTRACT

In order to shed light on the mechanism that underlies activity of bifunctional mononuclear Pt^{II} analogs of transplatin we examined in the present work a DNA binding mode of the analog of transplatin, namely *trans*-[$Pt(CH_3NH_2)_2Cl_2$], in which NH_3 groups were replaced only by a small, non-bulky methylamine ligand. This choice was made because we were interested to reveal the role of the bulkiness of the amines used to substitute NH_3 in transplatin to produce antitumor-active Pt^{II} drug. The results indicate that *trans*-[$Pt(CH_3NH_2)_2Cl_2$] forms a markedly higher amount of more distorting intrastrand cross-links than transplatin which forms in DNA preferentially less distorting and persisting monofunctional adducts. Also importantly, the accumulation of *trans*-[$Pt(CH_3NH_2)_2Cl_2$] in tumor cells was considerably greater than that of transplatin and cisplatin. In addition, the results of the present work demonstrate that the replacement of ammine groups by the non-bulky methylamine ligand in the molecule of ineffective transplatin results in a radical enhancement of its activity in tumor cell lines including cisplatin-resistant tumor cells. Thus, activation of the trans geometry in bifunctional mononuclear Pt^{II} complexes can be also accomplished by replacement of ammine groups in transplatin by non-bulky methylamine ligands so that it is not limited only to the replacement by relatively bulky and stereochemically more demanding amino ligands.

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1. Introduction

The platinum anticancer compounds currently in clinical use conform to the originally devised structure–pharmacological activity relationship delineated for platinum anticancer drugs soon after discovery of antitumor effects of cisplatin [1,2]. The paradigm for this original structure-pharmacological activity relationship of platinum complexes [3] was that the trans isomer of cisplatin (transplatin) was inactive at biologically relevant concentrations and that only neutral Pt^{II} complexes with two cis oriented inert ligands [such as am(m)ines carrying at least one H atom] and semi-labile ligands in the other positions would possess anticancer activity. Trans compounds and complexes having only one leaving group were considered inactive.

However, the search for platinum compounds having novel pre-clinical properties, such as activity in cisplatin-resistant cells or a pattern of cytotoxicity significantly different from that of cisplatin [4] casted doubts upon most of these early structure-activity rules. Several diverse classes of active platinum compounds that violate the classical structure–activity relationships for cisplatin have been identified, including Pt^{IV} complexes [5], polynuclear platinum complexes [6], and platinum compounds with a trans stereochemistry (for reviews, see Refs. [7–10]). Thus, to this end several new complexes of the trans structure have been identified that exhibit an enhanced toxicity in tumor cell lines, such that cytotoxicity is equivalent or even better than that of the analogous cis counterparts and, indeed, cisplatin itself (for reviews, see Refs. [7–12]). Examples of such new antitumor *trans*-platinum compounds are: (i) those of general formula *trans*-[$PtCl_2(L)(L')$] in which L and L′ represent planar or nonplanar heterocyclic ligands, aliphatic amines, heterocyclic aliphatic amines, iminoethers [7–10], and acetoxime [13]; (ii) *trans*-platinum(IV) complexes of general formula *trans*-$PtCl_2X_2(L)(L')$, where X represents chloride, hydroxide, carboxylate, or carbamate ligands, belong to the family of anticancer *trans*-platinum agents as well. Pt^{IV} compounds serve as prodrugs being reduced to the Pt^{II} analogs under physiological conditions (axial ligands of Pt^{IV} compounds can be detached during cellular activation by reductive elimination yielding antitumor active Pt^{II} analogs) [14]; (iii) bifunctional polynuclear Pt^{II} complexes with up to four positive charges [6] in which two monofunctional Pt^{II} spheres with the single chloride leaving group on each platinum are linked by

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http://dx.doi.org/10.1016/j.jinorgbio.2013.05.009

various bridging diamine ligands and the leaving chloride ligands are trans to the linker. Most antitumor *trans*-compounds display cytotoxicity in the micromolar (1–20 μM) range and the compounds consistently display cytotoxicity comparable or better than their cis oriented counterparts and in cisplatin-resistant cells [9].

DNA is the established primary target of platinum compounds in cells, making DNA adduct formation one of the key determinants of platinum-mediated cytotoxicity [3,15–17]. Thus, it appears important that due to this substitution, the analogs of transplatin altered the properties of DNA in a markedly different way than the parent compound. This is an intriguing finding, because the same replacement in the molecule of antitumor cisplatin often resulted in a reduced activity of the drug in both sensitive and resistant cell lines [18]. Thus, the latter observations represent an additional example of activation of trans geometry in bifunctional mononuclear Pt^{II} compounds, although the reasons for this activation have not been completely clarified. Mechanistic studies performed on analogs of transplatin suggest that one strategy to activate trans geometry in antitumor bifunctional Pt^{II} compounds is to chemically modify the ineffective transplatin, in a manner that will enhance formation of stable bifunctional adducts with the DNA. In general, bifunctional mononuclear Pt^{II} compounds may form in DNA intra- or interstrand cross-links and monofunctional adducts. Cisplatin forms a variety of adducts of which the 1,2-intrastrand cross-links between neighboring GG or AG residues predominate [19]. Other lesions including 1,3-GNG intrastrand and to a lesser extent the interstrand cross-link formed between G residues in the 5′-GC/5′-GC [20] sequences and monofunctional adducts represent minor DNA lesions of cisplatin. In contrast, geometric constraints prevent the formation of 1,2 cross-links by transplatin—instead 1,3-intrastrand cross-links are formed [21,22] which represent along with monofunctional adducts [23] major DNA lesions formed by transplatin. Minor interstrand cross-links of transplatin (~12%) are preferentially formed between G and C residues of the same base pair [24].

The slow rate of conversion of monofunctional to bifunctional lesions may account for the transplatin antitumor inactivity as well [16,23]. However, frequency of transplatin-like DNA adducts of antitumor-active analogs of transplatin and DNA sequence specificity of their formation are considerably different from that of transplatin [9], which may also be a factor contributing to improved toxicity of the transplatin analogs in tumor cells.

A common feature of amines used to substitute NH_3 in transplatin yielding its analogs exhibiting significantly improved toxicity in tumor cells is that these amines are also markedly bulkier than NH_3. Therefore, in order to shed light on the mechanism that underlies activity of bifunctional mononuclear Pt^{II} analogs of transplatin we examined in the present work DNA binding mode of the analog of transplatin, namely *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ (Fig. 1), in which NH_3 groups were replaced only by a small, non-bulky methylamine ligand. It is apparent that the bulkiness of the amine ligands in this new Pt^{II} complex is increased very little by replacement of one hydrogen atom in NH_3 groups by a methyl group in comparison with the bulkiness of the amines in the antitumor analogs of transplatin hitherto tested (such as pyridine-like ligands, iminoethers, cyclohexylamine, ramified aliphatic amines). This choice was made because we were interested to reveal the role of the bulkiness of the amines used to substitute NH_3 in transplatin to produce antitumor-active Pt^{II} drug. We also examined whether DNA binding modes of *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ and transplatin correlate with their toxicity in the cisplatin sensitive- and resistant tumor cell line.

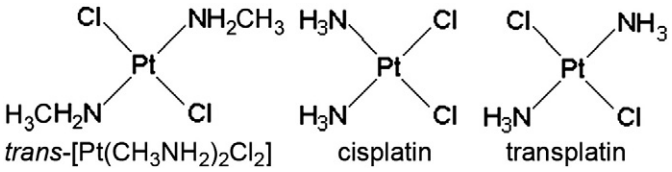

Fig. 1. Schematic representation of the Pt^{II} complexes used in the present work.

