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DNA interactions of dinuclear Ru^{II} arene antitumor complexes in cell-free media

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ABSTRACT

We recently synthesized and characterized water-soluble dinuclear Ru^{II} arene complexes, in which two {(η^6-p-isopropyltoluene)RuCl[3-(oxo-κO)-2-methyl-4-pyridinonato-κO_4]} units were linked by flexible chains of different length [$(CH_2)_n$ (n = 4, 6, 8, 12)]. These new dinuclear ruthenium drugs were found to exert promising cytotoxic effects in human cancer cells. In the present work DNA modifications by these new dinuclear Ru^{II} arene compounds, which differed in the length of the linker between the two Ru^{II} centers, were examined by biochemical and biophysical methods. The complexes bind DNA forming intrastrand and interstrand cross-links in one DNA molecule in the absence of proteins. An intriguing aspect of the DNA-binding mode of these dinuclear Ru^{II} compounds is that they can cross-link two DNA duplexes and also proteins to DNA—a feature not observed for other antitumor ruthenium complexes. Thus, the concept for the design of interhelical and DNA–protein cross-linking agents based on dinuclear Ru^{II} arene complexes with sufficiently long linkers between two Ru centers may result in new compounds which exhibit a variety of biological effects and can be also useful in nucleic acids research.

1. Introduction

Ruthenium(II) organometallic complexes have been gaining popular interest as potential anticancer agents [1–6]. One group of these compounds that exhibit anticancer activity, including activity against cisplatin (*cis*-diamminedichloridoplatinum(II)) resistant cancer cells, comprises organoruthenium complexes of the type [(η^6-arene)Ru^{II}(en)Cl]$^+$, where arene = benzene or a benzene derivative, and en = 1,2-diaminoethane [7,8]. This class of complexes readily interact with DNA, which is considered an important pharmacological target of a number of metal-based antitumor drugs [5,9,10],

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Abbreviations: bp, base pair; CD, circular dichroism; cisplatin, *cis*-diamminedichloridoplatinum(II); CL, cross-link; CT, calf thymus; DMS, dimethyl sulphate; EtBr, ethidium bromide; FAAS, flameless atomic absorption spectrometry; IC_{50}, the concentration of the compound that afforded 50% cell killing; ICD, induced circular dichroism; KF^-, Klenow fragment from DNA polymerase I, exonuclease minus mutated to remove the 3′ → 5′ proofreading activity; [PtCl(dien)]Cl, chloridodiethylenetriamineplatinum(II) chloride; r_b, the number of molecules of the metal-based compound bound per nucleotide residue; r_i, the molar ratio of free metal complex to nucleotides at the onset of incubation with DNA; Ru(4), 1,4-bis{chlorido[3-(oxo-κO)-2-methyl-4-pyridinonato-κO_4](η^6-p-isopropyltoluene)ruthenium}butane; Ru(6), 1,6-bis{chlorido[3-(oxo-κO)-2-methyl-4-pyridinonato-κO_4](η^6-p-isopropyltoluene)ruthenium}hexane; Ru(8), 1,8-bis{chlorido[3-(oxo-κO)-2-methyl-4-pyridinonato-κO_4](η^6-p-isopropyltoluene)ruthenium}octane; Ru(12), 1,12-bis{chlorido[3-(oxo-κO)-2-methyl-4-pyridinonato-κO_4](η^6-p-isopropyltoluene)ruthenium}dodecane; SDS, sodium dodecyl sulphate.

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and they can form stable monofunctional adducts with DNA. These adducts affect the DNA conformation and are recognized by downstream cellular systems in a unique way [11–13]. Therefore, much attention is being paid at present to the design of new ruthenium complexes that would exhibit new DNA-binding properties that differ from those already described [5,14].

An interesting class of metallodrugs that bind to DNA in a unique way, and consequently exhibit new biological properties is represented by polynuclear platinum or ruthenium complexes with flexible or sterically rigid linking groups [15–23]. Recently, water-soluble dinuclear Ru^{II} arene complexes, in which two {(η^6-p-isopropyltoluene)RuCl[3-(oxo-κO)-2-methyl-4-pyridinonato-κO_4]} units were linked by chains of different length [$(CH_2)_n$ (n = 4, 6, 8, 12)] (Fig. 1), were reported [24]. These new dinuclear ruthenium drugs were found to exert promising cytotoxic effects in human cancer cells being markedly more active than the mononuclear analogues [24] and even more interestingly, their potency increased with growing length of the linker between the ruthenium centers. In addition, several studies on chemical behavior of these dinuclear Ru(II) arene complexes have been conducted [25] and manuscript in preparation. The hydrolysis appears to be very fast and complete. These studies also demonstrate that both chloro groups in the dinuclear ruthenium complexes are replaced by water ligands. No evidence for cleavage of the dinuclear species was observed in these studies, and the hydrolysis was the only alteration of the original molecules. If the dinuclear ruthenium complexes reacted with monomeric nucleotides, a very fast reaction with guanosine- and adenosine-monophosphates was observed, but no reaction with cytidine, uridine, or thymidine monophosphates.

