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Modification of Natural, Double-Helical DNA by Antitumor *cis-* and *trans-*[$Cl_2(Me_2SO_4)_4Ru$] in Cell-Free Media

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ABSTRACT. Modifications of natural DNA in cell-free media by the antitumor ruthenium compounds *cis-* and *trans-*[$Cl_2(Me_2SO_4)_4Ru$] were studied by various biochemical and biophysical methods. These methods included: binding studies by means of flameless atomic absorption spectrophotometry, mapping of DNA adducts by means of transcription assay, use of ethidium bromide as a fluorescent probe of DNA adducts of metal complexes, an interstrand cross-linking assay employing gel electrophoresis under denaturing conditions, measurements of DNA unwinding by gel electrophoresis, differential pulse polarographic analysis of DNA conformation, and analysis of liquid crystalline dispersions of DNA by circular dichroism. The results indicated that both ruthenium compounds irreversibly coordinated to DNA; the rate of binding of the *cis* isomer was considerably lower than that of the *trans* isomer. The DNA-binding mode of *trans-*[$Cl_2(Me_2SO_4)_4Ru$] included formation of bifunctional adducts such as intrastrand cross-links between neighboring purine residues and a small amount (~1%) of interstrand cross-links. *cis-*[$Cl_2(Me_2SO_4)_4Ru$] formed mainly monofunctional lesions on natural DNA. Both ruthenium isomers induced conformational alterations of non-denaturational character in DNA, the *trans* compound being more effective. In addition, DNA adducts of *trans-*[$Cl_2(Me_2SO_4)_4Ru$] were capable of inhibiting RNA synthesis by DNA-dependent RNA polymerases, while the adducts of the *cis* isomer were not. Thus, several features of the DNA-binding mode of *trans-*[$Cl_2(Me_2SO_4)_4Ru$] were similar to those of antitumor *cis*-diamminedichloroplatinum (II), which may be relevant to the biological effects of this antitumor ruthenium drug. On the other hand, the different DNA-binding mode of *cis-*[$Cl_2(Me_2SO_4)_4Ru$] was consistent with its less pronounced biological effects. BIOCHEM PHARMACOL **60**;12:1761–1771, 2000. © 2000 Elsevier Science Inc.

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In recent years, metal-based antitumor drugs have played a relevant role in antiblastic chemotherapy [1–4]. Cisplatin† in particular is regarded as one of the most effective anticancer drugs used in the clinic. In spite of the great efficacy of cisplatin against several human tumors, this drug displays limited activity against some of the most common tumors, such as colon and breast cancers [4, 5]. In addition, a variety of adverse effects and acquired resistance are observed in patients receiving cisplatin chemotherapy. The great success of cisplatin on the one hand and these limitations on the other have initiated efforts to develop new metal-based agents that will display improved therapeutic properties.

Broadening the spectrum of antitumor drugs depends on understanding existing agents, with a view toward developing new modes of attack. After the discovery of cisplatin, the new platinum antitumor drugs so far introduced in the clinic were *cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II) (carboplatin) and (*trans-R,R*)1,2-diamminocyclohexaneoxalatoplatinum(II) (oxaliplatin) [5–7]. Carboplatin and oxaliplatin differ from cisplatin only in the more inert leaving group. Hence, as these new compounds are direct structural analogues of cisplatin that exhibit reactivity qualitatively similar to that of cisplatin [8, 9], it is not surprising that they also induce similar biological consequences [5–7].

One approach in the search for new, metal-based anticancer agents that would exhibit antitumor activity markedly different from that of cisplatin and its direct analogues is to examine complexes that would contain another transition metal. Possible advantages in using transition metal ions other than platinum may involve additional coordination sites, alterations in ligand affinity and substitution kinetics, changes in oxidation state, and photodynamic approaches to therapy [3]. In the design of these new drugs, ruthenium complexes have raised great interest [1, 3, 10].

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† *Abbreviations:* cisplatin, *cis*-diamminedichloroplatinum(II); Me_2SO_4, dimethyl sulfoxide; [Cl(dien)Pt]Cl, chlorodiethylenetriamineplatinum(II) chloride; CT, calf thymus; EtBr, ethidium bromide; PEG, poly(ethyleneglycol); r_b, the number of molecules of the ruthenium compound bound per nucleotide residue; FAAS, flameless atomic absorption spectrophotometry; r_i, the molar ratio of free ruthenium complex to nucleotide phosphates at the onset of incubation with DNA; TE buffer, 10 mM Tris · HCl plus 1 mM EDTA, pH 7.2; and DPP, differential pulse polarography.

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The antitumor activity of platinum and other metal-based drugs is frequently related to their binding to DNA [3, 11]. These drugs form adducts on DNA that block DNA and RNA synthesis and induce programmed cell death. Thus, intracellular interactions with these adducts are likely to be of importance in explaining their biological activity or at least some of its features. Dimethyl sulfoxide complexes of both Ru^{II} and Ru^{III} constitute a relatively new group of anticancer compounds [3, 12, 13]. For instance, these complexes exhibit antiblastic activity comparable to cisplatin at equitoxic dosage in animal models of metastasizing tumors, but with less severe side effects and prolonged host survival times [12, 14, 15]. In addition, these ruthenium compounds inhibit DNA replication, exhibit mutagenic activity, and induce the SOS repair mechanisms, which is consistent with the DNA binding of these compounds *in vivo* [16, 17].

A small series of complexes whose parent compounds are *cis-* and *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ constitute one class of dimethyl sulfoxide Ru^{II} compounds. The examination of their effect on primary tumor and on metastasis development has revealed antimetastatic activities superior to the effects on primary tumor growth [12, 14, 18]. The initial studies were performed with *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ because of its similarity to cisplatin [19]. However, the comparisons between the antitumor effects of *cis-* and *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ revealed the superiority of the latter [15]. *cis-* and *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ contain two chlorides in the octahedral structure [14, 20]. In *cis*-$[Cl_2(Me_2SO_4)_4Ru]$, the three Me_2SO_4 molecules are S-bound in a facial configuration and the fourth is O-bonded. In *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ all the Me_2SO_4s are S-bound. When dissolved in water, the *cis* isomer immediately undergoes loss of the O-bonded dimethyl sulfoxide ligand, whereas the *trans* compound rapidly loses two yielding *cis*-diaqua species. Both hydrolyzed isomers then undergo slow reversible chloride dissociation, forming cationic compounds. After this step, the *trans* compound contains three reactive groups, the *cis* isomer only two [14, 20]. In addition, the three remaining Me_2SO_4 ligands in the *cis* isomer represent a considerable steric hindrance, which makes the *cis*-aqua species relatively inert in contrast to the *trans* isomer. Importantly, this difference correlates with a higher potency of the *trans* isomer to act as the antitumor agent [21].

