Structural Characterization, DNA Interactions, and Cytotoxicity of New Transplatin Analogues Containing One Aliphatic and One Planar Heterocyclic Amine Ligand

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We report in the present work new analogues of clinically ineffective transplatin in which one ammine group was replaced by aliphatic and the other by a planar heterocyclic ligand, namely *trans*-[$PtCl_2$-(isopropylamine)(3-(hydroxymethyl)-pyridine)], **1**, and *trans*-[$PtCl_2$(isopropylamine)(4-(hydroxymethyl)-pyridine)], **2**. The new compounds, in comparison with parent transplatin, exhibit radically enhanced activity in tumor cell lines both sensitive and in particular resistant to cisplatin. Concomitantly, the DNA binding mode of **1** and **2** compared to parent transplatin and other antitumor analogues of transplatin in which only one ammine group was replaced is also different. The results also suggest that the reactions of glutathione and metallothionein-2 with compounds **1** and **2** do not play a crucial role in their overall biological effects. In addition, the monofunctional adducts of **1** and **2** are quenched by glutathione considerably less than the adducts of transplatin, which may potentiate cytotoxic effects of these new platinum complexes.

Introduction

Most of the drugs used in the treatment of cancer including *cis*-diamminedichloroplatinum(II) (cisplatin, Figure 1), which is the first antitumor drug used clinically, exhibit side effects, and their clinical use is limited by inherent and acquired resistance. Hence, the search for new, nonconventional metallodrugs has become a very active area of molecular pharmacology. For many years, cis geometry in mononuclear bifunctional platinum compounds has been thought to be essential for the development of antitumor platinum-based drugs. The discovery of the cytostatic activity of several analogues of transplatin (trans isomer of cisplatin) implied a very important change of the structure−pharmacological activity relationship for platinum compounds.[1−3]

Systematic study of the analogues of transplatin containing heterocyclic and aliphatic amines as nonleaving spectator ligands has confirmed that this class of analogues of transplatin exhibits cytostatic effects in a number of tumor cell lines including those resistant to cisplatin.[2−4] As a result of our continuous interest in the cytostatic activity of platinum complexes, we assume that the evaluation of a broad variety of ligands used to replace one or two ammine groups in parent transplatin to afford a cytotoxic agent may help understand mechanisms underlying cytostatic effects of this class of antitumor platinum compounds. Hence, to achieve a more complete library of antitumor analogues of transplatin and to contribute to better understanding molecular mechanisms underlying antitumor activity of platinum compounds, we also synthesized the analogues of transplatin containing one aliphatic and one planar heterocyclic amine. There have been reports of *trans*-diaminedichloroplatinum(II) complexes with two heterocyclic or two aliphatic amine ligands, which exhibited enhanced cytotoxicity in several tumor cell lines compared to ineffective parent transplatin.[5,5−7] However, to the best of our knowledge there are no reports on analogous cytotoxic *trans*-diaminedichloroplatinum(II) complexes containing one planar heterocyclic and one aliphatic ligand. The reason we combined the aliphatic and planar heterocylic amine ligands in one molecule of transplatin analogue was that replacement of one or both ammine groups in transplatin by either heterocyclic or aliphatic amine ligands also results in an enhancement of cytotoxicity (in comparison with parent transplatin) in a number of tumor cell lines,[1,8] but to a different extent. Hence, we were interested to examine whether a combination of two nonleaving ligands of distinctly different nature in *trans*-diaminedichloroplatinum(II) complex affords the compound exhibiting promising cytotoxic effects.

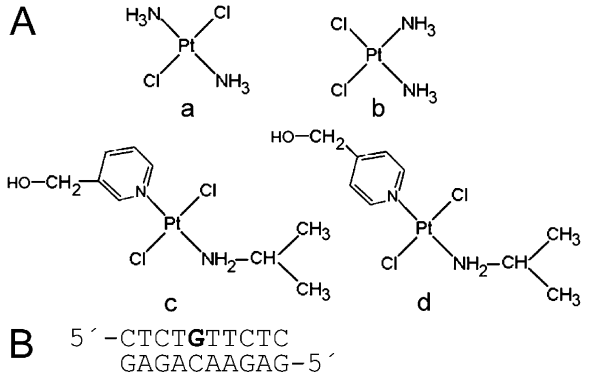

Figure 1. A. Structures of platinum complexes. a. cisplatin; b, transplatin; c. **1**; d. **2**. B. The sequence of the oligonucleotide duplex TGT. The top and bottom strand of the pair of oligonucleotides in B are designated "top" and "bottom", respectively, throughout. The boldface letter in the top strand of the duplex indicates the platinated residue of the monofunctional platinum adduct.

We report in the present work on new analogues of transplatin in which one ammine group was replaced by aliphatic and the other by the planar heterocyclic ligand, namely *trans*-[$PtCl_2$-(isopropylamine)(3-(hydroxymethyl)-pyridine)], **1**, and *trans*-[$PtCl_2$(isopropylamine)(4-(hydroxymethyl)-pyridine)], **2** (Figure 1c,d). The OH substituent on the heterocyclic ligand represents

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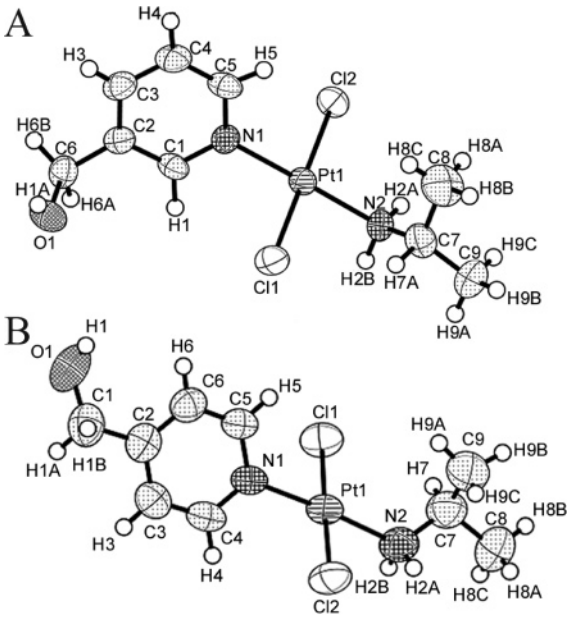

Figure 2. ORTEP view of compound **1**, *trans*-[$PtCl_2$(isopropylamine)-(3-(hydroxymethyl)-pyridine)] (A), and compound **2**, *trans*-[$PtCl_2$-(isopropylamine)(4-(hydroxymethyl)-pyridine)] (B).

a new concept in the design of new antitumor transplatin analogues focused on two main goals: (i) to increase the solubility of the complexes; (ii) to introduce a group, which would have important hydrogen-bond donor properties in the approach of the biological target or in the final structure formed when interacting with the target.[9] Moreover, the new complexes also make it possible to examine the effect of the different position of the hydroxy substituents in (hydroxymethyl)-pyridine ligands on the interaction with biological targets.

Results

Synthesis and Characterization of the New Analogues of Transplatin. *cis*-[$PtCl_2$(isopropylamine)$_2$] and the ligands L = 3-(hydroxymethyl)-pyridine or 4-(hydroxymethyl)-pyridine in water suspension at 85 °C afford the tetramine complexes with [Pt(isopropylamine)$_2$L$_2$]$^{4+}$. The addition of hydrochloric acid gives the corresponding complexes with the trans geometry, which exhibit enhanced solubility in water (~0.05 M at 37 °C) in comparison with transplatin (~1 mM) and some other antitumor analogues of this complex. The characterization of these complexes was carried out by usual techniques: elemental analyses, IR, ^{1}H, ^{13}C, and ^{195}Pt NMR spectra in $CDCl_3$ as solvent, and X-ray analysis.