2. Experimental

2.1. Starting materials and reagents

Pt^{II} complex *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ (Fig. 1) was synthesized and characterized using standard synthetic methods. In brief, potassium tetrachloroplatinate was converted to the tetraiodoplatinate with excess KI and was allowed to react with two equivalents of methylamine to form *cis*-$[PtI_2(CH_3NH_2)_2]$. *Cis*-$[PtI_2(CH_3NH_2)_2]$ was reacted with 2 equivalents of $AgNO_3$ and subsequently with an excess of methylamine to yield $[Pt(CH_3NH_2)_4]^{2+}$ which was refluxed in aqueous HCl from which *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ precipitated and was characterize by ^{195}Pt NMR $\delta = -2200$ ppm. Thus, the synthetic route for obtaining *trans*-$[Pt(CH_3NH_2)_2Cl_2]$, where first the tetraamine complex is prepared and characterized by ^{195}Pt NMR and then is reacted with HCl, yields exclusively the trans isomer. To ensure that we had pure trans isomer we collected the ^{195}Pt NMR spectrum for a very long time obtaining sufficient signal to noise ratio to observe sharp peaks with low line broadening and observed a single peak. There is a small difference between the cis and trans isomers in the ^{195}Pt NMR spectrum that can be detected when the spectrum is measured carefully. Moreover, we carried out the Kurnakov test [25] with an excess of thiourea that distinguishes between cis and trans isomers and the presence of cis isomer has not been observed. Cisplatin and transplatin were obtained from Sigma-Aldrich s.r.o. (Prague, Czech Republic). Chloridodiethylenetriamineplatinum(II) chloride ([PtCl(dien)]Cl) was a generous gift from Prof. G. Natile (University of Bari, Italy). The purity of platinum complexes was higher than 95% as established by combustion analysis carried out with a Hewlett-Packard 185 C, H, and N analyzer. The stock solutions of the platinum complexes were prepared in $NaClO_4$ (10 mM) and kept in the dark at 4 °C. The concentrations of platinum in the stock solutions and after dilution by water were determined by flameless atomic absorption spectrometry (FAAS). Calf thymus (CT) DNA (42% G + C, mean molecular mass approximately 20,000 kDa) was prepared and characterized as described previously [26,27]. Plasmids pUC19 [2686 base pairs (bp)] and pSP73KB (2455 bp) were isolated according to standard procedure. Restriction endonucleases, the Klenow fragment from DNA polymerase I and plasmid pBR322 (4361 bp) were purchased from New England Biolabs. T7, and SP6 RNA polymerases and RNasin ribonuclease inhibitor were purchased from Promega (Mannheim, Germany). Ribonucleotide and deoxyribonucleotide triphosphates were from Roche Diagnostics, GmbH (Mannheim, Germany). Ethidium bromide (EtBr) and thiourea were from Merck (Darmstadt, Germany). Agarose was from FMC BioProducts (Rockland, ME, USA). Radioactive products were from MP Biomedicals (Irvine, CA, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was from Calbiochem (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum (FBS), trypsin/EDTA and DMEM medium were from PAA (Pasching, Austria). Gentamycin was from Serva (Heidelberg, Germany). The cell-free extract (CFE) was prepared from the repair-proficient CHO-K1 cell line as reported previously [28].

2.2. DNA platination in cell free media

If not stated otherwise, CT or plasmid DNAs were incubated with platinum complexes in $NaClO_4$ (10 mM) at 37 °C in the dark. After 24 h, the samples were exhaustively dialyzed first against 1 M NaCl and then against the medium required for subsequent biochemical or biophysical analysis. Aliquots of these samples were used to determine the value of r_b (r_b is defined as the number of molecules of the platinum complex bound per nucleotide residue) by FAAS.

2.3. DNA melting

The melting curves of CT DNAs unplatinated or modified by *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] or transplatin were recorded by measuring the absorbance at 260 nm. The melting curves were recorded in a medium containing $NaClO_4$ (10 mM) with Tris–HCl (1 mM)/EDTA (0.1 mM, pH 7.4). The value of the melting temperature (t_m) was determined as the temperature corresponding to a maximum of the first derivative profile of the melting curves. The t_m values could be determined with an accuracy of ±0.3 °C.

2.4. Ethidium bromide fluorescence

Fluorescence measurements of CT DNA modified by platinum complexes in the presence of EtBr were performed at an excitation wavelength of 546 nm, and the emitted fluorescence was analyzed at 590 nm. The fluorescence intensity was measured at 25 °C in NaCl (0.4 M) to avoid secondary binding of EtBr to DNA [29,30]. The concentrations were 0.01 mg mL^{-1} for DNA and 0.04 mg mL^{-1} for EtBr, which corresponded to the saturation of all sites of EtBr in DNA [30].

2.5. Unwinding of negatively supercoiled DNA

Unwinding of closed circular supercoiled pUC19 plasmid DNA was assayed by an agarose gel mobility shift assay [31]. The unwinding angle Φ, induced per one DNA adduct of the platinum complex, was calculated upon the determination of the r_b value at which the complete transformation of the supercoiled to relaxed form of the plasmid was attained. Samples of plasmid DNA were incubated with the platinum complex at 37 °C in the dark for 24 h. The samples were subsequently subjected to electrophoresis on 1% native agarose gel running at 25 °C in the dark with TAE buffer [Tris acetate (40 mM, pH 8.0)/EDTA (1 mM)] and the voltage set at 18 V. The gels were then stained with EtBr, followed by photography with transilluminator.

2.6. Transcription mapping of DNA adducts in vitro

Transcription of the (NdeI/HpaI) restriction fragment of pSP73KB DNA treated with platinum complexes (r_b = 0.006) with DNA-dependent T7 RNA polymerase and electrophoretic analysis of transcripts were performed according to the protocols recommended by the manufacturer (Promega Protocols and Applications, 43–46, 1989/90) and described in detail previously [24]. The concentration of DNA used in this assay was 3.9×10^{-5} M (relative to the monomeric nucleotide content).

2.7. Interstrand (intramolecular) cross-linking assay

Platinum complexes were incubated for 24 h with 40 μg of pUC19 DNA after it had been linearized by *Eco*RI and 5′-end labeled with T4 polynucleotide kinase with [γ-^{32}P]ATP. The number of interstrand cross-links was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%). After the electrophoresis had been completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified. The frequency of interstrand cross-links was calculated as %ICL/Pt = XL / 5372 × r_b (pUC19 plasmid contained 5372 nucleotide residues), where %ICL/Pt is the number of interstrand cross-links per adduct multiplied by 100 and XL is the number of interstrand cross-links per molecule of the linearized DNA duplex and was calculated assuming a Poisson distribution of the interstrand cross-links as XL = −ln A, where A is the fraction of molecules running as a band corresponding to the non-cross-linked DNA.

2.8. Characterization of DNA adducts of trans-[Pt(CH_3NH_2)$_2$$Cl_2$] by thiourea

Double-stranded CT DNA (80 μg mL^{-1}) was incubated with transplatin or *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] at a drug to nucleotide ratio of r_i = 0.05 in $NaClO_4$ (10 mM) at 37 °C (r_i is defined as the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA). At various times the reaction was stopped by adjusting the NaCl concentration to 1 M and by immediate cooling to −20 °C. In parallel experiments, the reactions were stopped by addition of thiourea solutions (10 mM). These samples were incubated for 10 min at 25 °C and then quickly cooled to −20 °C. The samples were then exhaustively dialyzed against H_2O at 4 °C and the platinum content was determined by FAAS.

2.9. DNA repair synthesis by human cell extract

Repair DNA synthesis of CFEs was assayed using pUC19 plasmid. Each reaction of 50 μL contained unmodified pBR322 (600 ng) and unmodified or platinated pUC19 (600 ng), ATP (2 mM), KCl (30 mM), creatine phosphokinase (rabbit muscle) (0.05 mg mL^{-1}), dGMP (20 mM), dCTP (20 mM), TTP (20 mM), dATP (8 mM), [α-^{32}P] dATP (74 kBq) in the buffer composed of HEPES-KOH (HEPES = N-(2-hydroxyethyl)piperazine-N-ethanesulfonic acid) (40 mM, pH 7.8), $MgCl_2$ (5 mM), dithiothreitol (0.5 mM), creatine phosphate (22 mM), bovine serum albumin (1.4 mg mL^{-1}), and CFE (20 mg). Reactions were incubated for 3 h at 37 °C and terminated by adding EDTA to a final concentration of 20 mM, sodium dodecyl sulfate to 0.6%, and proteinase K to 250 μg mL^{-1} and then incubating for 20 min at 45 °C. The products were extracted with 1 vol of 1:1 phenol–chloroform. The DNA was precipitated from the aqueous layer by the addition of 0.02 vol NaCl (5 M), glycogen (5 mg), and 2.5 vol ethanol. After 20 min of incubation on dry ice and centrifugation at 12 000 *g* for 30 min at 4 °C, the pellet was washed with 0.5 mL 70% ethanol and dried in a vacuum centrifuge. DNA was finally linearized by EcoRI before electrophoresis on a 1% agarose gel. Gels were stained with EtBr for photodocumentation and the radioactivity associated with the bands was quantitated using a Typhoon FLA 9000 biomolecular imager with AIDA Image Analyzer. Experiments were performed in quadruplicate.