We analyzed in the present work the DNA-binding mode of these new dinuclear ruthenium arene complexes by various methods of molecular biology and biophysics and found that they bound to DNA in a unique way forming also interhelical and DNA–protein cross-links—a feature not observed for other ruthenium complexes hitherto tested for biological activity.

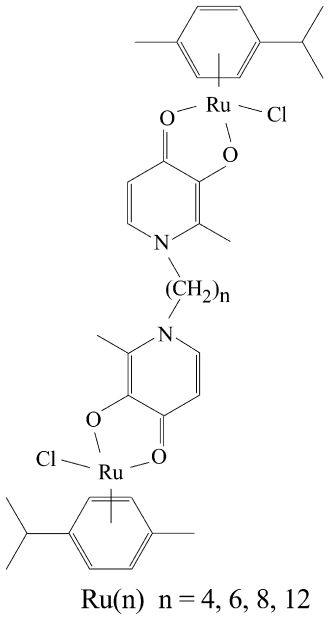

Fig. 1 – Structures of the dinuclear Ru^{II} arene complexes used in the present work and their abbreviations.

2. Materials and methods

2.1. Starting materials

The dinuclear Ru^{II} arene complexes (Fig. 1) were prepared and characterized as described previously [24,25]. Cisplatin was obtained from Sigma–Aldrich s.r.o. (Prague, Czech Republic). Chloridodiethylenetriamineplatinum(II) chloride ([PtCl(dien)]Cl) was a generous gift of Prof. G. Natile from the University of Bari. Stock solutions of metal complexes for the biophysical and biochemical studies were prepared at a concentration of 4×10^{-4} M in double distilled water and stored at −20 °C in the dark. The concentrations of ruthenium or platinum in the stock solutions were determined by flameless atomic absorption spectrometry (FAAS) or electron absorption spectrophotometry. Calf-thymus (CT) DNA (42% G + C, mean molecular mass ca. 2×10^7) was also prepared and characterized as described previously [26,27]. pSP73 [2464 base pairs (bp)] and pSP73KB (2455 bp) plasmids were isolated according to standard procedures. 107-bp, 221-bp, and 801-bp DNA fragments were isolated and purified in the same way as described recently [28]. Restriction endonucleases EcoRI, HpaI, NdeI, and PvuI, T4 polynucleotide kinase, and Klenow fragment from DNA polymerase I (exonuclease minus–KF^-, mutated to remove the 3′ → 5′ proofreading activity) were purchased from New England Biolabs (Frankfurt am Main, Germany). O'GeneRuler™ 1 kb Plus DNA Ladder Plus, ready-to-use was from Fermentas UAB (Vilnius, Lithuania) and histone H1 from Roche Diagnostics, GmbH (Mannheim, Germany). Dithiothreitol and ethidium bromide (EtBr) were from Merck KGaA (Darmstadt, Germany), sodium dodecyl sulphate (SDS) from Serva (Heidelberg, Germany), and proteinase K from Boehringer (Mannheim, Germany). Agarose and Metaphor® agarose were from FMC BioProducts (Rockland, ME). Wizard® SV and PCR Clean-Up System used to extract and purify 107-bp, 221-bp, and 801-bp DNA fragments (*vide infra*) were purchased from Promega (Mannheim, Germany). Nonidet P-30 was from Fluka (Prague, Czech Republic), and radioactive products were from MP Biomedicals, LLC (Irvine, CA).

2.2. Metalation reactions

CT DNA and plasmid DNAs were incubated with ruthenium or platinum complex in 10 mM $NaClO_4$ (pH ∼ 6) at 37 °C for 24 h in the dark, if not stated otherwise. The number of atoms of the metal-based compound bound per nucleotide residue (r_b values) were determined by FAAS and/or absorption spectrophotometry.

2.3. Preparation of proteins

The final composition of the storage buffers were: KF^-: 10 mM Tris pH 8, 0.5 mM EDTA, 100 μg bovine serum albumin/mL, 50% glycerol and 2 mM $MgSO_4$; histone H1: 10 mM Tris pH 7.9, 20 mM NaCl for histone H1. The commercially available sample of KF^- was in the manufacturer's storage buffer

containing dithiothreitol and was exchanged for that specified above using microcon concentrators.

2.4. DNA transcription by RNA polymerase in vitro

Transcription of the (NdeI/HpaI) restriction fragment of pSP73KB DNA with T7 RNA polymerase and electrophoretic analysis of the transcripts were performed according to the protocols recommended by Promega [Promega Protocols and Applications, 43–46 (1989/90)] [29,30]. The DNA concentration used in this assay was 2.2×10^{-4} M (related to the monomeric nucleotide content).

2.5. Fluorescence measurements

The measurements were performed on a Varian Cary Eclipse spectrofluorophotometer using a 1 cm quartz cell. Fluorescence measurements of DNA modified by ruthenium and platinum complexes at a 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 [31,32]. The concentrations were 0.01 mg/mL for DNA and 0.04 mg/mL for EtBr, which corresponds to the saturation of all intercalation sites of EtBr in DNA [31].

