DNA Interactions of Monofunctional Organometallic Osmium(II) Antitumor Complexes in Cell-Free Media

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This work is the first in-depth study of osmium binding to DNA and confirms the pharmacological activity of a new class of anticancer metallodrugs. We investigated the interactions between the potential biological target DNA and four osmium(II) arene complexes, of the type $[(\eta^6\text{-arene})Os(LL)Cl]^{n+}$, where arene = biphenyl or *p*-cymene and LL = ethylenediamine, picolinate, or oxinate in an effort to understand their mechanism of action. Most notably we show that these complexes bind to DNA. DNA adducts of the Os^{II} complexes that exhibit promising cytotoxic effects in ovarian tumor cell lines largely distort its conformation. The data are consistent with DNA binding of the complexes containing biphenyl as the arene ligand that involves combined coordination to guanine residues and noncovalent interactions between the arene ligand and DNA. The results also indicate both a mechanism of action and a detoxification mechanism for Os^{II} arene compounds different from those of cisplatin.

Introduction

Platinum coordination compounds are widely used as antitumor drugs. The clinical efficacy of these anticancer drugs is diminished by intrinsic and acquired tumor resistance and side effects. Owing to these limitations, there is an intense effort to design new transition metal-based compounds containing transition-metal ions other than platinum that are capable of overcoming problems associated with platinum chemotherapy while delivering the therapeutic effect. Possible advantages in using transition-metal ions other than platinum include the availability of additional coordination sites in octahedral complexes, the altered shape of the complex, alterations in ligand affinity and substitution kinetics, and changes in oxidation state. In the design of these new drugs, ruthenium complexes[1–3] and quite recently also osmium complexes[4,5] have attracted much interest.

Certain Ru^{II} arene complexes of the type $[(\eta^6\text{-arene})Ru(LL)(X)][Z]$ (where LL is a chelating ligand such as ethylenediamine (en)[a], X a leaving group such as Cl^-, and Z a counterion) exhibit both in vitro and in vivo activity, in some cases with activity comparable to that of cisplatin and carboplatin.[6–8] Similar to conventional cisplatin, these Ru^{II} arene complexes preferentially bind to guanine residues of DNA forming monofunctional DNA adducts that are recognized and repaired in the cell in a manner different from the bifunctional DNA adducts of cisplatin.[8] Nevertheless, in spite of the difference in chemical structure, DNA binding and downstream intracellular effects of cisplatin and organometallic Ru^{II} arene complexes, the formation and processing of their DNA adducts leads in both cases to cell death.

Recently, arene complexes of the heavier congener Os^{II} have been designed, and their chemical and cytotoxic activity has been described.[4,5,9,10] Interestingly, some half-sandwich Os^{II} arene complexes of the type $[(\eta^6\text{-arene})Os(XY)Cl]$ where arene = *p*-cymene (cym) or biphenyl (bip) and XY = N,O-chelating ligands such as picolinate (pico) showed promising activity toward human lung and ovarian cancer cells.[5]

DNA is an important potential biological target for many metal-based anticancer agents.[11] Distortions of DNA structure often correlate with anticancer activity.[2,12] Hence, it is of great importance to understand in detail DNA binding properties of these new osmium complexes and their possible relationship to cytotoxicity in different tumor cell lines. This may provide grounds for establishing new structure-pharmacological activity relationships for this class of metal-based complexes as new antitumor drugs. No work has been reported so far on the reactivity of Os arene complexes toward polymeric DNA. To address some fundamental questions about DNA binding modes of Os^{II} arene antitumor compounds, the experiments described in the present paper were carried out. More specifically, the interactions of polymeric B-DNAs with $[(\eta^6\text{-arene})Os(XY)Cl]$ where arene = *p*-cym or bip and XY = N,O-chelating ligands pico or 8-hydroxyquinolinate (oxinate) and $[(\eta^6\text{-bip})Os(en)Cl]BF_4$ (en = ethylenediamine; Figure 1) in cell-free media were investigated by various biochemical and biophysical methods with the goal of understanding their promising effects in cancer cell lines and to establish the foundations of structure-pharmacological relationships for this class of cytotoxic osmium compounds.

Results

Cytotoxicity. The cytotoxicity of complexes **1**−**4** toward both cisplatin-sensitive human ovarian A2780 and resistant (A2780cisR)

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[a] Abbreviations: bip, biphenyl; bp, base pair; cisplatin, *cis*-diamminedichloridoplatinum(II); CT, calf thymus; DEPC, diethyl pyrocarbonate; dienPt, chloridodiethylenetriamineplatinum(II) chloride; EtBr, ethidium bromide; en, ethylenediamine; FAAS, flameless atomic absorption spectrophotometry; HPLC, high pressure liquid chromatography; IC_{50}, concentration inhibiting cell growth by 50%; ICP OES, inductively coupled plasma optical emission spectroscopy; oxine, 8-hydroxyquinoline; PAGE, polyacrylamide gel electrophoresis; *p*-cym, *p*-cymene; pico, picolinate; r_b, the number of molecules of the metal complex bound per nucleotide residue; r_i, the molar ratio of free metal complex to nucleotide-phosphates at the onset of incubation with DNA; $t_{50\%}$, the times at which the binding reached 50%; t_m, DNA melting temperature.

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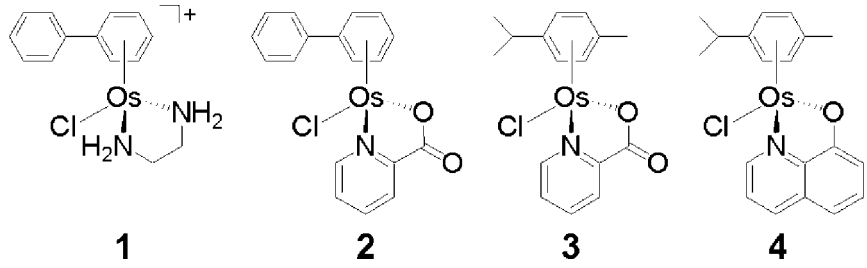

Figure 1. Structures of Os^{II} arene complexes. **1**, $[(\eta^6\text{-biphenyl})Os(ethylenediamine)Cl]^+$; **2**, $[(\eta^6\text{-biphenyl})Os(picolinate)Cl]$; **3**, $[(\eta^6\text{-}p\text{-cymene})Os(picolinate)Cl]$; **4**, $[(\eta^6\text{-}p\text{-cymene})Os(oxinate)Cl]$.

