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Unique DNA Binding Mode of Antitumor Trinuclear Tridentate Platinum(II) Compound

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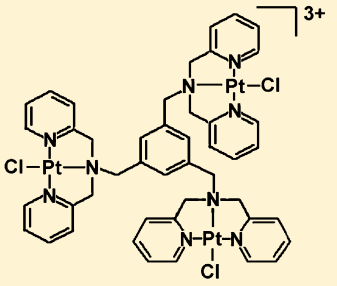
ABSTRACT: The new trinuclear tridentate Pt^{II} complex $[Pt_3Cl_3(hptab)]^{3+}$ (**1**; hptab = *N*,*N*,*N*′,*N*′,*N*″,*N*″-hexakis(2-pyridylmethyl)-1,3,5-tris(aminomethyl)benzene) exhibits promising cytotoxic effects in human and mouse tumor cells including those resistant to conventional cisplatin (*Dalton Trans.* **2006**, 2617; *Chem. Eur. J.* **2009**, *15*, 5245). The present study is focused on the molecular pharmacology of **1**, in particular on its interactions with DNA (which is the major pharmacological target of platinum antitumor drugs), to elucidate more deeply the mechanism underlying its antitumor effects. Results obtained with the aid of methods of molecular biophysics and pharmacology reveal new details of DNA modifications by **1**. Complex **1** binds to DNA forming in the absence of proteins and molecular crowding agents mainly trifunctional intrastrand cross-links. In these DNA adducts all three Pt^{II} centers of **1** are coordinated to DNA base residues, which leads to extensive conformational alterations in DNA. An intriguing aspect of the DNA-binding mode of this trinuclear Pt^{II} complex **1** is that it can cross-link proteins to DNA. Even more interestingly, **1** can cross-link in the presence of molecular crowding agent, which mimics environmental conditions in cell nucleus, two DNA duplexes in a high yield—a feature observed for the first time for antitumor trinuclear platinum complexes. Thus, the concept for the design of agents capable of forming intramolecular tridentate DNA adducts, DNA−protein and interduplex DNA−DNA cross-links based on trinuclear tridentate Pt^{II} complexes with semirigid aromatic linkers may result in new compounds which exhibit a variety of biological effects and can be also useful in nucleic acids research.



KEYWORDS: *platinum drug, antitumor, DNA binding, interduplex cross-links, DNA−protein cross-links*

INTRODUCTION

More than forty years after antitumor activity of cisplatin was discovered in 1969,[1] the search continues for platinum compounds having novel preclinical properties, in particular activity in tumor cells exhibiting inherent or acquired resistance to cisplatin and its clinically used analogues and/or reduced side effects. The findings include structures that significantly violate the original structure−pharmacological activity relationship, including cationic polynuclear Pt^{II} complexes. This class of new antitumor platinum compounds was designed as a result of systematically testing the hypothesis that there is a correlation between clinical efficacy of platinum compounds and their ability to induce a certain sort of damage or conformational change in target DNA. In other words, platinum drugs that bind to DNA in a manner fundamentally different from that of conventional cisplatin and its mononuclear analogues can exhibit altered biological properties including the spectrum and intensity of antitumor activity.[2,3] The antitumor effects of polynuclear platinum complexes may be modulated by the geometry and number of platinum centers and leaving groups in the coordination sphere of platinum atoms as well as the nature of the linkers bridging the platinum centers.[4,5] In addition, location of leaving ligands in relation to the linkers in polynuclear Pt^{II} compounds determines their susceptibility to deactivating metabolic decomposition by the sulfur nucleophiles since substitution of the bond between Pt^{II} center and leaving group (for instance Cl) by a trans-influencing S donor results in bridge cleavage.[6,7]

In an effort to design a new polynuclear platinum compound that is able to bind to DNA in a fundamentally new manner and consequently having novel preclinical properties, trinuclear trifunctional Pt^{II} complex $[Pt_3Cl_3(hptab)]^{3+}$ (**1**; hptab = *N*,*N*,*N*′,*N*′,*N*″,*N*″-hexakis(2-pyridylmethyl)-1,3,5-tris(aminomethyl)-benzene) was recently synthesized.[8] In this trinuclear and tridentate compound, the Pt^{II} centers are bridged by bulky semirigid aromatic linkers (Figure 1). This new trifunctional agent exerted promising cytotoxic effects in human and mouse tumor cell lines.[8,9] In addition, in contrast to another trinuclear but bidentate Pt^{II} complex $[\{trans\text{-}PtCl(NH_3)_2\}_2(\mu\text{-}trans\text{-}Pt(NH_3)_2\{NH_2(CH_2)_6NH_2\}_2)]^{4+}$ (BBR3464, Figure 1), which is in clinical trials,[4,5] the geometry of leaving groups in the

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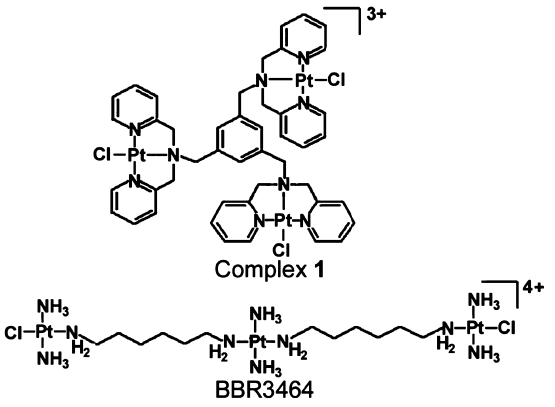

Figure 1. Structures of antitumor trinuclear platinum complexes.

coordination sphere of platinum atoms in **1** does not undergo decomposition of the polynuclear structure upon reaction with sulfur nucleophiles. Thus, also for this reason this trifunctional Pt^{II} complex can be considered a promising candidate for further preclinical and clinical testing.

In a continuing effort to design improved platinum antitumor agents, it is important to elucidate the biochemical and pharmacological factors that affect the cytotoxic properties of existing platinum complexes. The activity of Pt^{II} anticancer compounds involves their interaction with DNA in the nucleus. Reactions with DNA, the most nucleophilic sites of which are the N7 atoms of guanosine bases in the major groove, are the critical interactions relevant to antitumor effects which result in distortions of DNA conformation.[10] The entirely unique spatial configuration of leaving groups in **1**, semirigid linkers bridging three Pt^{II} centers and three reactive groups predispose this trifunctional complex to a unique DNA binding mode. Our primary objective in the present work was to understand more fully the DNA binding mode of **1** in a cell-free medium including also its unique intramolecular and intermolecular cross-linking capability. Comparisons of these results with those obtained earlier for conventional mononuclear cisplatin and other polynuclear Pt^{II} complex BBR3464 yielded new information that broadens the theoretical background needed for search and design of new, more effective antitumor metallodrugs.