2.6. Unwinding of negatively supercoiled DNA

Unwinding of closed circular supercoiled pSP73KB plasmid DNA was assayed by an agarose gel mobility shift assay [33]. The unwinding angle Φ, induced per DNA adduct of dinuclear Ru^{II} arene complex, was calculated upon the determination of the r_b value at which the complete transformation of the supercoiled plasmid to the relaxed form was attained. Samples of plasmid DNA at a concentration of 1.6×10^{-4} M (related to the monomeric nucleotide content) were incubated with complexes Ru(4), Ru(6), Ru(8), or Ru(12) at 37 °C in the dark for 24 h. All samples were precipitated by ethanol and redissolved in the TAE (Tris-acetate/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 set at 25 V. The gels were then stained with EtBr, followed by photography with a transilluminator. The other aliquot was used for the determination of r_b values by FAAS.

2.7. Circular dichroism (CD)

Isothermal CD spectra of CT DNA modified by dinuclear Ru^{II} arene complexes were recorded at 25 °C in 10 mM $NaClO_4$ by using a Jasco J-720 spectropolarimeter equipped with a thermoelectrically controlled cell holder with a cell pathlength of 1 cm. The spectra were recorded in the range of 230–500 nm in 0.5 nm increments with an averaging time of 0.25 s, speed 200 nm/min and 3 accumulations.

2.8. DNA interstrand cross-linking

The dinuclear Ru^{II} arene complexes were incubated with 1 μg of a 221-bp NdeI/HpaI fragment of pSP73 DNA. The DNA fragment was first 3′-end labeled by means of KF^- in the presence of [α-^{32}P]dATP. The ruthenated samples were precipitated by ethanol and analyzed for DNA interstrand CLs by previously published procedures [29,34]. After the ruthenation, the samples were precipitated by ethanol and the pellet was dissolved in 18 μL of a solution containing 30 mM NaOH, 1 mM EDTA, 6.6% sucrose and 0.04% bromophenol blue. The amount of interstrand cross-links was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%). After the electrophoresis was completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified by means of a bio-imaging analyzer.

2.9. Interhelical cross-linking

An equimolar mixture of pSP73 plasmid linearized by NdeI (2464 bp) and short 801-bp PvuI/NdeI restriction fragment of pSP73 uniquely 5′-end labeled at the NdeI restriction site was ruthenated in 0.08 M $NaClO_4$ at $r_b = 0.025–0.1$ in a reaction volume of 13 μL. The total amount of DNA in these mixtures was 1.0 pmol. Aliquots were subjected to electrophoresis in a 1% agarose gel in TAE buffer [35]. The location of the linearized pSP73 plasmid in the gel was visualized by fluorescence under UV irradiation after staining the gel with EtBr; subsequently, the gel was dried and subjected to autoradiography, revealing the location of the radioactively labeled fragment.

2.10. DNA–protein cross-linking

The ruthenated DNA (107-bp NdeI/EcoRI fragment of pSP73 plasmid at a concentration of 20 nM or 221-bp NdeI/HpaI fragment of pSP73 plasmid at a concentration of 10 nM) was incubated with proteins (KF^- or histone H1) at a concentration of 0.2 μM overnight in the dark at room temperature in the appropriate buffer: 10 mM Tris pH 8, 10 mM EDTA, 0.1 μM bovine serum albumin, 0.8% glycerol and 2 mM $MgSO_4$ (KF^-); 10 mM Tris pH 7.9 and 20 mM NaCl (histone H1). The ability to form cross-links between DNA fragments and proteins was assessed by 1.3% agarose (agarose and Metaphor® agarose 1:1) gel electrophoresis after mixing the samples with the loading buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and denaturing by heat at 60 °C for 5 min. Gels were electrophoresed for 3 h at 40 V, dried and visualized with a bio-imaging analyzer.

2.11. Other physical methods

Absorption spectra were measured with a Beckmann DU-7400 spectrophotometer. FAAS measurements were carried out with a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer. For FAAS analysis DNA was precipitated with ethanol and dissolved in double distilled water. The gels were visualized by using a BAS 2500 FUJIFILM bio-imaging analyzer, and the radioactivities associated with bands were quantitated with the AIDA image analyzer software (Raytest, Germany).

3. Results

3.1. DNA binding in cell-free media

Solutions of double-helical CT DNA at a concentration of 0.1 mg/mL were incubated at 37 °C with the complexes Ru(4), Ru(6), Ru(8), or Ru(12) in 10 mM $NaClO_4$ at an r_i value of 0.05 (r_i is defined as the molar ratio of free ruthenium complex to nucleotide-phosphates at the onset of incubation with DNA). At various time intervals, aliquots of the reaction mixtures were withdrawn, the reactions were stopped by adding 1.5 M NaCl (1/10 of the total volume), quickly cooled on an ice bath, DNA was precipitated by ethanol, and the content of ruthenium in the supernatant of these samples was determined by FAAS. The amount of DNA bound dinuclear Ru^{II} arene compounds (r_b) increased with time. All complexes bind rapidly (the times at which the binding reached 30% was ca. 10 s and the maximum binding was reached in less than ca.

