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DNA interactions of new cytotoxic tetrafunctional dinuclear platinum complex *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(piperazine)]

Viktor Brabec [a], *Petros Christofis* [a], *Martina Slámová* [a], *Hana Kostrhunová* [a], *Olga Nováková* [a], *Yousef Najajreh* [b,1], *Dan Gibson* [b,2], *Jana Kašpárková* [a,*]

[a] *Institute of Biophysics, Academy of Sciences of the Czech Republic, Kralovopolska 135, CZ-61265 Brno, Czech Republic*
[b] *Department of Medicinal Chemistry and Natural Products, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem 91120, Israel*

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ABSTRACT

A new tetrafunctional dinuclear platinum complex *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(piperazine)] with sterically rigid linking group was designed, synthesized and characterized. In this novel molecule, the DNA-binding features of two classes of the platinum compounds with proven antitumor activity are combined, namely *trans* oriented bifunctional mononuclear platinum complexes with a heterocyclic ligand and polynuclear platinum complexes. DNA-binding mode of this new complex was analyzed by various methods of molecular biology and biophysics. The complex coordinates DNA in a unique way and interstrand and intrastrand cross-links are the predominant lesions formed in DNA in cell-free media and in absence of proteins. An intriguing aspect of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(piperazine)] is that, using a semi-rigid linker, interstrand cross-linking is diminished relative to other dinuclear platinum complexes with flexible linking groups and lesions that span several base pairs, such as tri- and tetrafunctional adducts, become unlikely. In addition, in contrast to the inability of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(piperazine)] to cross-link two DNA duplexes, the results of the present work convincingly demonstrate that this dinuclear platinum complex forms specific DNA lesions which can efficiently cross-link proteins to DNA. The results substantiate the view that *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(piperazine)] or its analogues could be used as a tool for studies of DNA properties and their interactions or as a potential antitumor agent. The latter view is also corroborated by the observation that *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(piperazine)] is a more effective cytotoxic agent than cisplatin against human tumor ovarian cell lines.

* *Corresponding author*. Tel.: +420 541517174; fax: +420 541240499.
E-mail address: jana@ibp.cz (J. Kašpárková).

[1] Current address: Faculty of Pharmacy, Al-Quds University-Jerusalem, P.O. Box 20002, Palestinian Authority.

[2] Affiliated with the David R. Bloom, Center for Pharmacy at The Hebrew University of Jerusalem, Israel.

Abbreviations: 1,1/c,c, [{*cis*-[$PtCl(NH_3)_2$}$H_2N(CH_2)_{2-6}NH_2$]$^{2+}$; 1,1/t,t, [{*trans*-[$PtCl(NH_3)_2$}$H_2N(CH_2)_{2-6}NH_2$]$^{2+}$; 1,2/c,c, [{*cis*-$PtCl(NH_3)_2$}μ-$H_2N(CH_2)_6NH_2$-{*cis*-$PtCl_2(NH_3)$}]; BBR3464, [{*trans*-$PtCl(NH_3)_2$}$_2$μ-*trans*-$Pt(NH_3)_2${$H_2N(CH_2)_6NH_2$}$_2$]$^{4+}$; bp, base pair; cisplatin, *cis*-diamminedichloridoplatinum(II); CL, cross-link; CT, calf thymus; DMS, dimethyl sulfate; EtBr, ethidium bromide; FAAS, flameless atomic absorption spectrometry; IC_{50}, the concentration of the compound that afforded 50% cell killing; KF, Klenow fragment from DNA polymerase I (wild type); KF$^-$, Klenow fragment from DNA polymerase I, exonuclease minus mutated to remove the 3′ → 5′ proofreading domain; PAA, polyacrylamide; pz, piperazine; PAGE, polyacrylamide gel electrophoresis; [PtCl(dien)]Cl, chlorodiethylenetriamineplatinum(II) chloride; r_b, the number of molecules of the platinum compound bound per nucleotide residue; transplatin, *trans*-diamminedichloridoplatinum(II); r_i, the molar ratio of free platinum complex to nucleotides at the onset of incubation with DNA; t_m, DNA melting temperature

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1. Introduction

A number of low-molecular-mass compounds of biological significance interact with DNA. Hence, their biological effects may be associated with the changes that they induce in DNA and with recognition of these alterations by the components of downstream cellular systems. In addition, various DNA-binding low-molecular-mass compounds serve as tools or probes for studies of DNA properties and interactions. One class of such compounds is represented by platinum coordination complexes. Some of these compounds are effective cross-linking agents capable of linking DNA to itself [1–3] or forming DNA–protein cross-links (CLs) [4]; some exhibit remarkable antitumor or antiviral effects [5–7]. Therefore, a great deal of attention is being paid at present to the design of new platinum complexes that would exhibit new DNA-binding properties that differ from those already described.

A DNA-binding platinum complex that has been most extensively studied is the anticancer drug *cis*-diamminedichloridoplatinum(II) (cisplatin) (Fig. 1a). It binds to DNA forming various intrastrand, interstrand CLs [1,8] as well as DNA–protein CLs [9–13]. Recently two interesting classes of platinum complexes that bind to DNA differently than cisplatin, and consequently exhibit different biological properties, were described. One class is represented by mononuclear analogues of the clinically inactive transplatin (*trans*-diamminedichloridoplatinum(II), Fig. 1b) containing: (i) heterocyclic ligands, such as quinoline, thiazole, piperidine, piperazine (pz) and picoline (the structure of *trans*-$[PtCl_2(NH_3)(pz)]$ is shown in Fig. 1c) [2,14,15], (ii) aliphatic amines [16] or (iii) iminoethers [17]. The second class consists of polynuclear platinum complexes with flexible [2,18,19] or sterically rigid [20,21] linking groups (the structures of dinuclear $[cis\text{-}\{PtCl(NH_3)_2\}_2H_2N(CH_2)_{2-6}NH_2]^{2+}$ (1,1/c,c) and $[\{cis\text{-}PtCl(NH_3)_2\}\mu\text{-}H_2N(CH_2)_6NH_2\text{-}\{cis\text{-}PtCl_2(NH_3)\}]^{+}$ (1,2/c,c) containing flexible aliphatic linking groups are shown in Fig. 1e and f, respectively as examples).

In the present work we designed, synthesized and characterized a new tetrafunctional dinuclear platinum complex *trans*,*trans*-$[\{PtCl_2(NH_3)\}_2(pz)]$ (Fig. 1d) thereby combining in one molecule the DNA-binding features of two classes of the platinum compounds with proven antitumor activity, namely *trans* oriented bifunctional mononuclear platinum complexes with a heterocyclic ligand and polynuclear (dinuclear) platinum complexes. An interesting attribute of this new tetrafunctional dinuclear platinum complex is also that the linker (piperazine) between the two platinum units is more rigid in contrast to the previously tested antitumor polynuclear platinum complexes, such as $[\{trans\text{-}PtCl(NH_3)_2\}_2\mu\text{-}trans\text{-}Pt(NH_3)_2\{H_2N(CH_2)_6NH_2\}_2]^{4+}$ (BBR3464), $[\{trans\text{-}[PtCl(NH_3)_2\}H_2N(CH_2)_{2-6}NH_2]^{2+}$ (1,1/t,t) or (1,1/c,c) which contain a relatively flexible aliphatic linker. Moreover, DNA-binding properties of *trans*,*trans*-$[\{PtCl_2(NH_3)\}_2(pz)]$ could be uniquely affected by the ability of the piperazine linker to adopt either chair or boat conformation. We analyzed DNA-binding mode of this new complex by various methods of molecular biology and biophysics and found that it bound to DNA in a unique way. The results of this work also demonstrate that *trans*,*trans*-$[\{PtCl_2(NH_3)\}_2(pz)]$ is a more effective cytotoxic agent than cisplatin in ovarian tumor cell lines.