Microanalytical data are consistent with the empirical formula $C_9H_{16}ON_2PtCl_2$ for both complexes. The assignment of the trans geometry is supported by IR spectral data revealing single band at 340 and 339 cm^{-1} for compounds with 3-(hydroxymethyl)-pyridine (compound **1**) and 4-(hydroxymethyl)-pyridine (compound **2**), respectively. In the IR spectra of **1** and **2**, one band at 444 and 442 cm^{-1}, respectively, is also observed, assigned to ν asymmetric Pt−N. The structures of **1** and **2** were also determined by X-ray analysis. Selected bond lengths (Å) and angles (deg) for compounds **1** and **2** are summarized in the Supporting Information.

The ORTEP diagrams for compounds **1** and **2** are shown in Figure 2. The platinum atom is in a square-planar environment where the angles do not differ from those found in other trans platinum complexes.[10−12] The values of Pt−N and Pt−Cl lengths are also comparable with those found in other analogues of transplatin containing planar amines.[13] Importantly, compounds **1** and **2** show three different intermolecular hydrogen bonds in the crystal structure (see the Supporting Information).

Table 1. IC_{50} Mean Values (±SD) (μM) Obtained for the Platinum Compounds Tested in the Present Work[a]

	A2780	A2780cisR	CH1	CH1cisR
1	0.7 ± 0.1	3.5 ± 0.6	1.3 ± 0.3	7.4 ± 0.5
2	1.0 ± 0.2	4.4 ± 0.5	1.7 ± 0.3	8.0 ± 0.3
cisplatin	1.3 ± 0.4	28 ± 4	2.1 ± 0.2	17 ± 2
transplatin	>300	>300	>300	>300

[a] Drug-treatment period was 72 h.

Cytotoxic Activity. The cytotoxic activity of the new analogues of transplatin tested in the present work was determined against two pairs of cisplatin sensitive and resistant cancer cell lines (see Table 1). These pairs of cell lines were selected on the basis of encompassing the known major mechanisms underlying resistance to cisplatin, A2780cisR being resistant through a combination of decreased uptake, enhanced DNA repair/tolerance, and elevated reduced glutathione levels,[14] and CH1cisR through enhanced DNA repair/tolerance.[15] The compounds were incubated for 72 h with the tumor cell lines, and the cell survival in the culture treated with the platinum compounds was evaluated as described previously.[6,16] It is shown in the Table 1 that the IC_{50} values obtained for compounds **1** and **2** in CH1 cells resistant to cisplatin are somewhat lower than those of cisplatin (2.2 times) while those in A2780 cells resistant to cisplatin are lower considerably more pronouncedly (7.2 times). Even more interestingly, the IC_{50} values found for compounds **1** and **2** in the cells sensitive to cisplatin are lower than those found for cisplatin and markedly lower than those for parent (clinically ineffective) transplatin. In this respect, the new compounds **1** and **2** exhibit better cytotoxic effects than antitumor cisplatin in particular in cisplatin resistant cells. Thus, a major benefit from using compounds **1** and **2** as cytotoxic agents appears their cytotoxicity in tumor cell lines resistant to cisplatin. In other words, the replacement of both ammine ligands in transplatin by one aliphatic and one planar heterocyclic ligand results in new cytotoxic platinum compounds exhibiting markedly enhanced activity in cancer cell lines relative to parent transplatin or other transplatin analogues so far tested with two either heterocyclic or aliphatic amine ligands.

DNA Binding. Solutions of double-helical CT DNA at a concentration of 0.1 mg/mL were incubated with **1** and **2** at an r_i of 0.01 in 10 mM $NaClO_4$ 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 differential pulse polarography[17] for platinum not bound to DNA. The amount of platinum bound to DNA, r_b (r_b is defined as the number of molecules of the platinum compound bound per nucleotide residue) was calculated by subtracting the amount of free (unbound) platinum from the total amount of platinum 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 these binding reactions, the times at which the binding reached 50% ($t_{50\%}$) were 105 min for both complexes, i.e., **1** and **2** reacted with DNA somewhat faster than cisplatin or transplatin ($t_{50\%}$ is 120 min[18]). In further experiments, CT DNA was incubated with both complexes at r_i = 0.1, and essentially the same rates of the binding were observed as at r_i = 0.01. The binding experiments of the present work indicate that the modification reactions resulted in the

irreversible coordination of **1** and **2** to polymeric double helical DNA, which also facilitates sample analysis. Hence, it is possible to easily and precisely prepare the samples of DNA modified by the platinum complex at a preselected value of r_b. The samples of DNA modified by the platinum compounds and analyzed further by biophysical or biochemical methods were prepared in 10 mM $NaClO_4$ at 37 °C. If not stated otherwise, after 24 h of the reaction of DNA with the complex, the samples were precipitated in ethanol and dissolved in the medium necessary for a particular analysis, and the r_b value in an aliquot of this sample was determined by flameless atomic absorption specrophotometry (FAAS). In this way, the analyses described in the present paper were performed in the absence of unbound (free) platinum complex.

Sequence Preference of DNA Adducts. This procedure involved the extension by TaKaRa Taq DNA polymerase (which exhibits the extreme termostability) at the 3′ end of the 5′ end-radioactively labeled primer up to the metal adduct on the template strand of pSP73 plasmid (2464 bp). The products of the linear amplification were then examined on DNA sequencing gels, and the sequence specificity of the platinum adduct formation was determined to the exact base pair.

In vitro DNA synthesis on double-stranded templates containing the adducts of **1** or **2** generated a population of DNA fragments, indicating that these adducts terminate duplex synthesis (Figure 3A, lanes 1 and 2). Sequence analysis of the termination sites produced by **1** and **2** (Figure 3B) suggests a sequence preference for dG sites and some preference also for dA sites in double-helical DNA (Figure 3B). Both complexes exhibit sequence dependence of the inhibition clearly different from that of transplatin. The new compounds form less blocks on DNA for DNA polymerase than transplatin and some of them at different sequences. In addition, this mapping did not reveal dC as a strong termination site produced by the compounds **1** or **2** found for transplatin.[19] These results are consistent with a more regular sequence specificity of **1** and **2** in comparison with parent transplatin. The faster DNA binding of **1** and **2** may be also responsible for their enhanced sequence specificity.

Characterization of DNA Adducts by Ethidium Bromide (EtBr) Fluorescence. EtBr as a fluorescent probe has been used to characterize perturbations induced in DNA by bifunctional adducts of several platinum compounds.[20,21] Binding of EtBr to DNA by intercalation is blocked by formation of the bifunctional adducts of a series of platinum complexes including cisplatin and transplatin, which results in a loss of fluorescence intensity. On the other hand, modification of DNA by monodentate platinum complexes (having only one leaving ligand) only results in a slight decrease of EtBr fluorescence intensity as compared with nonplatinated DNA−EtBr complex.

Double-helical CT DNA was modified so that the levels of the modification corresponded to the values of r_b in the range between 0 and 0.1. The modification of DNA resulted in a decrease of EtBr fluorescence (Figure 4). In accordance with the results published earlier,[20,21] monodentate chlorodiethylenetriamineplatinum(II) chloride ([Pt(dien)Cl]Cl) decreased the fluorescence only to a small extent (not shown), whereas the decrease induced by the DNA adducts of cisplatin or transplatin was more pronounced (Figure 4). The decrease induced by adducts of **1** or **2** was pronouncedly more pronounced than that induced by the DNA adducts of transplatin, but slightly less than that induced by cisplatin (Figure 4). Thus, these results suggest that **1** and **2** form more bifunctional adducts (cross-links, CLs) than transplatin.

