Correlation between Cytotoxicity and DNA Binding of Polypyridyl Ruthenium Complexes†

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Received March 17, 1995; Revised Manuscript Received July 6, 1995⊗

ABSTRACT: The cytotoxicity of chloropolypyridyl ruthenium complexes of structural formulas [Ru(terpy)(bpy)Cl]Cl, *cis*-[Ru(bpy)$_2$Cl$_2$], and *mer*-[Ru(terpy)Cl$_3$] (terpy = 2,2′:6′2″-terpyridine, bpy = 2,2′-bipyridyl) has been studied in murine and human tumor cell lines. The results show that *mer*-[Ru(terpy)Cl$_3$] exhibits a remarkably higher cytotoxicity than the other complexes. Moreover, investigations of antitumor activity in a standard tumor screen have revealed the highest efficiency for *mer*-[Ru(terpy)Cl$_3$]. In a cell-free medium, the ruthenium complexes coordinate to DNA preferentially at guanine residues. The resulting adducts can terminate DNA synthesis by thermostable Vent$_R$ DNA polymerase. The reactivity of the complexes to DNA, their efficiency to unwind closed, negatively supercoiled DNA, and a sequence preference of their DNA adducts (studied by means of replication mapping) do not show a correlation with biological activity. On the other hand, the cytotoxic *mer*-[Ru(terpy)Cl$_3$] exhibits a significant DNA interstrand cross-linking, in contrast to the inactive complexes which exhibit no such efficacy. The results point to a potential new class of metal-based antitumor compounds acting by a mechanism involving DNA interstrand cross-linking.

Complexes of transition elements of the platinum group hold a promise in the design of new anticancer agents [for general reviews, see Clarke (1993), Frühauf and Zeller (1991), Heim (1993), Köpf-Maier (1994), Loehrer and Einhorn (1984), and Reedijk (1992)]. *cis*-Diamminedichloroplatinum(II) (cisplatin)[1] and *cis*-diammine(cyclobutane-1,1-dicarboxylato)platinum(II) (carboplatin) are the first antitumor drugs of this type which are currently in clinical use. At present, a research effort is directed to the synthesis of other platinum complexes which could help to overcome clinical problems associated with the relatively limited activity of cisplatin and carboplatin against the broad spectrum of human malignancies, acquired resistance, and side effects.

In attempts to find a new, metal-based anticancer drug with activity complementary to cisplatin, the complexes containing a ruthenium center, mostly in its lower oxidation states, have also been prepared and tested for antitumor activity [for reviews, see Clarke (1993), Keppler (1989), and Mestroni et al. (1994)]. Several ruthenium compounds with nitrogen ligands not only have been shown to exhibit good activity in screening studies, but many also preferentially localize in tumor tissue. The significant structural differences between ruthenium and most platinum-based antitumor drugs give a promise that ruthenium-based drugs could be suitable alternatives to cisplatin and carboplatin. Antitumor ruthenium compounds usually possess octahedral, six-coordinated geometry as opposed to the square-planar arrangement of the ligands of cisplatin or carboplatin. In addition, the two additional coordination sites for ruthenium as opposed to the platinum(II) center in cisplatin or carboplatin may allow for new modes of binding to intracellular targets and, with some ligands, provide for chirality in the complexes and in their interactions with the target structure.

In the last decade, particular attention has been paid to the ruthenium complexes of polypyridyl ligands. Some of these complexes exist as chiral molecules capable of enantioselective recognition of DNA. Thus, DNA binding and cleavage properties of various polypyridyl ruthenium compounds have been intensively investigated since they have been proposed as possibly useful probes of DNA conformation (Barton, 1986; Erikson et al., 1994; Grover et al., 1992; Satyanarayana et al., 1993) or DNA cleavage agents (Grover et al., 1994; Gupta et al., 1992, 1993; Neyhart et al., 1993). The analogues of these ruthenium complexes containing, besides polypyridyl ligands, aqua or chloro groups have been also synthesized and were found to bind DNA covalently in cell-free media (Barton & Lolis, 1985; Grover et al., 1992, 1994). The aqua or chloro ligands in these complexes represent leaving ligands in contrast to the kinetically more stable pyridyl groups (Grover et al., 1994; Moyer & Meyer, 1981). In spite of the fact that these polypyridyl ruthenium complexes coordinate to DNA and that several ruthenium

† Supported in part by the Internal Grant Agency of the Academy of Sciences of the Czech Republic, the Grant Agency of the Czech Republic (Grant No. 203/93/0052), and the Internal Grant Agency of the Ministry of Health of the Czech Republic (Grant No. 1893-3). This joint research is also a part of the European Cooperation in the field of Scientific and Technical Research (COST) network (COST Projects D1/0002/92 and D1/0001/95).

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⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: bp, base pair; bpy, 2,2′-bipyridyl; carboplatin, *cis*-diammine(cyclobutane-1,1-dicarboxylato)platinum(II); cisplatin, *cis*-diamminedichloroplatinum(II); DPP, differential pulse polarography; EtBr, ethidium bromide; FAAS, flameless atomic absorption spectroscopy; ID_{50}, the concentration of the drug required to inhibit cell growth by 50%; r_b, the ratio of metal atoms fixed per nucleotide; $r_b(c)$, the value of r_b at which the supercoiled and relaxed forms of DNA comigrate; r_i, the molar ratio of free metal complex to nucleotide phosphates at the onset of incubation; $t_{1/2}$, half-time; T/C, the ratio of the mean survival times of treated over nontreated tumor-bearing mice; T_m, melting temperature; terpy, 2,2′:6′,2″-terpyridine; XCL, cross-linked fraction of DNA; XL/A, number of interstrand cross-links per adduct.

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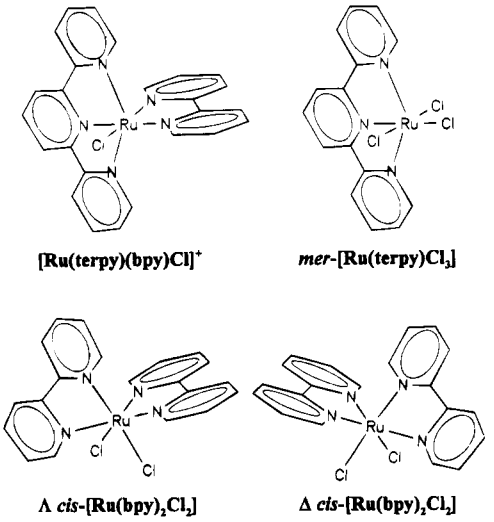

FIGURE 1: Schematic structures of ruthenium complexes used in this study. In the case of *cis*-[Ru(bpy)$_2$Cl$_2$], a racemic mixture or the complex enriched by the Δ-enantiomer was used (see the text).

complexes of different types exhibit antitumor activity, the biological effects of polypyridyl ruthenium complexes and the underlying biochemical mechanisms have not yet been investigated.

In this work, we describe cytotoxicity in murine and human tumor cell lines of the three chloropolypyridyl ruthenium complexes (Figure 1), which differ in the number of leaving ligands. The results of these investigations are compared with DNA binding in a cell-free medium. We find a clear correlation between the cytotoxicity of the polypyridyl ruthenium complexes and their DNA interstrand cross-linking capability.