2. Materials and methods

2.1. Reagents

2.1.1. Starting materials

Cisplatin was obtained from Sigma–Aldrich s.r.o. (Prague, Czech Republic). Chlorodiethylenetriamineplatinum(II) chloride ([PtCl(dien)]Cl) was a generous gift of Prof. G. Natile from University of Bari. The dinuclear tetrafunctional complex *trans*,*trans*-$[\{PtCl_2(NH_3)\}_2(pz)]$ was prepared and characterized as described below. Stock solutions of platinum compounds for the biophysical and biochemical studies were prepared at the concentration of 5×10^{-4} M in 10 mM $NaClO_4$ and stored at 4 °C in the dark. Stock solutions of platinum compounds for the cytotoxicity studies were prepared in DMSO and used immediately after dissolution. The concentrations of platinum in the stock solutions were determined by flameless atomic absorption spectrophotometry (FAAS). Calf thymus (CT) DNA (42% G + C, mean molecular mass ca. 2×10^7) was also prepared and characterized as described previously [22,23]. pSP73KB (2455 bp), pUC19 (2686 bp), and pBluescript SK^- (2958 bp) plasmids (superhelical density $\sigma = -0.063$, -0.055, and -0.058, respectively) were isolated according to standard procedures. The synthetic oligodeoxyribonucleotides (23-mers) were purchased from VBC-Genomics (Vienna, Austria) and purified as described previously [24,25]. The 23-bp duplex formed from these oligonucleotides contains central DNA consensus sequence of NF-κB protein. In the present work their molar concentrations are related to the duplexes. Restriction endonucleases *Eco*RI, *Nde*I and T4 polynucleotide kinase were purchased from New England Biolabs, NF-κB (p50 dimer) from Active Motif (Rixensart, Belgium), histone H1 from Roche Diagnostics, GmbH (Mannheim, Germany), and Klenow

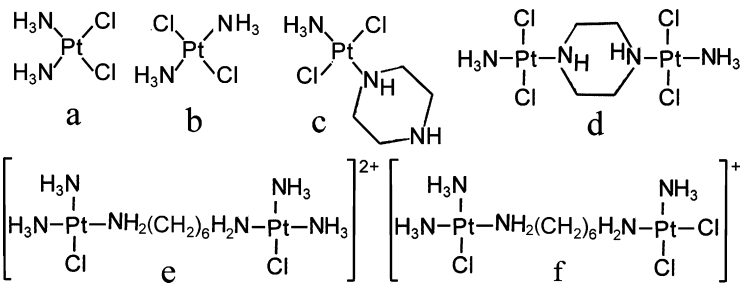


Fig. 1 – Structures of platinum complexes. (a) Cisplatin; (b) transplatin; (c) *trans*-$[PtCl_2(NH_3)(piperazine)]$; (d) *trans*,*trans*-$[\{PtCl_2(NH_3)\}_2(pz)]$; (e) $[cis\text{-}\{PtCl(NH_3)_2\}_2H_2N(CH_2)_6NH_2]^{2+}$ (1,1/c,c); (f) $[\{cis\text{-}PtCl(NH_3)_2\}\mu\text{-}H_2N(CH_2)_6NH_2\text{-}\{cis\text{-}PtCl_2(NH_3)\}]^{+}$ (1,2/c,c).

fragment from DNA polymerase I (wild type, KF or exonuclease minus, KF$^-$, mutated to remove the 3′ → 5′ proofreading domain) from Takara (Japan). Acrylamide, bis(acrylamide), dithiothreitol, NaCN, ethidium bromide (EtBr) and thiourea (TU) from Merck KgaA (Darmstadt, Germany), sodium dodecyl sulfate (SDS) from Serva (Heidelberg, Germany), and proteinase K from Boehringer (Mannheim, Germany). Agarose and Metaphor® agarose were from FMC BioProducts (Rockland, ME). Wizard® SV and PCR Clean-Up System used to extract and purify 213-bp DNA fragment (*vide infra*) was purchased from Promega. Nonidet P-30 was from Fluka (Prague, Czech Republic). Radioactive products were from MP Biomedicals, LLC (Irvine, CA).

2.1.2. *Synthesis of trans,trans-[{PtCl$_2$(NH$_3$)}$_2$(pz)]*

One hundred milligrams (0.333 mmol) of cisplatin were suspended in 20 ml of distilled deionized water. While stirring, 2 equiv. of piperazine (57.5 mg, 0.666 mmol) were added, and the mixture was heated to reflux for 3 h. At this stage another 100 mg (0.333 mmol) of cisplatin were added and the reflux of the mixture continued until the yellow suspension turned colorless (3 h). The reaction mixture was cooled to room temperature and all insoluble traces were filtered off. One ml of concentrated hydrochloric acid was added to the filtrate and the acidic mixture was refluxed for 3 h. After additional 2 h the solution was cooled to room temperature and the insoluble precipitate was collected by filtration and washed with 10 ml of distilled deionized water and 10 ml of DMF, 10 ml diethyl ether and suction dried. Yield: 77%. ^{195}Pt NMR (DMF): −2170 ppm. Elemental analysis for $C_4H_{16}Cl_4N_4Pt_2$ (C, H, N): calc. (7.37, 2.47, 8.59), found (7.15, 2.89, 9.02).

2.2. *Methods*

2.2.1. *Platination reactions*

DNA was incubated with platinum complex in 10 mM $NaClO_4$ at 37 °C for 48 h in the dark if not otherwise stated. The number of molecules of the platinum compound bound (coordinated) per nucleotide residue (r_b values) was determined by FAAS or by differential pulse polarography [23]. For example, CT DNA at a concentration of 0.32 mg/ml was incubated with platinum complex at an initial r_i of 0.08 in 10 mM $NaClO_4$ at 37 °C (r_i is defined as the molar ratio of free platinum complex to nucleotides at the onset of incubation with DNA). At various time intervals an aliquot of the reaction mixture was withdrawn and assayed by differential pulse polarography for platinum not bound to DNA. The amount of platinum bound to DNA (r_b) was calculated by subtracting the amount of free (unbound) platinum from the total amount of platinum present in the reaction.

2.2.2. *DNA transcription by RNA polymerases in vitro*

Transcription of the (*NdeI*/*HpaI*) restriction fragment of pSP73KB DNA with T7 RNA polymerase and electrophoretic analysis of transcripts was performed according to the protocols recommended by Promega (Promega Protocols and Applications, 43–46 (1989–1990) and previously described in detail [26,27].

2.2.3. *Fluorescence measurements*

These measurements were performed on a Shimadzu RF 40 spectrofluorophotometer using a 1 cm quartz cell. Fluorescence measurements of DNA modified by platinum 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 0.4 M NaCl to avoid secondary binding of EtBr to DNA [28,29]. 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 [28].

2.2.4. *DNA interstrand cross-linking*

Platinum complexes at varying concentrations were incubated with 1 μg of pSP73KB DNA linearized by *Eco*RI. The linear DNA was first 3′-end labeled by means of KF in the presence of [α-^{32}P]dATP. The platinated samples were precipitated by ethanol and analyzed for DNA interstrand CLs by previously published procedures [26,30]. After the platination, the samples were 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% bromophenol blue. The amount of interstrand CLs was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%). After the electrophoresis was completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified by means of a bioimaging analyzer.