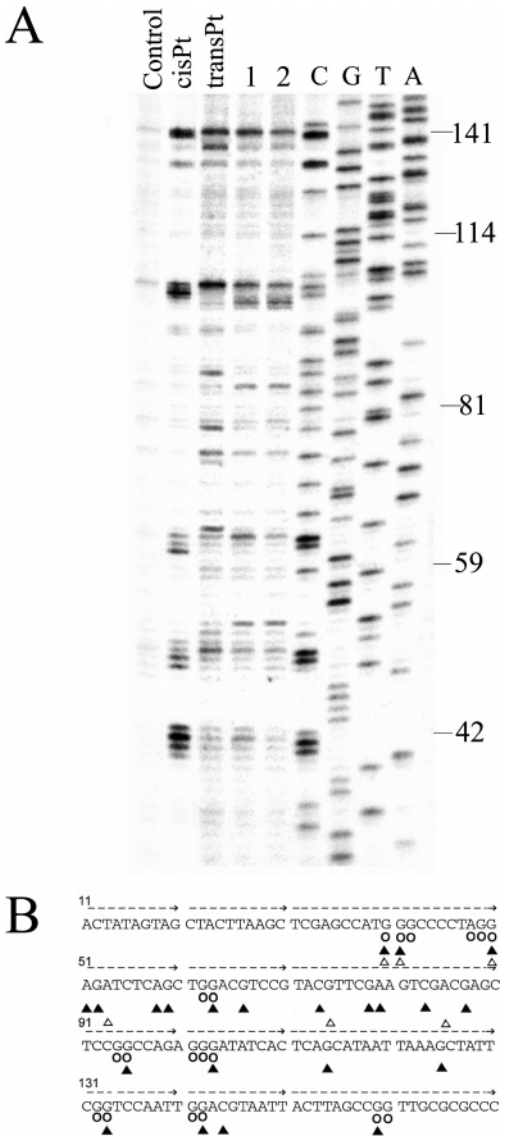

Figure 3. A: Autoradiogram of 6% polyacrylamide/8 M urea sequencing gel showing inhibition of DNA synthesis by TaKaRa Taq DNA polymerase on the pSP73 plasmid DNA linearized by *Hpa*I restriction enzyme and subsequently modified by platinum complexes. The gel contained the linear amplification products of control, nonplatinated DNA, and DNA treated with **1**, **2**, or with transplatin at $r_b = 0.006$. Lanes: Control, unmodified template; cisPt, DNA modified by cisplatin; transPt, DNA modified by transplatin; 1, DNA modified by **1**; 2, DNA modified by **2**; C, G, T, A, chain-terminated marker DNAs (note that these dideoxy sequencing lanes give the sequence complementary to the template strand). The numbers correspond to the nucleotide sequence numbering of panel B. B: Schematic diagram showing a portion of the sequence used to monitor inhibition of DNA synthesis on the template containing adducts of **1**, **2**, transplatin, or cisplatin. The arrows indicate the direction of the synthesis. Circles, major stop signals from panel A, lane cisPt; solid triangles, major stop signals from panel A, lane transPt; open triangles, major stop signals from panel A, lane 1. The numbering of the nucleotides in this scheme corresponds to the numbering of the nucleotides in the pSP73 nucleotide sequence map.

Characterization of Platinum Adducts by Thiourea (TU). Cisplatin, transplatin, and analogous bifunctional platinum compounds coordinate to DNA in a two-step process, forming first the monofunctional adducts preferentially at guanine residues, which subsequently close to bifunctional lesions.[22,23] TU is used to labilize monofunctionally bound transplatin from DNA.[24,25] The displacement of transplatin is initiated by

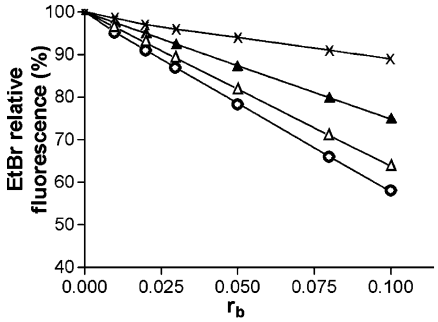

Figure 4. Dependence of the EtBr fluorescence on r_b for DNA modified by various platinum complexes in 10 mM $NaClO_4$ at 37 °C for 48 h. Circles, cisplatin; solid triangles, transplatin; open triangles, **1**; crosses, dienPt. Data are the average of three independent experiments.

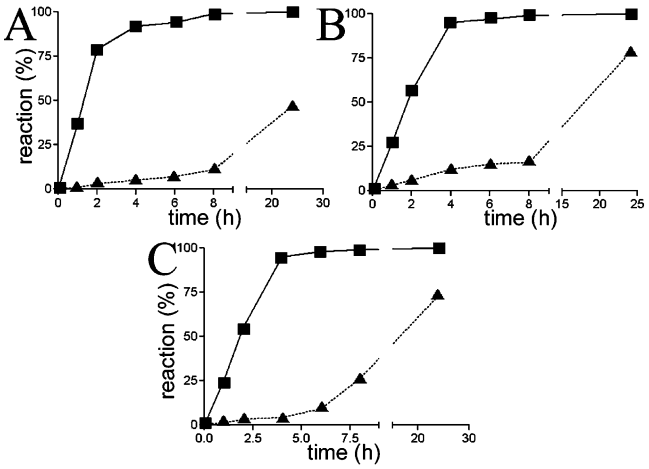

Figure 5. Kinetics of reaction of transplatin (A), **1** (B), and **2** (C) with double-helical DNA at $r_i = 0.1$ in 10 mM $NaClO_4$ at 37 °C. DNA concentration was 0.15 mg/mL. Reactions were stopped with (squares) or without (triangles) 10 mM TU (10 min), and platinum associated with DNA was assessed by FAAS. Data points measured in triplicate varied ±2% from their mean.

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.[24]

Double-stranded CT DNA at the concentration of 0.15 mg/mL was incubated with transplatin or its analogues **1** and **2** at a drug-to-nucleotide ratio of $r_i = 0.1$ in 10 mM $NaClO_4$ at 37 °C. At various time intervals the aliquots were withdrawn in which 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 10 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 (Figure 5). TU displaced ca. 90−95% *trans*-platinum compounds from DNA at early time intervals (4 h). At longer incubation times (24 h), TU was considerably less efficient in removing transplatin analogues from DNA (only 20 and 25% of the adducts of **1** and **2** was removed from DNA, respectively, Figures 5B,C). It can be concluded that at this time interval ca. 80% or 75% monofunctional adducts of **1** and **2**, respectively, had evolved to bifunctional lesions. Hence, monofunctional

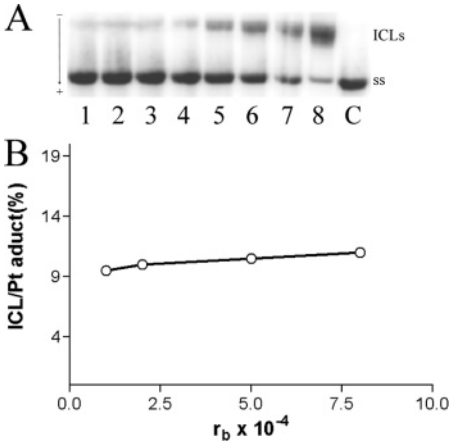

Figure 6. The formation of interstrand CLs by **1** in linearized pSP73 plasmid. (A) Autoradiogram of denaturing 1% agarose gels of linearized DNA which was 3′-end labeled. The interstrand cross-linked DNA appears as the top bands migrating on the gel more slowly than the single-stranded DNA (contained in the bottom bands). Plasmid linearized by *Eco*RI was incubated for 48 h with **1** at r_b values of 0 (control) (lanes C in Figure 5A) and 2×10^{-5}, 5×10^{-5}, 7×10^{-5}, 1×10^{-4}, 2×10^{-4}, 3×10^{-4}, 5×10^{-4}, 7×10^{-4} (lanes 1−8 in Figure 5A, respectively). (B) Dependence on r_b of the number of interstrand CLs per adduct (% ICL/adduct) formed by **1** in linearized DNA after 48 h. The ratio of interstrand CLs to total platinum complex bound was calculated as described previously. % ICL/Pt was then calculated by multiplying this ratio by 100. Data are the average of two independent experiments.

adducts of **1** and **2** close to become bifunctional lesions with a considerably higher rate than those of parent transplatin (only ca. 40% monofunctional adducts of transplatin had evolved to bifunctional lesions, Figure 5A). It was verified that 5−60 min incubations with 10 mM TU gave the same results as those described above. We have also verified that the different amount of DNA adducts of transplatin and its analogues removed from DNA by TU is not due to a different efficiency of TU to displace the monofunctional adducts of these different trans compounds from DNA in the same way as in our recent work.[25]

Interstrand Cross-Linking. The amounts of interstrand CLs formed by **1** or **2** in linear DNA were measured in pSP73 plasmid which was first linearized by *Eco*RI (*Eco*RI cuts only once within pSP73 plasmid) and subsequently modified by **1** or **2** to various r_b. The samples were analyzed for the interstrand CLs by agarose gel electrophoresis under denaturing conditions.