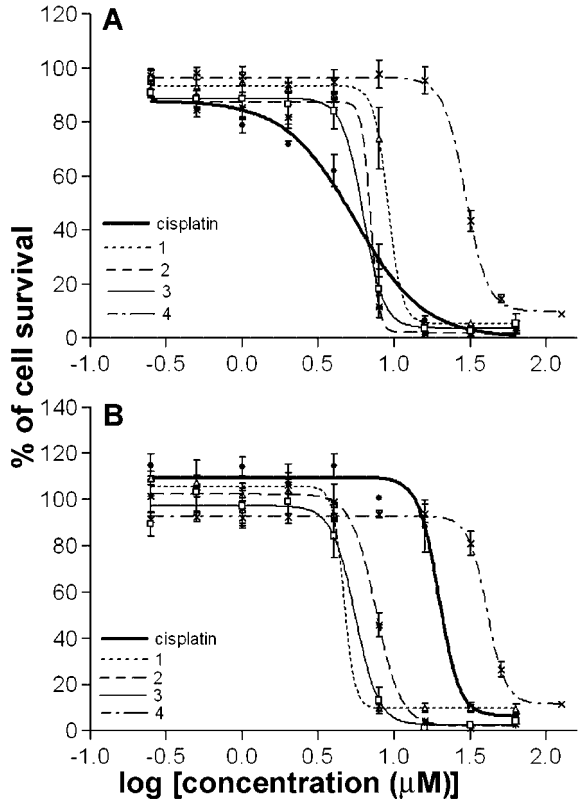

Figure 2. Dose response effects on the survival of A2780 (A) and A2780cisR (B) cancer cell lines. The cells were exposed to the Os^{II} arene complexes and cisplatin for 72 h in the concentration range of 0 to 128 μM. Cell death was determined by MTT assay. The drug concentrations causing 50% inhibition (IC_{50}) were calculated. The results are expressed as mean ± standard deviations of four independent experiments; all concentrations were tested in three replicates.

Table 1. In Vitro Growth Inhibition of Human Ovarian Cisplatin Sensitive and Resistant A2780 Cells, IC_{50} (μM)[a]

complex	sensitive	resistant[b]
cisplatin	3.6 ± 0.3	21.4 (5.9)
1	9.0 ± 0.6	5.0 (0.55)
2	6.8 ± 0.4	7.7 (1.13)
3	5.9 ± 0.4	5.6 (0.95)
4	30.3 ± 0.9	36.3 (1.2)

[a] Drug-treatment period was 72 h. Each value represents the mean ± SEM for three independent experiments. [b] Resistance factor, defined as IC_{50} (resistant)/IC_{50} (sensitive), is given in parentheses.

cancer cell lines was investigated. All complexes showed activity (Figure 2), and their corresponding IC_{50} (concentration inhibiting cell growth by 50%) values are reported in Table 1. Similar activity was found for complexes **1**−**3** with IC_{50} values ranging from 5.0 to 9.0 μM, with $[\eta^6\text{-}p\text{-cym})Os(pico)Cl]$ (**3**) showing the highest activity in cells sensitive to cisplatin and with $[\eta^6\text{-bip})Os(en)Cl]^+$ (**1**) in cells resistant to cisplatin. In contrast the complex $[\eta^6\text{-}p\text{-cym})Os(oxinate)Cl]$ (**4**) was the least potent with IC_{50} values of 30 and 36 μM in sensitive and resistant cells, respectively. Notably these complexes show similar potency in both the cisplatin-sensitive and resistant A2780 cell lines, indicating a different detoxification mechanism than cisplatin. Intriguingly, complexes **1** and **3** actually show higher activity in the cisplatin resistant A2780 cell line (5.0 and 5.6 μM, respectively) compared to the cisplatin sensitive cells (9.0 and 5.9 μM).

Table 2. Osmium and Cisplatin Uptake in A2780 Cells[a]

complex	uptake[b]
cisplatin	11.4 ±0.2
1	13.3 ± 1.0
2	14.0 ± 1.2
3	34.9 ± 2.3
4	31.6 ± 1.8

[a] Cellular osmium and cisplatin accumulation was measured by ICP OES after 6 h of treatment at equimolar concentrations of the indicated compound. Each point represents the mean ± SEM for three independent experiments. [b] Each value shown in this table is in pmole Os(Pt)/10^6 cells/μM.

Cellular Uptake. A factor that is usually thought to contribute to metallodrug cytotoxicity is cellular uptake. To examine accumulation of complexes **1**−**4**, the cellular levels of these compounds were measured after a 6 h exposure of human ovarian A2780 cancer cells to equimolar concentrations of the drugs. The uptake of these compounds was comparable with that of cisplatin for complexes **1** and **2** and approximately 2−3 times higher for complexes **3** and **4** (Table 2).

Kinetics of Binding to Calf Thymus (CT) DNA. Reactions of the cytotoxic complexes **1**−**4** with polymeric DNA were investigated, as binding to DNA is often associated with the cytotoxic action of metal anticancer drugs.[2,12] The rate of binding of the osmium complexes to CT DNA was determined at different ratios of r_i (molar ratio of free Os complex to nucleotide phosphate), 0.05 and 0.1, in 10 mM $NaClO_4$ at 37 °C in the dark. The Os^{II} complexes were incubated with the CT DNA and aliquots removed at various time intervals, rapidly cooled, and precipitated out by addition of ethanol and the Os content of the supernatant determined by inductively coupled plasma optical emission spectroscopy (ICP OES). The times at which the binding reached 50% ($t_{50\%}$) in these binding reactions and total % bound after 48 h can be found in Table 3. Intriguingly, complexes **2** $[(\eta^6\text{-bip})Os(pico)Cl]$ and **4** $[(\eta^6\text{-}p\text{-cym})Os(oxinate)Cl]$ bind rapidly ($t_{50\%}$ ca. 2 h) and almost quantitatively, whereas the complex $[(\eta^6\text{-}p\text{-cym})Os(pico)Cl]$ (**3**) binds most slowly ($t_{50\%}$ 4.9 and 8.3 h at r_i 0.05 and 0.1, respectively), and only ca. 75% is bound after 48 h.

Transcription Mapping. Cutting of pSP73KB DNA by *Nde*I and *Hpa*I restriction endonucleases yielded a 212-bp fragment

Table 3. Kinetics of Binding of Osmium(II) Arene Complexes to Calf Thymus DNA[a]

	$t_{50\%}$[b] (h) at $r_i = 0.05$	48 h (%) at $r_i = 0.05$	$t_{50\%}$[b] (h) at $r_i = 0.1$	48 h (%) at $r_i = 0.1$
1	2.1 ± 0.2	76.0 ± 0.7	4.6 ± 0.2	72.1 ± 0.8
2	1.8 ± 0.2	98.5 ± 0.6	2.1 ± 0.2	94.8 ± 0.7
3	4.9 ± 0.2	76.8 ± 0.8	8.3 ± 0.2	71.8 ± 0.8
4	0.9 ± 0.1	87.2 ± 0.7	1.6 ± 0.1	84.9 ± 0.6

[a] The concentration of DNA was 32 μg/mL. Each value represents the mean ± SEM for three independent experiments. [b] The times at which the binding reached 50%.

(a substantial part of its nucleotide sequence is shown in Figure 3B). This fragment contained the T7 RNA polymerase promotor. In vitro RNA synthesis by RNA polymerases on these DNA templates modified by osmium arene complexes **1**−**4** at the same level of metalation ($r_b = 0.005$) can be prematurely terminated at the level or in the proximity of adducts (Figure 3A). Interestingly, monofunctional DNA adducts of several platinum complexes are unable to terminate RNA synthesis.[13−15] The major stop sites, primarily guanine residues, with some adenine bases, were roughly identical for all Os complexes. The profiles are similar to that obtained for DNA treated with the anticancer drug cisplatin (lane Cisplatin in Figure 3A) and also to those reported previously for the ruthenium arene compounds, such as $[(\eta^6\text{-arene})\text{Ru(en)Cl}]^+$.[16] The major stop sites for DNA modified by **3** are demonstrated in Figure 3B. Intriguingly the distribution of the stop sites produced by biphenyl ethylenediamine complex **1** is rather in favor of shorter fragments, which is consistent with the view that the adduct of this complex presents the most difficult obstacle for RNA polymerase.