■ MATERIALS AND METHODS

Chemicals. Cisplatin was obtained from Sigma (Prague, Czech Republic) (purity was ~99.9% based on elemental and ICP trace analysis). Trinuclear Pt^{II} complex **1** was prepared as described previously.[8,9] The purity of **1** was higher than 95% as established by combustion analysis carried out with a Hewlett-Packard 185 C, H, and N analyzer. Chloridodiethylenetriamineplatinum(II) chloride ([PtCl(dien)]Cl) was a generous gift from Prof. G. Natile (University of Bari, Italy). The stock solutions of platinum compounds were prepared in 10 mM $NaClO_4$ and stored at −20 °C in the dark. The concentrations of platinum complexes in the stock solutions were determined by flameless atomic absorption spectrometry (FAAS). The concentrations of the platinum complexes indicated in the present work are related to the whole compound (not to the Pt content in the case of the trinuclear complex). Calf thymus (CT) DNA (42% G + C, mean molecular mass ca. 20,000 kDa) was prepared and characterized as described previously.[11,12] Plasmids pUC19 [2686 base pairs (bp)] and pSP73KB (2455 bp) were isolated according to standard procedures. The oligodeoxyribonucleotide duplex containing 50 base pairs bp 5′-CCAGATCTGATATCATC-GATGAATTCGAGCTCGGTACCCGGGGATCCTCC/ 5′-GGAGGATCCCCGGGTACCGAGCTCGAATTCATC-GATGATATCAGATCTGG was from VBC-GENOMICS (Vienna, Austria). The purity of the oligonucleotides was verified by either high-performance liquid chromatography (HPLC) or gel electrophoresis. The duplex was formed by heating the mixture of the complementary single-stranded oligonucleotides at equal concentrations at 90 °C for 5 min followed by incubation at 25 °C for 4 h. The Klenow fragment from DNA polymerase I (exonuclease minus, mutated to remove the 3′−5′ proofreading domain) and restriction endonucleases were purchased from New England Biolabs (Beverly, MA). DNase I from bovine pancreas, nuclease P1 from *Penicillium citrinum* and alkaline phosphatase from calf intestine were from Sigma-Aldrich (Prague, Czech Republic). Agarose was from FMC BioProducts (Rockland, ME). Electrophoresis-grade acrylamide, *N*,*N*′-methylenebisacrylamide, ethidium bromide (EtBr), urea, NaCN and dithiothreitol were from Merck KgaA (Darmstadt, Germany). Proteinase K and ATP were from Boehringer (Mannheim, Germany). Sodium dodecyl sulfate (SDS) was from Serva (Heidelberg, Germany). NF-κB protein (p50 homodimer) was kindly provided by Professor M. Vasak (University of Zurich, Switzerland). Histone H1 was from Roche Diagnostics, GmbH (Mannheim, Germany). Radioactive products were from Amersham (Arlington Heights, IL, USA).

DNA Binding. If not stated otherwise the binding of **1** to double-helical CT DNA was measured with the aid of differential pulse polarography (DPP).[13] Since the polarographic technique determines the concentration of unbound platinum in the reaction mixture, the binding determined using this method corresponds to the initial (monofunctional) binding step.

Characterization of DNA Adducts by Thiourea. Incorporation of [^{14}C]thiourea into DNA under controlled conditions (8.7×10^{-4} M, 10 min, 25 °C[14]) was used to quantitate platinum-DNA mono- or bifunctional adducts of **1**. The measurements were carried out in 10 mM $NaClO_4$ at 25 °C. The molar ratio of free complex **1** to nucleotide-phosphates at the onset of incubation with CT DNA, r_i, was 0.05 (DNA concentration was 0.24 mg mL^{-1} (0.75 mM related to the phosphorus content), and the concentration of **1** was 3.75×10^{-5} M). Samples (120 μL) were withdrawn at various time intervals, and each sample was divided into two aliquots (60 μL). In one aliquot, conversion of the monofunctional adducts to bifunctional CLs and eventual subsequent conversion of bifunctional adducts to trifunctional CLs were blocked by addition of NaCl (final concentration was 0.15 M) and quick cooling to −20 °C. The unbound complex **1** was removed from these samples by centrifugation through a Sephadex G50 coarse column, and a molar ratio of covalently bound molecules of complex **1** per nucleotide residue, r_b, was determined by FAAS. In the other aliquot, the conversion of the adducts was blocked by addition of [^{14}C]thiourea (final concentration was 8.7×10^{-4} M, and specific activity was 2.5 mCi $mmol^{-1}$) and NaCl (final concentration was 0.15 M) so that the final volume of these samples was 1.0 mL. The samples were further incubated at 25 °C for 10 min and subsequently layered on Millipore filters (diameter of pores was 0.1 μm); the unreacted thiourea and complexes formed between unbound **1** and thiourea were removed by washing

the filters with 15 mL of 5% (v/v) trichloroacetic acid (TCA). The filters were dried under an infrared lamp and transferred to glass tubes, to which 5 mL of toluene scintillator was added. The radioactivity was measured on a liquid scintillation analyzer TriCarb 2800 TR (Perkin-Elmer) (2 × 2 min). The content of free coordination sites in DNA adducts of **1** not involved in the binding to DNA was determined as the amount (%) of radioactive thiourea bound to platinated DNA; the concentration of thiourea corresponding to 3-fold concentration of **1** (having three potential DNA binding sites) bound to DNA in each sample (in each time interval) determined by FAAS (r_b) was taken as 100%. It was also verified that, at 8.7×10^{-4} M thiourea, complete saturation of monofunctional or bifunctional adducts was obtained with no apparent reversal of platination.[15,16]

Characterization of DNA Adducts by EtBr Fluorescence. These measurements were performed on a Varian Cary fluorescence spectrophotometer using a 0.5 cm quartz cell. Fluorescence measurements 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.[17] 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 intercalation sites of EtBr in DNA.[17]

HPLC Analyses of Enzymatically Digested DNA. These analyses were performed using a Waters Breeze liquid chromatograph system equipped with 2489 UV/visible detector, 1525 binary HPLC pump, and a DiscoveryHSF5 HPLC column, 5 μm particle size (Sigma Aldrich-Supelco). The products were separated by reverse phase (RP) HPLC (isocratic elution with ammonium acetate (0.1 M, pH 4.5), in CH_3CN (3.9%) at 1 mL min^{-1} flow rate). The following enzymatic digestion protocol was used in order to characterize the platinated 50-bp DNA fragment. The samples [1 nmol (30.7 μg) of the DNA] were incubated with 60 units of DNase I at 37 °C. After 10 h, nuclease P1 (12 μg) was added, and the reaction was allowed to continue at 37 °C for 24 h. Finally, alkaline phosphatase (5 units) was added and the incubation continued for an additional 4 h at 37 °C. The samples were digested with the enzymes in the buffers recommended by the manufacturers. The digested samples containing constituent nucleosides were then heated for 30 min at 50 °C and centrifuged, the enzymes were separated by filters Nanosep NMWL:30K (Pall Filtron, Inc., Northborough, MA, USA) and the supernatant was analyzed by RP-HPLC. Each analysis was performed three times, and the data varied on average by ±1% from their mean.