Both *cis-* and *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ bind to DNA in cell-free media [14, 21]. Whereas DNA modifications by platinum antitumor drugs have been studied systematically and described in detail [22], the modifications of high-molecular-mass natural DNA by *cis-* and *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ have been much less explored [3]. Some early studies based on the analysis of circular dichroism (CD) spectra of DNA suggested that coordination of the *cis* isomer to DNA does not significantly alter the conformation of B-DNA [14, 23]. The *trans* isomer binds to DNA more rapidly, with some changes in the CD spectra indicating conformational alterations [14]. Studies of the interactions of both isomers with monomeric constituents of nucleic acids and very short single-stranded oligonucleotides (di- and tetranucleotides) have revealed that both isomers may have some preference for bifunctional binding to neighboring guanine residues at their N7 atoms, with the *trans* isomer being more effective [24–27]. This suggestion has been indirectly corroborated by the restriction enzyme analysis of plasmid DNA modified by these ruthenium compounds [21]. These studies showed that DNA modified by *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ was protected most effectively from cutting by *Bam*HI restriction endonuclease, which contains two neighboring guanine residues in its recognition sequence.

In order to address further fundamental questions about the DNA-binding modes of ruthenium antitumor compounds containing Me_2SO_4 ligands, the experiments described in the present paper were carried out. More specifically, the interactions of polymeric natural DNAs with *cis-* and *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ in cell-free media were investigated by various methods of molecular biophysics, the aim being to contribute to the understanding of the differences in the biological effects of these isomers.

MATERIALS AND METHODS

Starting Materials

cis- and *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ were synthesized, recrystallized, and characterized as previously reported [20]. Cisplatin and [Cl(dien)Pt]Cl were synthesized and characterized in Lachema. Stock solutions of the ruthenium and platinum complexes (5×10^{-4} M in 10 mM $NaClO_4$) were prepared in the dark at 25°. CT DNA (42% G + C, mean molecular mass *ca.* 20,000 kDa) was also prepared and characterized as described previously [28, 29]. CT DNA used for the formation of liquid crystalline dispersions was depolymerized by ultrasound, so that its average molecular mass was ~500 kDa (determined by agarose gel electrophoresis). Plasmids pSP73 (2464 bp) and pSP73KB (2455 bp [30]) were isolated according to standard procedures and banded twice in CsCl/EtBr equilibrium density gradients. Restriction endonucleases were purchased from New England Biolabs. Klenow fragment of DNA polymerase I was from Boehringer Mannheim Biochemica. Riboprobe Gemini System II for transcription mapping containing T7 and SP6 RNA polymerase was purchased from Promega. EtBr and agarose were from Merck KgaA. PEG (molecular mass 4600 kDa) was purchased from Sigma. The radioactive products were from Amersham.

Metallation Reactions

CT or plasmid DNAs were incubated with the ruthenium or platinum complex in 10 mM $NaClO_4$ at 37° in the dark if not stated otherwise. After 48 hr, 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 exhaus-

tively dialyzed against such a medium. An aliquot of these samples was used to determine the r_b value by FAAS.

The dependencies of the r_b values on time for the ruthenium complexes tested in the present work were obtained in the following way. Solutions of CT DNA at the concentration of *ca.* 0.1 mg/mL were incubated with the ruthenium complex at an r_i value of 0.1. At the various time intervals, aliquots were withdrawn and the reaction was stopped by adding 1/10 of a volume of 1.5 M NaCl, including quick cooling to −20°. DNA was then precipitated by ethanol. The amount of the free ruthenium complexes (not bound to DNA) in supernatant was determined by FAAS. The amount of ruthenium bound to DNA (r_b) was calculated by subtracting the amount of ruthenium remaining in solution (determined by FAAS) from the total amount of ruthenium present in the reaction.

DNA Transcription by RNA Polymerase In Vitro

Transcription of the (*Nde*I/*Hpa*I) restriction fragment of pSP73KB DNA with DNA-dependent T7 and SP6 RNA polymerases and electrophoretic analysis of the transcripts were performed according to the protocols recommended by Promega (Promega Protocols and Applications, 43–46 [1989/90]) and previously described in detail [30, 31].

Interstrand Cross-Link Assay

If not stated otherwise, *cis-* and *trans-*$[Cl_2(Me_2SO_4)_4Ru]$ at varying concentrations were incubated with 2 μg of pSP73 DNA either in negatively supercoiled form or after it had been linearized by *Eco*RI. The modified samples were precipitated by ethanol, and the circular DNA already modified by ruthenium complex was subsequently linearized by *Eco*RI. The linear duplexes were then analyzed for DNA interstrand cross-links in the same manner as described in several recent papers [31, 32]. The linear duplexes were first 3′-end labeled by means of Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. The samples were deproteinized by phenol, precipitated by ethanol, and the pellet dissolved in 18 μL of 30 mM NaOH with 1 mM EDTA, 6.6% sucrose, and 0.04% bromophenol blue. The amount of interstrand cross-links was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%). After the electrophoresis was completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified by means of a Molecular Dynamics PhosphorImager (Storm 860 system with ImageQuant software). The frequency of interstrand cross-links, F (the number of interstrand cross-links per adduct), was calculated as $F = \frac{XL}{4928 \cdot r_b}$ (pSP73 plasmid contained 4928 nucleotide residues). XL is the number of interstrand cross-links per one molecule of the linearized DNA duplex which was calculated assuming Poisson distribution of the interstrand cross-links as XL = −ln A, where A is the fraction of molecules running as a band corresponding to the non-cross-linked DNA [32].

Fluorescence Measurements

Fluorescence measurements of DNA modified by ruthenium complexes in the presence of EtBr were performed at an excitation wavelength of 546 nm, and the emitted fluorescence was analyzed at 590 nm. The fluorescence intensity was measured at 25° in 0.4 M NaCl to avoid secondary binding of EtBr to DNA [33, 34]. 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 [33, 34]. These measurements were performed on a Shimadzu RF 40 spectrofluorophotometer using a 1-cm quartz cell.