2.10. Cell lines

Human ovarian carcinomas A2780 (cisplatin sensitive), and A2780cisR (resistant to cisplatin) were obtained from Prof. Keppler from University of Vienna, Austria. The acquired resistance of A2780cisR cells was maintained by supplementing the medium with cisplatin (1 μM) every second passage. A2780 and A2780cisR cells were cultured in RPMI 1640 medium supplemented with gentamycin and 10% heat-inactivated FBS.

2.11. Cytotoxicity assay

The cells were seeded in 96-well tissue cultured plates at a density of 10 000 cells/well. After overnight incubation, the cells were treated with the compounds tested. After 72 h of incubation 10 μL of MTT (2.5 mg mL^{-1}) was added to each well and incubated for 4 h in culture conditions. At the end of the incubation period the medium was removed and the formazan product was dissolved in DMSO (100 μL). The cell viability was evaluated by measurement of the absorbance at 570 nm, using an Absorbance Reader SUNRISE TECAN SCHOELLER. IC_{50} values (compound concentration that produces 50% of cell death) were calculated from curves constructed by plotting cell survival (%) versus drug concentration (μM). All experiments were made in triplicate. The reading values were converted to the percentage of control (% cell survival).

2.12. Cellular platinum complex accumulation

Cellular accumulation of *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$], transplatin and cisplatin was measured in A2780 cells. The cells were seeded in 100 mm tissue culture dishes (30,000/cm^2). After overnight incubation, the cells were treated with the Pt^{II} complex (10 μM) for 24 h [these concentrations were still verified by the measurement of platinum in the growing medium by FAAS or inductively coupled plasma mass spectroscopy (ICP-MS)]. The attached cells were washed twice with PBS (4 °C) and the pellet stored at −80 °C. The pellets were digested by a high pressure microwave digestion system (MARS5, CEM) with HCl to give a fully homogenized solution, and final platinum content was determined by FAAS. The results of cellular platinum uptake were corrected for adsorption effects [32]. For other details, see the Section 3.10. All experiments were performed in triplicate.

2.13. Other physical methods

Absorption spectra were measured with a Beckmann DU-7400 spectrophotometer. FAAS measurements were carried out with a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer. The measurements of fluorescence were performed on a Varian Cary Eclipse spectrofluorophotometer using a 1 cm quartz cell. The gels were dried and visualized using a FUJIFILM BAS 2500 bioimaging analyzer or Typhoon FLA 9000 biomolecular imager, and the radioactivities associated with bands were quantitated with AIDA Image Analyzer.

3. Results and discussion

3.1. DNA binding

A role for DNA binding is implicated in the mechanism of action of antitumor transplatin analogs. Thus, it is appealing to consider that the reason for activity of this class of Pt^{II} complexes lies at the DNA level. It has been shown in related studies that antitumor transplatin analogs induce a different array of lesions in comparison to transplatin. An altered mode of DNA binding may be inherently more cytotoxic than the lesions induced by transplatin. Thus, the complex discussed here may also be an example of a structural class capable of molecular interactions not accessible to transplatin. In the present study, we have applied some methodologies previously developed for cisplatin and its analogs to investigate the reaction products of *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$] with natural, high-molecular-mass DNA in cell-free media. The first experiments were aimed at quantifying the binding of *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$] to mammalian DNA. Solutions of double-helical CT DNA were incubated with *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$] or transplatin as described in the Section Experimental (Characterization of DNA adducts of *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$] by thiourea). At various time intervals, an aliquot of the reaction mixture was withdrawn, the reaction was stopped by adjusting the NaCl concentration to 1 M and by immediate cooling to −20 °C. The samples were then exhaustively dialyzed against H_2O at 4 °C and the platinum content was determined by FAAS (Fig. 2A, the lines passing through open and full triangles). This result indicates that the rate of binding of *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$] and transplatin to natural double-helical DNA is approximately the same and that both complexes were quantitatively bound after 24 h similarly as cisplatin [33,34].

The binding experiments of the present work indicate that the modification reactions resulted in the irreversible coordination of *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$] to polymeric double-helical DNA, which also facilitates sample analysis. Hence, it is possible to prepare easily and precisely the samples of DNA modified by the platinum complex at a preselected value of r_b. The samples of DNA modified by *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$] or transplatin and analyzed further by biophysical or biochemical methods were prepared in $NaClO_4$ (10 mM) at 37 °C as described in Experimental. In this way, the analyses described in the present paper were performed in the absence of unbound (free) platinum complex.

3.2. DNA melting

CT DNA was modified by *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$] or transplatin to the values of $r_b = 0.05$ or 0.1 in $NaClO_4$ (10 mM) at 37 °C for 24 h. The melting temperature (t_m) increased with growing level of platination (r_b) (Table 1). The efficiency of *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$] to enhance t_m was lower than that of parent transplatin. It should be pointed out that growing level of modification of DNA by cisplatin decreased t_m under identical experimental conditions [35].

Previously, three major factors have been invoked to account for the thermal stability of DNA modified by Pt^{II} complexes capable of DNA cross-linking and the observed change in melting temperature of DNA as a consequence of its platination reflects the relative proportion and contribution of these three factors [35]. These major factors are (i) a destabilizing effect of conformational distortions due to the formation of cross-links induced in DNA by platinum coordination; (ii) stabilizing effects of DNA interstrand cross-links which prevent dissociation of DNA strands; (iii) the positive charge on the Pt^{II} centers (introduction of a positive charge into the DNA molecule, e.g. by binding of positively charged ligands such as Pt^{II} moieties of platinum antitumor compounds, results in a stabilization of the DNA duplex by decreasing electrostatic repulsion of negative charges of phosphate groups located at the complementary strands). Thus, it is reasonable to conclude that

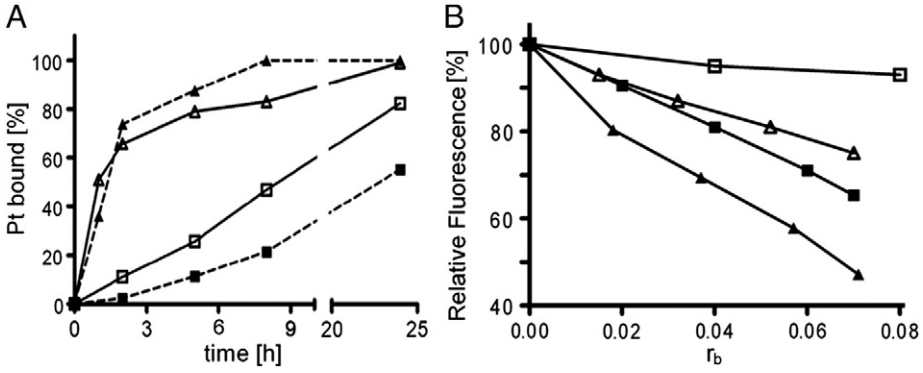

Fig. 2. A. Kinetics of the reaction of *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$] (open symbols) or transplatin (closed symbols) with double-helical CT DNA at $r_i = 0.05$ in $NaClO_4$ (10 mM) at 37 °C. DNA concentration was 80 μg mL^{-1}. Reactions were stopped with thiourea (10 mM, 10 min) (squares) or NaCl (1 M, −20 °C) (triangles), and the amount of platinum associated with DNA was determined by FAAS. For other details, see the text. B. Dependencies of ethidium bromide fluorescence on r_b for double-helical CT DNA modified by various platinum complexes in $NaClO_4$ (10 mM) at 37 °C for 24 h. (▲), *trans*-[Pt(CH$_3$NH$_2$)$_2$Cl$_2$]; (■), cisplatin; (△), transplatin; (□), monofunctional [PtCl(dien)]Cl.