2.2.5. *Assay for interhelical cross-linking*

An equimolar mixture of linearized pSP73KB (2455 bp) and short 213-bp *NdeI*/*Eco*RI restriction fragment of pUC19 uniquely 5′-end labeled at the *Eco*RI restriction site was platinated in 0.01 or 0.1 M $NaClO_4$ at r_b = 0.01–0.1 in a reaction volume of 20 μl. The total concentration of DNA in these mixtures was in the range of 0.25–0.70 mg/ml. Aliquots were subjected to electrophoresis in an 0.8% agarose gel in TBE (Tris–borate/EDTA) buffer [31]. Location of the pUC19 plasmid in gel was visualized by fluorescence under UV irradiaton after staining the gel with EtBr; subsequently, the gel was dried and subjected to autoradiography, revealing the location of the radioactively labeled fragment.

Another assay used was based on using negatively supercoiled pUC19 plasmid. The plasmid was platinated in 0.01 or 0.1 M $NaClO_4$ and subsequently linearized by *Eco*RI (which cuts only once within pUC19 plasmid). The resulting products were analyzed by electrophoresis in a 0.8% agarose gel in TBE buffer and their location in gel was visualized by fluorescence under UV irradiation after staining the gel with ethidium bromide.

2.2.6. *DNA melting*

The melting curves of CT DNAs were recorded by measuring the absorbance at 260 nm. The melting curves of unplatinated or platinated DNA were recorded after Tris–HCl/EDTA buffer and $NaClO_4$ were added so that the resulting media contained 0.01 M or 0.2 M $NaClO_4$ with 1 mM Tris–HCl/0.1 mM EDTA, pH 7.4. The value of the melting temperature (t_m) was determined as the temperature corresponding to a maximum on the first-derivation profile of the melting curves. The t_m values could be thus determined with an accuracy of ±0.3 °C.

2.2.7. *Unwinding of negatively supercoiled DNA*

Unwinding of closed circular supercoiled pBluescript SK$^-$ plasmid DNA was assayed by an agarose gel mobility shift

assay [22]. The unwinding angle Φ, induced per platinum-DNA adduct 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 *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] at 37 °C in the dark for 48 h. All samples were precipitated by ethanol and redissolved in the TAE (Tris–acetate/EDTA) buffer. An aliquot of the precipitated sample was subjected to electrophoresis on 1% agarose gels running at 25 °C in the dark with TAE buffer and the voltage set at 25 V. The gels were then stained with EtBr, followed by photography with transilluminator. The other aliquot was used for the determination of r_b values by FAAS.

2.2.8. *Preparation of proteins*

The final composition of the storage buffers: KF^-: 10 mM Tris pH 8, 0.5 mM EDTA, 100 μg/ml BSA, 50% glycerol and 2 mM $MgSO_4$; histone H1: 10 mM Tris pH 7.9, 20 mM NaCl, 0.1 mM PMSF for histone H1; NF-κB protein (p50 dimer): 25 mM Tris–HCl pH 8.0, 50 mM NaCl. The commercially available sample of KF^- was in the manufacturer's storage buffer containing dithiothreitol; the manufacturer's storage buffer was exchanged for that specified above using microcon concentrators.

2.2.9. *DNA–protein reactions*

Platinated DNA (23-bp oligodeoxyribonucleotide duplex or 213-bp *NdeI*/*Eco*RI fragment of pUC19 plasmid) at the concentration of 10 nM was incubated with the proteins (KF^-, histone H1 or NF-κB) at the concentration of 100 nM overnight at room temperature in the appropriate buffer: 10 mM Tris pH 8, 10 mM EDTA, 0.1 μM bovine serum albumin, 0.8% glycerol and 2 mM $MgSO_4$ (KF^-); 10 mM Tris pH 7.9 and 20 mM NaCl (histone H1); 42 mM HEPES, 42 mM KCl, 1 mM $MgCl_2$, 0.02 mM EDTA, 210 mM DDT, 2.5% glycerol and 2% Ficoll (NF-κB). The ability to form CLs between oligonucleotide duplexes (23-bp) and proteins was assessed by 10% SDS/polyacrylamide (PAA) gel electrophoresis after mixing the samples with the loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and denaturing by heat at 90 °C for 5 min. Gels were electrophoresed for 1–2 h at 140 V, dried and visualized by using the bio-imaging analyzer. The ability to form CLs between 213-bp DNA fragment and proteins was assessed by 1% agarose (agarose and Metaphor® agarose 1:1) gel electrophoresis after mixing the samples with the loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and denaturing by heat at 90 °C for 5 min. Gels were electrophoresed for 2 h at 50 V, dried and visualized by bio-imaging analyzer.

2.2.10. *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. For FAAS analysis DNA was precipitated with ethanol and dissolved in 0.1 M HCl. Purification of oligonucleotides with the aid of HPLC was carried out on a Waters HPLC system consisting of Waters 262 Pump, Waters 2487 UV detector and Waters 600S Controller with MonoQ HR 5/5 column. The gels were visualized by using the BAS 2500 FUJIFILM bioimaging analyzer, and the radioactivities associated with bands were quantitated with the AIDA image analyzer software (Raytest, Germany).

2.2.11. *Cytotoxicity*

The human ovarian tumor cell lines A2780 (parent cisplatin sensitive) and A2780cisR (with acquired cisplatin resistance) were cultured in RPMI 1640 medium (Gibco), supplemented with 10% FBS, 2 mM glutamine, 50 μg/ml gentamycin at 37 °C in an atmosphere of 95% of air and 5% CO_2. Cell death was evaluated by using a system based on the tetrazolium compound MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] which is reduced by living cells to yield a soluble formazan product that can be detected colorimetrically [32]. Cells were seeded in 96-well sterile plates at a density of 10^4 cells per well in 100 μl of medium and were incubated 16 h. The compounds were added to final concentrations from 0 to 256 μM in a volume of 100 μl per well. Seventy-two hours later 10 μl of a freshly diluted MTT solution (2.5 mg/ml) was pipetted into each well and the plate was incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 5 h the medium was removed and the formazan product was dissolved in 100 μl of DMSO. The cell viability was evaluated by measurement of the absorbance at 570 nm, using an Absorbance Reader SUNRICE TECAN SCHOELLER. IC_{50} values (compound concentration that produces 50% of cell growth inhibition) were calculated from curves constructed by plotting cell survival (%) versus drug concentration (μM). All experiments were made in quadruplicate.