Differential Pulse Polarography

DPP curves of DNA were measured after non-modified DNA or DNA modified by the ruthenium complex was redissolved in the medium of 0.3 M ammonium formate plus 0.05 M phosphate (Na_2HPO_4/NaH_2PO_4) buffer, pH 6.8 [35]. DPP curves were recorded with the aid of an EG&C PARC Electrochemical Analyzer, Model 384B at 25° using the following apparatus settings: voltage scan rate 2 mV/sec, pulse amplitude of 5 mV, drop time of 1.0 sec. The potentials are against the saturated calomel reference electrode.

Unwinding of Negatively Supercoiled DNA

Unwinding of closed circular supercoiled pSP73 plasmid DNA was assayed by an agarose gel mobility shift assay [36]. 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 *cis-* or *trans-*$[Cl_2(Me_2SO_4)_4Ru]$ for 48 hr, precipitated by ethanol, and redissolved in TAE buffer (0.04 M Tris–acetate + 1 mM EDTA, pH 7.0). An aliquot of the precipitated sample was subjected to electrophoresis on 1% agarose gels running at 25° in the dark with TAE buffer with a 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.

Liquid Crystalline Dispersions of DNA

Liquid crystalline dispersions of DNA modified by the metal complexes were formed by mixing DNA and PEG solutions as described earlier [37, 38]. Briefly, 1 mL of non-modified DNA or DNA modified by the metal complex dissolved in 0.01 M $NaClO_4$ at the concentration of

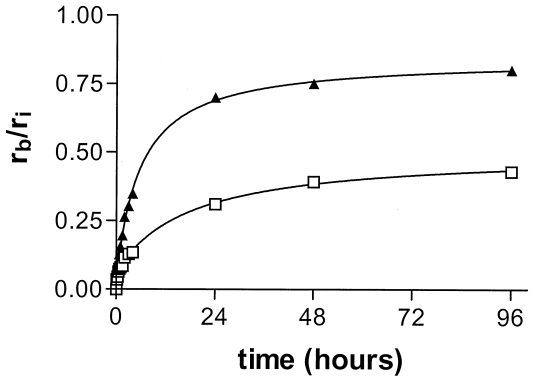

FIG. 1. Kinetics of the binding of *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ (□) and *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ (▲) to CT DNA. Medium: 10 mM $NaClO_4$ at 37°. The binding was determined by FAAS after precipitation by ethanol. The concentration of DNA was 0.1 mg/mL, $r_i = 0.1$. Data measured in triplicate varied on average ±3% from their mean. For other details, see the text.

0.06 mg/mL was mixed vigorously with 3 mL PEG at the concentration of 200 mg/mL (also dissolved in 0.3 M $NaClO_4$) for 1 hr.

Other Methods

Absorption spectra were measured with a Beckmann DU-8 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. CD spectra were recorded at 25° using a JASCO spectropolarimeter, Model J720.

RESULTS

DNA Binding

Solutions of double-helical CT DNA at a concentration of 0.1 mg/mL were incubated with *cis-* and *trans-*$[Cl_2(Me_2SO_4)_4Ru]$ at r_i values of 0.1 at 37° in three media: 10 mM $NaClO_4$, TE buffer, or 1 mM phosphate buffer (NaH_2PO_4/Na_2HPO_4, pH 7.0). At various time intervals, an aliquot of the reaction mixture was withdrawn; the reaction was then terminated by adjusting NaCl concentration to 0.15 M followed by quick cooling to −20° and precipitating DNA by ethanol. The supernatant was assayed by FAAS for the amount of free ruthenium not bound to DNA. The amount of ruthenium bound to DNA, r_b, was calculated by subtracting the amount of ruthenium remaining in solution from the total amount of ruthenium present in the reaction. Figure 1 shows a plot of r_b against the time of DNA incubation with *cis-* or *trans-*$[Cl_2(Me_2SO_4)_4Ru]$ in 10 mM $NaClO_4$. The amount of ruthenium coordinated to DNA increased with time. After 48 hr, the binding reached its maximum values, which corresponded to approximately 40 or 75% of the molecules of *cis-* or *trans-*$[Cl_2(Me_2SO_4)_4Ru]$, respectively, present in the reaction mixtures. Importantly, both ruthenium compounds reacted with DNA at a similar rate, with r_i values in the range of 0.001–0.1. Thus, the rate of binding to natural polymeric DNA of both ruthenium compounds tested in the present work was lower than that of cisplatin [39]. These results also confirmed that DNA binding of the *cis-*$[Cl_2(Me_2SO_4)_4Ru]$ is considerably less effective than that of its *trans* isomer.

The binding studies described above were performed in the medium of 10 mM $NaClO_4$, which is used as a standard medium if DNA interactions of antitumor platinum complexes are investigated in cell-free media. For comparative purposes, the binding of the two ruthenium compounds to DNA was also investigated in TE or phosphate buffers, i.e. in the media used in some previous studies of DNA modifications by ruthenium complexes. The components of these buffers are potential ligands of transition metal complexes that compete with DNA for metal [40]. This fact can significantly affect the rate of the binding of ruthenium complexes to DNA. DNA binding of *trans-*$[Cl_2(Me_2SO_4)_4Ru]$ measured after 24 hr in TE or phosphate buffers was decreased from 70% in 10 mM $NaClO_4$ to 20 or 50%, respectively. Similarly, DNA binding of the *cis* isomer under the same experimental conditions was decreased from 30% to 5 or 10%, respectively. Identical results were obtained when the binding of both isomers was measured in the media containing TE or phosphate buffers at ten times lower concentrations. On the other hand, if DNA was first modified by *cis-* or *trans-*$[Cl_2(Me_2SO_4)_4Ru]$ in 10 mM $NaClO_4$ to an r_b value in the range of 0.001–0.05, the level of the modification (r_b) remained unchanged even after DNA modified by ruthenium complex was transferred into the media in which their rate of DNA binding is noticeably decreased (*vide supra*). This observation was also consistent with the results of the following experiment. CT DNA modified by the ruthenium complex in 10 mM $NaClO_4$ was precipitated by ethanol, redissolved in the medium of TE or phosphate buffer (containing no ruthenium complex), further incubated at 37° for an additional 48 hr, again precipitated by ethanol, and the content of ruthenium in these samples (r_b) determined by FAAS. No changes in r_b values were noticed.