Table 1
Δt_m values[a] of CT DNA modified by *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] or transplatin.[b,c]

Compound	$r_b = 0.05$	$r_b = 0.1$
trans-[Pt(CH_3NH_2)$_2$$Cl_2$]	7.2 ± 0.2	9.3 ± 0.3
transplatin	7.9 ± 0.2	12.5 ± 0.3

[a] Δt_m is defined as the difference between the t_m values of platinated and unmodified DNAs.
[b] The t_m values were measured in the medium containing $NaClO_4$ (0.01 M), Tris–HCl (1 mM, pH 7.4) and EDTA (0.1 mM).
[c] Values shown in this table are the means ± SEM of three separate experiments.

the increases in t_m are caused by the interstrand cross-links formed by both trans-platinum compounds and by positive charges on platinum moieties and that these stabilizing factors dominate over destabilizing conformational distortions. Owing to the fact that both *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] and transplatin carry the same 2+ charge on platinum moiety and form approximately identical amount of interstrand cross-links (13 or 12%, respectively, vide infra), the differences in values of t_m yielded by the adducts of both complexes may be attributed mainly to differences in destabilizing effects of conformational distortions induced by these adducts. Hence, the results of DNA melting experiments (Table 1) are consistent with the thesis that DNA adducts of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] distort and destabilize double helix of DNA more than major DNA adducts of transplatin.

3.3. Characterization of DNA adducts by EtBr fluorescence

EtBr as a fluorescent probe has been used to characterize perturbations induced in DNA by bifunctional adducts of several mononuclear platinum compounds [36–40]. Double-helical CT DNA was first modified by bifunctional cisplatin or transplatin, monofunctional [PtCl(dien)]Cl, or *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] for 24 h. The levels of the modification corresponded to the values of r_b in the range between 0 and 0.08. Modification of DNA by all platinum complexes resulted in a decrease of EtBr fluorescence (Fig. 2B). The decrease caused by the adducts of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] was markedly more pronounced than that induced by the DNA adducts of cisplatin or transplatin at equivalent r_b values. Modification of DNA by monofunctional platinum complexes results in only a slight decrease of EtBr fluorescence intensity as compared with the control DNA–EtBr complex [36–40]. The structures of DNA adducts of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] may arise from mono- or bifunctional substitutions on the polynucleotide. Comparison with [PtCl(dien)]Cl, cisplatin and transplatin suggests that the conformational distortion induced in DNA by the adducts of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] is much more delocalized and extends over considerably more base pairs around the platination sites than in the case of the adducts of mononuclear complexes such as cisplatin or transplatin.

3.4. Unwinding of negatively supercoiled DNA

Electrophoresis in native agarose gel was used to quantify the unwinding induced in pUC19 plasmid by *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] or transplatin by monitoring the degree of supercoiling (Fig. 3). A compound that unwinds the DNA duplex reduces the number of supercoils so that the superhelical density of closed circular DNA decreases. This decrease upon binding of unwinding agents causes a decrease in the rate of migration through agarose gel, which makes it possible that the unwinding can be observed and quantified. Fig. 3A and B shows agarose gels in which increasing amounts of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] or transplatin, respectively, have been bound to a mixture of relaxed and supercoiled pUC19 DNA. Interestingly, the *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] accelerated the mobility of the relaxed form in a way similar to that of cisplatin, whose bifunctional binding to DNA shortens and condenses the DNA helix [41,42]. This observation is in contrast to DNA binding of clinically ineffective transplatin to relaxed forms of plasmid DNAs since the mobility of the relaxed forms is affected by transplatin considerably less

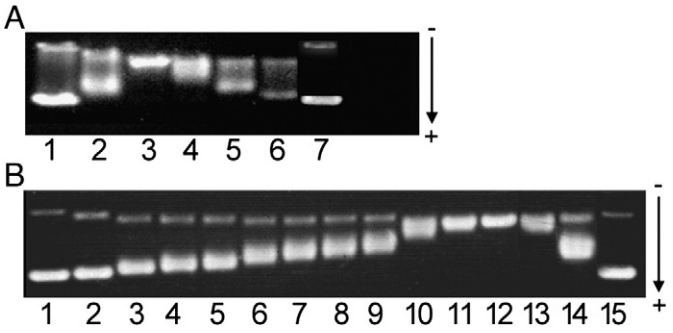

Fig. 3. Unwinding of supercoiled pUC19 plasmid DNA by *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] (A) or transplatin (B). The top bands correspond to the nicked form of the plasmid and the bottom bands to the closed, negatively supercoiled plasmid. The plasmid was incubated with Pt^{II} complexes at various r_b values in $NaClO_4$ (10 mM) at 37 °C for 24 h. Lanes 1–7 in panel A, DNA modified by *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] at r_b = 0, 0.02, 0.039, 0.06, 0.08, 0.1, 0, respectively. Lanes 1–12 in panel B, DNA modified by transplatin at r_b = 0, 0.01, 0.015, 0.02, 0.025, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.085, 0.09, 0.1, 0, respectively.

in accord with the previously published studies [41–44]. The unwinding angle is given by $\Phi = -18\sigma / r_b(c)$ where σ is the superhelical density and $r_b(c)$ is the value of r_b at which the supercoiled and relaxed forms comigrate [31]. Under the present experimental conditions, σ was calculated to be −0.043 on the basis of the data of cisplatin for which the $r_b(c)$ was determined in this study and $\Phi = 13°$ was assumed. By using this approach, we determined a DNA unwinding angle of 20 ± 3° for *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] and 9 ± 1° for transplatin. The DNA unwinding angle determined for transplatin is in excellent agreement with the previously published results [31]. Thus, the unwinding angle produced by the adducts of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] is more than double of the value of 9° determined for the adducts of transplatin, but also higher than that produced by the adducts of cisplatin (13°, [31]). This result provides another good experimental evidence for a DNA binding mode of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] different from that of transplatin and that DNA adducts of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] distort double-helical DNA more than DNA adducts of transplatin.

3.5. Transcription mapping of DNA adducts

Cutting of pSP73KB DNA by NdeI and HpaI restriction endonucleases yielded a 212-bp fragment (a substantial part of its nucleotide sequence is shown in Fig. 4B). This fragment contained a T7 RNA polymerase promoter [in the upper strand close to its 3′-end (Fig. 4B)]. The first experiments were carried out using this linear DNA fragment, randomly modified by cisplatin, transplatin or *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] at r_b = 0.006, for RNA synthesis by T7 RNA polymerase (Fig. 4A, lanes cisPt, transPt or 1, respectively). RNA synthesis on the template modified by the platinum complexes yielded fragments of defined sizes (Fig. 4A), which indicates that RNA synthesis on these templates was prematurely terminated. The sequence analysis revealed that the major bands resulting from termination of RNA synthesis by the adducts of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] were more similar to those produced by transplatin adducts rather than to those produced by cisplatin adducts (Fig. 4B). On the other hand, the sequence dependence of the inhibition of RNA synthesis by the adducts of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] is somewhat more regular than that by the adducts of transplatin.