DNA Melting. The melting curves of CT DNAs at the concentration of 32 μg mL^{-1} were recorded by measuring the absorbance at 260 nm. The melting curves of unmodified or metalated DNA were recorded in the medium containing $NaClO_4$ (0.01 or 0.1 M) with Tris-HCl (1 mM, pH 7.4)/EDTA (0.1 mM). The melting temperature (t_m) was determined as the temperature corresponding to a maximum on the first-derivative profile of the melting curves. The t_m values could be thus determined with an accuracy of ±0.3 °C.

Unwinding of Negatively Supercoiled DNA. Unwinding of closed circular supercoiled pUC19 plasmid DNA was assayed by an agarose gel mobility shift assay.[18] 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 trans-illuminator.

Interstrand (Intramolecular) Cross-Linking. Complex **1** at varying concentrations was incubated for 24 h with 0.5 μg of a linear 2686-bp fragment of pUC19 plasmid linearized by *Eco*RI. The linear fragment was first 3′-end labeled by means of the Klenow fragment of DNA polymerase I in the presence of [α^{32}P]dATP. The platinated samples were analyzed for DNA interstrand CLs by previously published procedures.[19,20] The number of interstrand CLs 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 CLs was calculated as % ICL/Pt = XL/5372r_b (the DNA fragment contained 5372 nucleotide residues), where % ICL/Pt is the number of interstrand CLs per adduct multiplied by 100, and XL is the number of interstrand CLs per molecule of the linearized DNA duplex and was calculated assuming a Poisson distribution of the interstrand CLs as XL = −ln *A*, where *A* is the fraction of molecules running as a band corresponding to the non-cross-linked DNA.

Electrophoretic Mobility Shift Assays with HMGB1 Protein. Radioactively labeled 50-bp DNA probe with a random nucleotide sequence was titrated with full-length HMGB1 protein. The fragments (0.1 pmol) were incubated with the protein in 10 μL sample volumes in a buffer composed of HEPES (10 mM, pH 7.5), $MgCl_2$ (10 mM), LiCl (50 mM), NaCl (0.1 M), spermidine (1 mM), bovine serum albumin (0.2 mg mL^{-1}), and Nonidet P40 (0.05% v/v). For all gel mobility shift experiments, samples were incubated on ice for 1 h and made 7% in sucrose and 0.017% in xylene cyanol before loading on running, precooled (4 °C), prerun (300 V, 1−2 h) 5% native PAA gels (29:1 acrylamide:bisacrylamide, 0.5× Tris-borate-EDTA buffer, Tris-HCl (45 mM), boric acid (45 mM), and Na_2H_2EDTA (1 mM, pH 8.3). Gels were electrophoresed at 4 °C and 300 V for ∼1.5 h, dried, exposed to a molecular imaging plate, and analyzed on a Fujifilm bioimaging analyzer. The radioactivities associated with the bands were quantitated with the AIDA image analyzer software. Other details have been published previously.[21,22]

Interduplex Cross-Linking. The double-stranded linearized pUC19 DNA (2686 bp) 3′-end-labeled with [α^{32}P]dATP was platinated by **1** at r_b = 0.0001−0.005 or by cisplatin at r_b = 0.001−0.05 in the medium containing sodium acetate (0.2 M, pH 5.5) and ethanol (70%) in a reaction volume of 15 μL at 37 °C for 48 h. Each reaction contained 500 ng of plasmid DNA. Then samples were stored on dry ice for 30 min and centrifugated at 14000*g* for 30 min at 4 °C; supernatant was removed, and pellet was dried and dissolved in TAE buffer. The amount of interduplex CLs was analyzed by electrophoresis in native agarose gel (0.5%). After the electrophoresis had been completed, the intensities of the bands corresponding to interduplex cross-linked and non-interduplex cross-linked duplexes were quantified. The frequency of interduplex CLs (% IICL/Pt) was calculated as % IICL/Pt = IXL/5372·r_b (the DNA fragment contained 5372 nucleotide residues), where % IICL/Pt is the number of interduplex CLs per adduct

multiplied by 100, IXL is the number of interduplex CLs per molecule of the linearized DNA duplex and was calculated assuming a Poisson distribution as IXL = −ln *B*, where *B* is the fraction of molecules running as a band corresponding to the non-interduplex cross-linked DNA.

DNA−Protein Cross-Linking. Platinated DNA (213-bp *Nde*I/*Eco*RI restriction fragment of pUC19 plasmid) at a concentration of 10 nM was incubated with the proteins (histone H1 or NF-κB (p50 dimer)) at a concentration of 100 nM overnight at room temperature in the appropriate buffer: Tris-HCl (10 mM, pH 7.9) and NaCl (20 mM) (histone H1); HEPES (42 mM), KCl (42 mM), $MgCl_2$ (1 mM), EDTA (0.02 mM), DTT (210 mM), glycerol (2.5%), and Ficoll (2%) (NF-κB). The ability to form CLs by **1** or cisplatin between the 213-bp DNA fragment and proteins was assessed by 2% agarose (agarose and Metaphor agarose 1:1) after mixing the samples with the loading buffer (Tris-HCl (50 mM, pH 6.8), SDS (2%), bromophenol blue (0.1%), glycerol (10%)) and denaturing by heat at 90 °C for 5 min. Gels were electrophoresed for 3 h at 40 V, dried and visualized with a bioimaging analyzer.

Other Physical Methods. Absorption spectra were measured with a Beckman 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 HCl (0.1 M). DPP was performed with an EG&G Princeton Applied Research Corporation model 384B polarographic analyzer. HPLC purification of oligonucleotides was carried out on a Waters HPLC system consisting of a Waters 262 pump, Waters 2487 UV detector, and Waters 600S controller with MonoQ HR 5/50 GL 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).