MATERIALS AND METHODS

Chemicals. [Ru(terpy)(bpy)Cl]Cl, *cis*-[Ru(bpy)$_2$Cl$_2$], and *mer*-[Ru(terpy)Cl$_3$] (Figure 1) (terpy = 2,2′:6′,2″-terpyridine, bpy = 2,2′-bipyridyl) were synthesized as described in the literature (Adcock et al., 1984; Sullivan et al., 1978; Takeuchi et al., 1984). Their stock solutions (5×10^{-4} M) were made in double-distilled water, kept in the dark, and used after 48 h of equilibration at 37 °C. *cis*-[Ru(bpy)$_2$Cl$_2$] enriched by the Δ-isomer was prepared by the procedure based on a preferential binding of Λ-isomers of polypyridyl chlororuthenium complexes to B-DNA (Barton & Lolis, 1985; Grover et al., 1992). An initial cycle of this procedure involved mixing calf thymus double helical DNA at the concentration of 0.32 mg/mL with racemic *cis*-[Ru(bpy)$_2$Cl$_2$] at $r_i = 0.1$ in the medium of 10 mM $NaClO_4$ at 37 °C in the dark (r_i is defined as the molar ratio of free metal complex to nucleotide phosphates at the onset of incubation). After 2 h, the mixture was ultrafiltered in 10 mL of cells from Amicon with an 8000 molecular mass cutoff membrane, and the resulting solution was concentrated by vacuum distillation. In the next cycle, double helical DNA was added to this filtrate so that its concentration was 0.32 mg/mL and r_i was 0.1. This mixture was again incubated and filtered as in the preceding step. The preferential binding of Λ-isomer was quantified by measuring circular dichroism spectra using a JASCO spectropolarimeter, Model J-720. The enrichment of the *cis*-[Ru(bpy)$_2$Cl$_2$] sample by the Δ-isomer was calculated using the molar ellipticity yielded by the filtrates at 280 nm and their comparison with the published molar ellipticity of the Δ-isomer at the same wavelength (Arce Sagüés et al., 1980). These cycles were repeated until the value of molar ellipticity of the filtrate at 280 nm no longer changed. The sample designated in the following text as *cis*-[Ru(bpy)$_2$Cl$_2$] enriched by the Δ-enantiomer contained ca. 75% Δ-isomer and ca. 25% Λ-isomer.

Fischer's and Dulbecco's modified Eagle's (DME) media, bovine insulin, penicillin, Streptomycin, and *Micrococcus lysodeikticus* DNA were from Sigma. Bovine fetal calf serum was purchased from Sebak (Germany). Calf thymus DNA was isolated and characterized as previously described (Brabec & Paleček, 1976). Plasmid pSP73 (2464 base pairs) was either purchased from Promega or prepared by transformation of the plasmid into Sure competent cells (*Escherichia coli* strain) from Stratagene, amplification of a clone, and purification using a Plasmid maxi kit from Qiagen (Germany). The prepared supercoiled DNA was identified by electrophoresis on a 1% agarose gel running with Promega plasmid pSP73. The sample of the plasmid prepared in this laboratory contained a 90% negatively supercoiled form. The CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit with Vent$_R$(exo$^-$) or Vent$_R$(exo$^+$) DNA polymerases, the Klenow fragment of DNA polymerase I, and restriction endonucleases were purchased from BioLabs. T4 polynucleotide kinase was from Boehringer. A primer 5′-GATTTAGGTGACACTATA-3′ was from BioVendor (Brno, Czech Republic). All radioactive products were from Amersham.

Physical and Physicochemical Methods. Absorption spectra were collected on a Beckman DU-8B UV/VIS spectrophotometer. Flameless atomic absorption spectroscopy (FAAS) measurements were carried out on a Unicam 939 AA spectrometer with a graphite furnace. Differential pulse polarographic (DPP) analysis was performed with the aid of an EG&G PARC Polarographic Analyzer, Model 384B, equipped with an EG&G Static mercury drop electrode, Model 303A; platinum wire and saturated calomel electrode (SCE) were used as counter and reference electrodes, respectively. DPP analysis was performed at the following apparatus settings: drop time, 1.0 s; scan rate, 2 mV/s; and pulse amplitude, −50 mV.

In Vitro Cytotoxicity Assays. Wild-type L1210 murine leukemia and human cervix carcinoma HeLa cells were cultured as suspensions in Fischer's or DME's medium, respectively, supplemented with 10% bovine fetal calf serum from Sebak (Germany), 10 μg of bovine insulin/mL, 100 units of penicillin/mL, and 100 μg of streptomycin/mL at 37 °C. For testing purposes, cells in the logarithmic growth phase were diluted to 1×10^5 cells/mL, and 4.5 mL of cell suspension was aliquoted to Müller's tissue culture tubes. Test compound, dissolved in 1 mM phosphate buffer, pH 7.0, was then added to the appropriate tubes (0.5 mL/tube) to attain the final concentration, and cells were maintained under growth conditions. After the time allowed for ruthenium complex exposure at 37 °C in the dark, the concentrations of L1210 or HeLa cells were determined by using a Coulter counter or were first harvested with trypsinisation and counted using Bürker chambers, respectively.

Cellular Ruthenium Complex Uptake. Experiments were performed with logarithmic growth phase L1210 cells from a culture maintained and treated with ruthenium complexes as described above. Ruthenium complexes were tested at a concentration of 3×10^{-5} M, and the time allowed for the treatment of the cells was 11 h. Unassociated ruthenium

complex was separated from the cell sample by repetitive centrifugation and resuspension of the sample in 0.15 M NaCl with 10 mM phosphate buffer, pH 7.0 (three times). The washed cell pellet was resuspended in 0.4 mL of 0.1% Triton X-100 and was sonicated for 30 s with a Dynatech sonicator set at 100 W. Ruthenium associated with the cell pellet was measured with FAAS.

In Vivo Efficacy Studies. Female (BALB/c × C_{57}BL/6)-F_1 mice weighing 20−23 g were obtained from the Institute of Biophysics, Brno, and housed in an environment having controlled humidity, temperature, and photoperiods. The animals had food and water available *ad libitum*, the wood chip bedding was changed daily, and they were randomized before treatment. The transplantable LS/BL ascitic tumor, widely disseminating lymphosarcoma of C_{57}BL mice, was maintained by successive intraperitoneal (ip) isologous transplants of 10^6 cells at 7-day intervals in (BALB/c × C_{57}-BL)F_1 mice. For testing purposes, 10^6 tumor cells were inoculated ip (day 0) and mice were administered five doses of test compound dissolved in 0.5 mL of 5 mM phosphate buffer, pH 7.0 ip, on days 1, 3, 5, 7, and 9. Mice in the control group were administered only pure buffer in the same schedule. Animals (10 mice in each group) were observed daily for signs of toxicity, and deaths and the day of death were recorded for each animal that died during the 40-day observation period. The efficacy of each dose of compound tested was evaluated by calculating the percent increased life span, which was determined by dividing the mean survival time of treated mice (using the day of death of only those animals that died during the 40-day period) by the mean survival time of nontreated tumor-bearing control animals (% T/C). Compounds exhibiting a % T/C of >140 are considered to have significant antitumor activity (Farrell et al., 1990a).