3.6. Interstrand cross-linking

Bifunctional platinum compounds that covalently bind to DNA form various types of interstrand and intrastrand cross-links and monofunctional adducts. Considerable evidence suggests that the antitumor efficacy of platinum compounds is the result of the formation of these lesions, but their relative efficacy remains unknown. Therefore, first we have decided to quantitate the interstrand cross-linking efficiency of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] in linearized pUC19 plasmid

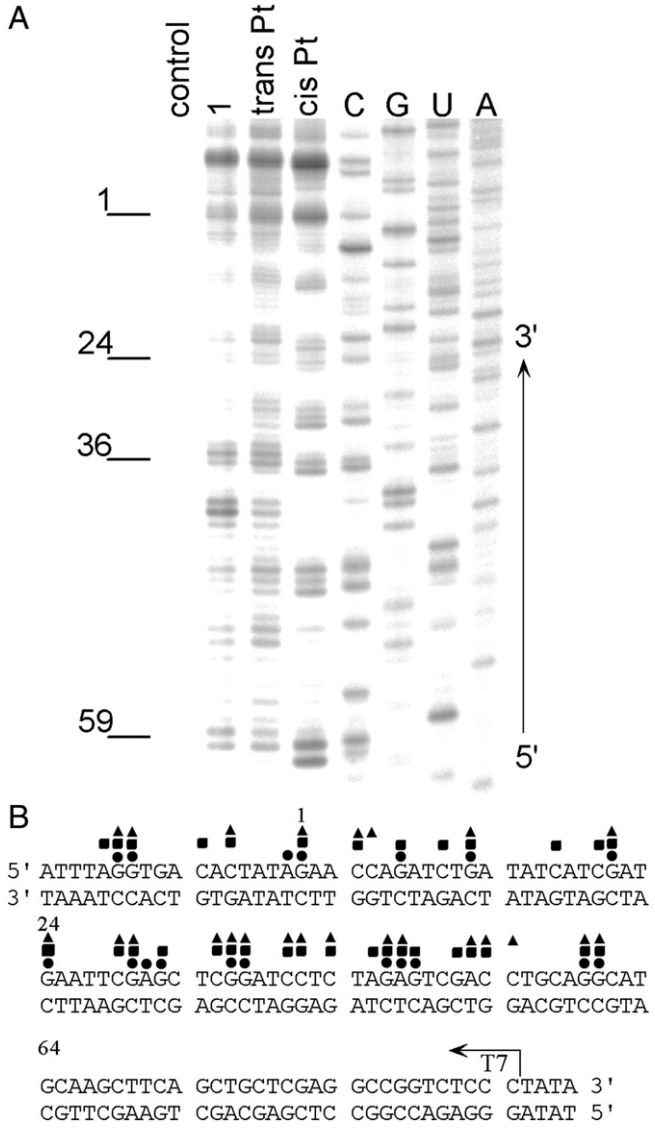

Fig. 4. RNA synthesis by T7 RNA polymerase on the NdeI/HpaI fragment of pSP73KB plasmid modified by cisplatin, transplatin, or *trans*-$[Pt(CH_3NH_2)_2Cl_2]$. A. Autoradiogram of 6% polyacrylamide/8 M urea sequencing gel. Lanes: control, nonplatinated template; C, G, U, and A, chain terminated marker RNAs; cisPt, transPt, 1, template modified by cisplatin, transplatin, or *trans*-$[Pt(CH_3NH_2)_2Cl_2]$, respectively at $r_b = 0.006$. B. Sequence of the NdeI/HpaI fragment of the pSP73KB plasmid. The arrow indicates the start of T7 RNA polymerase, which was used as a template. Numbers correspond to nucleotide numbering in the sequence of the pSP73KB plasmid. Circles, squares and triangles indicate stop signals from panel A, lanes cisPt, transPt and 1, respectively.

(2686 bp). This plasmid DNA was linearized by *Eco*RI (*Eco*RI cuts only once within the pUC19 plasmid) and modified by *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ at various r_b values. The samples were analyzed for the interstrand cross-links by agarose gel electrophoresis under denaturing conditions [45]. Upon electrophoresis, the 3′-end-labeled strands of linearized pUC19 plasmid containing no interstrand cross-links migrate as a 2686-base single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species (Fig. 5). The experiments were carried out with DNA samples that were modified by *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ for 24 h at various r_b values. The bands corresponding to the more slowly migrating interstrand-crosslinked fragments were seen for r_b values as low as 5×10^{-5} (Fig. 5, lane 2). 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-crosslinked or cross-linked

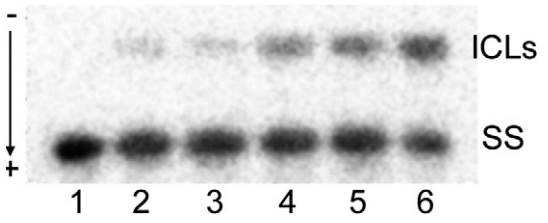

Fig. 5. The formation of interstrand cross-links (ICLs) by *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ in pUC19 plasmid DNA linearized by EcoRI. Autoradiograms of a denaturing 1% agarose gels of linearized DNA which was 3′-end-labeled; the interstrand cross-linked DNA appears as the top bands migrating on the gels more slowly than the single-stranded (ss) DNA (contained in the bottom bands). Lanes 1–6, DNA modified by *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ at $r_b = 0, 5 \times 10^{-5}, 1 \times 10^{-4}, 2 \times 10^{-4}, 3 \times 10^{-4}, 5 \times 10^{-4}$, respectively.

DNA under each condition. The frequency of intramolecular interstrand cross-links (% ICL per one molecule of the Pt complex) was calculated using the Poisson distribution from the fraction of non-cross-linked DNA in combination with the r_b values and the fragment size. The DNA interstrand cross-linking efficiency (% ICL per one molecule of the Pt complex) of *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ was almost independent of r_b and was 13%; thus the DNA interstrand cross-linking efficiency of *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ was similar to that of mononuclear transplatin (12%, [24]), but higher than that of cisplatin (6%, [24]). The samples of linearized DNA modified by *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ at $r_b = 0.001$ and 0.01 were also analyzed in 1% nondenaturing agarose gel (not shown). No new, more slowly migrating bands were observed, which indicates that no CLs between DNA strands belonging to different duplexes were formed under these experimental conditions ($NaClO_4$, 10 mM).

3.7. Quantification of monofunctional platinum adducts by thiourea assay

Cisplatin, transplatin, and analogous bifunctional platinum compounds coordinate to DNA in a two-step process, forming first monofunctional adducts, preferentially at guanine residues, which subsequently close to bifunctional lesions [15,33,46,47]. Thiourea is used to labilize monofunctionally bound transplatin from DNA [23]. The displacement of transplatin is initiated by coordination of thiourea trans to the nucleobase. Owing to the strong trans effect of sulfur, the nucleobase nitrogen–platinum bond is weakened and thus becomes susceptible to further substitution reactions. Consequently, transplatin in monofunctional DNA adducts is effectively removed, whereas bifunctional adducts of transplatin are resistant to thiourea treatment [23].

The experiments aimed at the characterization of DNA adducts of *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ were conducted employing thiourea as a probe for DNA monofunctional adducts formed by this trans-platinum compound (Fig. 2A). The reaction of DNA with *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ was complete after 24 h (Fig. 2A). Thiourea displaced approximately 75% of *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ or approximately 90% of transplatin from DNA at early time intervals (1–5 h; Fig. 2A). At longer incubation times (24 h), thiourea was considerably less efficient in removing *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ or transplatin from DNA, which suggests that after 24 h a considerable fraction of monofunctional adducts of both complexes had closed to a bifunctional lesion. Thus, after a reaction period of 24 h only approximately 20% of *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ or 50% of transplatin was displaced from double-stranded DNA (Fig. 2A), which implies that approximately 80% of monofunctional adducts of *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ and only 50% of transplatin had evolved to bifunctional lesions. Thus, the result of this experiment suggests monofunctional adducts of *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ close to bifunctional lesions with a significantly higher rate than those of transplatin.

In aggregate, considering the fact that *trans*-$[Pt(CH_3NH_2)_2Cl_2]$ and transplatin form approximately identical amount of interstrand cross-links (13 or 12%, respectively), the results of quantification of monofunctional platinum adducts by thiourea assay enable us to

conclude that *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] forms (after 24 h) mainly intrastrand cross-links (~67%), presumably 1,3-intrastrand crosslinks, 13% interstrand cross-links and only ca. 20% of adducts remain monofunctional. In contrast, transplatin forms (after 24 h) mainly monofunctional adducts (~50%), 12% interstrand cross-links and ca. 38% 1,3-intrastrand crosslinks. Interestingly, cisplatin forms (after 24 h) mainly 1,2-intrastrand cross-links (~90%), interstrand cross-links, 1,3-intrastrand crosslinks and monofunctional adducts represent minor adducts [19].