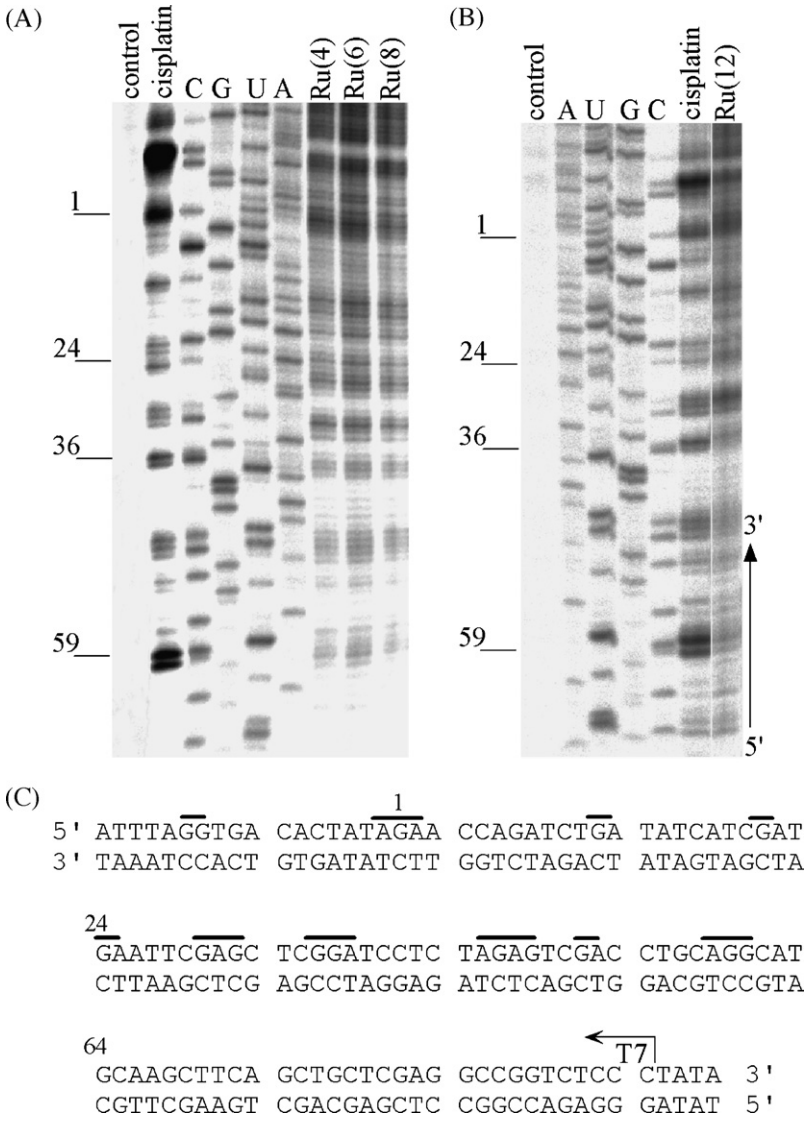

Fig. 2 – Inhibition of RNA synthesis by T7 RNA polymerase on the NdeI/HpaI fragment of pSP73KB plasmid modified by the dinuclear Ru^{II} arene complexes and cisplatin. (A and B) Autoradiograms of a 6% polyacrylamide/8 M urea sequencing gel showing the inhibition of RNA synthesis by T7 RNA polymerase on the NdeI/HpaI fragment containing adducts of Ru^{II} arene complexes and cisplatin. Lanes in left panel: control, unmodified template; A, U, G and C, chain terminated marker DNAs; cisplatin, Ru(4), Ru(6), Ru(8), the template modified by cisplatin, dinuclear Ru^{II} arene complexes Ru(4), Ru(6), Ru(8) at r_b = 0.01, respectively. Lanes in right panel: control, unmodified template; A, U, G and C, chain terminated marker DNAs; cisplatin, Ru(12), the template modified by cisplatin and dinuclear Ru^{II} arene complex Ru(12) at r_b = 0.01, respectively. (C) Schematic diagram showing the portion of the nucleotide sequence used to monitor the inhibition of the RNA synthesis by cisplatin and the dinuclear Ru^{II} arene complexes. The arrow indicates the start of the T7 RNA polymerase, which used the upper strand of the NdeI/HpaI fragment of pSP73KB as template. The short lines above the sequence represent major stop signals for DNA modified by cisplatin or dinuclear Ru^{II} arene complexes, respectively. The numbers correspond to the nucleotide numbering in the sequence map of the pSP73KB plasmid.

10 min), but not quantitatively, ca. 60% of the complexes Ru(4), Ru(6), and Ru(8) and ca. 75% of complex Ru(12) were bound after 40 h. Similar results were obtained if solutions of DNA were incubated with the complexes at an r_i value of 0.08.