Chemical Probes. A 21-base pair (bp) DNA duplex (for its sequence, see Figure 4B) was site-specifically modified with osmium arene complexes **1**-**3** so as to form a single monofunctional G-adduct in the middle of the top, pyrimidine-rich strand. The duplex containing the DNA adduct of the *p*-cymene oxinate osmium complex **4** was impossible to prepare, purify and isolate apparently because of the instability of this adduct during the high pressure liquid chromatography (HPLC) purification process. The metalated duplexes were subsequently treated with the chemical agents $KMnO_4$, diethyl pyrocarbonate (DEPC), and bromine that are used as tools for monitoring the existence of conformations other than canonical B-DNA. These agents react preferentially with base residues in single stranded and/or in distorted double stranded DNA but not with the base residues in intact, double-stranded DNA.[17,18] The pattern and degree of reactivity toward the chemical probes were identical for the adducts formed by all three osmium arene complexes **1**−**3** (Figure 4A), indicating a similar character of the conformational distortion. The results shown in Figure 4 also suggest that the adducts formed with the DNA cause distortions which extend 4 base pairs around the adduct and that these distortions are more pronounced in the base pairs containing the metalated adduct and that containing the thymine residue flanking this adduct on its 5′ side (Figure 4B). Also interestingly, the adduct of the complex $[(\eta^6\text{-}p\text{-cym})\text{Os(pico)Cl}]$ (**3**) appears to distort DNA less than the adducts of the biphenyl complexes **1** and **2**.

Electrophoretic Mobility of Multimers of 21 bp Oligonucleotides. Intrinsic bending of DNA duplexes results in the abnormal electrophoretic mobility of DNA fragments. A gel migration anomaly has been found for DNA fragments containing bidentate adducts formed by cisplatin at the d(GG), d(AG), and d(GTG) sites.[19,20] On the other hand, the monofunctional binding of *cis*-$[\text{Pt}(\text{NH}_3)_2(\text{Am})\text{Cl}]^+$ cations, in which Am is a derivative of pyridine, pyrimidine, purine, or aniline at the d(G) site[19] and dienPt at the d(G) site,[21] keeps the helix rodlike. We have compared the electrophoretic mobility of the multimers of the ligated 21-mer duplex (for its sequence, see Figure 4B) with and without single monofunctional adducts of complexes **1**−**3** formed at the central G residue in the top strand. The corresponding multimers exhibit virtually no gel mobility shifts, migrating at almost exactly the same positions as the ladder of nonmodified multimers (results not shown). We can, therefore, conclude that no bending is induced in DNA containing monofunctional osmium adducts of **1**−**3**.

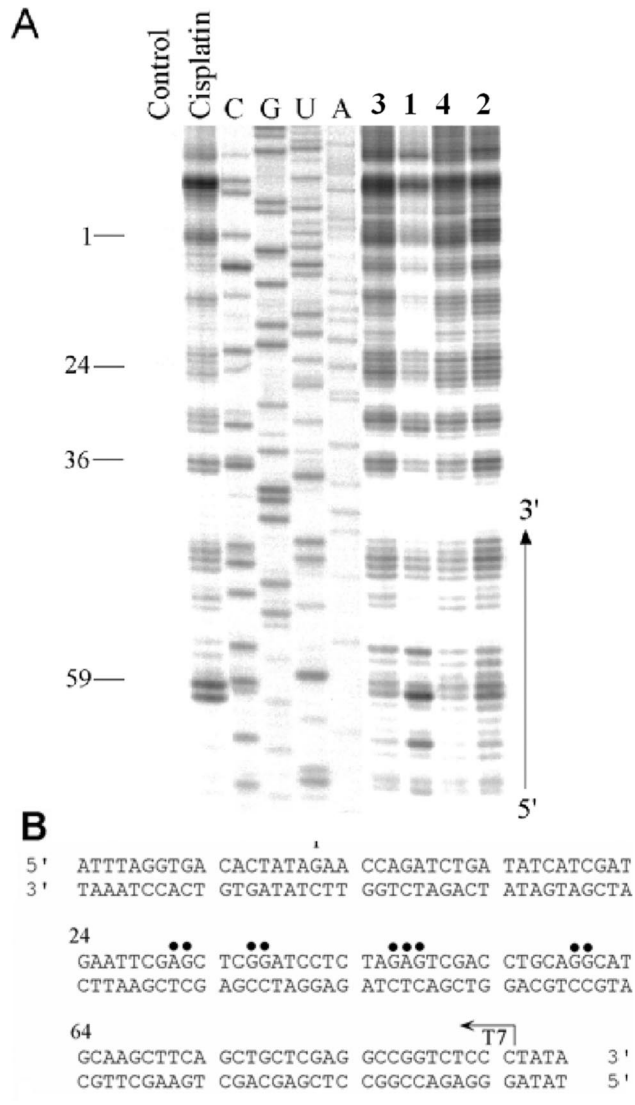

Figure 3. Inhibition of RNA synthesis by T7 RNA polymerase on the *Nde*I/*Hpa*I fragment of pSP73KB plasmid modified by Os^{II} arene complexes and cisplatin. (A) Autoradiogram of 6% polyacrylamide/8 M urea sequencing gel showing inhibition of RNA synthesis by T7 RNA polymerase on the *Nde*I/*Hpa*I fragment containing adducts of osmium complexes and cisplatin. Lanes: control, unmodified template; cisplatin, 1−4, the template modified by cisplatin and Os^{II} arene complexes **1**−**4** at $r_b = 0.005$, respectively; A, U, G, and C, chain terminated marker DNAs. (B) Schematic diagram showing the portion of the sequence used to monitor inhibition of RNA synthesis by cisplatin and osmium complexes. The arrow indicates the start of the T7 RNA polymerase, which was used as the template in the upper strand of the *Nde*I/*Hpa*I fragment of pSP73KB. The bullets represent major stop signals for DNA modified by **3**. The numbers correspond to the nucleotide numbering in the sequence map of the pSP73KB plasmid.