Modification of DNA by Ruthenium Complexes. If not stated otherwise, mammalian, bacterial, or plasmid DNAs were modified in 10 mM sodium perchlorate with 1 mM phosphate buffer, pH 7.0, for 24 h at 37 °C in the dark. The ratio of metal atoms fixed per nucleotide residue (r_b) was determined by DPP. The paper containing the details of this assay will be published elsewhere (Nováková et al., unpublished experiments) so that we describe in this paper only its main principles. DNA was mixed with the ruthenium complex in 10 mM $NaClO_4$ with 1 mM phosphate buffer, pH 7.0, so that the resulting DNA concentration was 0.3 mg/mL. At the time of this reaction chosen for the r_b measurement, the aliquot of 18 μL was withdrawn. Then 2 μL of 3 M sodium acetate was added to this aliquot, and DNA in this sample was precipitated by addition of 40 μL of ethanol and subsequent incubation at −20 °C. After centrifugation, 3 μL of the supernatant was added to 3.0 mL of 0.9 M ammonium formate with 0.15 M NaH_2PO_4, pH 5.4 (DPP buffer), and subjected to DPP analysis at 25 °C. This analysis is based on the observation that various ruthenium complexes, including those tested in this work, yield in this medium a DDP peak at around −1.35 V vs SCE. This DPP peak corresponds to the catalytic hydrogen current, and its height is linearly increased with the growing concentration of the ruthenium complex in the range of 5×10^{-10} to 1×10^{-8} M. The lower limit of the analytical determination of Ru complexes using this DDP activity is 10^{-9} M. In addition, if a reaction mixture containing free ruthenium and ruthenium−DNA complex is precipitated by ethanol, free ruthenium (not that bound to DNA) only yields the DPP catalytic hydrogen peak at around −1.35 V. Thus, the DPP analysis described above gives the amount of unreacted ruthenium. The amount of ruthenium bound to DNA (and from it the value of r_b) can be calculated by subtracting the unreacted amount from the amount of ruthenium present at the onset of the reaction. Importantly, the precipitation of DNA or ruthenium−DNA complex immediately stops the reaction of ruthenium with DNA and does not cause dissociation of ruthenium which had already been bound. Thus, this polarographic assay is also suitable for easy determination of the kinetics of DNA−ruthenium complexation. It was verified that this DPP assay gives r_b values identical with those obtained by FAAS.

Unwinding of Negatively Supercoiled DNA. Unwinding of closed circular supercoiled pSP73 plasmid DNA was assayed by an agarose gel mobility shift assay (Keck & Lippard, 1992). The unwinding angle Φ, induced per ruthenium−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 for 24 h at 37 °C in the dark. All samples were precipitated by ethanol and redissolved in the TBE buffer (Tris-borate/EDTA). An aliquot of the precipitated sample was subjected to electrophoresis on 1% agarose gels running at 25 °C in the dark with TBE buffer with a voltage set at 30 V. The gels were than stained with ethidium bromide (EtBr) and were followed by on Polaroid 667 film with a transilluminator. The other aliquot was used for the determination of r_b values by DPP.

Interstrand Cross-Link Assay. DNA interstrand cross-linking by the ruthenium complexes was determined by two methods. One procedure employed calf thymus DNA, and the cross-linking was measured by the EtBr fluorescence technique (Brent, 1984; Morgan & Pulleyblank, 1974). Control or Ru-modified DNAs at the concentration of 0.1 mg/mL were dialyzed against 10 mM $NaClO_4$ with 1 mM phosphate buffer, pH 7.0. This solution (0.2 mL) was added to 3.0 mL of EtBr (0.5 μg/mL) in 20 mM K_2HPO_4 with 0.4 mM EDTA, pH 11.8. The samples were incubated at 90 °C for 10 min and then rapidly cooled on an ice bath. The fluorescence was measured at 25 °C before and after this heating/cooling step (excitation and emission wavelengths were 525 and 580 nm, respectively). The cross-linked fraction of DNA (XCL) was calculated using the equation $XCL = (F_{Ru} - F_C)/(1 - F_C)$, where F_C and F_{Ru} are values of the fluorescence of control and ruthenium-modified DNAs, respectively, measured after the heating/cooling step divided by the fluorescence before this step.

Plasmid DNA was used in a further series of experiments. The ruthenium complexes at varying concentrations were incubated with 2 μg of closed circular pSP73 DNA or with this DNA linearized by *Eco*RI restriction enzyme in 10 mM $NaClO_4$ with 1 mM phosphate buffer, pH 7.0, for 24 h at 37 °C in the dark. The r_b values were determined by the DPP assay. Then the samples were precipitated by ethanol, and closed circular DNA already modified by the ruthenium complexes was subsequently linearized by *Eco*RI. The linear duplexes were 3′-end labeled by means of the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. The samples were deproteinized by phenol and precipitated by ethanol, and the pellet was dissolved in 18 μL of 10 mM

NaOH with 1 mM EDTA, 6.6% sucrose, and 0.04% bromophenol blue. The amount of cross-links was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%). After electrophoresis was complete, the bands corresponding to the single strands of DNA and interstrand cross-linked duplex were cut off, and their radioactivity was quantified on a LKB Wallac 1410 Betaspectrometer (Finland).

Sequence Specificity of Ruthenium−DNA Adducts. Mapping of DNA lesions induced by the ruthenium complexes in the pSP73 plasmid DNA was conducted in a way similar to that used for mapping of DNA adducts of cisplatin and its four platinum analogues (Murray et al., 1992a,b). The Circum Vent Thermal Cycle Dideoxy DNA Sequencing Kit with $Vent_R(exo^-)$ or (exo^+) DNA polymerases was used along with the protocol for thermal cycle DNA sequencing with 5′-end-labeled primer recommended by the manufacturer with small modifications. Two picomoles of 5′-end-labeled primer oligonucleotide 5′-GATTTAGGTGACACTATA-3′ (complementary to the SP6 promoter nucleotide sequence in the template strand of pSP73) was added to 100 ng of double-stranded template DNA (pSP73 plasmid linearized by the *Hpa*I restriction endonuclease). This DNA was modified by the ruthenium complexes or cisplatin at r_b = 0.05 or 0.01, respectively [eventual traces of free (unbound) metal complexes were removed from the template DNA by precipitation by ethanol]. These solutions containing control or modified DNA were further mixed with 1.5 μL of the sequencing buffer composed of 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl, pH 8.8, and 50 mM $MgSO_4$, 1 μL of 3% Triton X-100, 2 units of $Vent_R(exo^+)$ DNA polymerase, and distilled water to a total volume of 16 μL. A total of 3.2 μL of this reaction mixture was immediately added to 3 μL of the solution containing 100 μM dATP, dCTP, dGTP, and dTTP and overlaid with 50 μL of mineral oil. In the same experiment, dideoxy double-stranded DNA sequencing was performed using the same unmodified plasmid and primer and $Vent_R(exo^-)$ instead of (exo^+) DNA polymerase as DNA sequence standards. Linear amplification by thermal cycling was carried out at 95 °C for 30 s (time at the temperature), 47 °C for 30 s, and 72 °C for 30 s for 25 cycles in a Techne PHC-2 DNA Thermal Cycler. The reaction was terminated by the addition of 4 μL of stop/loading dye solution containing deionized formamide with 0.3% xylene cyanol, 0.3% bromophenol blue, and 0.37% EDTA (pH 7.0) to each tube, beneath the mineral oil. The total of 2 μL of the reaction mixture was loaded onto a 6% polyacrylamide/8 M urea DNA sequencing gel.