The binding experiments indicate that modification reactions resulted in the irreversible binding of the dinuclear Ru^{II} arene complexes to CT DNA, which thus facilitated further sample analysis, and it was possible to prepare samples of DNA modified by the complexes Ru(4), Ru(6), Ru(8), or Ru(12) at a preselected value of r_b. Thus, except where stated, samples of DNA modified by the dinuclear Ru^{II} arene compounds Ru(4), Ru(6), Ru(8), or Ru(12) were prepared in 10 mM $NaClO_4$ at 37 °C for further analysis by biophysical or biochemical methods. After 24 h of the reaction of DNA with the complex, the samples were precipitated in ethanol and dissolved in the medium necessary for a particular analysis, and the r_b value in an aliquot of this sample was checked by FAAS. Accordingly, all analyses were performed in the absence of unbound (free) dinuclear Ru^{II} arene complex.

3.2. *Transcription mapping of DNA adducts*

In vitro RNA synthesis by RNA polymerases on DNA templates containing several types of adducts of platinum or ruthenium complexes can be prematurely terminated at the level or in the proximity of adducts [11,29]. Cutting of pSP73KB DNA [29] by NdeI and HpaI restriction endonucleases yielded a 212-bp fragment (a substantial part of its nucleotide sequence is shown in Fig. 2C). This fragment contained T7 RNA polymerase promoter [in the upper strand close to its 3′-end (Fig. 2C)]. The experiments were carried out using this linear DNA fragment, modified at $r_b = 0.01$ by Ru(4), Ru(6), Ru(8), or Ru(12) and for comparative purposes also by cisplatin, for RNA synthesis by T7 RNA polymerase (Fig. 2A, lanes Ru(4), Ru(6), Ru(8), and cisplatin and Fig. 2B, lanes Ru(12) and cisplatin). RNA synthesis on the template modified by all ruthenium complexes and cisplatin yielded fragments of defined sizes, which indicates that RNA synthesis on these templates was prematurely terminated (Fig. 2A and B). The major stop sites, primarily guanine residues, were roughly identical for all dinuclear Ru^{II} arene complexes (Fig. 2A and B). The profiles are similar to that obtained for DNA treated with the anticancer drug cisplatin (lane cisplatin in Fig. 2A and B) and also to those reported previously for other type of mononuclear Ru^{II} arene complexes, such as $[(\eta^6\text{-arene})Ru(en)Cl]^+$ where en = 1,2-diaminoethane and arene = biphenyl, dihydroanthracene, tetrahydroanthracene, *p*-cymene, or benzene [11], but somewhat different from that obtained for DNA treated with the clinically inefficient transplatin [29]. The major stop sites for DNA modified by complex Ru(8) and cisplatin are demonstrated in Fig. 2C. These results suggest that the preferred binding sites for Ru(4), Ru(6), Ru(8), and Ru(12) on DNA are guanine residues.

3.3. *Ethidium bromide (EtBr) fluorescence*

The ability of the complexes to displace the DNA intercalator EtBr from CT DNA was probed by monitoring the relative fluorescence of the EtBr-DNA adduct after treating the DNA with varying concentrations of ruthenium or platinum complexes. Fig. 3 shows a plot of relative fluorescence versus r_b for the complexes Ru(4), Ru(6), Ru(8), and Ru(12), cisplatin and [PtCl(dien)]Cl. DNA intercalated EtBr was markedly more effectively replaced by adduct formation with the dinuclear Ru^{II} arene compounds than with monofunctional [PtCl(dien)]Cl. Complex Ru(12) was the most effective compound in replacing EtBr and it was even considerably more effective than the adducts of bifunctional cisplatin.

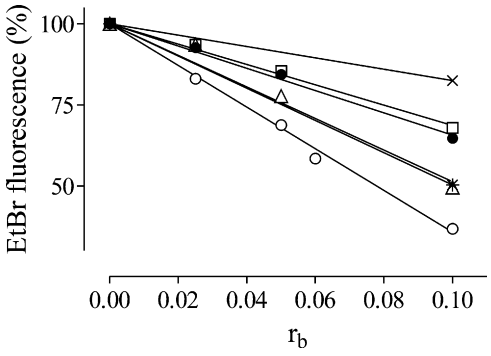

Fig. 3 – Plots of the EtBr fluorescence versus r_b values for calf-thymus DNA modified by cisplatin, [PtCl(dien)]Cl and the dinuclear Ru^{II} arene complexes in 10 mM $NaClO_4$ at 37 °C for 24 h: (×), [PtCl(dien)]Cl; (*), cisplatin; (Δ), Ru(4); (□), Ru(6); (●), Ru(8); (○) Ru(12). Data points were measured in triplicate and varied on average ± 3% from their mean.

3.4. *DNA unwinding*

The unwinding of supercoiled plasmid DNA induced by the four dinuclear Ru^{II} arene complexes Ru(4), Ru(6), Ru(8), and Ru(12) was determined by incubating the plasmid pSP73KB with the ruthenium complexes at various r_b values (Fig. 4). The resulting electrophoresis in native agarose gels of DNA modified by Ru(4) and Ru(12) are shown in Fig. 4 (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 value at which the supercoiled and nicked forms comigrate [33]. Under the present experimental conditions, σ was calculated to be −0.04 on the basis of the data of cisplatin for which the $r_b(c)$ was determined in this study and $\Phi = 13°$ was assumed [33]. It can be seen in Fig. 4 (bottom) that the complex Ru(12) causes an unwinding of the DNA (Φ) by 7 ± 1°, the comigration point of the modified supercoiled and nicked DNA, $r_b(c)$, was reached at $r_b = 0.1$. In contrast, complexes Ru(4), Ru(6) and Ru(8) do not unwind the DNA significantly, and the comigration point of the modified supercoiled and nicked DNA was not reached at an r_b value as high as 0.14 (shown for complex Ru(4) in Fig. 4, top).