3.8. DNA repair synthesis by mammalian cell extract

DNA repair is another key factor which significantly reduces the number of Pt-adducts on DNA in cells and thereby may significantly contribute to their biological activity [48]. Therefore, DNA repair efficiency in pUC19 plasmid (2686 bp) globally modified by *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$], transplatin or cisplatin at $r_b = 0.05$ was tested using CFE of repair-proficient CHO-K1 cells. Untreated plasmid pBR322 (4361 bp) was also included in each reaction to monitor damage-independent nucleotide incorporation. Repair activity was monitored by measurement of the amount of incorporated radiolabelled nucleotide. The incorporation of radioactive material was corrected for the relative DNA content in each band, determined by densitometry of EtBr stained gel. As illustrated in Fig. 6, damage-induced DNA repair synthesis determined for the plasmid modified by *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] or transplatin was lower than that found for cisplatin at the same level of modification (50 or 44%, respectively of that found for the plasmid modified by cisplatin, Fig. 6B).

3.9. Cytotoxicity

The cytotoxic activity of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] was determined against A2780 (parent cisplatin sensitive) and A2780cisR (with acquired cisplatin resistance) human ovarian carcinoma cell lines, commonly used to test cytotoxic activity of cisplatin analogs. A2780cisR cells are resistant to cisplatin through a combination of decreased uptake, enhanced DNA repair/tolerance, and elevated reduced glutathione levels [49,50]. The tumor cell lines were incubated for 72 h with the platinum compounds and the cell survival in the culture treated with the platinum compounds was evaluated as described in the Experimental section. Results (Table 2) show that in the sensitive A2780 cell line, *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] is about 5-fold less potent than cisplatin, but at least 18-fold more potent than transplatin. In the cisplatin-resistant A2780cisR cell line, the IC_{50} value of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] is slightly higher than that for cisplatin (~1.5-fold), although still at least 11-fold more potent than transplatin, but most importantly the resistance factor in the A2780/A2780cisR pair is 3-fold lower than that found for cisplatin. Thus, these results are consistent with the thesis that the trans geometry in Pt^{II}-dichloro compounds can be effectively activated by the replacement of two ammine ligand in transplatin by the non-bulky ligand, such as the shortest aliphatic amine, CH_3NH_2.

3.10. Cellular uptake

The factor that is usually thought to contribute to drug cytotoxicity is cellular uptake. To examine the accumulation of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$], transplatin and cisplatin the cellular levels of these compounds were measured after 24 h exposures of the A2780 cells to the drugs at the concentration of 10 μM. The accumulation of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] in the A2780 cells was, after 24 h exposures, approximately 14- and 4-fold greater than that of transplatin and cisplatin, respectively (Table 4). This enhanced accumulation of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] might be attributed to its enhanced hydrophobicity due to the presence of extra methyl groups although other factors such as uptake and/or efflux mediated by various transporters may be involved as well. These results also show that accumulation of *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] in tumor cells is markedly higher than that of cisplatin. This result implies that the cellular accumulation of cisplatin and *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] does not correlate with markedly higher toxicity of cisplatin in tumor cells in comparison with *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$]. In aggregate, these results are consistent with the thesis that there is a great deal of complexity at the DNA level including recognition and repair of DNA adducts that needs to be investigated.

4. Additional discussion and conclusions

The results of the present work demonstrate that the replacement of ammine groups by the non-bulky methylamine ligand in the molecule of ineffective transplatin results in a radical enhancement of its activity in tumor cell lines including cisplatin-resistant tumor cells (Table 2). Thus, the results of the present work demonstrate that

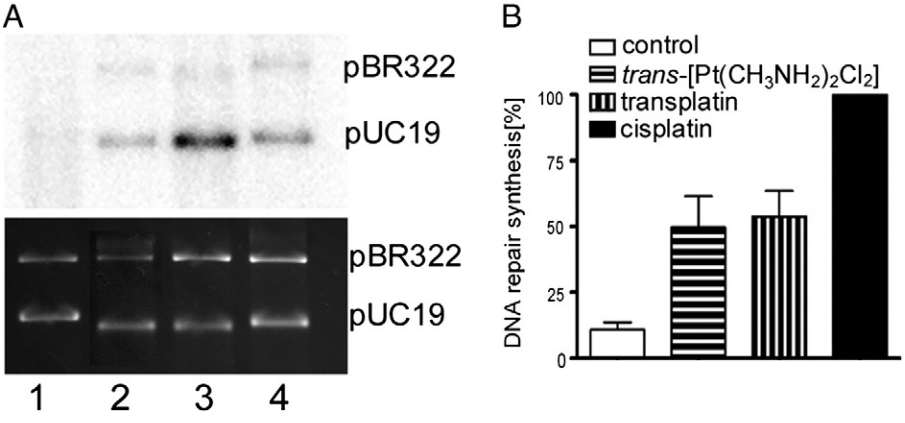

Fig. 6. In vitro DNA repair synthesis assay. DNA repair of pUC19 modified with cisplatin, transplatin, or *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] at $r_b = 0.05$ was mediated by the extract from the repair-proficient CHO-K1 cells. Unmodified pBR322 was also used as internal control. Repair efficiency is given by the radioactivity of incorporated [α-^{32}P]dATP normalized on the relative amount of DNA in the band determined from the ethidium bromide-stained gel. (A) Results of a typical experiment. Top panel: autoradiographic image of the gel showing radiolabel incorporation. Bottom panel: ethidium bromide-stained gel showing migration of undamaged control plasmid (pBR322, top bands) and platinated pUC19 (bottom bands). Lanes: 1: control, unmodified pUC19 plus unmodified pBR322; 2, pUC19 modified with *trans*-[Pt(CH_3NH_2)$_2$$Cl_2$] plus unmodified pBR322; 3, pUC19 modified with transplatin plus unmodified pBR322; 4, pUC19 modified with cisplatin plus unmodified pBR322. B. Incorporation of [α-^{32}P]dATP into unmodified or platinated pUC19 plasmid. For all quantifications representing the mean values of two separate experiments, incorporation of radioactive material is normalized on the relative DNA content in each band determined from the relative densities of the bands on the EtBr-stained gel. The radioactivity associated with incorporation of [α-^{32}P]dATP into DNA modified with cisplatin was arbitrarily set to 100%. Values shown in the graph are the means (±SEM) of two separate experiments, each conducted in duplicate.

Table 2
Cytotoxicity [IC_{50}mean values (μM)] obtained for *trans*-[$Pt(CH_3NH_2)_2Cl_2$], transplatin and cisplatin.[a]

Compound	A2780	A2780cisR
trans-[$Pt(CH_3NH_2)_2Cl_2$]	14.0 ± 0.9	21.5 ± 3.1 (1.5)
transplatin	>250	>250
cisplatin	2.8 ± 0.1	13.8 ± 0.1 (4.9)

[a] The results are expressed as mean ± SD of three independent experiments. Resistance factor, defined as IC_{50} (resistant)/IC_{50} (sensitive), is given in parentheses.

Table 4
Accumulation (ng of Pt/1 × 10^6 cells) in A2780 cells of Pt complexes.[a]

Compound	Accumulation
trans-[$Pt(CH_3NH_2)_2Cl_2$]	58 ± 6
Transplatin	4.2 ± 0.8
Cisplatin	14 ± 1

[a] The uptake of Pt complexes at their 10 μM concentrations after 24 h of cell treatment. Each value shown in the table (mean ± standard deviation) is in ng of Pt/1 × 10^6 cells. The experiments were performed in triplicate.

activation of trans geometry in bifunctional mononuclear Pt^{II} complexes by replacement of ammine groups in transplatin is not limited only to the replacement by relatively bulky and stereochemically more demanding amino ligands. A role for DNA binding is implicated in the mechanism of action of antitumor transplatin analogs [8,9]. Thus, it is appealing to consider that the reason for activity of *trans*-[$Pt(CH_3NH_2)_2Cl_2$] lies at the DNA level as it has been shown in related studies for cytostatic transplatin analogs containing bulky carrier amino ligands which induce a different array of lesions in comparison to transplatin. An altered modes of DNA binding may be inherently more cytotoxic than the lesions induced by transplatin.