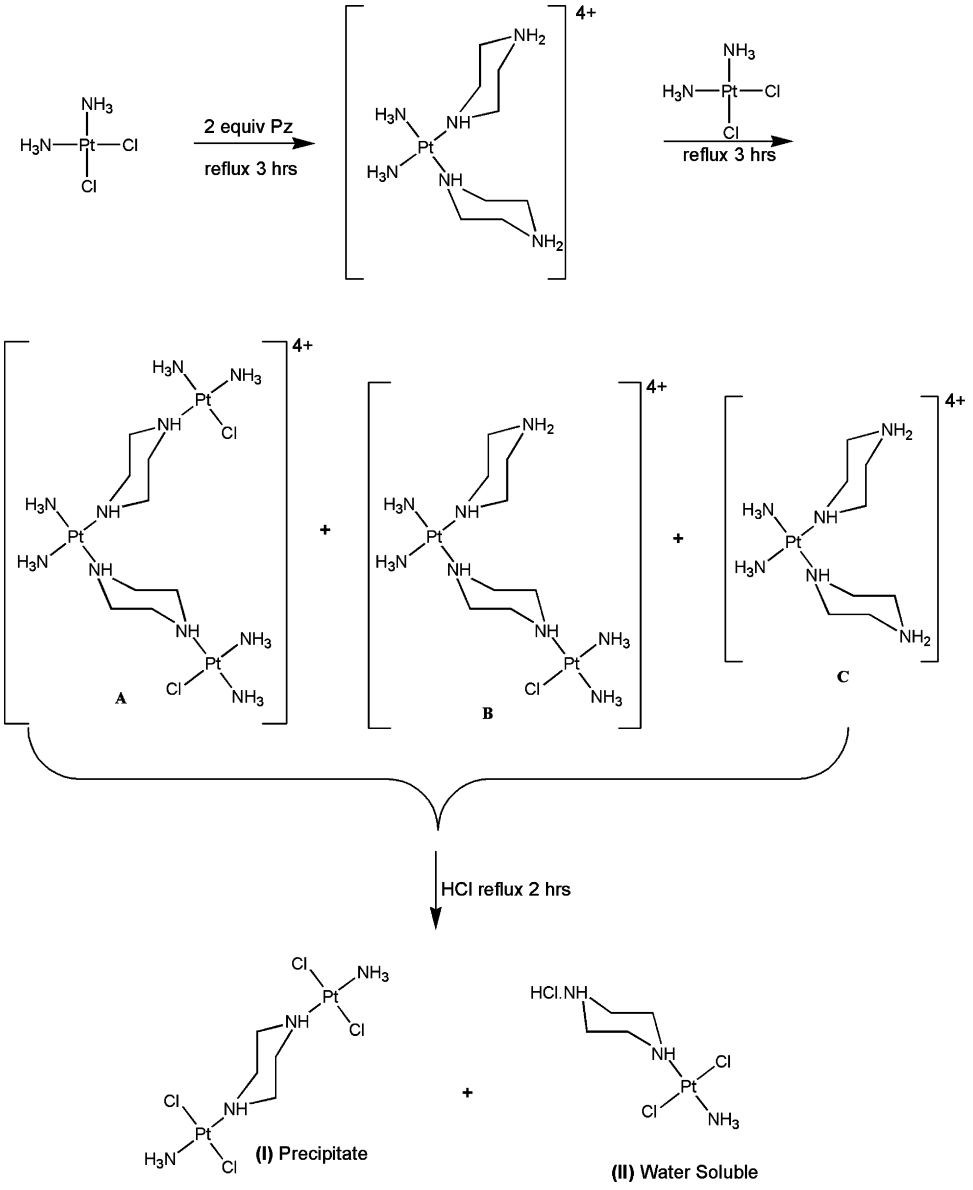
3. Results

3.1. *Synthesis of trans,trans-[{$PtCl_2(NH_3)$}$_2$(pz)]*

The synthesis of the compound is summarized in Scheme 1. Briefly, cisplatin was reacted with two equivalents of piperazine to form the *cis*-$[Pt(NH_3)_2(pz)_2]^{4+}$ (verified by ^{195}Pt NMR) to which two equivalents of cisplatin were added and the reaction mixture was refluxed for several hours. There are three possibilities: two cisplatin molecules will bind to the free amine of the pz; only one cisplatin molecule will bind to the free amine of the pz or no binding will occur (Scheme 1). Refluxing the reaction mixture with HCl could result in only two possibilities: obtaining the soluble monomer *trans*-$[PtCl_2(NH_3)(pz)]^+$ or the neutral insoluble binuclear complex *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)]. The compound was obtained in good yield (>70%).

3.2. *CT DNA binding*

The amount of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] complex coordinated to DNA increased rapidly with time and after approximately 10 h the complex was quantitatively bound (Fig. 2). The half-time ($t_{1/2}$) of this binding reaction was ~70 min. The binding of this electroneutral dinuclear tetrafunctional platinum complex is thus similar or faster than



Scheme 1

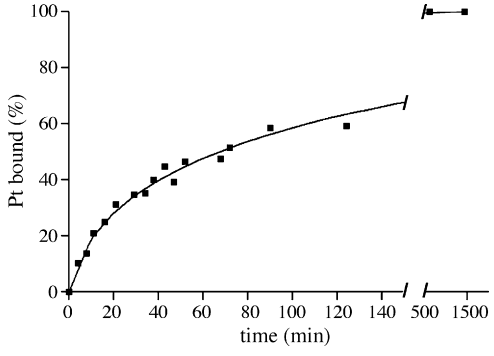


Fig. 2 – Kinetics of the binding of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] to calf-thymus DNA in the medium of 10 mM $NaClO_4$ at 37 °C determined by differential pulse polarographic assay. The concentration of DNA was 32 μg/ml and r_i was 0.08.

that of other positively charged dinuclear bifunctional platinum compounds, such as 1,1/t,t and 1,1/c,c [33]. This may be attributed to the availability of the two additional reactive groups for DNA binding, which may compensate a contribution to the rate of DNA binding of several positively charged bifunctional polynuclear platinum compounds with flexible linking groups due to attractive electrostatic interactions of the complex and DNA. No changes in the pH of the reaction mixture containing DNA and *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] were measured within 48 h after mixing DNA with the platinum complex. The rapid and essentially quantitative binding of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] facilitates sample analysis. The binding experiments indicate that such platination reaction resulted in the coordination of all molecules of the platinum complex, making it possible to prepare easily and precisely DNA samples modified at a preselected value of r_b.

3.3. *In vitro transcription of DNA containing platinum adducts*

In vitro RNA synthesis by RNA polymerases on DNA templates containing several types of bifunctional adducts of platinum complexes can be prematurely terminated at the level or in the proximity of adducts [26,34–38]. Interestingly, monofunctional DNA adducts of several platinum complexes are unable to terminate RNA synthesis [26,27,35]. Cutting of pSP73KB DNA by *Nde*I and *Hpa*I restriction endonucleases yielded a 212-bp fragment (a substantial part of its nucleotide sequence is shown in Fig. 3B). This fragment contained T7 RNA polymerase promotor. The experiments were carried out using this linear DNA fragment, modified by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] complex at r_b = 0.01, for RNA synthesis by T7 RNA polymerase (Fig. 3A, lane *trans*,*trans*-pz). RNA synthesis on the DNA template modified by these complexes yielded fragments of defined sizes, which indicates that RNA synthesis on these templates was prematurely terminated. The major stop sites produced by tetrafunctional complex *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] were at guanine residues, a few stop sites were also at adenine and cytosine residues. These results also suggest that the adducts formed by this complex are able to inhibit RNA polymerase.

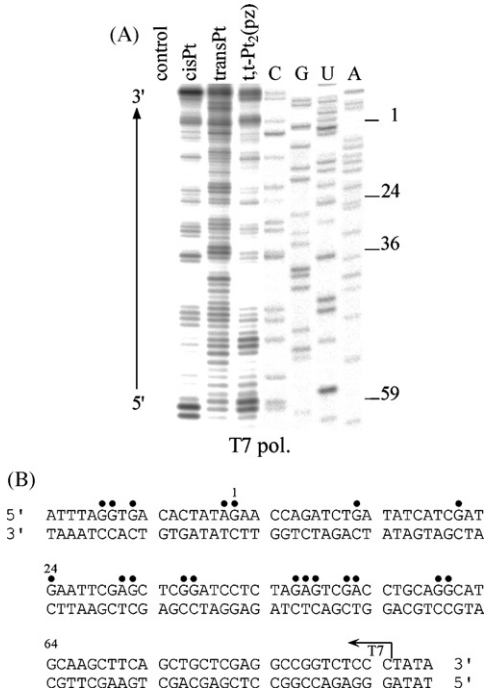


Fig. 3 – Inhibition of RNA synthesis by T7 RNA polymerase on the *Nde*I/*Hpa*I fragment of pSP73KB plasmid containing adducts of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)], cisplatin or transplatin. (A) Autoradiograms of a 6% PAA/8 M urea sequencing gel. Lanes: control, nonplatinated template; t,t-Pt_2 (pz), cisPt and transPt, DNA modified by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)], cisplatin or transplatin, respectively at r_b = 0.005; A, C, G and U, chain-terminated marker RNAs. The numbers correspond to the nucleotide sequence numbering of (B). (B) Schematic diagram showing the portion of the sequence used to monitor inhibition of RNA synthesis by the platinum complexes. The arrow indicates the start of T7 RNA polymerase, which used as template the upper strand of *Nde*I/*Hpa*I fragment of pSP73KB DNA. Full circles indicate the sites corresponding stop signals from (A), lane t,t-Pt_2 (pz). The numbers correspond to the nucleotide numbering in the sequence map of pSP73KB plasmid.