3.5. *Circular dichroism (CD)*

To gain further information on the structural changes induced by the dinuclear complexes in DNA, CD spectra of DNA

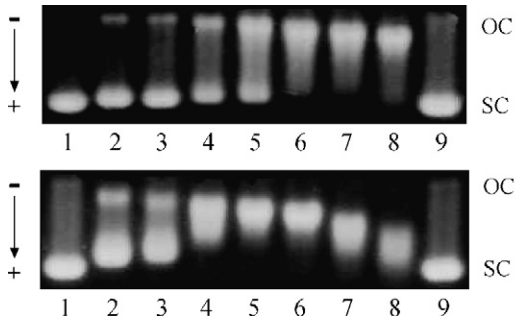

Fig. 4 – The unwinding of supercoiled pSP73KB plasmid DNA by Ru(4) (top) and Ru(12) (bottom). The plasmid was incubated with the dinuclear Ru^{II} arene complexes in 10 mM $NaClO_4$ at pH 6 for 24 h at 37 °C. Lanes in the top panel: (1 and 9) control, unmodified DNA; (2) r_b = 0.036; (3) r_b = 0.048; (4) r_b = 0.060; (5) r_b = 0.072; (6) r_b = 0.096; (7) r_b = 0.120; (8) r_b = 0.144. Lanes in the bottom panel: (1 and 9) control, unmodified DNA; (2) r_b = 0.043; (3) r_b = 0.058; (4) r_b = 0.072; (5) r_b = 0.086; (6) r_b = 0.115; (7) r_b = 0.144; (8) r_b = 0.173. The top bands in each panel correspond to the form of nicked plasmid, and the bottom bands, to the closed, negatively supercoiled plasmid.

modified by the complexes Ru(4), Ru(6), Ru(8), and Ru(12) were recorded (Fig. 5). These ruthenium complexes have no intrinsic CD signals so that any CD signal above 300 nm can be attributed to the interaction of complexes with DNA. Below 300 nm any change from the DNA spectrum is either due to the DNA induced CD (ICD) of the metal complex or due to the metal complex induced perturbation of the DNA spectrum. The signatures of complexes Ru(4), Ru(6), and Ru(8) bound to CT DNA are positive ICDs centered at around 325–335 nm (Fig. 5A–C). Complex Ru(12) bound to CT DNA yields a negative ICD at around 308 nm and a positive ICD centered at around 335 nm (Fig. 5D). Unfortunately these complexes also absorb in the DNA region (below 300 nm) so that the ICD signals in the DNA region are due to changes in both the intrinsic DNA CD and the ligand-induced CD, which impedes unambiguous interpretation of the CD spectra in Fig. 5 in the DNA region (<300 nm) in terms of alterations of DNA conformation or the DNA-binding mode of complexes Ru(4), Ru(6), Ru(8), and Ru(12).

3.6. *Interstrand cross-linking*

Bifunctional compounds that covalently bind to DNA may form various types of interstrand and intrastrand cross-links. Therefore, we have decided to quantitate the interstrand cross-linking efficiency of Ru(4), Ru(6), Ru(8), and Ru(12) in the 221-bp NdeI/HpaI fragment of the pSP73 plasmid. This fragment was 3′-end labeled by [α-^{32}P] ATP and modified at r_b = 0.01 by Ru(4), Ru(6), Ru(8), or Ru(12) and for comparative purposes also by cisplatin, which is known to form ca. 6% interstrand cross-links [29]. The samples were analyzed for the interstrand cross-links by agarose gel electrophoresis under denaturing conditions. Upon electrophoresis, the 3′-end-labeled strands of the DNA fragment containing no interstrand cross-links migrate as a 221-base single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular mass species (Fig. 6). The radioactivity associated with the individual bands in each lane