The DNA binding of *trans*-[$Pt(CH_3NH_2)_2Cl_2$] in comparison with transplatin is summarized in Table 3. The results of this paper indicate that the presence of the methylamine in this transplatin analog does not markedly change the rate of monofunctional coordination to DNA and the sequence preference of its binding (Fig. 4). In contrast, the presence of the methylamine markedly affects the overall rate of the rearrangement of monofunctional adducts to bifunctional lesions mainly to intrastrand cross-links (Fig. 2A). The latter adducts represent major DNA lesions formed by *trans*-[$Pt(CH_3NH_2)_2Cl_2$]. This is in contrast to transplatin which forms mainly monofunctional adducts which distort DNA conformation much less than cross-links. It has been shown that trans-platinum complexes cannot form due to their stereochemistry the cross-links between neighboring nucleotide residues in one strand of DNA, i.e. the cross-links which represent major lesions induced in DNA by antitumor cisplatin. Thus, as regards to intrastrand cross-links, trans-platinum compounds form instead 1,3-cross-links in which two platinated sites in one strand of DNA are separated by a third intervening nucleotide. It has been shown that these cross-links severely distort DNA conformation. The base pairing is disrupted over several base pairs around the adduct, the platinated 5′G residue and the central base residue are unpaired [51,52]. Thus, the results of DNA melting experiments (Table 1) and characterization of DNA adducts by EtBr fluorescence (Fig. 2B) indicating that DNA globally modified by *trans*-[$Pt(CH_3NH_2)_2Cl_2$] is distorted more than DNA modified by transplatin are consistent with the observation that *trans*-[$Pt(CH_3NH_2)_2Cl_2$] forms a higher amount of more distorting intrastrand cross-links. In addition, internal coordinate molecular modeling study proposed structures for 1,3-intrastrand cross-link of transplatin in which considerable unwinding is localized within a short segment centered on the platinum binding site [53]. Hence, more efficient DNA unwinding produced by the global modification of DNA by *trans*-[$Pt(CH_3NH_2)_2Cl_2$] in comparison with the modification by transplatin appears again to be consistent with enhanced efficiency of DNA adducts of *trans*-[$Pt(CH_3NH_2)_2Cl_2$] to form 1,3-intrastrand cross-links capable of efficient local DNA unwinding.

Table 3
Summary of DNA binding characteristics of *trans*-[$Pt(CH_3NH_2)_2Cl_2$], transplatin and cisplatin.[a]

	trans-[$Pt(CH_3NH_2)_2Cl_2$]	transplatin	cisplatin
% interstrand CLs/adduct	13	12	6
% monofunctional lesions/adduct	20	50	~1
% intrastrand CLs/adduct	67	38	~90
Unwinding angle/adduct	20°	9°	13°
Melting temperature (Δt_m) $r_b = 0.1$	9.3 °C	12.5 °C	−8.0 °C
EtBr fluorescence decrease	High	Medium	Medium

[a] For other details, see the text.

The enhanced rate of conversion of monofunctional to bifunctional cross-links was also previously noticed if NH_3 group in transplatin was replaced by several bulkier amines as carrier ligands [18,54–57], but reasons for this effect have not been fully clarified. For instance, the unexpected high rate of cross-link formation by such transplatin analogs relative to that determined for parent transplatin has been suggested [54,56] to be associated with conformational changes in double-stranded DNA, induced by the altered amine ligand in a monofunctional adduct which may modulate the second binding step and facilitate formation of cross-link. This interpretation might be applicable to monofunctional adducts of *trans*-[$Pt(CH_3NH_2)_2Cl_2$] as well, although data on conformational alterations induced in DNA by monofunctional adducts of *trans*-[$Pt(CH_3NH_2)_2Cl_2$] are not available. We also hypothesize that the NH_3 groups of the monofunctional DNA-transplatin adducts may form hydrogen bonds with the DNA (perhaps mediated by water molecules). This might slow down the ability of the monofunctional adducts to close to cross-links and when a methyl group is added this might break up the ability to form such bonds. The work focused on clarification of the mechanism underlying the enhanced rate of conversion of monofunctional to bifunctional cross-links due to replacement of NH_3 group in transplatin by methylamine which is in progress in our laboratory and will be published in a separate communication.

DNA repair is another key factor which may significantly affect toxicity of platinum drugs in tumor cells at the DNA level [48,58]. As illustrated in Fig. 6, damage-induced DNA repair synthesis detected in the plasmid modified by *trans*-[$Pt(CH_3NH_2)_2Cl_2$] was only slightly lower than that found for transplatin at the same level of modification. Thus, DNA repair appears to be an unlikely factor responsible for the markedly enhanced toxicity in tumor cells due to the replacement of ammine groups by the non-bulky methylamine ligand in the molecule of ineffective transplatin.

The results of the present work also suggest that enhanced toxicity of *trans*-[$Pt(CH_3NH_2)_2Cl_2$] in A2780 cells in comparison with transplatin correlates with increased cellular accumulation of the former platinum complex. Hence, the latter factor appears to be responsible for differences in toxicity of *trans*-[$Pt(CH_3NH_2)_2Cl_2$] and parent transplatin in tumor cells as well.

In aggregate, we find that activation of the trans geometry in bifunctional mononuclear Pt^{II} complexes can be also accomplished by replacement of ammine groups in transplatin by non-bulky methylamine ligands so that it is not limited only to the replacement by relatively bulky and stereochemically more demanding amino ligands. Moreover, we demonstrate that DNA binding mode and cellular accumulation of *trans*-[$Pt(CH_3NH_2)_2Cl_2$] and transplatin correlate with their toxicity in the cisplatin sensitive- and resistant tumor cell line. Hence, the results of the present work may expand the theoretical background needed to understand more fully mechanisms underlying anticancer effects of trans-platinum complexes and should provide a more rational basis for the design of new antitumor metallodrugs and chemotherapeutic strategies. Further investigations of other phases of the mechanism(s) of antitumor effects of trans-platinum complexes are warranted to unravel the origin of antitumor activity of platinum complexes with leaving ligands in the *trans* configuration.

Abbreviations

A2780	human ovarian carcinoma cells
A2780cisR	human ovarian carcinoma cells (cisplatin resistant)
bp	base pair
CFE	cell-free extract
CT DNA	calf thymus DNA
dien	diethylenetriamine
DMEM	Dulbecco's modified eagle medium
EtBr	ethidium bromide
FAAS	flameless atomic absorption spectrometry
FBS	fetal bovine serum
HEPES	N-(2-hydroxyethyl)piperazine-N-ethanesulfonic acid
ICL	interstrand cross-link
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
[PtCl(dien)]Cl	chloridodiethylenetriamineplatinum(II) chloride
r_b	the number of molecules of the platinum complex bound per nucleotide residue
r_i	the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA
RPMI	Royal Park Memorial Institute (culture medium)
t_m	melting temperature

Acknowledgments

This research was supported by the Czech Science Foundation (Grants P301/10/0598 and 13-08273S). Research of M.F. was also supported by the student project of the Palacky University in Olomouc (Grant PrF 2013 017). J.K.'s and V.B.'s research was also supported by Operational Program Education for Competitiveness—European Social Fund (CZ 1.07/2.3.00/20.0057) of the Ministry of Education, Youth and Sports of the Czech Republic.