RESULTS

Cytotoxicity and Antitumor Activity Studies. In vitro cyctotoxicity of all ruthenium complexes in tissue cultures was examined first in a murine L1210 tumor cell line (Figure 2A). The results show that *mer*-[Ru(terpy)Cl_3] exhibits markedly higher cytotoxicity than racemic *cis*-[Ru(bpy)$_2Cl_2$] or [Ru(terpy)(bpy)Cl]Cl. This higher cytotoxicity of *mer*-[Ru(terpy)Cl_3] was observed in the range of the final concentrations of the ruthenium complexes of 1×10^{-6} to 2×10^{-5} M. To confirm that this observation applies to human tumors, the compounds were also assayed for cytotoxicity in a human cervix carcinoma HeLa cell line (Figure 2B). There is a similarity in the behavior pattern of the complexes

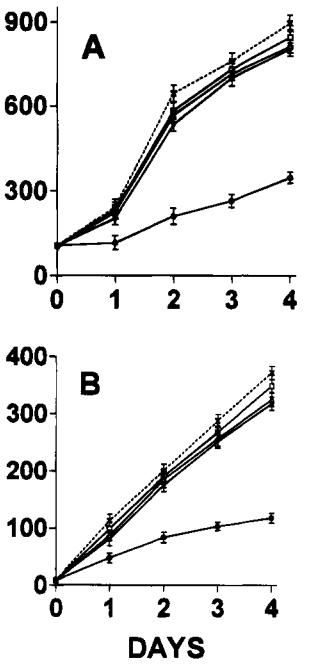

FIGURE 2: *In vitro* cytotoxicity of ruthenium complexes in murine L1210 leukemia (A) and human cervix carcinoma HeLa cells (B). The cells were treated with the complexes at their final concentration of 1×10^{-5} M: (x− −x), control; (□−□), [Ru(terpy)(bpy)Cl]Cl; (△−△), racemic *cis*-[Ru(bpy)$_2Cl_2$]; (▽−▽), *cis*-[Ru(bpy)$_2Cl_2$] enriched by the Δ-enantiomer (75% Δ-isomer + 25% Λ-isomer); (●−●), *mer*-[Ru(terpy)Cl_3].

toward this clinically relevant human cell line and the murine L1210 leukemia results. The cytotoxicity of the ruthenium complexes tested in this work can also be compared by means of the ID_{50} values, i.e. the concentrations of the drug required to inhibit cell growth by 50%. This value could be determined only for *mer*-[Ru(terpy)Cl_3]. For instance, after 72 h of ruthenium exposure, ID_{50} in L1210 and HeLa cells was 8 and 7 μM, respectively. Unfortunately, the ID_{50} values for racemic *cis*-[Ru(bpy)$_2Cl_2$] or [Ru(terpy)(bpy)Cl]Cl were impossible to determine because of the limited solubility of these compounds. Nevertheless, 50% inhibition of the cell growth by the treatment with racemic *cis*-[Ru(bpy)$_2Cl_2$] or this complex enriched by its Δ-enantiomer and [Ru(terpy)(bpy)Cl]Cl for 72 h was not attained if their concentrations were even 70 times higher than the value of ID_{50} of *mer*-[Ru(terpy)Cl_3]. Interestingly, intracellular ruthenium uptake was determined in L1210 cells and was found to be almost identical for racemic *cis*-[Ru(bpy)$_2Cl_2$] and *mer*-[Ru(terpy)Cl_3] and about 7 times higher for [Ru(terpy)(bpy)Cl]Cl (Table 1).

The antitumor activity of polypyridyl ruthenium complexes has been initially studied on murine lymphosarcoma LS/BL (Table 2). The objective was to ascertain which ruthenium complexes examined in this work show *in vivo* activity in standard murine screen. The present preliminary studies indicate that the complex *mer*-[Ru(terpy)Cl_3], which showed a pronounced *in vitro* cytotoxicity (Figure 2), also exhibits significant antitumor activity. On the other hand, the activities of [Ru(terpy)(bpy)Cl]Cl, racemic *cis*-[Ru(bpy)$_2Cl_2$], or this complex enriched by its Δ-enantiomer, which all exhibited only a slight *in vitro* cytotoxicity, were very small under identical conditions. A larger series of murine and human tumors and regimes of administration are being extensively tested and will be the subject of a more

Table 1: L1210 Cell Uptake of Polypyridyl Ruthenium Complexes[a]

compound[b]	pmol of Ru (10^6 cells)$^{-1}$ μM^{-1}[c]
[Ru(terpy)(bpy)Cl]Cl	0.58 ± 0.07
racemic *cis*-[Ru(bpy)$_2$Cl$_2$]	0.08 ± 0.01
cis-[Ru(bpy)$_2$Cl$_2$] enriched by its Δ-isomer[d]	0.08 ± 0.01
mer-[Ru(terpy)Cl$_3$]	0.08 ± 0.02

[a] The period of the treatment was 11 h; for other details, see Material and Methods. [b] The Ru complexes were at the concentration of 3×10^{-5} M. [c] The values represent mean ± SEM of six determinations from two independent experiments. [d] 75% Δ-isomer plus 25% Λ-enantiomer.

Table 2: Summary of Antitumor Activity of Polypyridyl Ruthenium Complexes in LS/BL Tumor Cells[a]

compound	dose, mg/kg[b]	% T/C
[Ru(terpy)(bpy)Cl]Cl	5 × 5	108
	10 × 5	110
	15 × 5	110
racemic *cis*-[Ru(bpy)$_2$Cl$_2$]	5 × 5	104
	10 × 5	101
	15 × 5	106
cis-[Ru(bpy)$_2$Cl$_2$] enriched by its Δ-isomer[c]	5 × 5	107
	10 × 5	102
	15 × 5	103
mer-[Ru(terpy)Cl$_3$]	5 × 5	134
	10 × 5	157
	15 × 5	142

[a] Tests conducted as described in Materials and Methods. [b] The appropriate dose was administered on day 1, 1 day after tumor inoculation. The second, third, fourth, and fifth doses were administered on days 3, 5, 7, and 9, respectively. [c] 75% Δ-isomer plus 25% Λ-enantiomer.

detailed paper confirming the antitumor activity of *mer*-[Ru(terpy)Cl$_3$].

cis-[Ru(bpy)$_2$Cl$_2$] complex is chiral (its racemic mixture contains equal amounts of Δ-and Λ-enantiomers). We have also tested a hypothesis that cellular uptake of these enantiomers could be different. A mixture of the two isomers of *cis*-[Ru(bpy)$_2$Cl$_2$] was prepared which was enriched by the Δ-enantiomer (75% Δ-enantiomer + 25% Λ-enantiomer). Importantly, the amounts of ruthenium found in L1210 cells treated with the racemic complex or the complex enriched by the Δ-isomer under conditions specified in the legend to Table 1 were identical. The cytotoxicity (Figure 2) and antitumor activity (Table 2) of the racemic or enriched *cis*-[Ru(bpy)$_2$Cl$_2$] were almost identical. These results are consistent with the view that, in the experiments described in this work, there was no substantial difference in the intracellular ruthenium uptake and cytotoxicity for individual enantiomers of *cis*-[Ru(bpy)$_2$Cl$_2$].

DNA Binding. Given the cytotoxicity results, it is of particular interest to elucidate the similarities and differences in the behavior of the polypyridyl chlororuthenium complexes toward isolated DNA in solution and to relate these properties to their biological effects. The DNA binding, unwinding, and interstrand cross-linking were examined by a combination of techniques of molecular biophysics including polarography and gel electrophoresis.