The binding experiments of the present work indicate that modification reactions resulted in the irreversible coordination of molecules of the ruthenium complexes to polymeric double-helical DNA. In addition, these results made it possible to prepare easily and precisely the samples of DNA modified by the ruthenium complexes at a preselected value of r_b. Importantly, it is also reasonable to assume that the levels of DNA modifications by the ruthenium complexes reached in 10 mM $NaClO_4$ remained unchanged after the sample of DNA modified by the ruthenium complex was transferred into the medium required for subsequent biophysical or biochemical analyses. Thus, the samples of DNA modified by the ruthenium complexes and analyzed further by biophysical or biochemical methods were prepared in 10 mM $NaClO_4$ at 37°. After 48 hr of the reaction of DNA with the complex, the samples were precipitated in ethanol, dissolved in the

medium necessary for a particular analysis, and the r_b value in an aliquot of this sample checked by FAAS. In this way, the analyses described in the present paper were performed in the absence of unbound (free) ruthenium complex.

Transcription Mapping of DNA Adducts

In vitro RNA synthesis by RNA polymerases on DNA templates containing several types of bifunctional adducts of metal complexes can be prematurely terminated at the level or in the proximity of adducts [30, 31, 41, 42]. Importantly, monofunctional DNA adducts of several platinum complexes are unable to terminate RNA synthesis.

Cutting of pSP73KB DNA [30, 31] by *Nde*I and *Hpa*I restriction endonucleases yielded a 212-bp fragment (a substantial part of its nucleotide sequence is shown in Fig. 2B). This fragment contained convergent T7 and SP6 RNA polymerase promoters (in the upper and lower strands, respectively, close to its 3′-ends [Fig. 2B]). The experiments were carried out using this linear DNA fragment, modified by *cis*-$[Cl_2(Me_2SO_4)_4Ru]$, its *trans* isomer, or cisplatin at $r_b = 0.01$, for RNA synthesis by T7 and SP6 RNA polymerases (Fig. 2A, lanes cisRu, transRu, or cisPt, respectively). RNA synthesis on the template modified by *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ or cisplatin yielded fragments of defined sizes (Fig. 2A, lanes transRu and cisPt), which indicates that RNA synthesis on these templates was prematurely terminated. The major stop sites produced by *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ were identical to those produced by cisplatin, i.e. mainly appearing at G sites and to a considerably lesser extent at A sites. These G and A sites were mostly contained in GG or AG sites, which are preferential DNA-binding sites of cisplatin. Interestingly, the bands produced by the *trans* ruthenium complex had a lower intensity than those yielded by cisplatin, which suggests that *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ also produces DNA adducts incapable of terminating RNA synthesis and that the amount of these DNA adducts (presumably monofunctional lesions) was considerably higher than that formed in the case of cisplatin. Taken together, the results of these mapping experiments suggest that *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ can also form, on double-helical DNA, an amount of bidentate adducts similar to DNA cross-links formed by cisplatin.

DNA adducts of *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ produced only faint bands corresponding to stop sites (Fig. 2A, lane cisRu). This observation is consistent with capability of *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ to form a considerably smaller amount of adducts on DNA that would be similar to those formed by *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ or cisplatin.

Characterization of DNA Adducts by EtBr Fluorescence

EtBr as a fluorescent probe has been used to characterize perturbations induced in DNA by adducts of several platinum compounds [33, 43–45]. Double-helical CT DNA was modified by cisplatin, monofunctional [Cl(dien)Pt]Cl, and

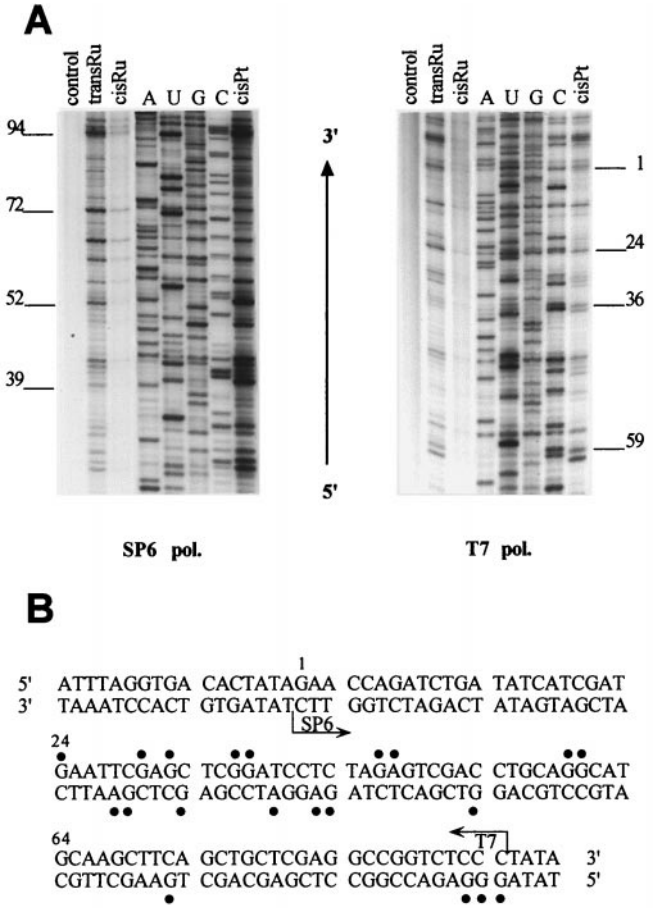

FIG. 2. Inhibition of RNA synthesis by SP6 (left) and T7 (right) RNA polymerases on the *Nde*I/*Hpa*I fragment of pSP73KB plasmid modified by ruthenium and platinum complexes. (A) Autoradiograms of 6% polyacrylamide/8 M urea sequencing gels. Lanes: control, non-modified template; cisPt, cisRu, and transRu, the templates modified by cisplatin, *cis*-$[Cl_2(Me_2SO_4)_4Ru]$, and *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ at $r_b = 0.01$, respectively. (B) Schematic diagram showing the portion of the nucleotide sequence of the template (upper) strand of the *Nde*I/*Hpa*I fragment used to monitor inhibition of RNA synthesis by *trans*-$[Cl_2(Me_2SO_4)_4Ru]$. The arrows indicate the start of the T7 or SP6 RNA polymerases. (●), major stop signals (from Fig. 2A, lanes transRu). The numbers correspond to the nucleotide numbering in the sequence map of pSP73KB plasmid.

cis- or *trans*-$[Cl_2(Me_2SO_4)_4Ru]$. The levels of the modification corresponded to the values of r_b in the range of 0–0.1. Modification of DNA by all-metal complexes resulted in a decrease in EtBr fluorescence as compared with the control DNA–EtBr complex (Fig. 3). The decrease caused by the adducts of *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ complexes was similar to that induced by the DNA adducts of cisplatin at equivalent r_b albeit somewhat smaller. On the other hand, modification of DNA by *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ resulted in a pronouncedly smaller decrease in EtBr fluorescence as compared with that due to the modification by *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ or cisplatin, but one that was still larger than that due to the modification by monofunctional [Cl(dien)Pt]Cl.