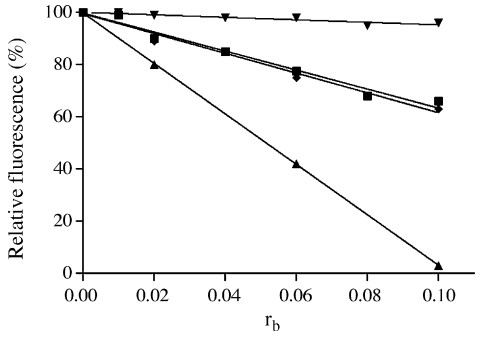


Fig. 4 – Dependences of the EtBr fluorescence on r_b for DNA modified by various platinum complexes in 10 mM $NaClO_4$ at 37 °C for 48 h. (▲) 1,1/t,t; (▼) [PtCl(dien)]Cl; (◆) cisplatin; (■) *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)].

3.4. *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 and polynuclear platinum compounds [33,34,39–41]. Double-helical DNA was first modified to an r_b in the range between 0 and 0.1 by cisplatin, monofunctional [PtCl(dien)]Cl, dinuclear bifunctional 1,1/t,t and dinuclear tetrafunctional *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)]. Modification of DNA by all platinum complexes resulted in a decrease of EtBr fluorescence (Fig. 4). The decrease caused by the adducts of dinuclear platinum complexes *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] was comparable to that induced by the adducts of cisplatin and markedly less pronounced than that caused by the DNA adducts of dinuclear complex 1,1/t,t at equivalent r_b. 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 [33,34,39–41]. Comparison with 1,1/t,t suggests that the conformational distortion induced in DNA by the adducts of dinuclear tetrafunctional complex *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] is localised and spans fewer base pairs around the platination sites, reminiscent of adducts of mononuclear bifunctional complexes like transplatin. These results show that formation of the long-range intra- or interstrand CLs or of other lesions that would extend over more than two base pairs, such as most of tri- or tetrafunctional adducts, by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] is unlikely. Thus, characterization of DNA adducts of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] by EtBr fluorescence supports the view that the DNA binding mode of the dinuclear tetrafunctional platinum compound with the semi-rigid linker is distinctly different from that of polynuclear complexes containing flexible aliphatic linkers, such as BBR3464, 1,1/t,t, or 1,1/c,c.

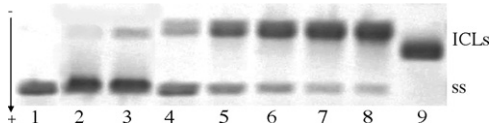


Fig. 5 – The formation of the interstrand CLs by platinum complexes in linear pSP73KB plasmid (2455 bp). Autoradiograms of denaturing 1% agarose gels of DNA fragment which was 3′-end labeled. The interstrand cross-linked DNA appears as the top bands migrating on the gel more slowly than the single-stranded DNA (contained in the bottom bands). DNA fragment was nonplatinated (control) (lane 1) or incubated for 24 h with *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] (lanes 2–9). r_b values: 0.00001 (lane 2), 0.00005 (lane 3), 0.0001 (lane 4), 0.0003 (lane 5), 0.0005 (lane 6), 0.0008 (lane 7), 0.001 (lane 8), and 0.005 (lane 9).

3.5. *Interstrand cross-linking*

To quantitate this feature pSP73KB plasmid (2455 bp) was linearized by *Eco*RI, 3′-end labeled and modified by *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] at various r_b values. The samples were analyzed for the interstrand CLs by agarose gel electrophoresis under denaturing conditions [26]. Upon electrophoresis under denaturing conditions, 3′-end labeled strands of DNA containing no interstrand CLs migrate as a 2455-nucleotide single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species. The bands corresponding to more slowly migrating interstrand-cross-linked 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-cross-linked or cross-linked DNA under each condition. The frequency of interstrand CLs 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 interstrand CL frequency was $\sim 44 \pm 3\%$. Thus, the DNA interstrand cross-linking efficiency of *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] was higher than that of cisplatin (6%; [26]), but significantly lower than that of the dinuclear bifunctional complexes, such as 1,1/t,t or 1,1/c,c (80–90%; [33,34]).

3.6. *Interhelical cross-linking*

A large number of DNA cross-linking agents are known, but the reactive moieties of such compounds usually bind to the same DNA duplex because binding of one moiety inevitably leaves the other in close proximity to other binding sites in the same duplex, leading to intramolecular cross-linking. However, if the reactive sites of the cross-linking agents are connected by a rigid linker that forces those sites to point in opposite directions, binding of both reactive sites into the same duplex may be more difficult unless the duplex is long enough to fold back on itself [42,43]. If the stereochemistry of the reactive sites of such cross-linking agents proves appropriate, such agents could bind to adjacent duplexes.

To asses whether the dinuclear *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] is able to form interhelical CLs, i.e. CLs between two DNA duplexes or between two remote double-helical regions of the same supercoiled DNA molecule, gel electrophoresis was used. In the first series of these experiments the radioactively labeled *Nde*I/*Eco*RI fragment of pUC19 (213 bp) and linearized pSP73KB plasmid (2455 bp) were mixed at 1:1 molar ratio, the mixture was platinated by *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] at r_b = 0.01–0.1 and the reaction products were analyzed by native agarose gel electrophoresis. In order to minimize the electrostatic repulsion between the sugar-phosphate backbones of two different duplexes, the mixture of the two DNA fragments was also platinated in a medium containing a high concentration of counter cations. Migration of the linearized pSP73KB DNA through the gel was detected by fluorescence under UV light after staining the gel with ethidium bromide (Fig. 6A). Subsequently, autoradiography of the same, but dried gel was

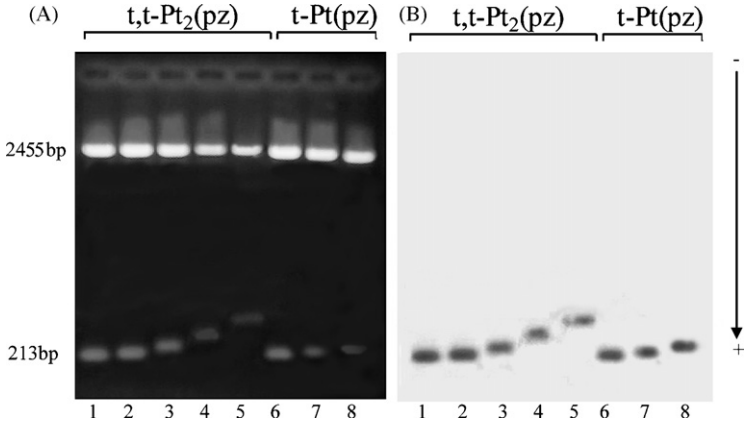


Fig. 6 – Interduplex cross-linking assay. The radioactively labeled *Nde*I/*Eco*RI fragment of pUC19 (213 bp) and linearized pSP73KB plasmid (2455 bp) were mixed at 1:1 molar ratio; the total concentration of DNA in this mixture was 0.32 mg/ml. This mixture was platinated in 0.1 M NaClO$_4$ at r_b = 0.05 by *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] and the reaction products were analyzed by native agarose gel electrophoresis. Migration of linearized pSP73KB was detected by fluorescence under UV irradiation after staining with EtBr (A). Migration of the labeled 213-bp fragment was detected by autoradiography of the dried gel (B). Migration of linearized pSP73KB and the labeled fragment is independent, indicating an absence of interduplex CLs.

performed to localize the labeled fragment (Fig. 6B). No discernible fraction of the labeled fragment was found to migrate along with the linearized plasmid.