RESULTS

DNA Binding. Solutions of double-helical CT DNA at a concentration of 0.032 mg mL^{-1} were incubated with trinuclear complex **1** at an r_i of 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 time intervals, an aliquot of the reaction mixture was withdrawn and assayed by DPP for platinum not bound to DNA. The amount of molecules of **1** bound to DNA (r_b) was calculated by subtracting the amount of free (unbound) molecules of **1** from the total amount of **1** present in the reaction. No changes in the pH of the reaction mixture containing DNA and platinum compounds were measured within 48 h after mixing DNA with the platinum complex. The amount of the platinum compounds bound to DNA increased with time. In this binding reaction, the time at which the binding reached 50% ($t_{50\%}$) was 60 min. The value of $t_{50\%}$ for the reaction of conventional mononuclear cisplatin and other antitumor trinuclear Pt^{II} complex BBR3464 with DNA under comparable conditions was ∼120 or 60 min, respectively.[23,24]

In further experiments, CT DNA was incubated with **1** at r_i = 0.1, and essentially the same rate of the binding was observed as when r_i was 0.05. The rapid and essentially quantitative binding of **1** facilitates sample analysis. The binding experiments indicate that such platination reactions resulted in the coordination of all molecules of **1**. This made it possible to prepare easily and precisely DNA samples modified at a preselected value of r_b. The samples of modified DNA which were further analyzed in this work by biochemical or biophysical techniques have been prepared by incubating DNA with **1** in $NaClO_4$ (10 mM) at 37 °C for 24 h except where stated (see also Materials and Methods). If it was necessary to transfer platinated DNA into a medium different from 10 mM $NaClO_4$ required for a particular analysis of DNA, the samples were precipitated in ethanol, and dissolved in this medium, and the r_b value in an aliquot of this sample was checked by FAAS. In this way, the analyses described in the present paper were performed in the absence of unbound (free) platinum complex.

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.[24−27] Double-helical DNA was first modified by bifunctional cisplatin, monofunctional [PtCl(dien)]Cl, or trinuclear **1** for 48 h. The levels of the modification corresponded to the values of r_b in the range between 0 and 0.04. Modification of DNA by all platinum complexes resulted in a decrease of EtBr fluorescence (Figure 2). The decrease caused by the adducts of trinuclear **1** was markedly more pronounced than that induced by the DNA adducts of cisplatin 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.[24−27] The structures of DNA adducts of **1** may arise from 1 to 3 monofunctional substitutions on the polynucleotide. Comparison with [PtCl(dien)]Cl suggests that the conformational distortion induced in DNA by the adducts of **1** 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.

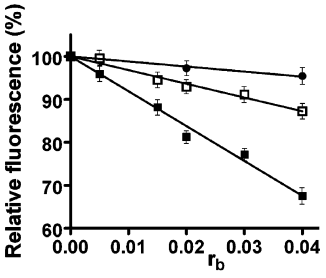

Figure 2. Ethidium bromide fluorescence. Dependences of the EtBr fluorescence on r_b for CT DNA modified by platinum complexes in 10 mM $NaClO_4$ at 37 °C for 24 h: (■) complex **1**; (□) cisplatin; (●) [PtCl(dien)]Cl. The experimental points represent mean ± standard deviations of three independent experiments.

Characterization of DNA Adducts by [^{14}C]Thiourea. Previous studies[14−16,28] have shown that under the appropriate conditions thiourea quantitatively reacts with DNA monoadducts of bifunctional Pt^{II} complexes without displacing platinum from the DNA. In other words, during the initial period of the reaction of DNA with bifunctional Pt^{II} complexes, when a significant part of the molecules is bound monofunctionally, the other coordination site can be blocked by thiourea.[16] This is so because, although Pt−N bond has higher thermodynamic stability than Pt−S(thioether) bond, thioether sulfur is kinetically more favorable than guanine nitrogen when binding to Pt^{II} drugs. Thus, incorporation of [^{14}C]thiourea into

DNA under controlled conditions[14] (see Materials and Methods) was used to quantitate platinum–DNA mono- or bifunctional adducts of **1**.

The adducts in which **1** still possessed free coordination sites not involved in the binding to DNA (which could be blocked by thiourea, presumably in mono- or bifunctional lesions) were quantitated at various times of incubation of **1** with CT DNA by [^{14}C]thiourea incorporation. The complete protocol is described in Materials and Methods.

The percent representation of free coordination sites in **1** not involved in the binding to DNA calculated in this manner is shown in Figure 3. The resulting data indicate that in the early

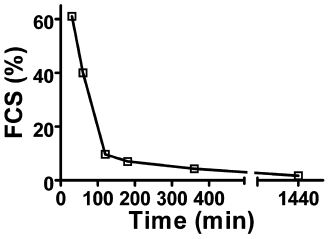

Figure 3. Kinetic study of conversion of the monofunctional binding of **1** to bi- and trifunctional attachment to CT DNA performed with incorporation of [^{14}C]thiourea into DNA. The figure shows a plot of the percent free coordination sites in **1** (FCS (%)) during its reaction with DNA on time. The complete protocol and other details are described in Materials and Methods and Results.

phase of the reaction of DNA with **1** lasting only 30 min approximately 40% coordination sites in all molecules of trifunctional Pt^{II} complex **1** present in the reaction were already involved in DNA adducts. The amount of these coordination sites in **1** rapidly decreased with time of incubation of DNA with **1**, and after 2 h only ~90% coordination sites in all molecules of **1** present in the reaction were already involved in DNA adducts. Interestingly, after 24 h, when all molecules of **1** were already bound to DNA (*vide supra*), approximately 98% coordination sites in all molecules of **1** present in the reaction were already involved in DNA adducts.

Characterization of DNA Adducts by HPLC Analysis of Digested DNA. A 50-bp deoxyribooligonucleotide duplex [with a random nucleotide sequence (G + C content was 54%; see Material and Methods)] was treated with **1** for 24 h; the level of platination corresponded to r_b = 0.023. The sample treated with **1** and the control (unmodified) sample were enzymatically digested to mononucleosides. RP-HPLC analysis of enzymatic digests of the oligonucleotide duplexes was performed by recording the optical density at 260 nm (Figure 4). Digestion of the unmodified duplex produced the well-resolved mononucleoside peaks whose areas integrated and normalized by their extinction coefficients reflected the proper proportions and content of the single mononucleosides in the unmodified 50-bp duplex. In contrast, digestion of the platinated sample resulted in a decrease of the integrated area of the deoxyriboguanosine peak by 17.8% and to a considerably smaller extent also in a decrease of the deoxyriboadenosine peak (by 8.1%); the peaks for deoxyribocytidine and thymidine were not affected. It was verified by FAAS that no product containing platinum coeluted with the peaks corresponding to unplatinated deoxyribonucleosides. The platinated products were not retained by the column under the conditions used, so they could not be identified and quantified. The r_b in the platinated

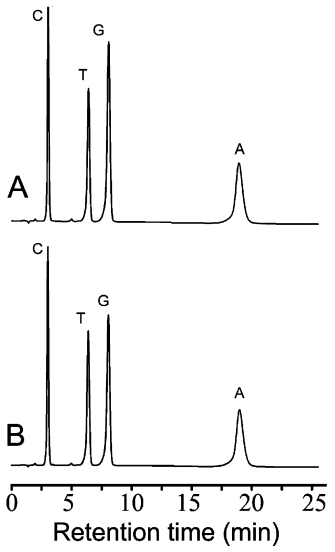

Figure 4. Reverse phase HPLC separation of the products of enzymatic digest of the 50-bp oligonucleotide duplex unmodified (A) or modified by **1** (B); r_b was 0.023. For other details, see the text.