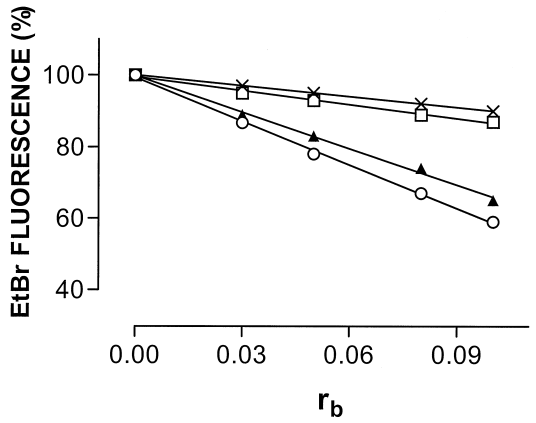

FIG. 3. Dependencies of ethidium bromide fluorescence on r_b for CT DNA modified by various metal complexes in 10 mM $NaClO_4$ at 37° for 48 hr. [Cl(dien)Pt]Cl (x), cisplatin (○), *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ (□), and *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ (▲). Data measured in triplicate varied on average ±2% from their mean.

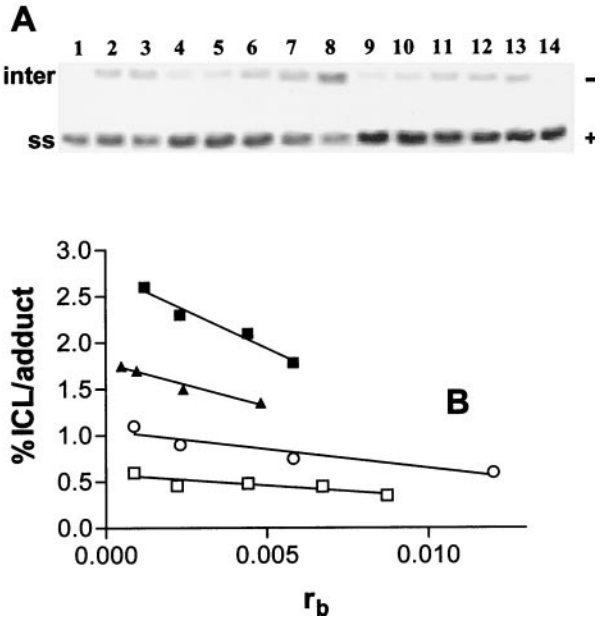

FIG. 4. The formation of interstrand cross-links by ruthenium complexes in negatively supercoiled and linearized pSP73 plasmid. (A) Autoradiogram of a denaturing 1% agarose gel of linearized DNA which was 3′-end labeled; the interstrand cross-linked DNA appears as the top bands migrating on the gels more slowly than the single-stranded (ss) DNA (contained in the bottom bands). The plasmid linearized by *Eco*RI was incubated with cisplatin (lanes 2 and 3), *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ (lanes 4–8), or *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ (lanes 9–13) for 48 hr at 37°; r_b values: 0 (control, non-modified DNA), lanes 1 and 14; 0.0005, lane 2; 0.001, lane 3; 0.0048, lane 4; 0.00096, lane 5; 0.0024, lane 6; 0.0048, lane 7; 0.0096, lane 8; 0.00087, lane 9; 0.0022, lane 10; 0.0044, lane 11; 0.0067, lane 12; 0.0087, lane 13. (B) Dependence on r_b of the percentage of interstrand cross-links (ICL) per adduct (interstrand cross-linking [%]) formed by *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ (▲, □) or *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ (■, ○) in supercoiled (closed symbols) or linearized (open symbols) DNA within 48 hr. Data measured in triplicate varied on average ±3% from their mean.

Interstrand Cross-linking

Transcription mapping experiments and EtBr fluorescence analysis (Figs. 2 and 3) were consistent with the capability of ruthenium complexes to form on polymeric DNA bidentate adducts, but these studies could not distinguish whether these adducts were intrastrand or interstrand cross-links. Therefore, further experiments were carried out to compare the amounts of the interstrand cross-links formed by *cis*- or *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ in negatively supercoiled or linear DNA. In these experiments, we used pSP73 plasmid (2464 bp, native supercoil density $\sigma = -0.063$), which was modified by *cis*- or *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ complexes in two ways. One series of samples was prepared by modifying the plasmid only after it had been linearized by *Eco*RI (*Eco*RI cuts only once within pSP73 plasmid). The samples of the other series were prepared by a modification of the supercoiled plasmid by ruthenium complexes, and only after the modification reaction was completed was the plasmid linearized by *Eco*RI. Thus, we prepared two types of linear DNA molecules of the same length and nucleotide sequence modified by ruthenium complexes, which could be differently affected by this drug if different DNA topology were to play a role during the modification reaction. The two samples were analyzed for interstrand cross-links by agarose gel electrophoresis under denaturing conditions in an attempt to reveal these differences. It was also verified by FAAS whether the amount of ruthenium complexes coordinated to the base residues in DNA was independent of DNA topology during the modification reaction over a broad range of r_i values (0.001–0.1) and at any reaction time.

An electrophoretic method for precise and quantitative determination of interstrand cross-linking by metal complexes in DNA has been described previously [31, 32]. Upon electrophoresis under denaturing conditions, 3′-end labeled strands of linearized pSP73 plasmid containing no interstrand cross-links migrate as a 2464-nucleotide single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher-molecular-mass species. The bands corresponding to more slowly migrating interstrand cross-linked fragments were observed if the *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ complex was used to modify DNA in both linearized and supercoiled forms at r_b as low as 5×10^{-3} (Fig. 4A). 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 cross-links (the amount of interstrand cross-links per one molecule of *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ complex coordinated to DNA) was calculated using the Poisson distribution in combination with the r_b values and the fragment size [32] (for more details, see Materials and Methods).