As the entropic factor involved in bringing two DNA molecules together is large, the capability of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] to form intermolecular CLs was also assessed using negatively supercoiled pUC19 plasmid. Two distant binding sites in supercoiled DNA can be localized in close proximity due to supercoiling. Thus, if these distant sites are cross-linked then subsequent linearization of the platinated plasmid results in a bent molecule or loop which can be resolved by gel electrophoresis. Negatively supercoiled pUC19 plasmid was modified by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] at r_b = 0.001–0.1 in 0.01 or 0.1 M $NaClO_4$ and subsequently linearized by *Eco*RI (which cuts only once within pUC19 plasmid). The resulting linear DNA co-migrated in the gel with the linear DNA produced by cleavage by *Eco*RI of the same, but nonmodified pUC19 plasmid (not shown). These results along with those described in the preceding paragraph can be interpreted to mean that formation of interduplex CLs by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] is unlikely.

3.7. Characterization of platinum adducts by thiourea (TU)

The characterization of DNA adducts of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] by EtBr fluorescence (*vide supra*) suggests that that formation of tri- and tetrafunctional adducts by this dinuclear complex is unlikely. Hence, the remaining adducts that could reasonably explain the results of the characterization of DNA adducts of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] by EtBr fluorescence (Fig. 4) are bifunctional CLs of this platinum compound. Each platinum unit of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] contains two leaving chloride groups in *trans* configuration. Hence, this complex can theoretically bind to DNA in a bidentate manner and form CLs via leaving groups belonging to one or both platinum units. In order to examine this eventuality we characterized DNA adducts formed by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] using TU assay [44]. Bifunctional platinum compounds that coordinate to DNA form CLs in a two-step process, forming first the monofunctional adducts preferentially at guanine residues, which subsequently close to bifunctional lesions [45,46]. TU is used to labilize monofunctionally bound transplatin from DNA. The displacement of transplatin is initiated by coordination of TU *trans* to the nucleobase. Because of the strong *trans* effect of sulfur in TU, 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 TU treatment [44]. Thus, if *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] binds to DNA in a bidentate manner only via one platinum unit then TU is unable to remove the adduct from DNA. On the other hand, if *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] would bind to DNA in a bidentate manner via two platinum units then each platinum unit would form DNA adduct leaving one reactive group *trans* to the nucleobase. Hence, DNA bidentate adducts of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] involving both platinum atoms coordinated to DNA (as well as monofunctional adducts) should be removed by TU whereas those involving only one platinum atom should be resistant to the TU treatment.

Double-stranded CT DNA at the concentration of 0.13 mg/ml was incubated with mononuclear *trans*-[$PtCl_2(NH_3)$(pz)] or *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] at r_b = 0.05 in 10 mM $NaClO_4$ at 37 °C. After 24 h the reaction was stopped by adjusting the NaCl concentration to 0.2 M and by immediate cooling to −20 °C. In parallel experiments, the reaction was stopped by addition of 10 mM TU solutions. These samples were incubated for 15 min at 37 °C and then quickly cooled to −20 °C. The samples were then exhaustively dialyzed against 0.2 M NaCl and subsequently against H_2O at 4 °C, and the platinum content was determined by FAAS. TU displaced 20% of the platinum from DNA modified by the mononuclear complex in accord with the previously published results [47] implying that this complex formed 80% CLs. On the other hand, TU displaced no platinum from DNA modified by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] so that it is reasonable to conclude that this dinuclear complex does not bind to DNA in a bidentate manner via coordination of two platinum atoms to DNA, but rather forms transplatin-like adducts.

3.8. DNA melting

CT DNA was modified by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] to various r_b (0–0.08) in 10 mM $NaClO_4$. Salt concentration was then further adjusted by addition of $NaClO_4$ to the values of 0.01 or 0.2 M. The effect on DNA melting temperature (t_m) is dependent both on the amount of platinum bound and the salt concentration. At low concentrations of $NaClO_4$ (0.01 M) an increase of t_m is observed even at relatively high levels of the modification of DNA by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] (Δt_m was ~10 °C at r_b = 0.08) (Fig. 7). At high salt concentrations of 0.2 M the modification resulted in an increase of t_m which became less pronounced compared to that induced at low concentration of $NaClO_4$ (Fig. 7). Thus, the dinuclear tetrafunctional compound *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] affected t_m qualitatively in different way than for instance dinuclear bifunctional compounds 1,1/t,t or 1,1/c,c [33,34], which destabilize DNA at high salt concentration. This behavior is in marked contrast to cisplatin, where the modification of DNA results in a decrease of t_m if DNA melting is measured at salt concentrations ranging from 0.01 to 0.2 M [48].

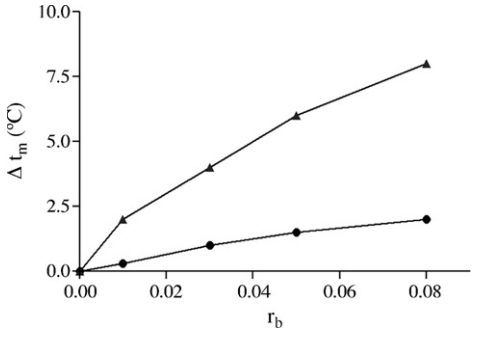


Fig. 7 – The plot of Δt_m values of CT DNA modified by *trans*,*trans*-[{$PtCl_2(NH)_3$}$_2$(pz)] on r_b measured in 1 mM Tris–HCl with 0.1 mM EDTA, pH 7.4 plus 0.01 M (▲) or 0.2 M (●) $NaClO_4$. Δt_m is defined as the difference between the t_m values of platinated and nonmodified DNAs.

Previously, three factors have been invoked to account for the thermal stability of DNA modified by platinum(II) complexes: stabilizing effects of the positive charge on the platinum(II) moiety and of DNA interstrand CLs, and a destabilizing effect of conformational distortions induced in DNA by platinum coordination resulting in various types of adducts [48]. The dependence of transition melting temperature on ionic strength was explained by competing electrostatic effects as salt concentration was varied. Under the incubation conditions, we expect bifunctional or polyfunctional platinum compounds to have produced a range of CLs and that the observed change in melting temperature will reflect the relative proportion and contribution of the two limiting factors. Inherently, we predict conformational alterations due to intrastrand cross-linking to destabilize the helix, as has been consistently observed in studies with cisplatin. In contrast, interstrand cross-linking is also predicted to stabilize the helix by preventing strand dissociation. At low ionic strength, it is reasonable to conclude that the increases in t_m are caused by the high percentage of interstrand CLs formed by the dinuclear compound and by positive charges on platinum moieties. The observation that high salt appears to result in stabilization by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] adducts seems to be a consequence of ''stabilizing'' effect of interstrand cross-links that then dominate over the conformational changes. At high salt concentration the stabilizing effects are reduced since electrostatic effects of the platinum compounds are apparently lowered with increasing concentration of Na^+ counter ions.