sample was 0.023. At this r_b, 2.3, 4.6, or 6.9 base residues in the 50-bp DNA fragment would be platinated in the case of the monofunctional, bifunctional or trifunctional binding of **1**, respectively. The 50-bp duplex employed in these analyses contained 27 guanine and 23 adenine residues. Thus, the decrease of the deoxyriboguanosine and deoxyriboadenosine peaks corresponds to the loss of ~4.8 guanine and ~1.9 adenine residues, i.e. in total ~6.7 base residues. This implies that, at a relatively low level of binding of **1** to DNA and after 24 h, this complex formed mainly trifunctional adducts, which is consistent with the characterization of DNA adducts of **1** by [^{14}C]thiourea (see the preceding paragraph). Moreover, characterization of DNA adducts of **1** by HPLC analysis of enzymatically digested DNA revealed that these adducts contained mainly guanine residues and to a lesser extent also adenine residues.

DNA Melting. CT DNA was modified by **1** to various r_b values (0–0.045) in 10 mM $NaClO_4$ at 37 °C for 24 h. Salt concentration was then further adjusted by the addition of $NaClO_4$ to the values of 0.01 or 0.1 M. The effect on t_m is dependent both on the amount of platinum complex bound and the salt concentration (Figure 5). At low concentrations of $NaClO_4$ (0.01 M) an increase of t_m was observed even at relatively high levels of the modification of

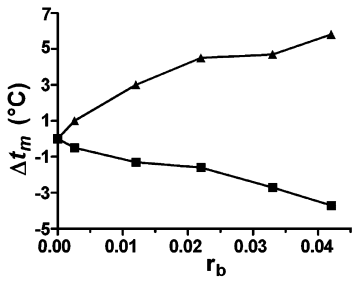

Figure 5. Plots of Δt_m values of CT DNA modified by **1** on r_b. The t_m values were measured in media containing 0.01 M (▲) or 0.1 M (■) $NaClO_4$; the solutions also contained Tris-HCl (1 mM, pH 7.4) and EDTA (0.1 mM) (Δt_m is defined as the difference between the t_m values of platinated and unmodified DNAs).

DNA by **1** (r_b = 0.045). At high salt concentrations of 0.1 M the modification of DNA by **1** resulted in a decrease of t_m which became more pronounced with increasing r_b values (Figure 5).

Unwinding of Negatively Supercoiled DNA. Electrophoresis in native agarose gel was used to quantify the unwinding induced in pUC19 plasmid by **1** by monitoring the degree of supercoiling (Figure 6). A compound that unwinds

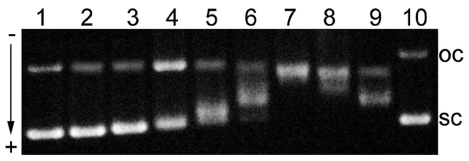

Figure 6. Unwinding of negatively supercoiled pUC19 plasmid DNA by **1**. The top bands (oc) correspond to nicked plasmid and the bottom bands (sc) correspond to the closed, negatively supercoiled plasmid. Lanes: 1 and 10, control, unmodified DNA; 2–9, r_b = 0.001, 0.003, 0.005, 0.008, 0.01, 0.015, 0.02, 0.03, respectively.

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. Figure 6 shows an agarose gel in which increasing amounts of **1** have been bound to a mixture of relaxed and supercoiled pUC19 DNA. Interestingly, the trinuclear complex 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.[29,30] This observation is in contrast to DNA binding of clinically ineffective transplatin (trans isomer of cisplatin) to relaxed forms of plasmid DNAs. It has been shown in previous studies[29−32] that transplatin affects the mobility of the relaxed forms only negligibly. 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.[18] Under the present experimental conditions, σ was calculated to be −0.0505 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 53 ± 3° for **1**.

Interstrand (Intramolecular) Cross-Linking. Bi- or multifunctional platinum compounds that covalently bind to DNA form various types of interstrand and intrastrand CLs. 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, we have decided to quantitate the interstrand cross-linking efficiency of **1** in linearized pUC19 plasmid (2686 bp). This plasmid DNA was linearized by *Eco*RI (*Eco*RI cuts only once within the pUC19 plasmid) and modified by **1** at various r_b values. The samples were analyzed for the interstrand CLs by agarose gel electrophoresis under denaturing conditions.[20] Upon electrophoresis, the 3′-end-labeled strands of linearized pUC19 plasmid containing no interstrand CLs migrate as a 2686-base single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species (Figure 7).

The experiments were carried out with DNA samples that were modified by **1** for 24 h at various r_b values. The bands corresponding to the more slowly migrating interstrand-cross-

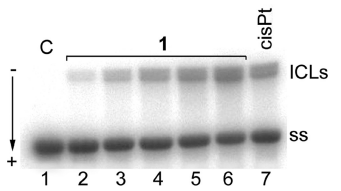

Figure 7. The formation of interstrand cross-links (ICLs) by platinum complexes in pUC19 DNA. Autoradiogram of the denaturing 1% agarose gel of linearized DNA which was 3′-end-labeled; the interstrand cross-linked DNA appears as the top bands (ICLs) migrating on the gel more slowly than the single-stranded (ss) DNA (contained in the bottom bands). Plasmid linearized by *Eco*RI was incubated for 24 h with **1** at r_b values of 0 (control, lane 1), 0.0002, 0.0004, 0.0006, 0.0008, 0.001 (lanes 2–6, respectively) or with cisplatin at r_b = 0.001 (lane 7).

linked fragments were seen for r_b values as low as 2×10^{-4} (Figure 7, 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 intramolecular interstrand CLs (% 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 **1** was almost independent of r_b and was only 9%; thus the DNA interstrand cross-linking efficiency of **1** was similar to that of mononuclear cisplatin.[20]

The samples of linearized DNA modified by **1** 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 (10 mM $NaClO_4$).

Recognition of Adducts by HMGB1 Protein. An important feature of the mechanism of action of cisplatin is that the altered structures produced by the bending of the helical axis induced in DNA by 1,2-intrastrand or 1,2-interstrand CLs of cisplatin attract HMG domain proteins and other proteins.[33−35] This binding of HMG domain proteins to cisplatin-modified DNA has been postulated to mediate or enhance the drug's antitumor properties.[35] Full-length HMGB1 or HMGB2 proteins and the domains A and B of HMGB1 protein (HMGB1a and HMGB1b, respectively) bind to 1,2-GG intrastrand CLs of cisplatin.