An electrophoretic method for precise and quantitative determination of interstrand cross-linking by platinum complexes in DNA was described previously.[19,21,26] Upon electrophoresis under denaturing conditions, 3′-end labeled strands of linearized pSP73 plasmid containing no interstrand CLs migrate as a 2464-base single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species. The bands corresponding to more slowly migrating interstrand-cross-linked fragments were noticed if **1** or **2** were used to modify linearized DNA at r_b as low as 5×10^{-6} (shown in Figure 6A for **1**). 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 (the amount of interstrand CLs per one molecule of **1** or **2** bound to DNA) was calculated using the Poisson distribution from the fraction of non-cross-linked DNA in combination with

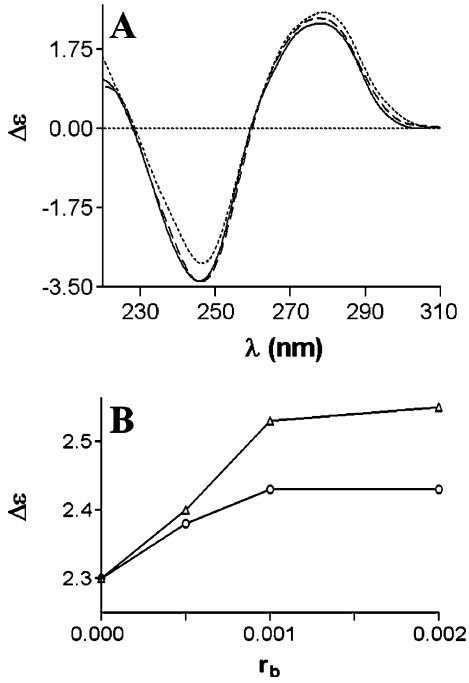

Figure 7. CD spectroscopy of CT DNA modified by compounds **1** and **2**. A. CD spectra for DNA modified by the compound **1**; DNA was in 10 mM $NaClO_4$. Curves: solid, control (nonmodified) DNA; dashed, $r_b = 0.002$; dotted, $r_b = 0.005$. (B) Changes in CD spectra of DNA at λ_{max} around 275 nm (at the wavelength at which the maximum of the positive CD band around 275 nm occurred) induced by the binding of **1** (triangles) and **2** (circles) plotted as the function of r_b.

the r_b values and the fragment size. **1** and **2** showed a similar cross-linking efficiency (~10−12%) as parent transplatin (12%[19]).

Circular Dichroism. CD spectral characteristics were compared for CT DNA in the absence and in the presence of **1** or **2** at $r_b = 0-0.002$. Upon the binding of these compounds to CT DNA, the conservative CD spectrum normally found for DNA in the canonical B-conformation is transformed at wavelengths below 300 nm. There was a slight increase in the intensity of the positive band around 280 nm if double-helical DNA was modified by **1** or **2** (Figure 7). Importantly, CD spectra of thermally denatured DNA remained unaffected as a consequence of its modification by **1** or **2** under the same conditions (not shown). This observation can be interpreted to mean that the pyridine ligand does not contribute to the CD produced by double-helical DNA modified by **1** or **2**.

These results indicate that both analogues modify DNA conformation differently than parent transplatin which decreases the intensity of the positive CD band around 280 nm.[27] On the other hand, a similar increase although greater was observed if DNA was under identical conditions modified by cisplatin.[27] Thus, on the basis of the analogy with the changes in the CD spectra of DNA modified by cisplatin and clinically ineffective transplatin,[27] it might be suggested that the binding of both transplatin analogues **1** or **2** results in the conformational alterations in double-helical DNA of nondenaturational character similar to those induced in DNA by cisplatin although to a lesser extent.

In addition, the changes in CD spectra of DNA induced by **1** and **2** (Figure 7A) are also consistent with DNA unwinding. More specifically, the origin of the nonconservative CD spectra, namely a reduction of the CD minimum at ~245 nm are similar

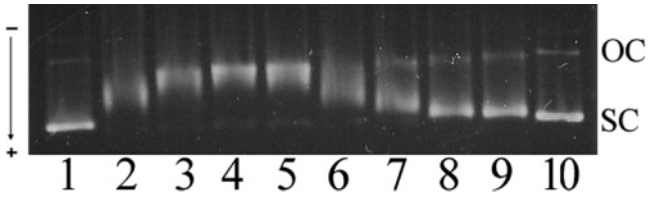

Figure 8. Unwinding of supercoiled pSP73 plasmid DNA by **1**. The top bands correspond to the form of nicked plasmid and the bottom bands to closed, negatively supercoiled plasmid. The plasmid was incubated with **1** with the r_b values of 0 (control) (lanes 1 and 10), 0.001, 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.055 (lanes 2−9, respectively).

to the changes in CD spectra observed during the transition of B-DNA into its A-form,[28,29] which is less tightly wound.

Unwinding Induced in Supercoiled Plasmid DNA. Electrophoresis in native agarose gel was used to quantify the unwinding induced in pSP73 plasmid by the platinum complex by monitoring the degree of supercoiling (Figure 8). A compound that unwinds the DNA duplex reduces the number of supercoils so that the superhelical density of closed circular DNA decreases. This decrease upon binding of unwinding agents causes a decrease in the rate of migration through agarose gel, which makes it possible that the unwinding can be observed and quantified.[30] Figure 8 shows electrophoresis gel in which increasing amounts of **1** have been bound to a mixture of relaxed and supercoiled pSP73 DNA. Interestingly, the platinum complex accelerated the mobility of the relaxed form similarly as does cisplatin, whose bifunctional binding to DNA shortens and condenses the DNA helix. 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. Under the present experimental conditions, σ was calculated to be −0.063 on the basis of the data of cisplatin for which the $r_b(c)$ was determined in this study and $\Phi = 13°$ was assumed. The value of $r_b(c)$ at which the supercoiled and relaxed forms modified by **1** comigrate was found to be 0.07. Hence, the unwinding angle for **1** was calculated as 16°. The same analysis performed with DNA modified by **2** yielded the identical result (not shown). Thus, the unwinding angle for both **1** and **2** is considerably greater than that induced in DNA by transplatin or cisplatin (9° or 13°, respectively[30]).

The values of unwinding angles are affected by the nature of the ligands in the coordination sphere of platinum and the stereochemistry at the platinum center. It has been shown[30] that platinum(II) compounds with the smallest unwinding angles (3−6°) are those that can bind DNA only monofunctionally {[PtCl(dien)]Cl or $[PtCl(NH_3)_3]Cl$}. The observation that the analogues of transplatin tested in the present work cannot be grouped with monofunctional platinum(II) compounds is readily understood in terms of adduct structures in which the complexes are preferentially coordinated to DNA in a bifunctional manner, which is consistent with the characterization of DNA adducts of **1** and **2** by TU (Figure 5). Interestingly, the unwinding angles produced by other antitumor analogues of transplatin in which one or both ammine groups were replaced by, for instance, the heterocyclic ligand were also higher than those produced by transplatin (9°).[8,20] This observation was explained[8] by the additional contribution to unwinding associated with the interaction of the heterocyclic ligand with the duplex upon covalent binding of platinum. Thus, in this way, also the heterocyclic moiety or aliphatic or both moieties in DNA adducts of **1** and **2** could be geometrically positioned to interact with the double helix.