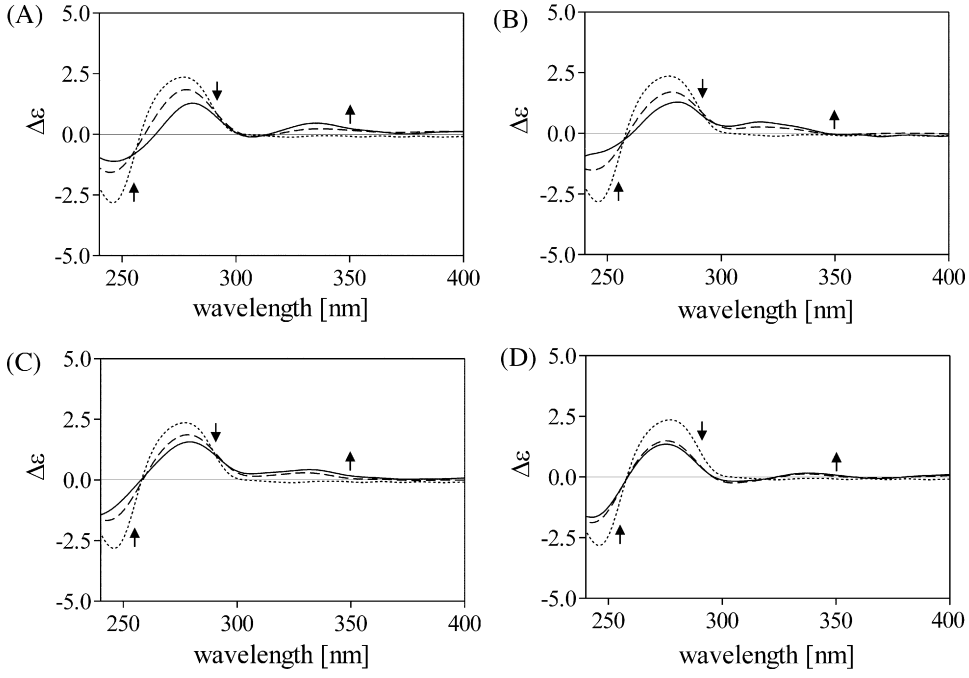

Fig. 5 – Circular dichroism (CD) spectra of calf-thymus DNA (1×10^{-4} M) modified by the dinuclear Ru^{II} arene complexes in 10 mM $NaClO_4$, pH 6. DNA was modified by Ru(4) (A), Ru(6) (B), Ru(8) (C) at r_b = 0 (dotted line), 0.05 (dashed line), 0.1 (solid line) and by complex Ru(12) (D) at r_b = 0 (dotted line), 0.05 (dashed line), 0.06 (solid line). The arrows in (A–D) show a change of CD with increasing r_b value.

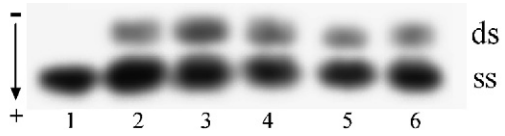

Fig. 6 – The formation of interstrand cross-links by dinuclear Ru^{II} arene complexes and cisplatin in the 221-bp NdeI/HpaI fragment of the pSP73 plasmid. Autoradiogram of a denaturing 1% agarose gel of the DNA fragment which was 3′-end labeled and modified at r_b = 0.01; the interstrand cross-linked DNA appears as the top bands migrating on the gels more slowly than the single-stranded DNA (contained in the bottom bands); lanes: (1–6) control, cisplatin, Ru(4), Ru(6), Ru(8), and Ru(12), respectively.

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 (% ICL/Pt) was calculated using the Poisson distribution from the fraction of non-cross-linked DNA in combination with the r_b values and the fragment size. The DNA interstrand cross-linking efficiency of Ru(4), Ru(6), Ru(8), or Ru(12) was ca. 5% and therefore similar to that of cisplatin.

3.7. *Interhelical cross-linking*

A large number of DNA cross-linking agents are known, but the reactive moieties of such compounds usually bind to the same DNA duplex because binding of one moiety inevitably leaves the other in close proximity to other binding sites in the same duplex, leading to intramolecular cross-linking. However, if the reactive sites of the cross-linking agents are connected by a linker that forces those sites to point in opposite directions and if the stereochemistry of the reactive sites of such cross-linking agents proves appropriate, such agents could bind to adjacent duplexes [36–38].

To assess whether the dinuclear Ru^{II} arene complexes are able to form interhelical cross-links, i.e., cross-links between two DNA duplexes, gel electrophoresis was used. The radioactively labeled NdeI/PvuI fragment of pSP73 (810 bp) (short fragment) and linearized pSP73 plasmid (2464 bp) (long fragment) were mixed at 1:1 molar ratio, the mixture was ruthenated by Ru(4), Ru(6), Ru(8), or Ru(12) at r_b = 0.025, 0.05, and 0.1 and the reaction products were analyzed by native agarose gel electrophoresis. In order to minimize the electrostatic repulsion between the sugar–phosphate backbones of two different duplexes, the mixture of the two DNA fragments was ruthenated in a medium containing a high concentration of counter cations (0.08 M Na^+). Migration of the linearized