As summarized in Fig. 4B, *trans*-$[Cl_2(Me_2SO_4)_4Ru]$

showed a relatively low interstrand cross-linking efficiency in both linear and negatively supercoiled DNA, i.e. approximately 6 times less than formed by cisplatin under identical conditions [31, 46]. Importantly, *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ forms an even smaller amount of interstrand cross-links (roughly half), which is consistent with a tendency of the *cis* isomer to preferentially form on double-helical DNA monofunctional adducts. These results indicate that the interstrand cross-links are only highly minor adducts in double-helical DNA modified by $[Cl_2(Me_2SO_4)_4Ru]$ complexes.

Differential Pulse Polarography

DPP analysis readily and with great sensitivity distinguishes between non-denaturational and denaturational conformational alterations induced in DNA by various physical or chemical agents [47, 48]. This analysis is based on the observation that intact double-helical DNA is polarographically inactive, because its reduction sites are involved in hydrogen bonds and are unable to make contact with the working electrode in a manner suitable for electron transfer. Electroreduction of adenine or cytosine residues present in distorted but still double-stranded (non-denatured) regions of DNA is responsible for the appearance of the small DPP peak II (Fig. 5A, curve 1). Base residues in these distorted regions become more accessible for electroreduction at the mercury electrode and can yield a small polarographic current. On the other hand, the appearance of a more negative peak III on DPP curves of DNA indicates the presence of single-stranded, denatured regions in the DNA molecule, in which hydrogen bonds between complementary bases have been broken [47, 48]. Differences in the adsorption properties of double-helical and denatured DNA at the mercury electrode have been suggested to give rise to the different reduction potentials that are observed for the two DNA conformations. Importantly, less than 1% denatured material in the excess of double-helical DNA can be determined by DPP [49].

DPP has already been used to analyze DNA modified by various physical or chemical agents, including platinum compounds with different clinical efficacy [50, 51]. It has been found that DNA globally modified by antitumor cisplatin or its analogues at r_b values up to 0.05 yields the DPP peak II, indicating that these antitumor drugs induce non-denaturational conformational changes in DNA [32, 35]. In contrast, the more negative DPP peak III is observed on DPP curves of DNA globally modified by clinically ineffective transplatin and other inactive platinum(II) complexes, indicating that the clinically ineffective platinum complexes induce denaturational conformational alterations in DNA [50, 51].

DPP analysis also sheds considerable light on the conformational basis for DNA binding of *cis*- or *trans*-$[Cl_2(Me_2SO_4)_4Ru]$. The modification of CT DNA by these ruthenium complexes at r_b of 0.005–0.02 resulted in an increase in DPP peak II with a growing level of modification (shown for the *trans* isomer in Fig. 5A). The more negative peak III was not detected even on the DPP curves recorded for DNA modified at the highest r_b value used in our experiments (0.02). It could be argued that the absence of peak III on the DPP curves recorded for the samples of DNA modified at relatively high r_b values (~0.02) could be due to an increase in the slope of the part of the DPP curve corresponding to the background electrolyte discharge (Fig. 5A, curve 4). The observation that peak III was not buried under the background electrolyte discharge curve was

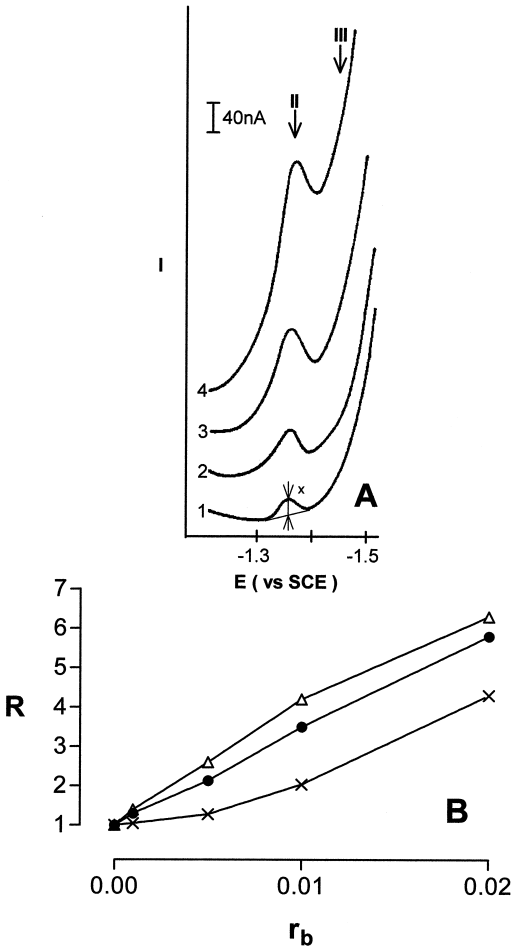

FIG. 5. Differential pulse polarographic analysis of CT DNA modified by ruthenium complexes. DNA at a concentration of 0.4 mg/mL in 0.3 M ammonium formate with 0.01 M phosphate buffer, pH 6.8. (A) DPP curves; DNA modified by *trans*-$[Cl_2(Me_2SO_4)_4Ru]$; r_b values: 0 (control, non-modified DNA), curve 1; 0.005, curve 2; 0.01, curve 3; and 0.02, curve 4. The arrows in Fig. 5A marked by II and III indicate potentials E (against saturated calomel electrode [SCE]) at which native or denatured DNA samples yielded DPP peaks II or III, respectively (see the text). The way in which the height of the DPP peak (x) was measured in the present work is shown in Fig. 5A, curve 1. (B) Dependence of the relative height of DPP peak II, R, yielded by DNA modified by the ruthenium complexes on r_b: (x—x), *cis*-$[Cl_2(Me_2SO_4)_4Ru]$; (●——●), *trans*-$[Cl_2(Me_2SO_4)_4Ru]$; and (△——△), cisplatin. The value of R was calculated as the ratio of the peak height yielded by the modified DNA over the peak height yielded by the control (non-modified) DNA.