Reactions with Sulfur-Containing Compounds. Platinum(II) compounds have a strong thermodynamic preference for binding to S-donor ligands. Hence, before antitumor platinum(II) drugs reach DNA in the nucleus of tumor cells or even after they bind to DNA, they may interact with various compounds including sulfur-containing molecules.[31] These interactions are generally believed to play a role in mechanisms underlying tumor resistance to platinum compounds, their inactivation and side effects.[32−35] In addition, a distinct difference between cisplatin and its clinically inefficient trans isomer is that transplatin is kinetically more reactive than cisplatin and more susceptible to deactivation. While cisplatin major adducts are intrastrand CLs between neighboring guanine residues, transplatin−DNA adducts are mainly interstrand CLs and a relatively large portion of adducts remains monofunctional (Figure 5 and ref 23). It has been also suggested[36] that in cells transplatin forms only a small amount of CLs because of the slow closure of the monofunctional adducts coupled to their trapping by intracellular sulfur nucleophiles. Therefore, interest in the interactions of platinum antitumor drugs with sulfur-containing molecules of biological significance has recently markedly increased.

Examples of endogenous thiols to which platinum(II) complexes bind when they are administered intravenously or after they enter the cell are glutathione (GSH) and metallothionein (MT). MTs are small (∼6−7 kDa) cellular metalloproteins that strongly bind metal ions. The metal ion binding domains of this protein consist of 20 cysteine residues arranged in two thiol (S)-rich sites.[37] In the present work, we investigated using UV absorption spectrophotometry[38] reactions of GSH and mammalian MT-2 with **1**, **2**, and transplatin free in the bulk solution or in the case of GSH with these platinum complexes coordinated only monofunctionally to short oligodeoxyribonucleotide duplex TGT (10 bp) (for its nucleotide sequence, see Figure 1B) or to its single-stranded top strand so that these oligonucleotides contained the single, site-specific monofunctional aduct of these platinum compounds.

Free **1**, **2**, or transplatin at the concentration of 33 μM were mixed with 16.5 mM GSH (this concentration of GSH represents physiologically relevant concentration) or with 2.1 μM MT-2 at 37 °C in the medium of 10 mM $NaClO_4$ plus 0.1 mM Tris-$HClO_4$ buffer, pH 7.0. The half-times of these reactions were found <1 min for the complexes **1** and **2**, whereas transplatin reacted with GSH and MT-2 much more slowly, with the half-time of 11 and 18 min, respectively. Thus, these results suggest that **1** and **2**, despite of their cytotoxicity in several tumor cell lines (Table 1), are inactivated by sulfur-containing compounds considerably faster than clinically inefficient transplatin. As the reaction of free **1** and **2** with GSH and MT-2 should contribute to the resistance of tumor cells to these complexes, it is reasonable to suggest that the faster reaction of free molecules of antitumor **1** and **2** with GSH and MT-2 does not play a decisive role in the overall biological effects of these new compounds. This conclusion is also consistent with the observation that these new transplatin analogues are radically more potent also in the tumor cell line resistant to cisplatin A2780cisR (Table 1) which is known to be resistant also through elevated levels of reduced glutathione.[14]

We have also examined the capacity of GSH to remove monofunctional adducts of compounds **1** and **2** and transplatin from DNA. The bifunctional platinum complexes react with DNA in a two-step process, first forming monofunctional adducts on DNA at guanine residues which may subsequently close to various CLs.[18] Hence, the monofunctional adducts of

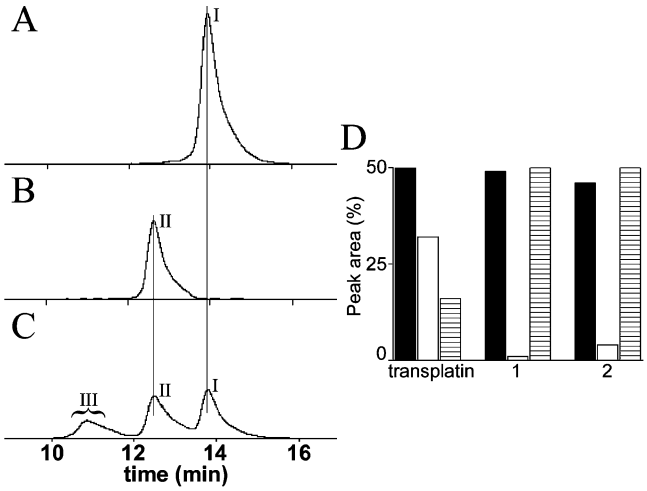

Figure 9. Reaction of the single-stranded oligonucleotides (the top strand of the TGT duplex, for its nucleotide sequence, see Figure 1B) containing the site-specific, monofunctional adduct of **1**, **2**, or transplatin with GSH. The oligonucleotides were at the concentration of 20 μM (A) or 10 μM (B−D) and GSH was at the concentration of 10 mM. After addition of GSH to the platinated oligonucleotides, the resulting solution was incubated for additional 90 min at 37 °C in 0.1 M $NaClO_4$. A. HPLC profile (absorbance at 260 nm versus retention time) of the unmodified oligonucleotide. B. HPLC profile of the oligonucleotide containing the site-specific, monofunctional adduct of transplatin. C. HPLC profile of the oligonucleotide containing the adduct of transplatin and treated with GSH (vide supra). D. The areas under the peaks from the HPLC profiles of the oligonucleotides containing the adduct of transplatin (C), **1**, or **2** and treated with GSH (vide supra) (as percentage of the total species); solid bars, monoadducted oligonucleotides (peak II); open bars, unmodified oligonucleotides (peak I); horizontal stripped bars, new products (peak III).

transplatin and its bifunctional analogues (having one group still accessible to interact with a nucleophile) could further react with sulfur-containing compounds which could, due to the trans effect, labilize these monofunctional adducts and remove them from DNA. We prepared 10-bp DNA duplex TGT and its single-stranded top strand (for their sequence, see Figure 1B) containing the single, site-specific monofunctional adduct of **1**, **2**, or transplatin. These oligonucleotides were analyzed by ion-exchange HPLC under conditions when the modified oligonucleotides were eluted considerably earlier than the unmodified oligonucleotides. Thus, the unmodified and platinated oligomers yielded well separated peaks I and II in the HPLC profiles (shown in Figures 9A,B for the single-stranded oligonucleotide unmodified or containing the adduct of transplatin). We demonstrate in Figure 9 the results obtained with the single-stranded oligomers, but basically identical results were also obtained for double-stranded oligonucleotides. The platinated oligonucleotides at the concentration of 10 μM were reacted at 37 °C for 90 min with GSH at the concentration of 10 mM and analyzed by HPLC. The area under the HPLC peak II corresponding to the oligonucleotides containing the adduct of transplatin was reduced with the concomitant occurrence of the peak I corresponding to the unmodified oligonucleotide (via comparisons with chromatograms of these compounds alone, and via spiking experiments) and several new peaks (peaks III) in the HPLC profile (shown for the single-stranded oligonucleotide in Figure 9C); these new peaks corresponded to the products which were eluted still earlier than the platinated oligonucleotide. The fraction of the products corresponding to the new peaks III was collected, and platinum FAAS and elemental analysis confirmed that these products contained platinum and sulfur atoms. These observations along with the

Table 2. Summary of DNA Binding Characteristics of *trans*-[$PtCl_2$(Isopropylamine)(3-(hydroxymethyl)-pyridine)] (**1**), *trans*-[$PtCl_2$(Isopropylamine)(4-(hydroxymethyl)-pyridine)] (**2**), Transplatin, Cisplatin, and *trans*-[$PtCl_2$(4-Picoline)(piperazine)]$^+$ (trans-pic,pz)

	1[a]	**2**[a]	transplatin	cisplatin	trans-pic,pz[b]
DNA binding ($t_{50\%}$, min)[c]	105	105	120[d]	120[d]	10
sequence preference	G, A	G, A	G, C[e,f]	GG, AG[e]	ND
% interstrand CLs/adduct	~10−12	~10−12	12[f]	6[f]	6
% monofunctional lesions/adduct after 24 h	20	25	~60[a]	~1−4[e]	18
% intrastrand CLs/adduct	~69	~64	~28[a,f]	~90[e]	76
% decrease of EtBr fluorescence at $r_b = 0.1$	37	37	24	42[a]	ND
unwinding angle/adduct	16°	16	9°[g]	13°[g]	16°
CD band at 278 nm	increase	increase	decrease[h]	increase[h]	increase

[a] This work. [b] Najajreh et al.[56] [c] The time at which the binding reached 50%. [d] Bancroft et al.[18] [e] Eastman.[23] [f] Brabec and Leng.[19] [g] Keck and Lippard.[30] [h] Brabec et al.[27]

fact that these products absorbed at 260 nm may be interpreted to mean that reaction of the single- or double-stranded oligonucleotides containing monofunctional adducts of transplatin with GSH resulted in some type of the ternary complexes DNA-Pt-GSH. The occurrence of the unmodified oligonucleotides among the products of the reaction of the oligonucleotides containing the adduct of transplatin with GSH also indicates that GSH labilized, due to the trans effect, the monofunctional adducts of transplatin to the extent that a part of these adducts was even removed from DNA.