Unwinding of Supercoiled DNA. The unwinding of supercoiled plasmid DNA induced on binding the four osmium

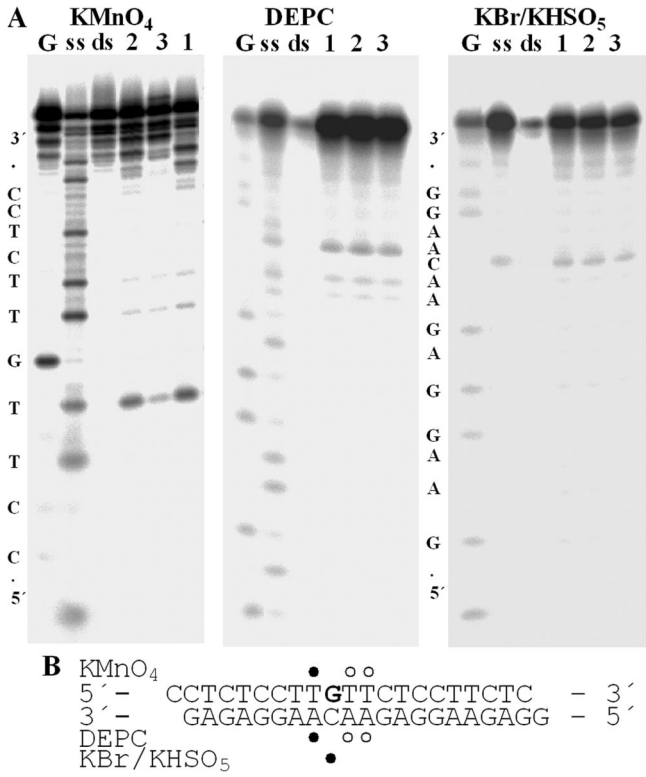

Figure 4. Chemical probes of DNA conformation. (A) Piperidine-induced specific strand cleavage at $KMnO_4$-modified, $KBr/KHSO_5$-modified, and DEPC-modified bases in the 21-bp duplex (shown at the bottom of this figure) nonmodified or containing single, site-specific monofunctional adduct of **1**−**3**. Lanes: ss, the nonmodifed strand; ds, the nonmodified duplex; 1, 2, 3, the duplex containing a unique monofunctional adduct of **1**, **2**, **3**, respectively; G, a Maxam−Gilbert specific reaction for the unplatinated duplex. The oligomers were 5′-end labeled at either the top (left panel marked $KMnO_4$) or bottom strand (middle and right panels marked DEPC and $KBr/KHSO_5$, respectively). (B) Summary of the reactivity of chemical probes with the 21-bp duplex containing single, site-specific monofunctional adduct of **1**−**3**. Closed and open circles designate strong and weak reactivity, respectively.

complexes **1**−**4**, respectively, was determined by incubating the plasmid with the osmium complex for 24 h at 37 °C at various r_b (different lanes in the gel). The resulting electrophoresis native agarose gels of DNA modified by **3** and **4** are shown in Figure 5 (top and bottom panels, respectively) as examples. A decrease in the rate of migration is the result of unwinding the DNA as this reduces the number of supercoils. The mean unwinding angle is calculated from the equation $\Phi = -18\sigma/r_b(c)$, where σ is the superhelical density and $r_b(c)$ is the r_b at which the supercoiled and nicked forms comigrate.[22] It can be seen in Figure 5 (top) that the complex [(η^6-*p*-cym)Os(pico)Cl] (**3**) causes a significant unwinding of the DNA ($\Phi = 21°$, the comigration point of the modified supercoiled and nicked DNA, $r_b(c)$, was reached at $r_b = 0.040$, Table 4). In contrast, the oxinate complex, [(η^6-*p*-cym)Os(oxinate)Cl] (**4**), does not unwind the DNA significantly, and the comigration point of the modified supercoiled and nicked DNA was not reached at as high r_b as 0.36 (Figure 5, bottom). The data are summarized in Table 4, and it can be seen that the unwinding is greatest for biphenyl ethylenediamine complex **1** (27°), followed by the biphenyl picolinate complex **2** (24°) and *p*-cymene picolinate complex **3** (21°). The high level of unwinding induced by osmium arene complexes **1**−**3** is notable.

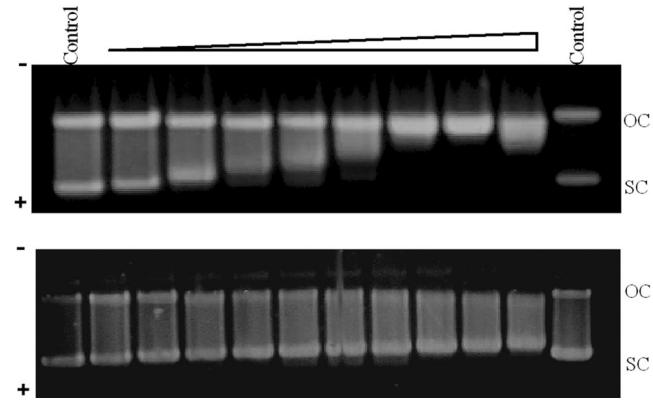

Figure 5. Unwinding of supercoiled pUC19 plasmid DNA by the compounds **3** (top) and **4** (bottom). The plasmid was incubated with the Os^{II} arene complexes for 24 h at 37 °C. Lanes in the top panel: 1 and 10, control, unmodified DNA; 2, $r_b = 0.01$; 3, $r_b = 0.015$; 4, $r_b = 0.021$; 5, $r_b = 0.028$; 6, $r_b = 0.032$; 7, $r_b = 0.036$; 8, $r_b = 0.04$; 9, $r_b = 0.048$. Lanes in the bottom panel: 1 and 12, control, unmodified DNA; 2, $r_b = 0.02$; 3, $r_b = 0.03$; 4, $r_b = 0.05$; 5, $r_b = 0.08$; 6, $r_b = 0.1$; 7, $r_b = 0.14$; 8, $r_b = 0.2$; 9, $r_b = 0.25$; 10, $r_b = 0.30$; 11, $r_b = 0.36$. The top bands in each panel correspond to the form of nicked plasmid, and the bottom bands, to the closed, negatively supercoiled plasmid.

Table 4. Unwinding of Supercoiled Plasmid DNA by Osmium(II) Arene Complexes[a]

	r_b (c)	Φ (deg)[b]
1	0.032 ± 0.005	27 ± 2
2	0.035 ± 0.005	24 ± 2
3	0.040 ± 0.005	21 ± 2
4	>0.36	<2.5

[a] Plasmid was incubated with the osmium complex for 24 h in 10 mM $NaClO_4$ at 37 °C. Each value represents the mean ± SEM for three independent experiments. [b] The unwinding angle was calculated as described in the text.

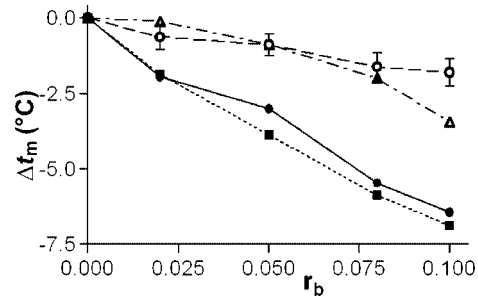

Figure 6. Plots showing the dependence of Δt_m values on r_b for CT DNA modified by Os^{II} arene complexes, **1** (Δ), **2** (○), **3** (●), and **4** (■). The melting curves were measured in 10 mM $NaClO_4$ plus 1 mM Tris-HCl with 0.1 mM EDTA, pH 7.4. Δt_m is defined as the difference between the values of metalated and nonmodified DNAs. Data measured in triplicate varied on average ± 5% from their mean.