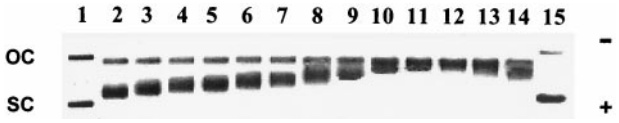

FIG. 6. Unwinding of negatively supercoiled pSP73 plasmid DNA by ruthenium complexes. The plasmid was incubated with *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ for 48 hr at 37° and precipitated by ethanol. r_b values: 0 (control, non-modified DNA), lanes 1 and 15; 0.06, lane 2; 0.07, lane 3; 0.08, lane 4; 0.09, lane 5; 0.1, lane 6; 0.11, lane 7; 0.12, lane 8; 0.13, lane 9; 0.14, lane 10; 0.15, lane 11; 0.16, lane 12; 0.2, lane 13; and 0.26, lane 14. The top bands (oc) correspond to the form of nicked plasmid and the bottom bands (sc) to the closed, negatively supercoiled plasmid.

verified using the sample of DNA modified by *cis-* or *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ at $r_b = 0.02$ to which 0.8% thermally denatured CT DNA was added. This sample yielded a small, more negative peak III on the DDP curve (recorded under conditions specified for curve 4 in Fig. 5A) (not shown). Thus, the absence of the peak III on the DPP curves of DNA modified by *cis-* or *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ suggests that these ruthenium complexes, as with antitumor cisplatin and other antitumor analogues of this drug, induce non-denaturational conformational distortions in DNA at relatively low levels of the global modification ($r_b \leq 0.02$). The DNA-binding mode of *cis-* or *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ is also in profound contrast to the modification of DNA by clinically ineffective transplatin [50, 51]. In addition, the relative increase in peak II due to the global modification by *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ was considerably smaller at the same level of DNA modification (r_b) (Fig. 5B) than the increase in peak II due to the modification by *trans*-$[Cl_2(Me_2SO_4)_4Ru]$. This finding supports the view that non-denaturational distortions of DNA due to the global binding of *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ are more extensive than those due to the binding of *cis*-$[Cl_2(Me_2SO_4)_4Ru]$.

Unwinding

Electrophoresis in native agarose gel was used to quantify the unwinding induced in pSP73 plasmid by the ruthenium complexes by monitoring the degree of supercoiling (Fig. 6). A compound that unwinds DNA duplex reduces the number of supercoils in closed, negatively supercoiled DNA so that the negative 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 for the unwinding to be observed and quantified [36]. Figure 6 shows an electrophoresis gel in which increasing amounts of *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ were bound to a mixture of relaxed and negatively supercoiled pSP73 DNA. Interestingly, *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ accelerated the mobility of the relaxed form in a similar fashion to cisplatin, whose bifunctional binding to DNA shortens and condenses the DNA helix [52, 53]. In contrast, *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ affected the mobility of the relaxed form markedly less (not shown), implying that its binding results in shortening or condensation of DNA to a very small extent. 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 co-migrate [36]. Under the present experimental conditions, σ was calculated to be -0.063 on the basis of the data for cisplatin, for which the $r_b(c)$ was determined in this study and $\Phi = 13°$ was assumed. Using this approach, the DNA unwinding angle of $7 \pm 1°$ was determined for *trans*-$[Cl_2(Me_2SO_4)_4Ru]$. On the contrary, the co-migration of the relaxed and negatively supercoiled DNAs was not observed even at such a high level of modification by *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ as is that corresponding to the r_b value of 0.25, indicating a negligible efficiency of the *cis* isomer to unwind DNA.

Liquid Crystals

The cholesteric liquid crystalline dispersions of DNA, which can simulate the principal properties of DNA molecules within cells, such as their spatial ordering in condensed and packed state, have been used as model systems *in vivo* [38, 54]. This model system was used in the present work to provide information on the possible consequences of the binding of *cis-* or *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ on the formation and stability of the condensed DNA. In the presence of PEG water-containing salt solutions, linear double-stranded DNA is condensed, forming helically twisted liquid crystalline dispersions of DNA molecules (the left-handed helicoidal structure of cholesteric phase from the right-handed DNA molecules). The peculiar properties of this phase caused by anisotropic orientations can be analyzed by measuring CD spectra. The occurrence of the cholesteric liquid crystalline form of DNA is accompanied by the origin of the intense negative CD band at *ca.* 275 nm (Fig. 7) [55–57]. The decrease in the amplitude of this band in the CD spectra of liquid crystalline dispersions of DNA modified by antitumor platinum complexes is associated with the disappearance of the helical twist of the liquid crystalline microphase due to alterations of DNA secondary structure, such as disturbances in the stacking interactions of bases [37, 55, 58]. Interestingly, clinically ineffective platinum complexes, such as *trans* isomer of cisplatin or monofunctional platinum(II) compounds, only negligibly or weakly affected the CD spectra of liquid crystalline dispersions of DNA. Thus, the CD spectra of liquid crystalline microphases of DNA modified by platinum compounds are very sensitive to the status of the antitumor activity of platinum compounds and have been suggested [37] for preliminary testing of the antitumor activity of novel metal-based drugs.

We recorded CD spectra of the liquid crystalline dispersions of sonicated CT DNA modified by *cis-* or *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ at a wide range of r_b values in the presence of PEG (shown for the *trans* isomer in Fig. 7A), and the data were compared with those obtained for DNA modified by cisplatin and monofunctional [Cl(dien)Pt]Cl. In the CD spectrum of the liquid crystalline dispersions of