In contrast, if the oligonucleotides containing the monofunctional adduct of compounds **1** and **2** were treated with GSH, the area under the HPLC peak II corresponding to the single- or double-stranded oligonucleotides containing the adduct was reduced noticeably less than in the case of the adducts of transplatin (shown for single-stranded oligonucleotides in Figure 9D). Concomitantly, several new peaks (peaks III) in the HPLC profile occurred which corresponded to the products, which were eluted earlier than the platinated oligonucleotide, presumably the ternary complexes DNA-Pt-GSH (vide supra). However, no additional peak I corresponding to the unmodified single- or double-stranded oligonucleotide was observed in the HPLC profile of the oligonucleotides containing the adduct of transplatin and treated with GSH. This observation may be interpreted to mean that reaction of the single- or double-stranded oligonucleotides containing monofunctional adducts of the compounds **1** or **2** with GSH only resulted in some type of the ternary complexes DNA-Pt-GSH although to a lesser extent in comparison with the reaction of the oligomers containing the adduct of transplatin. In other words, it seems likely that the monofunctional adducts of compounds **1** and **2** were more resistant to the labilization (due to the trans effect) and quenching by GSH than the adducts of parental transplatin.

In aggregate, our results are consistent with the view that the monofunctional adducts of the new antitumor analogues of transplatin **1** and **2** could persist on DNA in tumor cells for a significantly longer time than those of parent transplatin, which may potentiate cytotoxic effects of these new platinum complexes.

Discussion

The replacement of one ammine group by the heterocyclic planar ligand such as 3-(hydroxymethyl)-pyridine or 4-(hydroxymethyl)-pyridine and the other ammine group by aliphatic isopropylamine in the molecule of clinically ineffective transplatin affords the new compound exhibiting radically enhanced activity (related to parent transplatin) in tumor cell lines both sensitive and resistant to cisplatin (Table 1). An intriguing observation is that the enhancement of the activity is more pronounced in the tumor cells resistant to cisplatin in comparison with the parental cells, which is a feature seen previously for some other antitumor analogues of transplatin. The results of the biochemical and biophysical analysis of DNA interactions with compounds **1** and **2** are summarized in Table 2. They provide experimental support for the view that the binding of these new platinum complexes modifies DNA in a way which is distinctly different from the modification by transplatin (Table 2) and in several features also from that by other antitumor analogues of transplatin in which only one ammine group was replaced.[18]

As regards the comparisons of DNA binding modes of compounds **1** and **2** with other antitumor analogues of transplatin, we compare further in detail the behavior of **1** and **2** with *trans*-[$PtCl_2$(4-picoline)(piperazine)]$^+$ since the latter compound is the only antitumor analogue of transplatin in which both NH_3 groups were replaced and for which DNA binding studies similar to those described in the present work for **1** and **2** were carried out.

DNA binding mode of compounds **1** and **2** is in several features identical or similar to that of *trans*-[$PtCl_2$(4-picoline)-(piperazine)]$^+$ (Table 2). Even more interestingly, several features of DNA binding mode of **1** and **2** are also similar to those of cisplatin (Table 2). As DNA is a main pharmacological target of antitumor platinum(II) complexes, the results of the present work demonstrating DNA binding mode of compounds **1** and **2** are also consistent with the hypothesis that the altered DNA binding mode of compounds **1** and **2** relative to parent transplatin is an important factor responsible for altered biological (pharmacological) activity of these new platinum complexes in tumor cells.

An important property of compounds **1** and **2** or closely related *trans*-[$PtCl_2$(4-picoline)(piperazine)]$^+$ is that due to replacement of both ammine groups by aliphatic and/or planar heterocyclic ligands, interstrand cross-linking is relatively low (10−12%, Table 2). This is in contrast to the antitumor analogues of transplatin in which only one ammine group was replaced by either aliphatic or heterocyclic ligand, which exhibit markedly higher interstrand cross-linking efficiency.[8,20,39−41] The CLs are formed in DNA by bifunctional platinum compounds in a two-step process. In the first step, the monofunctional adducts are formed preferentially at guanine residues that subsequently close to the CLs. It is reasonable to suggest that the unexpectedly high rate of interstrand CL formation by transplatin analogues only containing one heterocyclic ligand relative to that determined for parent transplatin is associated with favorable conformational changes in double-stranded DNA, induced by the heterocyclic ligand in a monofunctional adduct which modulate the second binding step and facilitate formation of an interstrand CL. Hence, it is likely that the replacement of both ammine groups does not allow positioning of the nonleaving moieties in the adducts of these analogues that would be entirely favorable for their interaction with the double helix allowing the closure of the monofunctional adducts to interstrand CLs.

It is also interesting that similarly as in the case of closely related *trans*-[$PtCl_2$(4-picoline)(piperazine)]$^+$, compounds **1** and **2** unwind DNA only moderately (16°, Table 2), i.e., much less than the analogues of transplatin in which only one ammine group was replaced by a heterocyclic ligand [for instance *trans*-[$PtCl_2(NH_3)$(piperidine)] and *trans*-[$PtCl_2(NH_3)$(4-picoline)] unwind DNA by 30 and 26°, respectively[12]]. This may imply that heterocyclic or aliphatic amine ligands in compounds **1** and **2** or *trans*-[$PtCl_2$(4-picoline)(piperazine)]$^+$ do not allow positioning of their nonleaving moieties in the adducts of these analogues that would be entirely favorable for their interaction with the double helix also allowing more extensive unwinding of DNA. This may also imply that the marked enhancement of the unwinding angles produced by several analogues of transplatin in which only one ammine group was replaced by the heterocyclic ligands[8,20] is a feature of their DNA binding mode that affects the cytotoxic effects in tumor cell lines unfavorably.

Nevertheless, the most striking consequence of the replacement of both ammine ligands in parent transplatin by the heterocyclic or aliphatic ligands is a relatively high amount of stable intrastrand CLs (Table 2) (after 48 h the compounds **1** and **2** and *trans*-[$PtCl_2$(4-picoline)(piperazine)]$^+$ form 69%, 64% and 76% intrastrand CLs in double-helical DNA, respectively). The clinical ineffectiveness of the parent transplatin has been proposed to be also associated with its reduced capability to form bifunctional adducts in double-helical DNA.[36] A considerably higher interstrand cross-linking efficiency of transplatin analogues containing only one heterocyclic ligand in comparison with the parent transplatin and enhanced stability of their 1,3-GXG intrastrand CLs in double-helical DNA has been suggested to be important candidates for the factor(s) responsible for their higher activity in tumor cells.[8] Interestingly, the presence of two heterocyclic or one heterocyclic and one aliphatic nonleaving ligands in the analogues of transplatin further enhances the frequency of stable 1,3-intrastrand CLs (but not interstrand CLs) in double-helical DNA. This observation supports the view that in particular enhanced stability of intrastrand CLs is a factor associated with the enhanced potency of the transplatin analogues in which both (and not only one) ammine groups were replaced by the two heterocyclic or by one heterocyclic and one aliphatic ligands.