In order to demonstrate that the ruthenium complexes tested in this work coordinate to DNA, we used procedures slightly modified in comparison with those already applied to prove coordination to DNA of other polypyridyl ruthenium

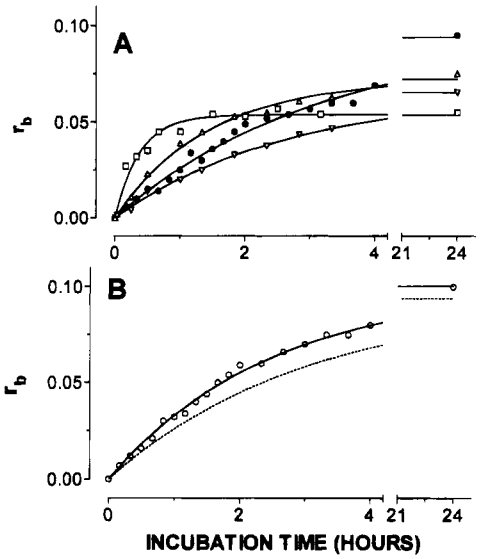

FIGURE 3: Formation of DNA adducts by ruthenium complexes as a function of incubation time. (A) Reaction of calf thymus DNA with [Ru(terpy)(bpy)Cl]Cl (□), racemic *cis*-[Ru(bpy)$_2$Cl$_2$] (△), *cis*-[Ru(bpy)$_2$Cl$_2$] enriched by the Δ-enantiomer (75% Δ-isomer + 25% Λ-isomer) (▽), or *mer*-[Ru(terpy)Cl$_3$] (●). (B) Reaction of *Micrococcus lysodeikticus* DNA with *mer*-[Ru(terpy)Cl$_3$] (○); for comparative purposes, the dashed line shows the dependence for calf thymus DNA with *mer*-[Ru(terpy)Cl$_3$] taken from panel A. See Materials and Methods for details.

complexes (Lolis & Barton, 1985; Grover et al., 1992). Solutions of calf thymus DNA at the concentration of 0.3 mg/mL were incubated with the ruthenium complex for various time intervals at r_i values of 0.1. Following incubation, ethanol was added to precipitate the DNA, with unbound ruthenium remaining in solution. An aliquot of this supernatant was added to the DPP buffer and assayed by DPP. The amount of ruthenium bound to DNA (r_b) was calculated by subtracting the amount of ruthenium remaining in solution (determined by DPP) from the total amount of ruthenium present in the reaction. Figure 3A shows a plot of r_b vs time upon incubation of DNA with *cis*-[Ru(bpy)$_2$Cl$_2$], [Ru(terpy)(bpy)Cl]Cl, and *mer*-[Ru(terpy)Cl$_3$] at 37 °C. Ruthenium associated with the precipitated DNA (i.e. which did not remain in the solution after precipitation of DNA) was already detected at short incubation times (several minutes), thus indicating its coordination to DNA. The amount of ruthenium bound to DNA increased with time and reached maximum after ca. 24 h. The maximum binding of *mer*-[Ru(terpy)Cl$_3$] corresponded to the binding of almost all molecules present in the reaction mixture. In contrast, the maximum modification by racemic *cis*-[Ru(bpy)$_2$Cl$_2$] or [Ru(terpy)(bpy)Cl]Cl was less extensive (75 or 55%, respectively) (Figure 3A). If r_i was decreased to 0.01 (at the concentration of DNA of 1 mg/mL), the fraction of *cis*-[Ru(bpy)$_2$Cl$_2$] or [Ru(terpy)(bpy)Cl]Cl molecules, which remained unbound after 24 h, was found to be the same. Half-times ($t_{1/2}$) for the binding reactions can be estimated from Figure 3. The $t_{1/2}$ values of 16, 59, 111, and 129 min were found for DNA binding of [Ru(terpy)(bpy)Cl]Cl, racemic *cis*-[Ru(bpy)$_2$Cl$_2$], *cis*-[Ru(bpy)$_2$Cl$_2$] enriched by the Δ-enantiomer, and *mer*-[Ru(terpy)Cl$_3$], respectively in 10 mM $NaClO_4$ with 1 mM phosphate buffer, pH 7.0, at 37 °C. Thus, our results on the binding of the ruthenium complexes to DNA are consistent with the earlier work on other aqua or chloropolypyridyl ruthenium complexes (Barton & Lolis,

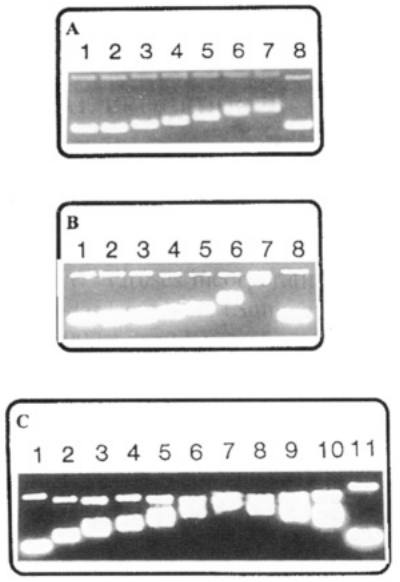

FIGURE 4: Unwinding of supercoiled pSP73 plasmid DNA by ruthenium complexes. See also Table 3. In each panel, the top bands correspond to the form of nicked plasmid and the bottom bands to closed, negatively supercoiled plasmid. In panel A, the plasmid was incubated with [Ru(terpy)(bpy)Cl]Cl with r_b values of 0 (control), 0.03, 0.06, 0.08, 0.11, 0.14, 0.17, and 0 (lanes 1−8, respectively). In panel B, *cis*-[Ru(bpy)$_2$Cl$_2$] was studied in the same way with r_b values of 0 (control), 0.004, 0.008, 0.023, 0.038, 0.053, 0.075, and 0 (lanes 1−8, respectively). In panel C, DNA was incubated with *mer*-[Ru(terpy)Cl$_3$] with r_b values of 0 (control), 0.09, 0.11, 0.13, 0.15, 0.17, 0.18, 0.19, 0.20, 0.21, and 0 (lanes 1−11, respectively).

1985; Grover et al., 1992, 1994) and with our preliminary work describing coordination of *mer*-[Ru(terpy)Cl$_3$] to DNA with the aid of spectrophotometric assay (van Vliet et al., 1995).

The metal complexes were also reacted with *M. lysodeikticus* DNA, which contains more guanine + cytosine (G + C) than calf thymus DNA (72 and 42%, respectively). The initial rate of the binding of the ruthenium complexes to DNA increased as a consequence of the higher G + C content in DNA (shown for *mer*-[Ru(terpy)Cl$_3$] at $r_i = 0.1$ in Figure 3B).

The DNA binding experiments were also conducted with the ruthenium complexes from their stock solutions which were supplemented with NaCl to reach its concentration of 0.5 M and subsequently incubated for 48 h at 37 °C in the dark. The reaction of these ruthenium complexes with DNA was allowed to proceed in 0.5 M NaCl with 1 mM phosphate buffer, pH 7.0, at 37 °C for 48 h and assayed by DPP. In this case, all ruthenium remained in solution after precipitation of DNA, indicating no coordination of ruthenium to DNA. This result suggests the importance of hydrolysis of the polypyridyl chlororuthenium complexes, involving exchange of their chloride ligands, for binding to DNA.

Unwinding Induced in DNA by Ru Binding. Agarose gel electrophoresis was used to determine the unwinding induced in pSP73 plasmid by *cis*-[Ru(bpy)$_2$Cl$_2$], [Ru(terpy)(bpy)Cl]Cl, or *mer*-[Ru(terpy)Cl$_3$] by monitoring the degree of supercoiling (Keck & Lippard, 1992) (Figure 4). 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. Figure 4 shows electrophoresis gels in which increasing amounts of the ruthenium complexes have been bound to be relaxed and supercoiled pSP73 DNA. The complexes *cis*-[Ru(bpy)$_2$Cl$_2$] and [Ru(terpy)(bpy)Cl]Cl did not apparently alter the mobility of the relaxed form, implying that their binding does not result in shortening or condensation of DNA. This result is consistent with the formation of monofunctional DNA adducts of *cis*-[Ru(bpy)$_2$Cl$_2$] and [Ru(terpy)(bpy)Cl]Cl. In contrast, *mer*-[Ru(terpy)Cl$_3$] accelerated the mobility of the relaxed form in a manner similar to that of cisplatin, whose bifunctional binding to DNA shortens and condenses the DNA helix (Cohen et al., 1979; Scovell & Collart, 1985). 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 (Bellon et al., 1991; Keck & Lippard, 1992). The unwinding angle was also determined under the same experimental conditions for *cis*-[Ru(bpy)$_2$Cl$_2$] enriched by the Δ-isomer (not shown). The value of the angle obtained was the same as it was for the racemic complex. This result suggests that there was no marked difference in the effectivity of the individual isomers of *cis*-[Ru(bpy)$_2$Cl$_2$] in unwinding DNA duplex. Unwinding angles for the ruthenium complexes tested in this work are listed in Table 3 with unwinding ability in the order *cis*-[Ru(bpy)$_2$Cl$_2$] > *mer*-[Ru(terpy)Cl$_3$] > [Ru(terpy)(bpy)Cl]Cl.