Melting Temperature of Modified CT DNA. CT DNA was modified by the osmium arene complexes **1**−**4** at various r_b values (0−0.1) in 10 mM $NaClO_4$. The effect on the DNA melting temperature (t_m) is dependent on the nature of the osmium complex and the amount of osmium bound (r_b), as can be seen in Figure 6. In general DNA modified by osmium was destabilized and to a greater extent with increasing r_b. The destabilizing effect of the Os^{II} arene complexes on DNA is more pronounced in the case of the *p*-cymene complexes (**3** and **4**) than in the case of the biphenyl complexes (**1** and **2**). On the

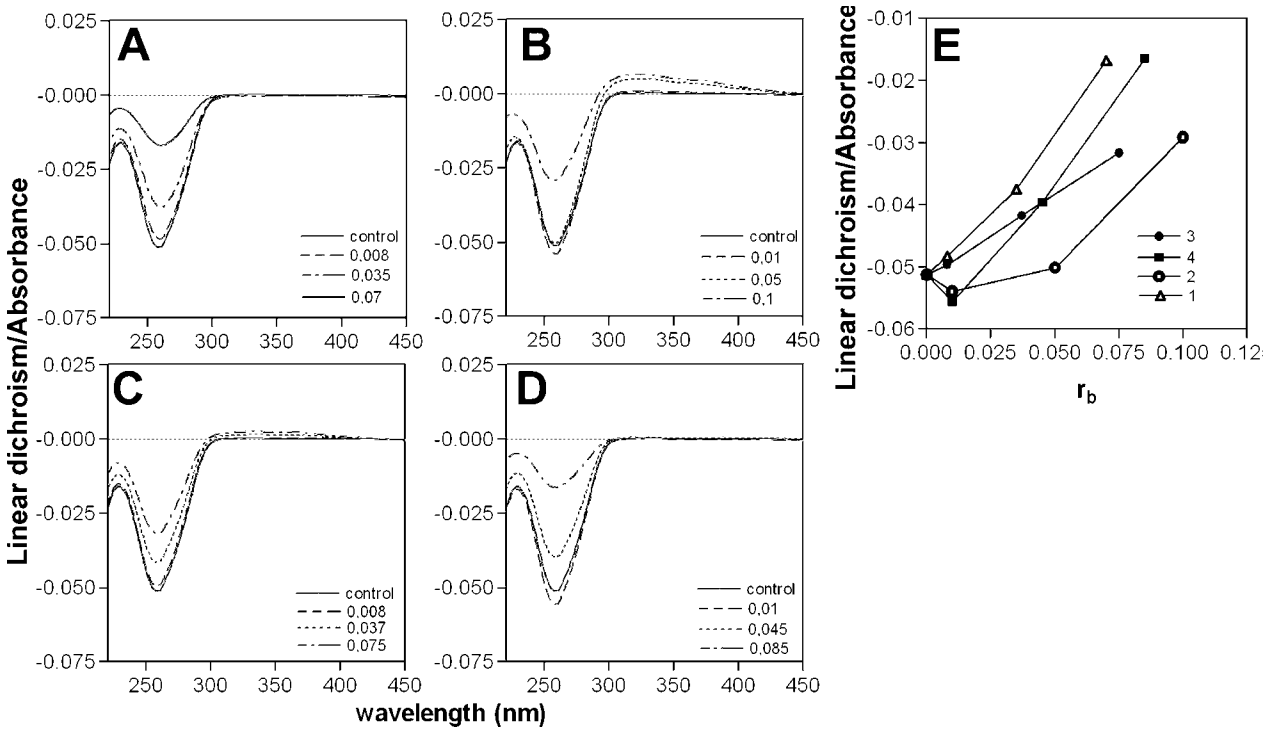

Figure 7. Linear dichroism spectra of CT DNA modified by Os^{II} arene complexes. LD spectra were recorded for DNA in 10 mM $NaClO_4$, 20 mM NaCl, and 10 mM sodium cacodylate, pH 7.0. The concentration of DNA was 0.1 mg/mL. (A−D) LD spectra of CT DNA modified by **1** (A) (thick solid line, control, nonmodified DNA; dashed line, $r_b = 0.008$; dash-dotted line, $r_b = 0.035$; solid line, $r_b = 0.07$); **2** (B) (thick solid line, control, nonmodified DNA; dashed line, $r_b = 0.01$; dotted line, $r_b = 0.05$; dash-dotted line, $r_b = 0.1$); **3** (C) (thick solid line, control, nonmodified DNA; dashed line, $r_b = 0.008$; dotted line, $r_b = 0.037$; dash-dotted line, $r_b = 0.075$); and **4** (D) (thick solid line, control, nonmodified DNA; dashed line, $r_b = 0.01$; dotted line, $r_b = 0.045$; dash-dotted line, $r_b = 0.085$). (E) Plots of the intensity of the band in LD spectra at 258 nm of DNA modified by complexes **1** (Δ), **2** (○), **3** (●), and **4** (■) versus r_b.

contrary, there is little difference between the complexes carrying the same axial ligand, either *p*-cymene or biphenyl.

Linear Dichroism. Binding of all three osmium complexes to CT DNA was also monitored by linear dichroism spectroscopy (Figure 7). It is well established that the magnitude of the LD signal measured within the DNA absorption band (e.g., at the 258 nm maximum) is a function of its persistence length. It is known that changes in flexibilities, or the formation of rigid bends or kinks induced by strongly bound compounds, can manifest themselves as decreases in the abilities of the modified DNA molecules to align themselves in the hydrodynamic flow gradient of the LD cell. The magnitudes of the LD signals at 258 nm decrease as a function of r_b for all Os^{II} arene complexes **1**−**4** (Figure 7E). These results suggest that the formation of strongly bound adducts derived from Os^{II} arene complexes is accompanied by the appearance of flexible hinge joints at the site of the lesion. Another eventuality, such as appearance of rigid bends or kinks, is unlikely based on the results of gel electrophoresis analysis of multimers of site-specifically modified oligonucleotides (vide supra). In addition, treatment of the DNA with complexes **2** and **3** produces a new and weak positive band at 330 nm, which increases more significantly for the biphenyl picolinate complex **2** compared to the *p*-cymene picolinate complex **3**.

Ethidium Bromide (EtBr) Fluorescence. The ability of a complex to displace the DNA intercalator EtBr from CT DNA was probed by monitoring the relative fluorescence of the EtBr−DNA complex after treating the DNA with varying concentrations of the Os^{II} arene complexes **1**−**4**. Figure 8 shows a plot of relative fluorescence versus r_b for complexes **1**−**4**, cisplatin, and monofunctional dienPt (chloridodiethylenetriamineplatinum(II) chloride). The adducts of all four monofunctional Os^{II} arene complexes competitively replaced intercalated EtBr markedly more effectively than the adducts of monofunctional dienPt. The adducts of biphenyl complexes **1** and **2** are most potent. The adducts of the other two Os^{II} *p*-cymene complexes reduced EtBr fluorescence less effectively but still slightly more than the adducts of bifunctional cisplatin.

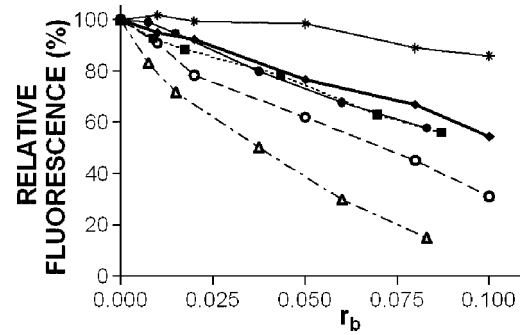

Figure 8. Plots of the EtBr fluorescence versus r_b for DNA modified by cisplatin, dienPt, and Os^{II} arene complexes in 10 mM $NaClO_4$ at 37 °C for 24 h: (♦) cisplatin, (*) [PtCl(dien)]Cl, **1** (Δ), **2** (○), **3** (●), and **4** (■). Data points measured in triplicate varied on average ± 3% from their mean.