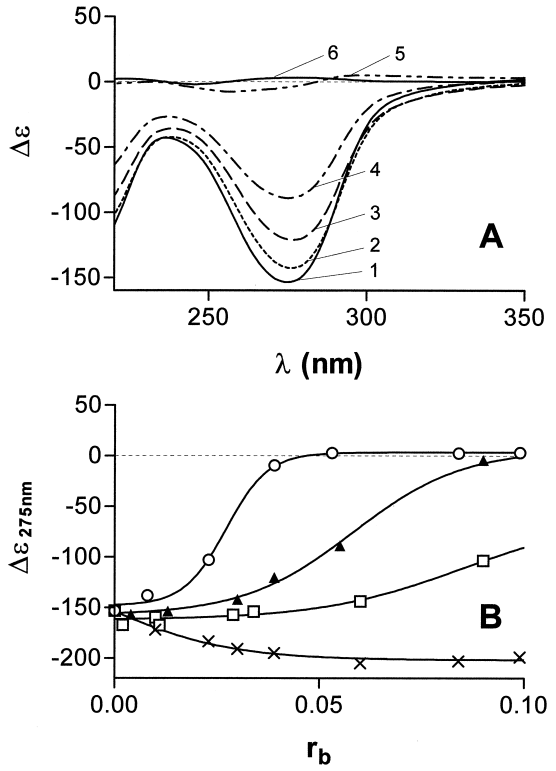

FIG. 7. (A) Circular dichroism spectra of liquid crystalline dispersions formed from non-modified CT DNA and CT DNA modified by *trans*-[$Cl_2(Me_2SO_4)_4Ru$] in the presence of 150 mg of PEG/mL. r_b values: 0 (control, non-modified DNA), curve 1; 0.03, curve 2; 0.039, curve 3; 0.055, curve 4; 0.098, curve 5; and 0.12, curve 6. (B) The dependence on r_b of the relative amplitude of the negative circular dichroism band at ~275 nm, $\Delta\epsilon_{275nm}$, of liquid crystalline dispersions formed from CT DNA modified by [Cl(dien)Pt]Cl (x), cisplatin (○), *cis*-[$Cl_2(Me_2SO_4)_4Ru$] (□), and *trans*-[$Cl_2(Me_2SO_4)_4Ru$] (▲) in the presence of 150 mg PEG/mL. For other details, see the text.

the control, non-modified DNA, an intense negative band at around 275 nm was observed. The amplitude of this band decreased as a consequence of the increasing amount of *cis*- or *trans*-[$Cl_2(Me_2SO_4)_4Ru$] bound to DNA (Fig. 7B). At the level of binding of *trans*-[$Cl_2(Me_2SO_4)_4Ru$] corresponding to $r_b \geq 0.1$, the negative band disappeared. It is also evident from Fig. 7B that *cis*-[$Cl_2(Me_2SO_4)_4Ru$] was markedly less efficient in decreasing the amplitude of the negative CD band at 275 nm than its *trans* isomer.

The results shown in Fig. 7B also indicate that *cis*-[$Cl_2(Me_2SO_4)_4Ru$] only disturbs DNA liquid crystals weakly, but more than monofunctional [Cl(dien)Pt]Cl. On the other hand, although *trans*-[$Cl_2(Me_2SO_4)_4Ru$] disturbs these crystals markedly more than its *cis* isomer, its efficiency is in this respect lower than that of cisplatin. Hence, these results are consistent with the view that disturbance of the secondary structure of DNA by *trans*-[$Cl_2(Me_2SO_4)_4Ru$] has some features similar to those induced by cisplatin, which might be relevant to the antitumor activity observed for this ruthenium compound earlier. On the other hand, *cis*-[$Cl_2(Me_2SO_4)_4Ru$] could be rather grouped with clinically less effective or ineffective metal-based compounds that alter conformation of DNA in a way only resulting in a negligible or weak disturbance of its liquid crystalline dispersions.

DISCUSSION

The present work demonstrates that antitumor *cis*- and *trans*-[$Cl_2(Me_2SO_4)_4Ru$] irreversibly coordinate to the residues in high-molecular-mass DNA. The rate of DNA binding of *cis*-[$Cl_2(Me_2SO_4)_4Ru$] is considerably lower than that of the *trans* isomer. The lower reactivity of the *cis* isomer is very likely associated with the presence of three bulky Me_2SO_4 ligands after this compound is dissolved in water, in contrast to only two Me_2SO_4 groups remaining in the dissolved molecules of the *trans*-isomer. Importantly, the reactivity of these metal-based compounds toward DNA is strongly affected by potential ligands of ruthenium complexes that may be present in the reaction medium, for instance as components of the reaction buffers.

Some details about the nature of the adducts formed by *cis*- and *trans*-[$Cl_2(Me_2SO_4)_4Ru$] on natural, double-helical DNA have emerged from the present work, such as transcription mapping of these adducts, the analysis of DNA by the EtBr fluorescent probe, and the DNA interstrand cross-linking assay. The results of these investigations are consistent with the view that *trans*-[$Cl_2(Me_2SO_4)_4Ru$], like antitumor cisplatin, forms a significant amount of intrastrand cross-links in DNA between neighboring purine residues, but their amount was considerably smaller than that formed by the platinum drug. Interestingly, *trans*-[$Cl_2(Me_2SO_4)_4Ru$] can also form DNA interstrand cross-links, a previously unobserved phenomenon, although their amount is very low (~1%). *cis*-[$Cl_2(Me_2SO_4)_4Ru$] preferentially forms on DNA monofunctional lesions.

One of the important features of the DNA-binding mode of metal-based antitumor drugs relevant to their biological effects is the conformational alteration induced by the formation of the adduct. The assays based on differential pulse polarographic analysis, gel electrophoresis of negatively supercoiled and nicked plasmids, and the formation of liquid crystalline dispersions of DNA revealed that both ruthenium complexes are capable of inducing local conformational alterations of a non-denaturational character. The extent of these alterations induced by the *trans* isomer was noticeably higher than that induced by the *cis* isomer. Thus, the global character of conformational changes induced in DNA by the two ruthenium isomers is similar to that induced by antitumor cisplatin and different from distortions induced in DNA by clinically ineffective transplatin or monofunctional platinum complexes such as [Cl(dien)Pt]Cl or [$Cl(NH_3)_3Pt$]Cl.

The results of these studies also demonstrate that bifunctional DNA adducts of *trans*-[$Cl_2(Me_2SO_4)_4Ru$] inhibit RNA synthesis and specifically block RNA polymerases at the sites of the modified guanine and, to a lesser extent, adenine residues. Importantly, DNA adducts of therapeutically less effective *cis*-[$Cl_2(Me_2SO_4)_4Ru$] are unable to

inhibit RNA synthesis, very likely because this ruthenium compound binds DNA in a monodentate fashion, a binding mode expected to result in antitumor activity only in rare cases.

Taken together, several features of the DNA-binding mode of *trans*-$[Cl_2(Me_2SO_4)_4Ru]$ described in the present work are similar to those of antitumor cisplatin, which may be relevant to the biological effects of this antitumor ruthenium drug. DNA binding of *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ mainly results in the formation of monofunctional adducts, and the extent of conformational distortions induced in DNA is considerably smaller in comparison with distortions induced by its *trans* isomer. This observation is consistent with the less pronounced biological effects of *cis*-$[Cl_2(Me_2SO_4)_4Ru]$ in comparison with its *trans* isomer. Whatever the detailed mechanism, however, the ruthenium compounds are interesting from a mechanistic point of view and, therefore worthy of additional testing. Further studies should reveal the extent to which ruthenium complexes hold promise as clinically useful antitumor compounds.

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