3.9. *Unwinding induced in supercoiled DNA*

Electrophoresis in native agarose gel was used to quantify the unwinding induced in pBluescript SK^- plasmid by the platinum complex *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] by monitoring the degree of supercoiling. Fig. 8 shows electrophoresis gels in which increasing amounts of *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] have been bound to a mixture of nicked and supercoiled pBluescript SK^- DNA. Interestingly, the dinuclear complexes *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] accelerated the mobility of the relaxed form similarly as do several other bifunctional complexes, whose bifunctional binding to DNA shortens and condenses the DNA helix [49,50]. 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 [51]. Under the present experimental conditions, σ was calculated to be -0.058 on the basis of the data of cisplatin for which the $r_b(c)$ was determined in this study and

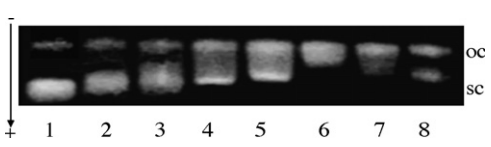


Fig. 8 – Unwinding of supercoiled pBluescript SK^- plasmid DNA modified by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)]. Lanes: (1) control, nonmodified DNA; (2–8) r_b = 0.03, 0.04, 0.045, 0.05, 0.055, 0.06, 0.07, respectively. The top bands correspond to the form of nicked plasmid (oc) and the bottom bands to closed negatively supercoiled plasmid (sc).

$\Phi = 13^\circ$ was assumed [51,52]. Using this approach the DNA unwinding angle of $17 \pm 2^\circ$ was determined for complex *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)]. The observation that *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] unwinds DNA markedly more than 1,1/c,c or 1,1/t,t compounds (13°) can be understood in terms of adduct structures in which the complex is preferentially coordinated to DNA in a bifunctional manner and is again consistent with the view that dinuclear tetrafunctional complex *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] with the rigid linker form on DNA adducts different from their bifunctional polynuclear counterparts containing the flexible linker.

3.10. *DNA–protein complex formation*

The dinuclear tetrafunctional platinum complex *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] was also investigated for its ability to act as a DNA–protein cross-linking agent. The proteins chosen for these studies bind to DNA with a relatively high affinity. KF^- and the linker histone H1 were chosen as the representatives of non-sequence specific DNA-binding proteins with enzymatic or structural function, respectively, whereas transcription factor NF-κB (p50 dimer) was chosen as the representative of a sequence-specific DNA-binding protein with a regulation function. The 23-bp duplex 5′-end-labeled at its top strand was used (its nucleotide sequence shown in Fig. 9C contains central DNA consensus sequence of NF-κB). It was globally modified by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] for 24 h so that one molecule of the complex was bound per duplex on average (r_b = 0.022). The platinated duplexes (10 nM) were mixed with either KF^-, histone H1, or NF-κB (the molar ratio protein/duplex was 10).

Ternary DNA–Pt–protein cross-linking efficiency was assessed by SDS/PAGE shift assay (PAGE, polyacrylamide gel electrophoresis). Fractions were detected by SDS/PAGE with significantly retarded mobility compared with that of the free probe (Fig. 9A and B). The intensity of the bands with the retarded mobility increased with the incubation time and at a given time of this incubation (in the range of 1–24 h) it also increased with growing r_b value (not shown). These more slowly migrating fractions were eliminated after treatment with NaCN or proteinase K converting them to those of the unmodified probes (not shown). These results suggest that the species is a protein–DNA CL tethered by platinum–DNA and platinum–protein covalent bonds.

The yields of DNA–protein cross-linking by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] using short 23-bp duplexes were relatively low (12%, 3%, and 18% for NF-κB, KF^-, and histone H1, respectively) (Fig. 9A and B). Therefore, we also used for similar studies DNA fragment 213-bp long. This fragment had a random nucleotide sequence and we examined its cross-linking to histone H1 and KF^-. The 213-bp duplex 3′-end-labeled at one strand was globally modified by *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] for 24 h to the r_b value of 0.0023. The platinated fragment (10 nM) was mixed with histone H1 or KF^- (the molar ratio protein/duplex was 10) and incubated overnight. Ternary DNA–Pt–protein cross-linking efficiency was assessed by 1% agarose (agarose and Metaphor® agarose 1:1) gel shift assay. As in the similar experiments using short duplexes the fractions were detected with significantly retarded mobility compared with that of the free probe (not

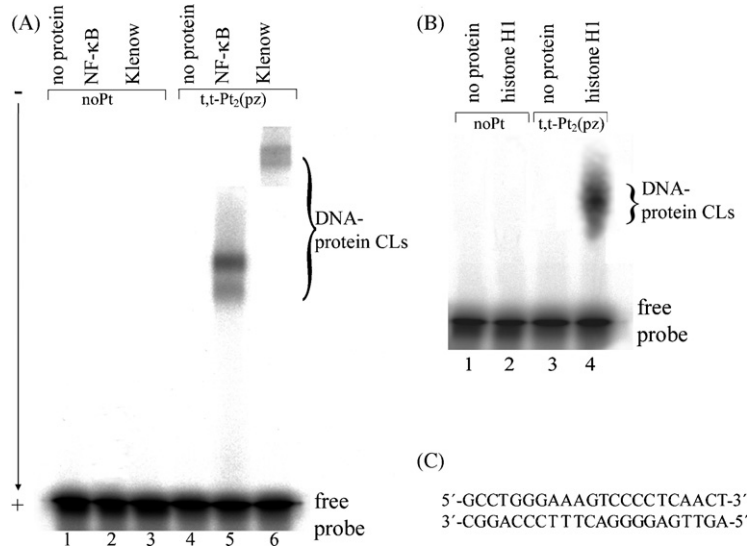


Fig. 9 – Formation of ternary DNA–Pt–Pt–protein complexes of 23-bp oligodeoxyribonucleotide duplexes modified by *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] with KF$^-$, NF-κB (A), or histone H1 (B) assessed by SDS/PAA gel electrophoresis. The duplexes were globally modified by *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] so that 1 molecule of the complex was bound per duplex on average (r_b = 0.022). Lanes in (A): (1–3) control, unplatinated duplex; (4–6), the duplex modified by *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)]; (1 and 4) no protein added; (2 and 5) NF-κB added; (3 and 6) KF$^-$ added. Lanes in (B): (1 and 2) control, unplatinated duplex; (3 and 4) the duplex modified by *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)]; (1 and 3) no protein added; (2 and 4) histone H1 added. (C) The sequence of the 23-bp synthetic oligodeoxyribonucleotide duplex used in this study; its top and bottom strands are designated 'top' and 'bottom', respectively, throughout.

shown). These more slowly migrating fractions were also eliminated after treatment with NaCN or proteinase K. Importantly, the amount of radioactivity associated with the bands corresponding to DNA–protein CLs formed by *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] (55%, 30%, and 33% for NF-κB, KF$^-$, and histone H1, respectively) was considerably higher than that observed in the case of the short 23-bp fragments. In aggregate, the experiments with the 213-bp fragment confirm that *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] is an efficient agent capable of forming ternary DNA–Pt–protein CLs.