Discussion

The four complexes investigated differ from each other in the following ways. First of all complex **1**, $[(\eta^6\text{-bip})Os(en)Cl]^+$, is the only positively charged complex and after hydrolysis (believed to activate the complex) would possess an overall

positive charge of +2. Clearly its electrostatic interactions with negatively charged DNA will be different than for the remaining three complexes. Second the chelated NH_2 groups in complex **1** are capable of hydrogen bonding.[23] In contrast, in the remaining complexes the N-donor group is a pyridine, which is unable to take part in hydrogen bonding. Complexes **2** and **3** differ from one another in that the arene varies, extended biphenyl arene in **2** (capable of intercalation in the adducts of analogous Ru^{II} arene complexes containing a symmetrical *N*,*N*-chelating ligand[16]) and a single ring arene with bulky substituents (*p*-cymene) in **3**. Complex **4** differs from **3** primarily in the nature of the O-donor group, which in the latter case is an aryloxide donor as opposed to the carboxylate group in **3**. A major difference is the pK_a of this chelated oxygen (its acidity) and the higher partial negative charge donated to the osmium. Clearly the chemistry of these four complexes is different,[5,9] and consequently we would anticipate that their effects on DNA would be different as well.

We report in the present work the first detailed DNA binding study of Os^{II} arene complexes, which have been shown[5,24] to be a potential new class of anticancer agents (Table 1). In addition, we investigated the effects and extent of changes induced in the DNA on binding of osmium and compared these observations with other metal-based anticancer agents.

Though we have previously reported the binding of these complexes to nucleobases,[4,5,9] it is notable that these complexes all bind polymeric DNA. Binding to DNA has often been associated with the cytotoxic action of metal-based anticancer agents,[2,11,12] and therefore DNA may be a possible biological target for this class of Os^{II} arene complexes. The cell uptake studies (Table 2) also suggest that the type of DNA lesion is important for activity since, despite its low activity, the cellular levels of Os from **4** are higher than those of complexes **1** or **2**.

CT DNA was treated with osmium solutions, yet their subsequent rates of reaction with DNA (Table 3) do not correlate with their rates of hydrolysis. Rates of irreversible binding to DNA increase in the order **3** (binds most slowly) < **1** ~ **2** < **4** (rapid binding), whereas their rates of hydrolysis, previously determined at 25 °C and pH 2, are such that **1** hydrolyzes most slowly, followed by **2** and then **3**, with **4** hydrolyzing too rapidly to be measured by ^{1}H NMR.[4,5,9] Therefore although hydrolysis may be rate-determining for some complexes (e.g., **4**) other factors such as electrostatic interactions may also play a role.

The rate of binding to DNA compares well with that determined for the anticancer drug cisplatin ($t_{1/2}$ ca. 2 h under similar conditions),[25] for which DNA binding is thought to be responsible for its cytotoxic properties. In contrast, the Ru^{II} analogue of **1**, $[(\eta^6\text{-bip})Ru(en)Cl]^+$, which has also been shown to be cytotoxic to cancer cells,[6–8] reacts much more rapidly with DNA under similar conditions (t_{50} ca. 10 min).[16] The slower kinetics of osmium binding (28 times slower comparing **1** with its lighter Ru^{II} analogue) may allow more of it to reach its target in vivo than the ruthenium analogue, which is more reactive and likely to be deactivated by reacting with other biological molecules before reaching the target DNA within the cell nucleus. The Os^{II} biphenyl picolinate complex, $[(\eta^6\text{-bip})Os(pico)Cl]$ (**2**), reacts almost quantitatively with the DNA and is the only complex to do so. In addition, replacing the extended biphenyl arene by the single ring arene, *p*-cymene, as in **3**, results in a marked decrease in DNA binding (to ca. 76%). For all the osmium complexes, >90% of the equilibrium had been reached within the first 24 h.

Osmium binding to DNA inhibits RNA synthesis in a similar fashion and with similar stopsites to cisplatin and the ruthenium analogue of **1**, $[(\eta^6\text{-bip})Ru(en)Cl]^+$, and in both cases is thought to be significant in their cytotoxic mechanism of action (Figure 3). The major stopsites were guanine residues, which agrees well with the small molecule binding studies performed previously which show that complex **1** binds selectively to monomeric guanine[9] and complex **3** binds more strongly and selectively to monomeric guanine in competition experiments.[5]

The distortions induced on binding to DNA extend 4 base pairs around the adduct for the three Os^{II} arene complexes studied (**1**−**3**) (Figure 4B), which is similar compared to DNA binding of ruthenium arene analogues containing multiring arene ligands.[16]

The significant result obtained from the ligation experiment was that formation of the DNA adducts of Os^{II} arene compounds does not result in DNA bending. As this bending and subsequent binding of HMG (high mobility group) proteins to damaged/bent DNA is thought to be responsible for the cytotoxic action of cisplatin in tumor cells,[12,26] we can conclude that the cytotoxic mechanism of action of Os^{II} arene complexes is different from that of cisplatin.

The binding of Os^{II} complexes **1**−**3** to DNA results in a significantly large degree of unwinding (21−27°; Table 4), much larger than that observed for the Ru^{II} complexes $[(\eta^6\text{-arene})Ru(en)Cl]^+$ (7−14°)[16] or cisplatin (6° and 13° for mono- or bifunctional adducts, respectively).[22] Similar large unwinding angles in the range of 17−30° have been observed for the adducts of several antitumor platinum compounds containing heterocyclic planar or nonplanar ligands.[27,28] Thus, the large unwinding angles produced by the adducts of Os^{II} arene compounds **1**−**3** can be explained by the additional contribution to unwinding associated with the interaction of the arene ligand with the duplex upon strong binding of osmium. Complex **4** consistently behaves differently to the other Os^{II} arene complexes, which is similar to the reports of its aqueous solution behavior (rapid hydrolysis and high acidity of coordinated water). Most notably it was reported that the chelated oxygen atom is readily protonated about physiological pH, and a dynamic pH-dependent ring-opening process at the osmium center was observed.[5] This suggests that adducts of **4** on DNA would be less stable and the chelate ring opening would not allow the osmium to enforce any significant constraints on the DNA. In other words, the arene moiety in DNA adducts of Os^{II} arene compounds **1**−**3** could be geometrically well-positioned to interact with the double helix. In contrast, the oxinate complex, $[(\eta^6\text{-}p\text{-cym})Os(oxinate)Cl]$ (**4**), does not unwind the DNA significantly (<2.5°). The explanation behind this phenomenon is unclear, nevertheless it may be hypothesized that the presence of the oxinate chelating ligand in Os^{II} arene complexes is not favorable for the interaction of the arene rings in these complexes with the double helix. In summary, it seems reasonable to suggest that the ligands in **4** do not interact with the double helix in a way similar to other Os^{II} complexes **1**−**3**, thus also supporting a different DNA binding mode for this compound in comparison with the other three Os^{II} arene complexes studied in the present work. In particular, complexes **1** and **2** containing the extended biphenyl arene capable of intercalating were potent at replacing the EtBr intercalator compared to complexes **3** and **4** containing the single arene ring, *p*-cymene.