Table 3: Unwinding of Supercoiled pSP73 DNA by Ruthenium Complexes

compound	$r_b(c)$	unwinding angle (deg)[a]
[Ru(terpy)(bpy)Cl]Cl	≫0.17[b]	≪7
cis-[Ru(bpy)$_2$Cl$_2$]	0.075	15
mer-[Ru(terpy)Cl$_3$]	0.18	6

[a] The unwinding angle was calculated as described in the text. [b] The comigration of supercoiled and relaxed DNA did not occur even at an r_b value as high as 0.17. At higher levels of the modification by this complex, DNA precipitated.

Interstrand Cross-Linking. The interstrand cross-linking was assayed by two independent procedures. The EtBr techniques is based on the observation that the fluorescence of this compound is markedly enhanced if it is intercalated in double-stranded DNA. A little intercalation occurs in denatured DNA. In addition, if DNA contains covalent interstrand adduct, it renatures in a markedly higher extent than DNA without such lesions. Thus, the enhanced EtBr fluorescence yielded by the samples of DNA modified by ruthenium in comparison with the control (unmodified) DNA, after they were denatured and subsequently treated with the intercalator, can be used to quantify DNA interstrand cross-links (Brent, 1984; Morgan & Pulleyblank, 1974). A very small enhancement of the EtBr fluorescence was only noticed in the case of calf thymus DNA modified by *cis*-[Ru(bpy)$_2$Cl$_2$] (racemic complex or the complex enriched by the Δ-enantiomer) or [Ru(terpy)(bpy)Cl]Cl at $r_b = 0.005-0.02$. This enhancement gave the amount of interstrand cross-linked DNA, calculated as described in Materials and Methods, less than 2%. On the other hand, this enhancement observed in the case of calf thymus DNA modified by *mer*-[Ru(terpy)Cl$_3$] gave the amount of interstrand cross-linked

DNA as 16 or 34% at r_b of 0.005 or 0.01, respectively. These initial studies indicated that *mer*-[Ru(terpy)Cl_3] was markedly more efficient in DNA interstrand cross-linking than [Ru(terpy)(bpy)Cl]Cl or both isomers of *cis*-[Ru(bpy)$_2Cl_2$].

A more precise method for determination of DNA interstrand cross-linking by the polypyridyl ruthenium complexes exploited the linearized pSP73 DNA as described previously (Brabec & Leng, 1993; Lemaire et al., 1991). In one series of experiments, plasmid DNA was modified by varying concentrations of the ruthenium complexes after it had been linearized by *Eco*RI restriction endonuclease. In the other series, plasmid DNA was modified in the closed, negatively supercoiled form, and only after it had been modified by the metal complex was it linearized by *Eco*RI. Upon electrophoresis under denaturing conditions, noninterstrand cross-linked 3′-end-labeled strands of linearized pSP73 plasmid migrate as a 2464-base single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species.

No bands corresponding to the fragments migrating more slowly than the 2464-base single strand were observed if superhelical or linearized pSP74 DNAs were modified in a broad range of r_i values up to 0.1 by the complexes [Ru(terpy)(bpy)Cl]Cl or *cis*-[Ru(bpy)$_2Cl_2$] (racemic or the complex enriched by the Δ-enantiomer) (shown for racemic *cis*-[Ru(bpy)$_2Cl_2$] in Figure 5A). This result confirmed that [Ru(terpy)(bpy)Cl]Cl and the isomers of *cis*-[Ru(bpy)$_2Cl_2$] exhibited, under the conditions used, no DNA interstrand cross-linking efficiency. On the other hand, the bands corresponding to the more slowly migrating interstrand cross-linked fragments were noticed if DNA was modified by *mer*-[Ru(terpy)Cl_3] at as low an r_b value as 5×10^{-4} (Figures 5B,C). The intensity of this band increased with the growing level of the modification. Quantitation of the cross-links per adduct formed was carried out in essentially the same manner as previously described (Farrell et al., 1990b). The radioactivity associated with each band 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 was calculated using the Poisson distribution from the fraction of non-cross-linked DNA in combination with the r_b values and the fragment size. As summarized in Figure 5D, *mer*-[Ru(terpy)Cl_3] showed a much higher frequency of interstrand cross-links in superhelical DNA in comparison with their formation in the linearized form. In addition, while the number of interstrand cross-links formed in the linearized DNA per one ruthenium adduct was independent of r_b, the frequency of the interstrand cross-links formed in superhelical DNA increased with a decreasing level of the modification.

Sequence Preference of DNA−Ruthenium Adducts. This procedure involved the extension by Vent$_R$(exo$^+$) DNA polymerase (which exhibits extreme thermostability) at the 3′-end of the 5′-end radioactively labeled primer up to the metal adduct on the template strand of pSP73 plasmid. Using thermal cycling, this process was repeated many times in order to amplify the signal. The products of this linear amplification were then examined on DNA sequencing gels, and the sequence specificity of ruthenium adduct formation was determined to the exact base pair.

Vent$_R$(exo$^+$) DNA polymerase has active 3′ → 5′ exonuclease similar to that of several other native DNA polymerases. Vent$_R$(exo$^-$) DNA polymerase is genetically engineered from Vent$_R$(exo$^+$) DNA polymerase, retaining the polymerase activity but having no 3′ → 5′ exonuclease activity. The absence of exonuclease activity makes it suitable for the finest DNA sequencing so that we used Vent$_R$(exo$^-$) DNA polymerase for dideoxy sequencing of control-unmodified DNA. On the other hand, it has been shown (Comess et al., 1992) that, for mapping studies to detect the location of metal−DNA adducts in a heterogeneous population, DNA polymerases, which have active 3′ → 5′ exonuclease, are suitable. It is so because these enzymes permit only a very weak translesion synthesis in contrast to their counterparts, which have no associated 3′ → 5′ exonuclease activity. Therefore, we used Vent$_R$(exo$^+$) DNA polymerase for mapping of ruthenium−DNA adducts.