References

[1] M.J. Cleare, J.D. Hoeschele, Bioinorg. Chem. 2 (1973) 187–210.
[2] M.J. Cleare, Coord. Chem. Rev. 12 (1974) 349–405.
[3] S.E. Sherman, S.J. Lippard, Chem. Rev. 87 (1987) 1153–1181.
[4] T. Fojo, N. Farrell, W. Ortuzar, H. Tanimura, J. Weinstein, T.G. Myers, Crit. Rev. Oncol. Hematol. 53 (2005) 25–34.
[5] M.D. Hall, H.R. Mellor, R. Callaghan, T.W. Hambley, J. Med. Chem. 50 (2007) 3403–3411.
[6] J.B. Mangrum, N.P. Farrell, Chem. Commun. 46 (2010) 6640–6650.
[7] J.-M. Perez, M.A. Fuertes, C. Alonso, C. Navarro-Ranninger, Crit. Rev. Oncol. Hematol. 35 (2000) 109–120.
[8] M. Coluccia, G. Natile, Anti-Cancer Agents Med. Chem. 7 (2007) 111–123.
[9] S.M. Aris, N.P. Farrell, Eur. J. Inorg. Chem. 2009 (2009) 1293–1302.
[10] A.G. Quiroga, J. Inorg. Biochem. 114 (2012) 106–112.
[11] N. Farrell, in: A. Sigel, H. Sigel (Eds.), Metal Ions in Biological Systems, vol. 32, Marcel Dekker, Inc., New York, Basel, Hong Kong, 1996, pp. 603–639.
[12] V. Brabec, Prog. Nucleic Acid Res. Mol. Biol. 71 (2002) 1–68.
[13] S. Zorbas-Seifried, M.A. Jakupec, N.V. Kukushkin, M. Groessl, C.G. Hartinger, O. Semenova, H. Zorbas, V.Y. Kukushkin, B.K. Keppler, Mol. Pharmacol. 71 (2007) 357–365.
[14] E. Wexselblatt, D. Gibson, J. Inorg. Biochem. 117 (2012) 220–229.
[15] N.P. Johnson, J.-L. Butour, G. Villani, F.L. Wimmer, M. Defais, V. Pierson, V. Brabec, Prog. Clin. Biochem. Med. 10 (1989) 1–24.
[16] E.R. Jamieson, S.J. Lippard, Chem. Rev. 99 (1999) 2467–2498.
[17] L. Kelland, Nat. Rev. Cancer 7 (2007) 573–584.
[18] J. Kasparkova, V. Marini, Y. Najajreh, D. Gibson, V. Brabec, Biochemistry 42 (2003) 6321–6332.
[19] A.M.J. Fichtinger-Schepman, J.L. Van der Veer, J.H.J. Den Hartog, P.H.M. Lohman, J. Reedijk, Biochemistry 24 (1985) 707–713.
[20] M.A. Lemaire, A. Schwartz, A.R. Rahmouni, M. Leng, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 1982–1985.
[21] A. Eastman, M.M. Jennerwein, D.L. Nagel, Chem. Biol. Interact. 67 (1988) 71–80.
[22] S.F. Bellon, S.J. Lippard, Biophys. Chem. 35 (1990) 179–188.
[23] A. Eastman, M.A. Barry, Biochemistry 26 (1987) 3303–3307.
[24] V. Brabec, M. Leng, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 5345–5349.
[25] J. Arpalahti, B. Lippert, Inorg. Chim. Acta Bioinorg. Chem. 138 (1987) 171–173.
[26] V. Brabec, E. Palecek, Biophys. Chem. 4 (1976) 76–92.
[27] V. Brabec, E. Palecek, Biophysik 6 (1970) 290–300.
[28] J.T. Reardon, A. Vaisman, S.G. Chaney, A. Sancar, Cancer Res. 59 (1999) 3968–3971.
[29] J.L. Butour, J.P. Macquet, Eur. J. Biochem. 78 (1977) 455–463.
[30] J.L. Butour, P. Alvinerie, J.P. Souchard, P. Colson, C. Houssier, N.P. Johnson, Eur. J. Biochem. 202 (1991) 975–980.
[31] M.V. Keck, S.J. Lippard, J. Am. Chem. Soc. 114 (1992) 3386–3390.
[32] A.E. Egger, C. Rappel, M.A. Jakupec, C.G. Hartinger, P. Heffeter, B.K. Keppler, J. Anal. At. Spectrom. 24 (2009) 51–61.
[33] D.P. Bancroft, C.A. Lepre, S.J. Lippard, J. Am. Chem. Soc. 112 (1990) 6860–6871.
[34] L. Zerzankova, H. Kostrhunova, M. Vojtiskova, O. Novakova, T. Suchankova, M. Lin, Z. Guo, J. Kasparkova, V. Brabec, Biochem. Pharmacol. 80 (2010) 344–351.
[35] R. Zaludova, V. Kleinwächter, V. Brabec, Biophys. Chem. 60 (1996) 135–142.
[36] V. Brabec, J. Kasparkova, O. Vrana, O. Novakova, J.W. Cox, Y. Qu, N. Farrell, Biochemistry 38 (1999) 6781–6790.
[37] A. Zakovska, O. Novakova, Z. Balcarova, U. Bierbach, N. Farrell, V. Brabec, Eur. J. Biochem. 254 (1998) 547–557.
[38] V. Brabec, O. Vrana, O. Novakova, V. Kleinwachter, F.P. Intini, M. Coluccia, G. Natile, Nucleic Acids Res. 24 (1996) 336–341.
[39] L. Zerzankova, T. Suchankova, O. Vrana, N.P. Farrell, V. Brabec, J. Kasparkova, Biochem. Pharmacol. 79 (2010) 112–121.
[40] R. Olivova, J. Kasparkova, O. Vrana, M. Vojtiskova, T. Suchankova, O. Novakova, W. He, Z. Guo, V. Brabec, Mol. Pharm. 8 (2011) 2368–2378.
[41] G.L. Cohen, W.R. Bauer, J.K. Barton, S.J. Lippard, Science 203 (1979) 1014–1016.
[42] W.M. Scovell, F. Collart, Nucleic Acids Res. 13 (1985) 2881–2895.
[43] W.M. Scovell, L.R. Kroos, Biochem. Biophys. Res. Commun. 104 (1982) 1597–1603.
[44] J.P. Macquet, J.L. Butour, Biochimie 60 (1978) 901–914.
[45] N. Farrell, Y. Qu, L. Feng, B. Van Houten, Biochemistry 29 (1990) 9522–9531.
[46] V. Brabec, V. Kleinwächter, J.L. Butour, N.P. Johnson, Biophys. Chem. 35 (1990) 129–141.
[47] A. Eastman, Pharmacol. Ther. 34 (1987) 155–166.
[48] V. Brabec, J. Kasparkova, in: N. Hadjiliadis, E. Sletten (Eds.), Metal Complex—DNA Interactions, Wiley, Chichester, UK, 2009, pp. 175–208.
[49] L.R. Kelland, C.F.J. Barnard, K.J. Mellish, M. Jones, P.M. Goddard, M. Valenti, A. Bryant, B.A. Murrer, K.R. Harrap, Cancer Res. 54 (1994) 5618–5622.
[50] L.R. Kelland, S.Y. Sharp, C.F. ONeill, F.I. Raynaud, P.J. Beale, I.R. Judson, J. Inorg. Biochem. 77 (1999) 111–115.
[51] C.A. Lepre, L. Chassot, C.E. Costello, S.J. Lippard, Biochemistry 29 (1990) 811–823.
[52] M.F. Anin, M. Leng, Nucleic Acids Res. 18 (1990) 4395–4400.
[53] C. Prevost, M. Boudvillain, P. Beudaert, M. Leng, R. Lavery, F. Vovelle, J. Biomol. Struct. Dyn. 14 (1997) 703–714.
[54] Y. Najajreh, J. Kasparkova, V. Marini, D. Gibson, V. Brabec, J. Biol. Inorg. Chem. 10 (2005) 722–731.
[55] F.J. Ramos-Lima, O. Vrana, A.G. Quiroga, C. Navarro-Ranninger, A. Halamikova, H. Rybnickova, L. Hejmalova, V. Brabec, J. Med. Chem. 49 (2006) 2640–2651.
[56] A. Halámiková, P. Heringová, J. Kašpárková, F.P. Intini, G. Natile, A. Nemirovski, D. Gibson, V. Brabec, J. Inorg. Biochem. 102 (2008) 1077–1089.
[57] T. Suchankova, M. Vojtiskova, J. Reedijk, V. Brabec, J. Kasparkova, J. Biol. Inorg. Chem. 14 (2009) 75–87.
[58] D. Wang, S.J. Lippard, Nat. Rev. Drug Discov. 4 (2005) 307–320.