Since the DNA conformational changes caused by **1** are very different from those caused by cisplatin, experiments were performed to determine if differences exist in the recognition of the adducts of **1** by full-length HMGB1 protein. The interactions of the HMGB1 protein, which is considered the prototype of this family of proteins, with the adducts of **1** were investigated using a gel mobility shift assay.[21,36] In these experiments, the 50-bp fragment of DNA (with blunt ends) was modified by **1** or cisplatin at r_b = 0.02 so that it contained 2 adducts per duplex on average. The binding of the HMGB1 proteins to these DNA probes was detected as bands of reduced electrophoretic mobility on the gels.[21,36] The protein exhibited negligible binding to the unmodified DNA fragment, whereas HMGB1 protein recognized and bound to the fragment containing the adducts of cisplatin. These results

indicate that HMGB1 protein did not bind the probes under conditions where this protein associated with the DNA fragment containing the adducts of cisplatin (Figure 8).

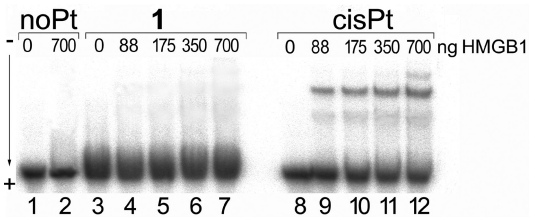

Figure 8. Autoradiograms demonstrating gel mobility shift assay analysis of the interaction of a 50-bp DNA fragment randomly modified by **1** or cisplatin at r_b = 0.02 with full-length HMGB1 protein. Radioactively labeled DNA fragments (0.1 pmol) were incubated with HMGB1 (the amount in ng is indicated above each lane). Lanes: 1, 2, control (unplatinated) DNA; 3−7, DNA modified by **1**; 8−12, DNA modified by cisplatin.

Hence, either the adducts of **1** are not recognized by HMG domain proteins or the affinity of these proteins is markedly lower for the adducts of **1** than for the adducts of cisplatin.

Interduplex Cross-Linking. At least two types of DNA interstrand cross-linking by multifunctional platinum complexes can exist, depending on whether the platinum complex coordinates to the bases in one DNA molecule (intraduplex interstrand CLs) or coordinates to the bases in separate DNA molecules (interduplex interstrand CLs). 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 via one leaving group inevitably leaves the other in close proximity to other binding sites in the same duplex, leading to intraduplex interstrand cross-linking. However, if the reactive sites of the cross-linking agents are connected by a more rigid linker that forces those sites to point in appropriate directions and if the stereochemistry of the reactive sites of such cross-linking agents proves appropriate, such agents could bind to adjacent duplexes.[37−39] Such cross-linking agents might be effective interduplex cross-linkers in cases of two fragments of double-helical DNA molecules forced to lie together, i.e. for instance during recombination, at replication forks or sites of topoisomerase action[38,40] or more generally in cellular environmental conditions.[41,42]

To assess whether the trinuclear Pt^{II} complex **1** is able to form interduplex CLs in cellular especially nuclear environmental conditions, DNA was platinated under molecular crowding conditions in a medium containing ethanol which is a commonly used crowding agent[43,44] similar to polyethylene glycol, Ficolls and dextrans.[45,46] The radioactively labeled double-stranded linearized pUC19 DNA (2686 bp) was platinated by **1** or cisplatin for comparative purposes in a medium containing 70% ethanol, 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 DNA fragments were platinated in a medium containing a high concentration of counter cations (0.2 M Na^+). Upon electrophoresis, the 3′-end-labeled double-stranded linearized pUC19 plasmid not cross-linked to other double-stranded DNA molecule migrates as a 2686-bp DNA molecule, whereas the interduplex cross-linked duplexes (two or more) migrate more slowly as higher molecular mass species (Figure 9). The intensity of the bands corresponding to these interduplex cross-linked duplexes increased with a growing level of the modification by **1** or cisplatin. The radioactivity associated with the individual bands in each lane was measured to obtain estimates of the fraction of non-interduplex cross-linked or interduplex cross-linked molecules under each condition. The frequency of interduplex CLs (% IICL/Pt) was calculated as described in Materials and Methods. The DNA interduplex cross-linking efficiency of **1** was 21%. Thus, interduplex cross-linking efficiency of the trinuclear Pt^{II} complex **1** under molecular crowding conditions was considerably higher than that found for mononuclear cisplatin, ∼1% (Figure 9).

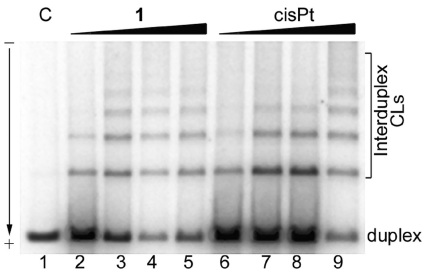

Figure 9. Formation of interduplex cross-links. The radioactively labeled linear plasmid DNA was modified by **1** or cisplatin in sodium acetate (0.2 M) and ethanol (70%), and the reaction products were analyzed by native 0.5% agarose gel electrophoresis. Lanes: 1, control, unmodified DNA; 2−5, DNA was modified by **1** at r_b = 0.0001, 0.0005, 0.001, 0.005, respectively; 6−9, DNA was modified by cisplatin at r_b = 0.001, 0.005, 0.01, 0.05, respectively.

DNA−Protein Cross-Linking. Complex **1** was also investigated for its ability to form ternary DNA−protein complexes covalently linked by the platinum moiety. The proteins were chosen for these studies that bind to DNA with a relatively high affinity. The linker histone H1 was chosen as the representative of non-sequence specific DNA-binding proteins with structural function, whereas transcription factor NF-κB (p50 dimer) was chosen as the representative of a sequence-specific DNA-binding protein with a regulation function. The 213-bp *Nde*I/*Eco*RI restriction fragment of pUC19 plasmid contains at least two 10-bp NF-κB DNA binding sequences very similar to canonical DNA consensus sequences of NF-κB to which this transcription factor also specifically binds with a relatively high affinity.[47−49] This restriction fragment 3′-end-labeled was globally modified by **1** or cisplatin for 24 h so that ∼10 molecules of the Pt^{II} complex were bound per fragment on average (r_b = 0.025). The fragment (13 nM) modified by **1** or cisplatin was mixed with histone H1 or NF-κB (the molar ratio protein/duplex was 8 or 34, respectively).