As it is mentioned above, most of the features of DNA binding of compounds **1** and **2** on one hand and *trans*-[$PtCl_2$-(4-picoline)(piperazine)]$^+$ on the other are very similar except the rate of binding. The extremely rapid binding of *trans*-[$PtCl_2$-(4-picoline)(piperazine)]$^+$ to DNA ($t_{50\%}$ = 10 min) (Table 2) suggests that the most important factor in determining the binding rate is the electrostatic attraction between the polyanionic DNA and the monocationic *trans*-[$PtCl_2$(4-picoline)-(piperazine)]$^+$. In the case of the less positively charged compounds **1** and **2**, these complexes diffuse to the vicinity of the DNA more slowly and only then the short-range interactions can affect the binding rates. Nonetheless, the cytotoxicity data (Table 1) suggest that the rate of DNA binding of both analogues of transplatin in which both ammine groups were replaced by either two heterocyclic ligands or by one heterocyclic and one aliphatic ligand is hardly associated in a fundamental way with their improved activity in tumor cell lines.

As also mentioned above, the results shown in Table 2 demonstrate that some features of the DNA binding mode of compounds **1** and **2** or *trans*-[$PtCl_2$(4-picoline)(piperazine)]$^+$ are similar to those of cisplatin. The intriguing similarity consists of the observation that cisplatin, its antitumor analogues, and the latter analogues of transplatin all induce in DNA nondenaturational alterations. This is in contrast to transplatin and several antitumor analogues of transplatin containing only one heterocyclic and one ammine nonleaving ligand, which induce in DNA denaturational conformational changes. We have interpreted the results demonstrating low interstrand cross-linking efficiency of compounds **1** and **2** and their only moderate efficiency to unwind DNA to mean that heterocyclic or aliphatic amine ligands in compounds **1** and **2** or *trans*-[$PtCl_2$(4-picoline)-(piperazine)]$^+$ do not allow positioning of their nonleaving moieties in the adducts of these analogues that would be entirely favorable for their interaction with the double helix. Hence, we speculate that this somewhat limited interaction of compounds **1** and **2** or *trans*-[$PtCl_2$(4-picoline)(piperazine)]$^+$ leads only to less severe nondenaturational alterations in DNA. On the other hand, the nonleaving heterocyclic moiety in the adducts of the analogues of transplatin containing only one heterocyclic and one ammine ligand is presumably positioned in the way that is favorable for its interaction with the double helix so that this interaction may lead to more severe denaturational alterations in DNA.

Hence, the lesions typical for analogues of transplatin in which both ammine groups were replaced by heterocyclic or aliphatic ligands may have critical consequences for the biological functions of DNA—the resulting conformational alterations in DNA may play an important role in altered antitumor effects of these new platinum complexes as compared with transplatin. Thus, "downstream" effects modulated by recognition and binding of other cellular components (such as proteins) are not likely to be the same as those modulated by the subset of cellular components which recognize transplatin.

The mechanism underlying antitumor activity of platinum compounds is a complex process involving a number of factors.[42,43] Among these factors are also those that do not operate directly at the level of the DNA adducts, such as those affecting the amount of platinum complex that can reach DNA [by influencing either the cell accumulation of the complexes or the levels of intracellular platinophiles that act as detoxification agents (metallothionein and GSH)]. The results of the present work also suggest that the reaction of free molecules of antitumor compounds **1** and **2** with GSH and MT-2 does not play a dominant role in the overall biological effects of these new compounds. In addition, GSH labilizes, due to the trans effect, and quenches the monofunctional adducts of compounds **1** and **2** less efficiently than those of inactive parent transplatin so that the adducts of the new antitumor analogues **1** and **2** could persist on DNA in tumor cells for a significantly longer time than those of parent transplatin, which may potentiate cytotoxic effects of these new platinum complexes.

Nevertheless, it is generally accepted that DNA is an important and major pharmacological target of platinum compounds.[44] The present work correlates DNA binding mode in a cell-free medium of the bifunctional analogues of transplatin containing one aliphatic and one planar heterocyclic ligand with their activity in several tumor cell lines. The results offer strong experimental support for the view that another strategy of how to activate trans geometry in bifunctional mononuclear platinum(II) compounds and to circumvent resistance to cisplatin consists of a chemical modification of the "classical" transplatin which would result in an increased amount of stable bifunctional intrastrand adducts of these *trans*-platinum compounds in double-helical DNA. The present work also suggests that such a modification may also be accomplished by the replacement of both ammine groups, one by a planar heterocyclic and the other by an aliphatic ligand.

Generally, *trans*-diaminedichloroplatinum(II) analogues have lower solubility in aqueous solution than their cis counterparts, resulting in limited bioavailability. One way of increasing the aqueous solubility is by adding a charge to the complex.[7] The results of this work also suggest that the strategy of how to activate trans geometry in mononuclear platinum(II) complexes based on a combination of the aliphatic and planar heterocylic amine ligands in one molecule of transplatin analogue may also result in the design of new platinum anticancer drugs which have markedly enhanced solubility in water. This is a feature not always carefully considered when new antitumor analogues of transplatin are designed.

Experimental Section

Starting Material. Cisplatin was synthesized from K_2PtCl_4 supplied by Johnson Matthey. Transplatin was purchased from Sigma (Prague, Czech Republic). Compounds **1** and **2** were prepared in the following way. A suspension of *cis*-[$PtCl_2$(isopropylamine)$_2$] (0.390 mmol, 150 mg) in water (3 mL) was treated with 4 equiv of 3-(hydroxymethyl)-pyridine (1.56 mmol, 170 mg) or 2 equiv of 4-(hydroxymethyl)-pyridine (0.78 mmol, 85 mg). The mixture was stirred at 85 °C until the reaction mixture became clear and yellow. The yellow solution was allowed to cool at room temperature, hydrochloric acid (12 M, 0.5 mL) was added, and the solution was stirred for 48 h at 85 °C. After cooling at room temperature, the solution was evaporated until dryness. $CHCl_3$ was added to the resulting solid, and the *trans*-[$PtCl_2$(isopropylamine)(L)] [L = 3- or 4-(hydroxymethyl)-pyridine] was isolated in chloroform solution. The solution was concentrated until dryness and dissolved in water. The final water solution afforded a yellow precipitate overnight. The solid was washed with ice water and diethyl ether.

Compound **1**. (Yield: 30%): ν(Pt−Cl):340 cm^{-1}, FAB-MS 434.236, Anal. ($C_9H_{16}ON_2PtCl_2$) C, H, N. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C) (ppm): 1.45 d 6H (CH_3), 2.08 t 1H (CH_2OH), 3.43 hp 1H (CH), 3.52 bs 2H (NH_2), 4.72 d 2H (CH_2OH), 7.26 m 1H (*meta*), 8.78 m 1H (*para*), 8.89 m 2H (*ortho*). ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C) (ppm): 152.20 (C5), 151.70 (C4), 138.48 (C3), 136.49 (C2), 124.91 (C6), 61.57 (CH_2OH), 48.99 (CH), 23.99 (CH_3).^{195}Pt NMR (300 MHz, $CDCl_3$, 25 °C) (ppm): −2091.7.

Compound **2**. (Yield: 38%)ν(Pt−Cl):339 cm^{-1}, FAB-MS 434.236, Anal. ($C_9H_{16}ON_2PtCl_2$) C, H, N.^{1}H NMR (300 MHz, $CDCl_3$, 25 °C) (ppm): 1.45 d 6H (CH_3), 2.1 t 1H (CH_2OH), 3.45 hp 1H (CH), 3.48 bs 2H (NH_2), 4.76 d 2H (CH_2OH), 7.26 m 2H (*meta*), 8.78 d 2H (*ortho*).^{13}C NMR (300 MHz, $CDCl_3$, 25 °C) (ppm): 152.53 (C3 and C5), 155.19 (C4), 122.62 (C2 and C6), 62.59 (CH_2OH), 48.69 (CH), 24.01 (CH_3).^{195}Pt NMR (300 MHz, $CDCl_3$, 25 °C) (ppm): −2090.9.

ORTEP views of compounds **1** and **2** with the atomic numbering scheme are shown in Figure 2. Crystal data are summarized in Tables 7 and 8 of the Supporting Information. The bond lengths and angles for compounds **1** and **2** are summarized in Tables 3 and 4 of the Supporting Information, respectively.