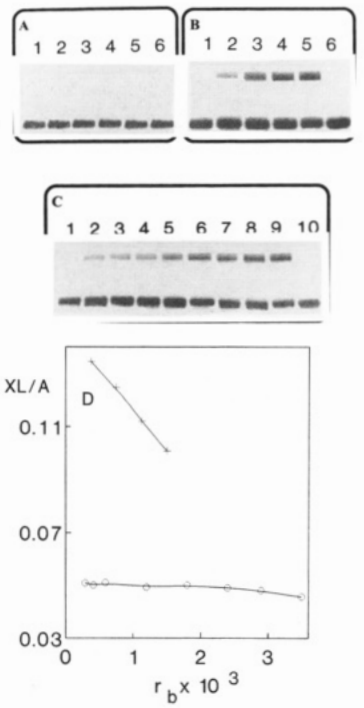

FIGURE 5: Interstrand cross-linked formation induced by ruthenium complexes in linearized or closed negatively supercoiled pSP73 plasmid (2464 bp). (A−C) Autoradiograms of a denaturing 1% agarose gel of DNA which was 3′-end labeled. The interstrand cross-linked DNA appears as the top bands migrating more slowly on the gel than the single-stranded DNA (contained in the bottom bands). (A) Linearized plasmid was incubated with *cis*-[Ru(bpy)$_2Cl_2$] with r_b values of 0 (control), 0.002, 0.005, 0.012, 0.023, and 0 (lanes 1−6, respectively). (B) Closed negatively supercoiled DNA was incubated with *mer*-[Ru(terpy)Cl_3] with r_b values of 0 (control), 0.000 34, 0.000 75, 0.001 25, 0.0015, and 0 (lanes 1−6, respectively); after the modification, the closed DNA was linearized by the *Eco*RI restriction enzyme. (C) Linearized DNA was incubated with *mer*-[Ru(terpy)Cl_3] with r_b values of 0 (control), 0.000 29, 0.000 41, 0.000 59, 0.0012, 0.0018, 0.0024, 0.0029, 0.0035, and 0 (lanes 1−10, respectively). (D) Dependence on r_b of the number of interstrand cross-links per adduct (XL/A) in the pSP73 plasmid modified by *mer*-[Ru(terpy)Cl_3]. DNA was either modified in closed negatively supercoiled form (and linearized by the restriction enzyme after the modification) (+), or already in linearized form (linearization of plasmid DNA was performed before its modification) (○). The ratio of interstrand cross-links to total ruthenium bound was calculated as described previously (Zou et al., 1993). XL/A was then calculated by dividing this ratio by the number of adducts per DNA molecule.

In vitro DNA synthesis on double-stranded templates containing the adducts of *cis*-[Ru(bpy)$_2Cl_2$], *mer*-[Ru(terpy)Cl_3], or [Ru(terpy)(bpy)Cl]Cl generated a population of DNA fragments indicating that these adducts terminate duplex synthesis (Figure 6A, lanes Ru1−3). Results identical to

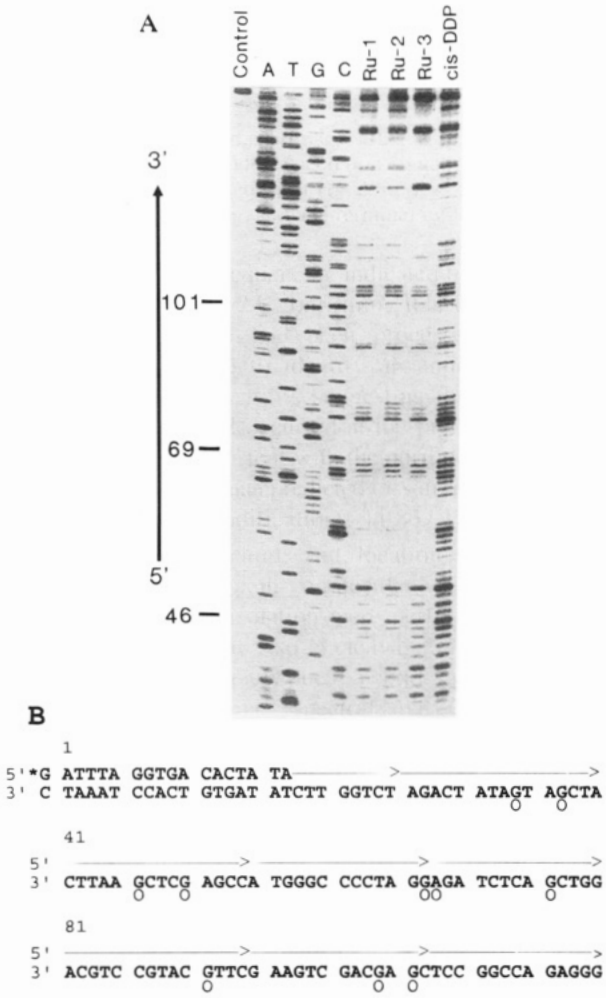

FIGURE 6: (A) Autoradiogram of 6% polyacrylamide/8 M urea sequencing gel showing inhibition of DNA synthesis by $Vent_R$ DNA polymerase on the pSP73 plasmid DNA linearized by *Hpa*I restriction enzyme and subsequently modified by metal complexes. The gel contained the linear amplification products of DNA treated with the three ruthenium analogues and cisplatin. Lanes: control, unmodified template; Ru-1, DNA modified by [Ru(terpy)(bpy)Cl]Cl at $r_b = 0.05$; Ru-2, DNA modified by *cis*-$[Ru(bpy)_2Cl_2]$ at $r_b = 0.05$; Ru-3, DNA modified by *mer*-$[Ru(terpy)Cl_3]$ at $r_b = 0.05$; cis-DDP, DNA modified by cisplatin at $r_b = 0.01$; and A, T, G, and C, 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 the ruthenium complexes or cisplatin. The star indicates the 5′-end labeling of the primer. The arrow indicates the start site of the DNA polymerase and the direction of the synthesis. ○ represents stop signals from panel A, lane Ru-1. Nucleotides 1 and 18 correspond respectively to nucleotides 2548 and 1 on the pSP73 nucleotide sequence map.

those shown for the racemic *cis*-$[Ru(bpy)_2Cl_2]$ (Figure 6A, lane Ru-2) were obtained if the DNA synthesis was performed on the template modified by this ruthenium complex enriched by its Δ-isomer (not shown). If the same synthesis was performed with $Vent_R(exo^-)$ DNA polymerase, the sequence dependence of the termination was considerably less regular (not shown). Sequence analysis of the termination sites produced by all ruthenium complexes suggests a sequence preference for isolated dG sites in double helical DNA (Figure 6B). This result is consistent with the observation that these complexes were bound with a higher initial rate to DNA, which had a higher content of G + C (Figure 3B). The sequence dependence of the inhibition is not identical to that found for cisplatin (Figure 6A, lane cis-DDP), indicating a sequence preference of DNA adducts of the ruthenium complexes somewhat different from that exhibited by antitumor cisplatin. Interestingly, the ruthenium complexes form less adducts effective in inhibiting DNA polymerase at d(GG) or oligo(dG) sites. In addition, at the same level of modification, the bands produced by the ruthenium complexes were considerably weaker. This result suggests that the ruthenium complexes tested in this work also form more types of DNA adducts and that some of these adducts are unable to inhibit DNA synthesis by $Vent_R(exo^+)$ DNA polymerase.

DISCUSSION

The purpose of this study was to examine cytotoxicity of several polypyridyl ruthenium complexes (Figure 1) and to compare this biological activity with their DNA binding. The results presented here show that *mer*-$[Ru(terpy)Cl_3]$ exhibits a significant cytotoxicity in both murine and human tumor cell lines and antitumor activity in a standard tumor screen. In contrast, [Ru(terpy)(bpy)Cl]Cl or both isomers of *cis*-$[Ru(bpy)_2Cl_2]$, i.e. the complexes with only one or two leaving chloride ligands, respectively, show markedly lower cytotoxic activities in the same tumor models.

A possible pathway of discrimination between *mer*-$[Ru(terpy)Cl_3]$ and inactive *cis*-$[Ru(bpy)_2Cl_2]$ or [Ru(terpy)(bpy)Cl]Cl is intracellular ruthenium uptake. However, the accumulation of the ruthenium complexes in the cells (Table 1) exhibited no apparent correlation with their cytotoxicity. This observation supports the view that a different cytotoxicity found for the ruthenium complexes tested in this work was not only due to a different extent of exposure of an intracellular target structure to these compounds.