Ternary DNA−Pt^{II} compound−protein cross-linking efficiency was assessed by agarose gel mobility shift assay. Fractions were detected with significantly retarded mobility (shown for cross-linking NF-κB protein in Figure 10, lanes 4, 6) compared with that of the free probes (in the absence of proteins) (Figure 10, lanes 3, 5). 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 coordination bonds. While the proteinase K and NaCN experiments clearly indicate that protein is the species cross-linked to DNA, the amino acids participating in the

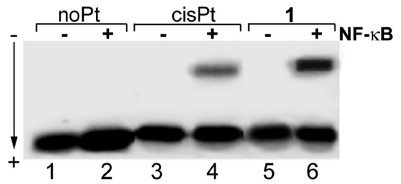

Figure 10. Formation of ternary DNA–Pt(–Pt)–protein complexes of unmodified or platinated DNA fragment globally modified by **1** or cisplatin with NF-κB. The 213-bp duplex was globally modified at r_b = 0.025. The ability to form DNA–protein CLs by **1** or cisplatin was assessed by 2% agarose gel electrophoresis after mixing the samples with the loading buffer containing 2% SDS and denaturing at 90 °C. Lanes: 1, 3, and 5, the DNA fragment unplatinated or modified by **1** or cisplatin, respectively, in the absence of the protein; 2, 4, and 6, the DNA fragment unplatinated or modified by **1** or cisplatin, respectively, incubated with the protein for 24 h at 25 °C. See the text for other details.

cross-linking reaction have not been determined. Importantly, the amount of radioactivity associated with the bands corresponding to DNA–Pt^{II} complex–protein CLs formed by **1** was higher than that by cisplatin (cf. Figure 10, lanes 4 and 6 for cross-linking NF-κB protein and Table 1) demonstrating

Table 1. Formation of the Ternary DNA–Pt(–Pt)–Protein Complexes of Histone H1 or NF-κB with Unmodified and Platinated[a] 213-bp DNA Fragment Assessed by Gel Electrophoresis

DNA cross-linked to the protein (%)[b]			
histone H1		NF-κB	
1	cisplatin	**1**	cisplatin
13 ± 1	5.1 ± 0.6	21 ± 1	8 ± 0.5

[a]The fragments were globally modified by **1** or cisplatin (r_b = 0.025). For other details, see the caption to Figure 10 and the text. [b]Each value represents the average of four samples, and standard errors are indicated.

that **1** exhibited a significantly higher efficiency (ca. 2.6-fold) to form ternary DNA–Pt^{II} complex–protein CLs than cisplatin.

DISCUSSION

Previous studies[9,50] employing short synthetic DNA duplexes, having preselected nucleotide sequences, have shown that **1** may potentially lead to novel DNA lesions, which can be important in the avoidance of cellular cross-resistance. We sought to extend this previous study and report in the present work the detailed study of binding of **1** to natural, high-molecular-mass DNA with emphasis on intramolecular and intermolecular cross-linking capability of this metallodrug.

Our study of the binding of **1** to natural double-helical CT DNA shows that the rate of binding of **1** to natural double-helical DNA is considerably higher than that of cisplatin and somewhat lower than that of other antitumor trinuclear Pt^{II} complex BBR3464. The results of RP-HPLC separation of the products of enzymatic digest of platinated DNA fragment have shown (Figure 4) that guanine residues are the preferential DNA binding sites of **1** when polymeric DNA is modified with this complex in a random fashion and that **1** can also bind adenine residues, but to a lesser extent.

EtBr as a fluorescent probe can be used to characterize DNA binding of small molecules such as platinum antitumor drugs.[24,51] The fluorescence of EtBr is markedly enhanced as a consequence of its intercalation into DNA, but binding of EtBr to DNA by intercalation is blocked in a stoichiometric manner by a wide spectrum of DNA-binding platinum drugs. On the other hand, the modification of DNA by monofunctional complexes, such as [PtCl(dien)]Cl, results in only a slight decrease of EtBr fluorescence intensity as compared with that for nonplatinated DNA. The molecules of trinuclear trifunctional complex **1** bound to CT DNA sterically block approach of molecules of EtBr to DNA and in this way hinder the molecules of EtBr to intercalate, which lowers EtBr fluorescence in comparison with the experiment in which unplatinated DNA was used (Figure 2). The structures of DNA adducts of trinuclear **1** may arise from 1 to 3 monofunctional substitutions on the polynucleotide. Comparison with monofunctional [PtCl(dien)]Cl suggests that the conformational distortion induced in DNA by the adducts of **1** 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. Thus, these results are consistent with capability of **1** to form DNA adducts which either span more base pairs around the binding sites or at least induce conformational distortions in DNA which extend over several base pairs (over more base pairs than in case of major CLs of cisplatin). In this respect, DNA binding mode of **1** resembles that of the other trinuclear Pt^{II} complex BBR3464 which also forms long-range CLs preferentially at G residues, although their nature and conformational alterations they induce in DNA are different.[24,52,53] This is so because spatial configuration of leaving groups in both trinuclear Pt^{II} complexes is markedly different.

DNA adducts of tridentate **1** could be either monoadducts or bi- or trifunctional CLs. It is reasonable to expect that similarly to bifunctional Pt^{II} complexes **1** forms first monofunctional adducts, which subsequently close to CLs. Incorporation of [^{14}C]thiourea into DNA modified by **1** under controlled conditions was used to quantitate conversion of DNA monoadducts to CLs. Thus, as expected, at early stages of the reaction of CT DNA with **1** a prevailing part of coordination sites in **1** bound to DNA were free (not involved in the binding to DNA) (e.g., 60% after 30 min of the reaction of DNA with **1**, Figure 3). The amount of these free coordination sites rapidly decreased with time of incubation of DNA with **1**. Interestingly, after 24 h, when all molecules of **1** were already bound to DNA (*vide supra*), approximately 98% coordination sites in all molecules of **1** present in the reaction were already involved in DNA adducts. This result is consistent with the thesis that after 24 h of incubation of DNA with **1** almost all DNA adducts of **1** were trifunctional, i.e. all three Pt^{II} centers of **1** were coordinated to DNA base residues. This conclusion is in an excellent agreement with the results of RP-HPLC separation of the products of enzymatic digest of DNA fragment modified by **1** (Figure 4) and is also consistent with previous experimental findings[9] indicating that this trinuclear Pt^{II} complex **1** formed this type of CL in short synthetic oligodeoxyribonucleotide duplexes. Moreover, the observation of the present work that **1** forms in natural DNA trifunctional adducts represents another proof that **1** forms delocalized CLs which span more than two base pairs. The latter long-range CLs can readily occur in DNA because of the distance between reactive sites: the Pt–Pt distances in **1** are in the range 0.73–0.84 nm.[8] Taking into account the approximate 0.34 nm distance of neighboring base residues in B-DNA, it is clear that

the molecule of **1** can easily span 3−4 base pairs. An expected result, then, for **1** is that DNA long-range cross-linking may occur.

It has been shown[54] that antitumor polynuclear Pt^{II} complexes form a markedly higher amount of intramolecular interstrand CLs than mononuclear cisplatin. We quantitated this feature for **1** in linearized plasmid DNA determining the frequency of intramolecular interstrand CLs. We found that the intramolecular DNA interstrand cross-linking efficiency of **1** was only 9%, although slightly higher than that found under identical conditions for cisplatin (6%[20]), but markedly lower than that found for trinuclear BBR3464 (20%[24]). Thus, another intriguing aspect of DNA binding mode of trifunctional **1** also is that intramolecular interstrand cross-linking is diminished relative to other polynuclear bifunctional Pt^{II} complexes with flexible linking groups. Hence, unique tridentate intrastrand rather than intramolecular interstrand CLs appear to be major adducts formed in natural high-molecular-mass DNA globally modified for 24 h by **1** (in the absence of proteins and molecular crowding agents).