EtBr as a fluorescent probe can be used to distinguish intercalating and nonintercalating ligands.[16,22,28] Binding of EtBr to DNA by intercalation is blocked in a stoichiometric manner by formation of a wide spectrum of DNA-binding ligands including intercalators. On the other hand, modification of DNA

by monofunctional nonintercalative ligands, such as dienPt, results in only a slight decrease of EtBr fluorescence intensity as compared with that for the complex of nonmodified DNA with EtBr. Competitive binding of other intercalators leads to a loss of fluorescence because of depletion of the DNA−EtBr complex (free EtBr is poorly fluorescent).

The adducts of all Os^{II} complexes replace the EtBr intercalator slightly or markedly more efficiently than those of cisplatin (Figure 8). The adducts of compounds **1**−**3** unwind DNA by 21−27° (Table 4); that is, the values of the unwinding angles are considerably higher than those produced by the monofunctional adducts of dienPt (unwinding angle 6° [16,22,28]). Thus, the results of unwinding experiments are consistent with the view that the arene ligands in **1**−**3** interact substantially with the double helix upon coordination of the osmium complex.[16,22,28] Hence, these results strengthen the case for combined noncovalent, perhaps intercalative and monofunctional coordination binding modes of **1**−**3**.

In contrast, the oxinate complex, [(η^6-*p*-cym)Os(oxinate)Cl] (**4**), does not unwind the DNA significantly (<2.5°, Table 4) but replaces the EtBr intercalator as efficiently as the Os^{II} complex **3**. In aggregate, it seems reasonable to suggest that the ligands in **4** do not interact with the double helix in a way similar to other Os^{II} complexes **1**−**3**, thus also supporting a different DNA binding mode for this compound in comparison with the other three Os arene complexes studied in the present work.

The noncovalent interactions of arenes, which may be involved in the binding of the Os^{II} arene compounds **1** and **2** to double-helical DNA (vide supra), may also affect its melting behavior (Figure 6). Previously,[16] two important factors have been invoked to account for the thermal stability of DNA modified by monofunctional Ru^{II} complexes in media of relatively low ionic strength (0.01 M Na^+): (i) a destabilizing effect of conformational distortions and (ii) a stabilizing effect of the positive charge on the ruthenium moieties and of noncovalent binding, such as changes in solvent structure and the counterion distribution around the phosphate groups of DNA which may help to overcome electrostatics unfavorable for the hybridization of the strands of the duplex.[29,30] Under the conditions of our experiments, we expect all Os^{II} arene complexes to have produced monofunctional adducts. Inherently, we predict that conformational distortions due to the formation of the adducts will destabilize the helix, as has been consistently observed in earlier studies with various ruthenium and platinum compounds. Hence, it is possible that the less pronounced decrease in t_m due to the modification by the Os^{II} compounds **1** and **2** (Figure 6) is a consequence of compensation of destabilizing effects of conformational changes. This stabilizing compensation might be associated with noncovalent interaction of the arene ligand with the duplex inferred from DNA unwinding (Figure 5, Table 4) and quenching EtBr fluorescence (Figure 8) and with the overall positive charge on these Os^{II} compounds. In addition, the stabilizing effects of the positive charge on the osmium atom of the compounds **3** and **4** might be considerably reduced due to a substantially different location of the osmium atom in the adducts of these compounds relative to the DNA sugar−phosphate backbone. This location might be unfavorable from the viewpoint of the efficiency of the positive charge on the osmium atom to neutralize negative charges of DNA phosphate groups. Thus, the solution behavior of the DNA adducts of Os^{II} arene complexes appears interesting and merits further study.

The results of DNA unwinding experiments (Figure 5, Table 4) suggest that the arene ligand (*p*-cymene) in **3** also interacts with the double helix upon coordination of the osmium complex. However, the adducts of this Os^{II} complex thermally destabilize DNA similarly as those of **4** [whose arene ligand (*p*-cymene) apparently does not interact noncovalently with DNA to induce distinct unwinding of its double helical structure]. The explanation of this is unclear but may be associated with a different DNA noncovalent binding mode of the *p*-cymene ligand in Os^{II} arene complexes compared to that of the biphenyl ligand. This hypothesis is corroborated by the observation that the single-ring *p*-cymene arene ligand (in contrast to double-ring biphenyl arene ligand) in analogous monofunctional Ru^{II} arene ethylenediamine complexes does not intercalate in the DNA base-pair stack.[16,31]

The results of the present work demonstrate cytotoxicity for these complexes in ovarian cell lines, and importantly, the activity in the cells sensitive and resistant to cisplatin was also determined (Figure 2 and Table 1). That the osmium(II) arene complexes show very similar activity in both cell lines is highly significant and indicates a different detoxification mechanism for this class of complexes. Intriguingly, complex **1**, [(η^6-bip)Os(en)Cl]$^+$, shows even greater activity in the cisplatin-resistant cell line (resistance factor of 0.55). Such results indicate promising compounds with which to tackle the common problem of developed cisplatin resistance which can occur during chemotherapy treatment. On the other hand, the markedly lower activity associated with complex **4**, [(η^6-*p*-cym)Os(oxinate)Cl], correlates with its different binding to DNA and with its different aqueous solution chemistry compared with the picolinate complexes (**2** and **3**).

Experimental Section

Starting Materials. The osmium complexes were prepared and characterized as described previously.[5,9] Cisplatin was obtained from Sigma-Aldrich sro (Prague, Czech Republic). dienPt was a generous gift of Professor G. Natile from University of Bari. Stock solutions of metal complexes for the biophysical and biochemical studies were prepared at the concentration of 2×10^{-4} M in 10 mM $NaClO_4$ and stored at 4 °C in the dark. Stock solutions of metal complexes for the cytotoxicity studies were prepared in DMSO and used immediately after dissolution. The concentrations of osmium or platinum in the stock solutions were determined by ICP OES. CT DNA (42% G + C, mean molecular mass ca. 2×10^7) was also prepared and characterized as described previously.[32,33] pSP73KB (2455 bp) and pUC19 (2686 bp) plasmids (superhelical density $\sigma = -0.063$ and -0.055, respectively) were isolated according to standard procedures. The synthetic oligodeoxyribonucleotides (21-mers) were purchased from VBC-Genomics (Vienna, Austria) and purified as described previously.[21,34] Restriction endonucleases *Eco*RI and *Nde*I and T4 polynucleotide kinase were purchased from New England Biolabs. Dimethyl sulfate (DMS), DMSO, $KMnO_4$, DEPC, KBr, and $KHSO_5$ were from Sigma (Prague, Czech Republic). Acrylamide, bis(acrylamide), and EtBr were from Merck KgaA (Darmstadt, Germany). Agarose was from FMC BioProducts (Rockland, ME). Radioactive products were from MP Biomedicals, LLC (Irvine, CA).