The Pt atom shows an square-planar environment with typical angles for square-planar platinum complexes.[10,12,45] The Pt−N(1) bond lengths (2.028 Å in **1** and 2.025 Å in **2**, respectively) are in the normal range of those found in similar complexes.[12,46] These distances are also similar to that observed in the complex *cis*-[$PtCl_2$(NH_3){2-(2-hydroxyethylpyridine)}] (2.028 Å),[47] which could indicate that the hydroxymethylpyridine ligand does not suffer from a high trans effect. The values of Pt−Cl lengths are comparable with those found in other *trans*-Pt complexes with planar amines.[12,13,46]

The packing of the molecules is completely determined by the N−H···Cl (1 and 2) and N−H···O (2) intermolecular hydrogen bonding. Three intermolecular hydrogen bonds were observed in the crystal of complex **1** (Table 5 of the Supporting Information), while in the complex **2** two different hydrogen bonds were only observed (see Table 6 of the Supporting Information). No intramolecular hydrogen bonding was observed in both complexes, in comparison with that observed in *trans*-[$PtCl_2$(dma)(ipa)].[12] The distances of these hydrogen bonds agree well with the values published for other Pt complexes with ligands containing N donor atoms.[12]

The stock solutions of the platinum complexes for biochemical, biophysical, and cytotoxicity studies were prepared in water in the dark at 25 °C. The 100-mm culture and micro well plates were obtained from NUNCLON (Roskilde, Denmark). MTT was purchased from Sigma (Prague, Czech Republic), and fetal calf serum (FCS) was supplied by GIBCO−BRL. Calf thymus (CT) DNA (42% G+C, mean molecular mass ca. 20 000 kDa) was prepared and characterized as described previously.[48] Thermally denatured CT DNA was prepared by heating in 10 mM $NaClO_4$ 10 min at 100 °C followed by quick cooling on an ice bath. Plasmid pSP73 was isolated according to standard procedures. The synthetic oligodeoxyribonucleotides (Figure 1B) were synthesized and purified as described previously.[49] Restriction endonuclease *Eco*RI, *Hpa*I, and T4 polynucleotide kinase were purchased from New England Biolabs. Klenow fragment of DNA polymerase I was from Boehringer-Mannheim Biochemica. Glutathione was purchased from Sigma (Prague) and rabbit metallothionein-2 (Zn_7-MT-2) was a kind gift of prof. M. Vasak from University of Zurich. Acrylamide, agarose, bis(acrylamide), EtBr, urea, TU, ethanol, and NaCN were from Merck KgaA (Prague, Czech Republic). The radioactive products were from Amersham.

Cytotoxicity. The human ovarian tumor cell lines (A2780/A2780cisR) and human ovarian carcinoma cell lines (CH1/CH1cisR) 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 which is reduced by living cells to yield a soluble formazan product [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] that can be detected colorimetrically.[16] Cells were seeded in 96-well sterile plates at a density of 10^4 cells/well in 100 μL of medium and were incubated for 3−4 h. The compounds were added to final concentrations from 0 to 200 μM in a volume of 100 μL/well. Twenty-four hours later 50 μL of a freshly diluted MTT solution (1/5 in culture medium) to a concentration of 1 mg/mL was pipetted into each well and the plate was incubated for 5 h at 37 °C in a humidified 5% CO_2 atmosphere. After 72 h the cell viability was evaluated by measurement of the absorbance at 570 nm, using a Absorbance Reader BIOTEC EL800-PC. 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.

DNA Platination. CT or plasmid DNAs were incubated with the platinum complex in 10 mM $NaClO_4$ at 37 °C in the dark. After 48 h, the samples of plasmid DNA were precipitated by ethanol and redissolved in the medium required for subsequent biochemical or biophysical analysis whereas the samples of CT DNA were exhaustively dialyzed against such a medium. An aliquot of these samples was used to determine the value of r_b by FAAS or differential pulse polarography (DPP).[17] The single-stranded 10-mer oligodeoxyribonucleotide [pyrimidine-rich strand containing central TGT sequence (Figure 1B) was reacted in a stoichiometric amount with transplatin and its analogues. The platinated oligonucleotides were repurified by ion-exchange FPLC. It was verified by platinum FAAS and by the measurements of the optical density that the modified oligonucleotides contained one platinum atom. It was also verified using DMS footprinting of platinum on DNA that one platinum molecule was coordinated to the central guanine residue at their N7 position.[49,50] Other details have been described previously.

Mapping of DNA Adducts. The fragment of pSP73 DNA linearized by *Hpa*I (*Hpa*I cuts only once within this plasmid) was obtained as previously described.[19,51] A 10 μg amount of pSP73 was treated with *Hpa*I to obtain linear plasmid. After deproteinization by phenol/chloroform, the modification of this fragment by

the platinum complex was carried out in 10 mM $NaClO_4$ for 48 h at 37 °C to obtain $r_b = 0.006$. TaKaRa Taq Cycle Sequencing Kit with TaKaRa Taq DNA polymerase was used along with the protocol for thermal cycle DNA sequencing with 5′ end-labeled primer recommended by the manufacturer with small modifications.[52]

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 recorded at 590 nm. The fluorescence intensity was measured at 25 °C in 0.4 M NaCl to avoid secondary binding of EtBr to DNA.[53,54] 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.[53]

DNA Interstrand CL Assay. The compounds **1** and **2** at varying concentrations were incubated with 2 μg of pSP73 DNA linearized by *Eco*RI. The platinated samples were precipitated by ethanol and analyzed for DNA interstrand CLs in the same way as described in several recent papers.[19,26] The linear duplexes were first 3′-end labeled by means of Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP. The samples were deproteinized by phenol and precipitated by ethanol, and the pellet was dissolved in 18 μL of a solution containing 30 mM NaOH, 1 mM EDTA, 6.6% sucrose, and 0.04% 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 (vide infra).

Unwinding of Negatively Supercoiled Plasmid DNA. Unwinding of closed circular supercoiled pSP73 plasmid DNA was assayed by an agarose gel mobility shift assay.[30] 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 pSP73 plasmid were incubated with **1** or **2** at 37 °C in the dark for 48 h. All samples were precipitated by ethanol and redissolved in the Tris-borate/EDTA (TBE) buffer. An aliquot of the precipitated sample was subjected to electrophoresis on 1% agarose gels running at 25 °C in the dark with TBE buffer and the voltage set at 30 V. The gels were then stained with EtBr, followed by photography on Polaroid 667 film with transilluminator. The other aliquot was used for the determination of r_b values by FAAS.

Reactions with Sulfur-Containing Compounds. Reactions of GSH and mammalian MT-2 with **1**, **2**, and transplatin free in the bulk of solution or in the case of GSH with these platinum complexes coordinated to oligodeoxyribonucleotides were investigated by monitoring UV absorption at 260 nm of solutions containing the platinum complex and GSH exactly as described in the previous work.[38,55] Free **1**, **2**, or transplatin were mixed with GSH or MT-2 at 37 °C in the medium of 10 mM $NaClO_4$ plus 0.1 mM phosphate buffer, pH 7.0, in the dark. Reactions were initiated by mixing the platinum complex with the buffer followed by immediate addition of GSH or MT-2. The capacity of GSH to remove from DNA monofunctional adducts of compounds **1** and **2** and transplatin was examined as described in the text (vide supra).

Other Physical Methods. Infrared spectra were recorded in Nujol mulls on CsI windows and KBr pellets in the 4000−200 cm^{-1} range with a Perkin-Elmer Model 283 Spectrophotometer. NMR spectra were recorded on a Brucker AMX-300 (300 MHz) spectrometer in $CDCl_3$ solution. Elemental analysis was performed on a Perkin-Elmer 2400 Series II microanalyzer. Absorption spectra were measured with a Beckmann DU-7400 spectrophotometer. FAAS measurements were carried out on a Unicam 939 AA spectrometer with a graphite furnace. For FAAS analysis, DNA was precipitated with ethanol and dissolved in 0.1 M HCl. DPP curves were recorded with the aid of an EG&C PARC Electrochemical Analyzer, Model 384B. HPLC analysis 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. CD spectra were recorded at 25 °C using a JASCO spectropolarimeter, Model J720. The gels were dried and 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).

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Supporting Information Available: X-ray crystallographic analysis of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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