3.11. *Cytotoxic activity*

The cytotoxic activity of the new dinuclear platinum compound tested in the present work was determined against cisplatin sensitive and resistant ovarian cancer cell lines A2780 and A2780cisR. A2780cisR cells are resistant to cisplatin through a combination of decreased uptake, enhanced DNA repair/tolerance and elevated reduced glutathione levels [53]. *Trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] and cisplatin were incubated for 72 h with the tumor cell line, and the cell survival in the culture treated with the platinum compounds was evaluated as described previously [32,54,55]. The IC$_{50}$ values obtained for *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] and cisplatin are summarized in the Table 1. These data suggest that the new dinuclear complex might be more effective than cisplatin in cisplatin sensitive ovarian tumor cells and markedly more effective than cisplatin in cisplatin resistant version of these tumor cells. A more definite conclusion on the activity of this new complex will be obtained by its testing in more tumor cell lines. These tests are in progress and their results will be reported in our future communication.

Table 1 – IC$_{50}$ mean values (±S.D.) (μM) obtained for the platinum compounds tested in the present work[a]

	A2780	A2780cisR
Trans,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)]	2.0 ± 0.3	4.3 ± 0.4
Cisplatin	3.6 ± 0.2	21 ± 2

[a] Drug-treatment period was 72 h.

4. Discussion

The biophysical studies performed here are entirely consistent with interstrand and intrastrand CLs as the predominant lesions formed in DNA by *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] in cell-free media and in absence of proteins (Table 2). An intriguing aspect of *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] is that, using a rigid linker, interstrand cross-linking is diminished relative to other dinuclear platinum complexes with flexible linking groups and delocalized long-range CLs or lesions that span several base pairs, such as tri- and tetrafunctional adducts, become unlikely.

Stop sites observed with the transcription mapping assay demonstrate that the CLs are formed preferentially between guanine residues. Few stop sites at adenine and cytosine residues are observed as well. Those at cytosine residues may be attributed to transplatin-like interstrand CLs formed between guanine and complementary cytosine residues [26].

Table 2 – Summary of DNA binding of dinuclear platinum complex *trans*, *trans*-[{PtCl$_2$(NH)$_3$}$_2$(pz)] (t,t-Pt$_2$(pz)) and comparison with DNA binding of dinuclear bifunctional complexes, mononuclear cisplatin, transplatin and *trans*-[PtCl$_2$(NH)$_3$(pz)] (transPt(pz))

	t,t-Pt$_2$(pz)[a]	transPt(pz)[b]	1,2/c,c[c]	1,1/c,c[d]	Cisplatin	Transplatin
DNA binding ($t_{1/2}$)	11 min	20 min[b]	20 min	~120 min	~120 min[e]	~120 min[e]
Nucleotide preference	G	G	G	G	GG, AG[f]	G, C[e]
Decrease of EtBr fluorescence	Medium	ND	Strong	Strong	Medium[a,g]	Medium[g]
% Interstrand CLs/adduct (after 48 h)	44	18	85	87–95	6[h]	12[h]
Unwinding angle/adduct	17°	17°	10°	10–12°	13°[i]	9°[i]
Melting temperature						
High ionic strength	Increase	Decrease	Decrease	ND	Decrease[j]	Increase[j]
Low ionic strength	Increase	Increase	Increase	Increase	Decrease[j]	Increase[j]
DNA–protein cross-linking	Yes	ND	Yes	ND	Yes[k]	No[k]

[a] This work.
[b] Ref. [47].
[c] Ref. [56].
[d] Ref. [33].
[e] Ref. [63].
[f] Ref. [45].
[g] Ref. [39].
[h] Ref. [26].
[i] Ref. [51].
[j] Ref. [48].
[k] Ref. [64].

Several polynuclear platinum compounds (for instance BBR3464, 1,1/t,t, 1,1/c,c or 1,2/c,c) form in DNA a large amount of long range CLs [33,34,39,56]. It is not possible to unambiguously rule out some formation of long range CLs by *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)], but characterization of its DNA adducts by EtBr fluorescence assay (Fig. 4) are not consistent with this eventuality. In addition, the results of studies using TU assay suggest that *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] does not bind to DNA in a bidentate manner via coordination of two platinum atoms to DNA, each in a monofunctional fashion. The results of studies using TU assay also demonstrate that *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] forms no monofunctional adducts.

Other biochemical and biophysical analyses of DNA interactions with *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] described in this report and summarized in Table 2 provide experimental support that binding of this platinum complex modifies DNA in a way which is different from that by cisplatin and other polynuclear platinum complexes. As DNA is an important target of biological effects of platinum(II) complexes [57], the unique DNA binding mode of *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] supports the suggestion that the altered DNA-binding mode might be a factor responsible for altered biological activity of this new dinuclear platinum complex.

The observation that *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)], in which two platinum units are linked by the rigid linker, readily forms the CLs within one duplex and no interhelical CLs deserves further discussion. For instance, bisintercalators of DNA with rigid and extended linkers of a sufficient length exhibit intermolecular DNA CLs [42,43,58]. Thus, the inability of *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] to form interduplex CLs (Fig. 6) can be interpreted to mean that the rigid linker (piperazine residue) in *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] is apparently too short to reach the binding sites of other DNA duplex. However, it cannot be excluded that dinuclear platinum analogues of *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] which contain a longer and rigid linker that forces the binding sites of these analogues to point in opposite directions will exhibit interhelical cross-linking efficiency so that they could become useful tools for probing DNA during its replication or recombination. On the other hand, binding of one platinum moiety of this dinuclear platinum complex to DNA inevitably leaves the other in close proximity to other DNA binding sites in the same duplex, leading to intramolecular cross-linking.

Interestingly, in contrast to the inability of *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] to cross-link two DNA duplexes, the results of the present work convincingly demonstrate that this dinuclear platinum complex forms specific DNA lesions which can efficiently cross-link proteins to DNA (Fig. 9). If such a ternary complex is formed, it is reasonable to expect that in the first step relatively flexible DNA-binding proteins (such as those tested in the present work) come into a close contact with DNA at the site of the platinum adduct. Then one molecule of *trans*,*trans*-[{PtCl$_2$(NH$_3$)}$_2$(pz)] can be coordinated simultaneously to a base residue in DNA and a reactive group in the protein only if the non-covalent preassociation complex is formed first. On the other hand, formation of a similar preassociation complex composed of two double-helical DNA duplexes or two double-helical segments of superhelically wound DNA molecule is much less likely also due to the repulsive negative charges on the surface of these rigid double-helical structures.

DNA–protein CLs are created following exposure of cells to a number of chemical or physical agents exhibiting various biological effects so that a number of these agents are cytotoxic, mutagenic or carcinogenic. The formation of platinum complex-mediated DNA–protein ternary complexes raises the possibility of ''suicide'' lesions, which may irreversibly sequester various DNA-binding proteins, such as transcription factors or repair proteins [59–61]. In addition, the cross-linking procedures involving these platinum agents

may provide a tool for identification of proteins or protein domains closely positioned to DNA including mapping of protein-binding sites on DNA *in vivo* [62]. Moreover, it is likely that structures and consequences of DNA conformational alterations and DNA–protein CLs will not only differ between mononuclear and polynuclear compounds [56], but also among polynuclear platinum complexes themselves suggesting further rational pathways to separate the biological consequences within the class of polynuclear platinum compounds.

Thus, the concept for the design of DNA–protein cross-linking agents based on tetrafunctional dinuclear platinum complexes with rigid linkers may result in new compounds which exhibit a variety of biological effects and can be also useful in nucleic acids research. Our observation (Table 1), that *trans*,*trans*-[{$PtCl_2(NH_3)$}$_2$(pz)] is more effective than cisplatin in ovarian tumor cell lines is consistent with this idea.

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