Metalation Reactions. CT DNA and plasmid DNAs were incubated with osmium or platinum complex in 10 mM $NaClO_4$ (pH ~ 6) at 37 °C for 48 h in the dark, if not stated otherwise. The number of atoms of the metal bound per nucleotide residue (r_b values) was determined by ICP OES (osmium) or FAAS (platinum).

The single-stranded oligonucleotide (the top, pyrimidine rich, strand containing a single central G of the TGT(21) duplex, Figure 4B) (5×10^{-4} M) was reacted in stoichiometric amounts with **1**, **2**, and **3**. The metalated oligonucleotides were purified by ion-exchange HPLC. It was verified by ICP OES and by absorbance

measurements that the modified oligonucleotides contained one osmium atom per mole. It was also verified using DMS footprinting[13] that one molecule of osmium complex was coordinated to the N7 atom of the single G in the top strand of each duplex.

DNA Transcription by RNA Polymerase in Vitro. Transcription of the (*Nde*I/*Hpa*I) restriction fragment of pSP73KB DNA with T7 RNA polymerase and electrophoretic analysis of the transcripts was performed according to the protocols recommended by Promega (Promega Protocols and Applications, 43-46 (1989/90)) and previously described in detail.[13,14] The DNA concentration used in this assay was 3.9×10^{-5} M (related to the monomeric nucleotide content).

Chemical Modifications. The modification of the metalated oligonucleotide duplexes by $KMnO_4$, DEPC, and $KBr/KHSO_5$ was performed as described previously.[18,35–37] The top or bottom strands of the oligonucleotide duplexes were 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Ligation and Electrophoresis of Oligonucleotides. Unmodified 21-mer single strand (bottom strand of the duplex described in the Results section, DNA Unwinding and Bending paragraph) were 5′-end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Then they were annealed with their phosphorylated complementary strands (unmodified or containing the monofunctional osmium adduct). The duplexes were allowed to react with T4 DNA ligase. The resulting samples along with ligated unmetalated duplexes were subsequently examined on 8% native PAA [mono:bis(acrylamide) ratio = 29:1] electrophoresis gels. Other details of these experiments were as described in previous papers.[19,34,38]

Unwinding of Negatively Supercoiled DNA. Unwinding of closed circular supercoiled pUC19 plasmid DNA was assayed by an agarose gel mobility shift assay.[22] The unwinding angle Φ, induced per osmium−DNA adduct, was calculated upon the determination of the r_b value at which the complete transformation of the supercoiled to relaxed form of the plasmid was attained. Samples of plasmid DNA at the concentration of 1.6×10^{-4} M (related to the monomeric nucleotide content) were incubated with complexes **1**−**4** at 37 °C in the dark for 24 h. All samples were precipitated by ethanol and redissolved in the TAE (Tris-aceate/EDTA) buffer. One aliquot of the precipitated sample was subjected to electrophoresis on 1% agarose gels running at 25 °C in the dark with TAE buffer, and the voltage was set at 25 V. The gels were then stained with EtBr, followed by photography with transilluminator. The other aliquot was used for the determination of r_b values by ICP OES.

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

Flow Linear Dichroism (LD). Flow LD spectra were collected by using a flow Couette cell in a Jasco J-720 spectropolarimeter adapted for LD measurements. Long molecules, such as DNA (minimum length of ∼250 bp), can be orientated in a flow Couette cell. The flow cell consists of a fixed outer cylinder and a rotating solid quartz inner cylinder, separated by a gap of 0.5 mm, giving a total path length of 1 mm. LD spectra of DNA at the concentration of 0.1 μg/mL modified by the osmium complexes were recorded at 25 °C in 10 mM $NaClO_4$ plus 20 mM NaCl and 10 mM sodium cacodylate, pH 7.0.[39,40]

Fluorescence Measurements. These measurements were performed on a Shimadzu RF 40 spectrofluorophotometer using a 1 cm quartz cell. Fluorescence measurements of DNA modified by osmium at the concentration of 32 μg/mL in the presence of EtBr were performed at an excitation wavelength of 546 nm, and the emitted fluorescence was analyzed at 590 nm. The fluorescence intensity was measured at 25 °C in 0.4 M NaCl to avoid secondary binding of EtBr to DNA.[41,42] 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.[41]

Other Physical Methods. Absorption spectra were measured with a Varian Cary 4000 UV−vis spectrophotometer equipped with a thermoelectrically controlled cell holder and quartz cells with a path length of 1 cm. Purification of oligonucleotides with the aid of HPLC was carried out on a Waters HPLC system consisting of Waters 262 pump, Waters 2487 UV detector, and Waters 600S controller with MonoQ HR 5/5 column. The analysis with the aid of ICP OES was perfomed using Jobin Yvon, Ultrace 170 equipment. The FAAS measurements were carried out on a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer. For ICP OES and FAAS analyses, DNA was precipitated with ethanol and dissolved in 0.1 M HCl. The gels were visualized by using a BAS 2500 FUJIFILM bioimaging analyzer, and the radioactivity associated with bands was quantitated with the AIDA image analyzer software (Raytest, Germany).

Cytotoxicity. The human ovarian tumor cell lines A2780 (parent, cisplatin-sensitive) and A2780cisR (with acquired cisplatin resistance) were cultured in RPMI 1640 medium (Gibco), supplemented with 10% FBS, 2 mM glutamine, 50 μg/mL gentamycin at 37 °C in an atmosphere of 95% air and 5% CO_2. Cell death was evaluated by using a system based on the tetrazolium compound MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] which is reduced by living cells to yield a soluble formazan product that can be detected colorimetrically.[43] Cells were seeded in 96-well sterile plates at a density of 10^4 cells/well in 100 μL of medium and were incubated for 16 h. Osmium complexes were dissolved in DMSO; the stock solutions were freshly prepared before use. The final concentration of DMSO in cell culture medium did not exceed 0.25%. The compounds were added to final concentrations from 0 to 128 μM in a volume of 100 μL/well. Seventy-two hours later 10 μL of a freshly diluted MTT solution (2.5 mg/mL) was pipetted into each well, and the plate was incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 5 h the medium was removed and the formazan product was dissolved in 100 μL of DMSO. The cell viability was evaluated by measurement of the absorbance at 570 nm, using an Absorbance Reader SUNRICE TECAN SCHOELLER. All experiments were made in triplicate. IC_{50} values (compound concentration that produces 50% of cell growth inhibition) were calculated from curves constructed by plotting cell survival (%) versus drug concentration (μM). All experiments were made in triplicate.

Cellular Os^{II} Arene Complex Uptake. Cellular uptake of Os^{II} arene compounds and cisplatin was measured in A2780 cells. The cells were seeded in 60 mm tissue culture dishes (30 000/cm^2). After overnight incubation, the cells were treated with the osmium compound or cisplatin for 6 h at equimolar concentration (10 μM); this concentration was verified by the measurement of osmium or platinum in the growing medium by ICP OES. The attached cells were washed twice with PBS (4 °C) and centrifuged at 2500 rpm, and the pellet was stored at −80 °C. Afterward, the pellets were digested with 12 M HNO_3, 30% H_2O_2, and 12.1 M HCl. Osmium and platinum content was determined by ICP OES.

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