Antitumor agents derived from the coordination complexes of the transition metals with nitrogen ligands and anionic leaving groups are assumed to have a nuclear DNA as the target site (Clarke, 1989; Johnson et al., 1989). It implies a role for DNA binding in the mechanism of cytotoxic action of the ruthenium complexes investigated in this work. In a cell-free system, the polypyridyl chlororuthenium complexes coordinate to DNA. *mer*-$[Ru(terpy)Cl_3]$ exhibited a slightly higher affinity to DNA at longer reaction times than the inactive complexes (Figure 3A) (after 24 h, *mer*-$[Ru(terpy)Cl_3]$ was bound 1.3 or 1.8 times more than *cis*-$[Ru(bpy)_2Cl_2]$ or [Ru(terpy)(bpy)Cl]Cl, respectively. It could be argued that, at the same intracellular concentrations of the ruthenium complexes, a higher cytotoxicity of *mer*-$[Ru(terpy)Cl_3]$ was due to a higher extent of the modification of DNA. However, on the basis of the ID_{50} values (see Results), *mer*-$[Ru(terpy)Cl_3]$ was found to be many times more cytotoxic than *cis*-$[Ru(bpy)_2Cl_2]$ (both racemic or enriched by the Δ-enantiomer) or [Ru(terpy)(bpy)Cl]Cl. Thus, the slightly different DNA binding of the ruthenium complexes (Figure 3A) can hardly explain their markedly distinct cytotoxicity in murine and human tumor cells observed in this work (Figure 3).

The binding to DNA occurs preferentially at isolated guanine residues (Figures 3B and 6) and results in conformational alterations in DNA as represented by unwinding of superhelical DNA (Figure 4). These results also indicate differences in the DNA adducts formed by the ruthenium

complexes but do not show a correlation with cytotoxicity exhibited by these compounds in the tumor models used in this work.

On the other hand, the results of this work show that the cytotoxic complex *mer*-[Ru(terpy)Cl_3] exhibits a significant DNA interstrand cross-linking in contrast to the inactive ruthenium complexes which exhibit no such efficiency (Figure 5). Thus, an important feature for biological activity of polypyridyl ruthenium complexes is DNA interstrand cross-linking.

It is for obvious reasons why the formally monofunctional [Ru(terpy)(bpy)Cl]Cl does not form on DNA the bifunctional interstrand cross-link. On the other hand, no DNA interstrand cross-linking capability of bifunctional *cis*-[Ru(bpy)$_2$$Cl_2$] reported in this work deserves a deeper discussion. Recently, thermal denaturation studies on DNA modified by a group of mono- and diaqua polypyridyl complexes of ruthenium including racemic *cis*-[Ru(bpy)$_2$(H_2O)$_2$]$^{2+}$ (Grover et al., 1994) were described. These recent investigations yielded the result that *cis*-[Ru(bpy)$_2$(H_2O)]$^{2+}$ increased the melting temperature of DNA (T_m) by 6.1 °C at r_b = 0.024. This observation was interpreted to mean that the bifunctional complex *cis*-[Ru(bpy)$_2$(H_2O)$_2$]$^{2+}$ formed DNA interstrand cross-links responsible for the increase of T_m (Grover et al., 1994). Since the nature of the DNA adducts of *cis*-[Ru(bpy)$_2$(H_2O)$_2$]$^{2+}$ and its dichloro counterpart has been proposed to be the same (Grover et al., 1992), it implies that *cis*-[Ru(bpy)$_2$$Cl_2$] should also be an effective DNA interstrand cross-linking agent. On the other hand, the conclusion on interstrand cross-linking by *cis*-[Ru(bpy)$_2$(H_2O)$_2$]$^{2+}$ has only been based on the T_m measurements, and no additional, more direct evidence has been presented (Grover et al., 1994). It is well-established that the increase of T_m due to the binding of a cationic compound to DNA does not necessarily imply DNA interstrand cross-linking [see Kleinwächter et al., (1969) and Waring (1965)]. Also importantly, there is no further published result except that based on the T_m measurements suggesting that *cis*-[Ru(bpy)$_2$(H_2O)$_2$]$^{2+}$ or *cis*-[Ru(bpy)$_2$$Cl_2$] forms in reaction with DNA or its monomeric constituents some type of disubstituted products. On the contrary, the single crystal X-ray analysis and ^{1}H nuclear magnetic resonance spectroscopy revealed that both *cis*-[Ru(bpy)$_2$(H_2O)$_2$]$^{2+}$ and *cis*-[Ru(bpy)$_2$$Cl_2$] with an excess of 9-ethylguanine or 9-methylhypoxanthine (1:10 mixture) gave only monosubstituted products in a chloride-free medium (van Vliet et al., 1994). A similar analysis of the products of the reaction of *mer*-[Ru(terpy)Cl_3] with these bases only in stochiometric amounts revealed two bases coordinated at the N7 site in a *trans* configuration (van Vliet et al., 1995).

Therefore, we reevaluated the DNA interstrand cross-linking capability of *cis*-[Ru(bpy)$_2$$Cl_2$] with the aid of two independent methods routinely used for sensitive and quantitative determination of DNA interstrand cross-links. In accordance with the results on the ruthenium adducts of monomeric nucleic acid bases (van Vliet et al., 1994, 1995), we find in this work that *mer*-[Ru(terpy)Cl_3] cross-linked the complementary strands in double helical DNA while both isomers of *cis*-[Ru(bpy)$_2$$Cl_2$] formed other types of adducts (Figure 5).

DNA interstrand cross-links formed by several cytotoxic or antitumor agents have been proposed to be relevant to their biological activity (Larminat et al., 1993; Lee & Gibson, 1993; Masuda et al., 1988; Zwelling et al., 1979). These bidentate adducts could inhibit DNA and/or RNA synthesis (Brabec et al., 1993; Lemaire et al., 1991) or can produce in DNA a specific lesion which could be an important structural motif in recognition of damaged DNA by some specific, DNA binding proteins. *mer*-[Ru(terpy)Cl_3] forms in linear DNA ca. 5% interstrand cross-links. This amount is almost identical with that formed under similar conditions by antitumor cisplatin (Brabec & Leng, 1993). Interestingly, *mer*-[Ru(terpy)Cl_3] forms markedly more interstrand cross-links in a closed, supercoiled DNA than in linear DNA (Figure 5). This observation might be associated with the fact that formation of the interstrand cross-link in double helical DNA by *mer*-[Ru(terpy)Cl_3] requires a distortion in this biomacromolecule. Distortions have already been described in DNA duplexes containing unique interstrand lesions of cisplatin or its *trans* isomer (Brabec et al., 1993; Malinge et al., 1994). The occurrence of such distortions in DNA may be assisted by the effect of negative supercoiling. The excess energy possessed by a negatively supercoiled molecule can be used to help provide the energy needed to distort double helical DNA. The enhanced interstrand cross-linking efficiency of *mer*-[Ru(terpy)Cl_3] in supercoiled DNA might be of particular importance for understanding some aspects of molecular mechanisms of cytotoxic activity of ruthenium complexes in cells. Most DNA present in the cells exists in the negative supercoiled state complexed with specific DNA binding proteins (Kornberg & Klug, 1981; McGhee & Felsenfeld, 1981; Morse & Cantor, 1985).

In summary, polypyridyl ruthenium complexes which exhibit cytotoxic effects in tumor cells may be designed. The results presented here further support the working hypothesis that ruthenium complexes may also exhibit their biological effects via their capability to form DNA interstrand cross-links. Further investigation of the mechanism of action of these species is warranted to define their clinical potential and to contribute to a rational approach for developing a new antitumor metal-based drug with antitumor properties complementary to those exhibited by the drugs already used in clinic.

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BI950605L