An intriguing aspect of modification of DNA by **1** in the absence of proteins and molecular crowding agents is that the plots of DNA melting temperatures on the level of modification of CT DNA by **1** have a fundamentally different character if they are recorded at low and high ionic strength (Figure 5). Whereas at low concentrations of Na^+ (0.01 M) an increase of t_m was observed even at relatively high levels of the modification of DNA by **1**, at high salt concentrations (0.1 M) the modification of DNA by **1** resulted in a decrease of t_m which became more pronounced with increasing r_b values. This behavior is in marked contrast to cisplatin,[55] where the modification of DNA results in a decrease of t_m if DNA melting is measured in salt concentrations ranging from 0.01 to 0.2 M. Thus, this difference supports the view that DNA binding mode of **1** is markedly different from that of cisplatin.

The different melting behavior of DNA modified by **1** and present in the media of low and high salt concentration (0.01 and 0.1 M $NaClO_4$, respectively) (Figure 5) can be explained in the following way. 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.[55] These major factors are (i) a destabilizing effect of conformational distortions due to the formation of CLs induced in DNA by platinum coordination; (ii) stabilizing effects of DNA interstrand (intramolecular) 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). However, it has been shown[56] that cations present in the medium also stabilize DNA against thermal denaturation by decreasing electrostatic repulsion of negative charges of phosphate groups located at the complementary strands. Hence, the stabilization effect of the positive charge on the Pt^{II} centers depends on the concentration of cations in the medium. It is stronger at low ionic strength and practically disappears at sufficiently high concentrations of the cations. Thus, at high ionic strength (0.1 M $NaClO_4$) when the electrostatic stabilizing effects of **1** are markedly weakened, the decreases in t_m are caused by destabilizing effects of conformational distortions induced by lesions of **1** which dominate the stabilizing effects of intramolecular interstrand CLs (in fact, **1** formed only 9% of intramolecular interstrand CLs (Figure 7). In contrast, when DNA modified by **1** was present in the medium of a low salt concentration (0.01 M $NaClO_4$), the electrostatic stabilizing effect dominated and therefore an increase of t_m was observed.

The formation of tridentate adducts in one strand of double-helical DNA may require a severe distortion of double-helical conformation of this nucleic acid.[50] Consistent with this suggestion is the observation that tridentate adducts formed in double-helical DNA globally modified by **1** markedly locally unwind DNA double helix (by 53 ± 3°, Figure 6) and thermally destabilize it (Figure 5). Further studies aimed at determining base residues involved in tridentate intrastrand adducts formed by **1** in double-helical DNA, the number of intervening base pairs separating platinated base residues in these adducts and characterization of resulting conformational distortions are warranted.

It has been suggested that HMG domain proteins play a role in sensitizing cells to cisplatin,[57] although these proteins have been shown to play no significant role in the mechanism of cisplatin-induced cytotoxicity in mouse embryonic cells.[58] It has been shown that HMG domain proteins recognize and bind to DNA CLs formed by cisplatin between bases in neighboring base pairs.[34,59,60] The molecular basis for this recognition is still not entirely understood, although several structural details of the 1:1 complex formed between the HMG domain proteins and the duplex containing the 1,2-GG intrastrand CL of cisplatin were elucidated.[59] The details of how the binding of HMG domain proteins to cisplatin-modified DNA sensitizes tumor cells to cisplatin are also still not completely resolved, but possibilities such as shielding cisplatin−DNA adducts from excision repair or that these proteins could be titrated away from their transcriptional regulatory function have been suggested[33,35,60,61] to explain how these proteins are involved in the antitumor activity. An important structural motif recognized by HMG domain proteins on DNA containing the major 1,2-GG intrastrand CL of cisplatin is a stable, directional bend of the helix axis toward the major groove.[60] No recognition of DNA adducts of **1** by HMGB1 protein was observed in the present work (Figure 8). A plausible explanation of this observation may be that the prebending due to the adducts of **1** is too small to be recognized by HMG domain proteins or that bulky adducts of **1** could restrict the additional DNA bending required for HMG domain binding.[59,60] Alternatively, this result may also be associated with some specific structural features of the adducts of **1**. Thus, from the results of this work, it is clear that the DNA adducts of the antitumor compound **1** may present a block to DNA or RNA polymerase, but are not a substrate for recognition by HMG domain proteins. These important findings may also apply to other nuclear proteins known to recognize severely distorted DNA, such as DNA damage recognition proteins belonging to DNA repair systems and transcription factors. From these considerations we conclude that the mechanism of antitumor activity of trinuclear **1** does not involve recognition by HMG domain proteins as a crucial step, in contrast to the proposals for cisplatin and its direct analogues.

Another interesting phenomenon specific for the DNA binding of this trifunctional Pt^{II} complex **1** is that it can also form specific DNA lesions which can efficiently cross-link proteins to DNA (Figure 10). 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, i.e., NF-κB and the linker histone H1) come into close contact with DNA at the site of platination. Then one molecule of **1** can be bound simultaneously to base residue(s) in DNA and reactive group(s) in the protein, but only if a noncovalent preassociation takes place first. The formation of DNA−protein ternary complexes mediated by **1** raises the possibility of "suicide" lesions, which may irreversibly sequester various DNA-binding proteins, such as transcription factors or repair proteins.[62,63]

A unique feature of the DNA binding of this trinuclear tridentate complex **1** also is that it forms, under molecular crowding conditions mimicking environmental conditions in cellular nucleus, another type of CL, namely, interduplex CLs (Figure 9). The frequency of these interhelical CLs tethered by platinum−DNA bonds was considerably higher (21%) than that found under identical conditions for mononuclear cisplatin, ∼1%. A plausible explanation of this observation is that crowding conditions in the nucleus allow interduplex contacts that are sufficient for the intermolecular (interduplex) cross-linking by **1**, but not by cisplatin.

In conclusion, the concept for the design of agents capable of forming intramolecular tridentate DNA adducts, DNA−protein and interduplex DNA−DNA CLs based on trinuclear tridentate Pt^{II} complexes with semirigid aromatic linkers may result in new compounds which exhibit a variety of biological effects and can be also useful in nucleic acid research. The earlier observation[8,9] that **1** is markedly more potent than cisplatin in human and mouse tumor cell lines is consistent with this idea. Such results indicate promising compounds with which to tackle the common problem of developed resistance to clinically used platinum drugs, frequently occurring during chemotherapy.

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