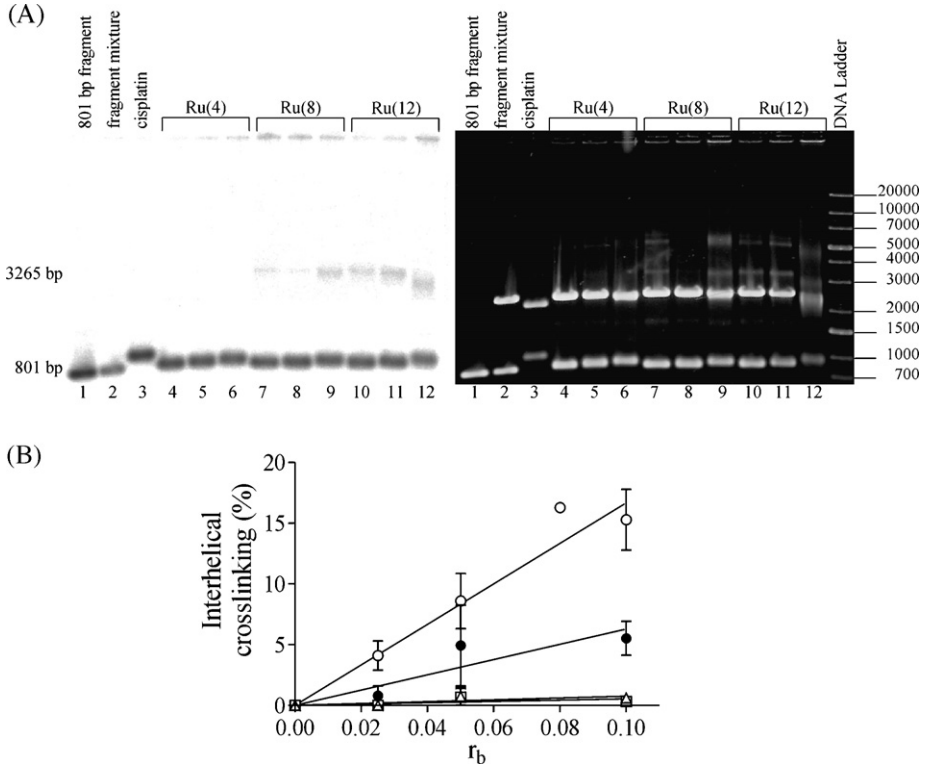

Fig. 7 – Formation of interhelical cross-links. The radioactively labeled NdeI/PvuI fragment of pSP73 (810 bp) and linearized pSP73 plasmid (2464 bp) (not labeled) were mixed at 1:1 molar ratio, the mixture was ruthenated by Ru(4), Ru(8), or Ru(12) at r_b = 0.025, 0.05, and 0.1 and the reaction products were analyzed by native 1% agarose gel electrophoresis. The mixture of the two DNA fragments was ruthenated in 0.08 M $NaClO_4$. (A) The migration of the labeled fragment was detected by autoradiography of the gel (left). The linearized pSP73 as well as the molecular size markers, derived from a lambda phage digest of HindIII, were detected by flourescence under UV irradiation after staining with EtBr (right). (B) Plots of interhelical cross-linking (%) of 810-bp and 2464-bp fragments by dinuclear Ru^{II} complexes versus the r_b values: (Δ), Ru(4); (□), Ru(6); (●), Ru(8); (○) Ru(12).

pSP73 DNA (long fragment) through the gel was detected by fluorescence under UV light after staining the gel with EtBr (right part of Fig. 7A). Subsequently, autoradiography of the same, but dried gel was performed to localize the labeled short fragment (left part of Fig. 7A). Discernible bands corresponding to fraction of the labeled short fragment were found to migrate along with the linearized plasmid. The intensity of these bands increased with a growing level of the modification by each dinuclear ruthenium complex and the maximum efficiency of these complexes to cross-link short and long fragments was observed for Ru(12) (Fig. 7B). It should be pointed out that at higher levels of the ruthenation not all of the labeled DNA enters the gel. It is assumed that this may be due to interhelical cross-linking of a mixture of DNA fragments which results in a DNA matrix of high molecular weight. Such structures have been hypothesized for cisplatin [39] and observed for this antitumor mononuclear platinum complex under conditions of mild hyperthermia [40].

3.8. *DNA–protein cross-linking*

The dinuclear Ru^{II} arene complexes were investigated for their ability to form ternary DNA–protein complexes. For these studies proteins were chosen that bind to DNA with a relatively high affinity. KF^- and the linker histone H1 were selected as representatives of non-sequence specific DNA-binding proteins with enzymatic or structural function. One strand 3′-end-labeled 221-bp duplex (NdeI/HpaI fragment of pSP73 plasmid) was globally modified by Ru(4), Ru(6), Ru(8), or Ru(12) at $r_b = 0.03$. The fragment modified by either Ru complex (10 nM) was mixed with KF^- or histone H1 (the molar ratio protein/duplex was 10) and incubated overnight. Ternary DNA–(Ru–Ru)–protein cross-linking efficiency was assessed by a 1.3% agarose (agarose and Metaphor® agarose 1:1) gel shift assay. Fractions were detected with significantly retarded mobility (shown for KF^- in Fig. 8, lanes 9–12) and compared with that of the free probe (Fig. 8, lane 7). These more slowly migrating fractions were also eliminated after treatment with proteinase K (not shown) converting them to those of the unmodified probes. Importantly, the amount of radioactivity associated with the bands corresponding to ternary DNA–protein complexes formed by cisplatin was markedly lower (Fig. 8, lane 8). The same experiments were performed with the shorter 107-bp duplex (NdeI/EcoRI fragment of pSP73 plasmid) globally modified with the dinuclear Ru^{II} arene complexes. The yields of the DNA–protein cross-linking reactions were considerably lower (not shown).

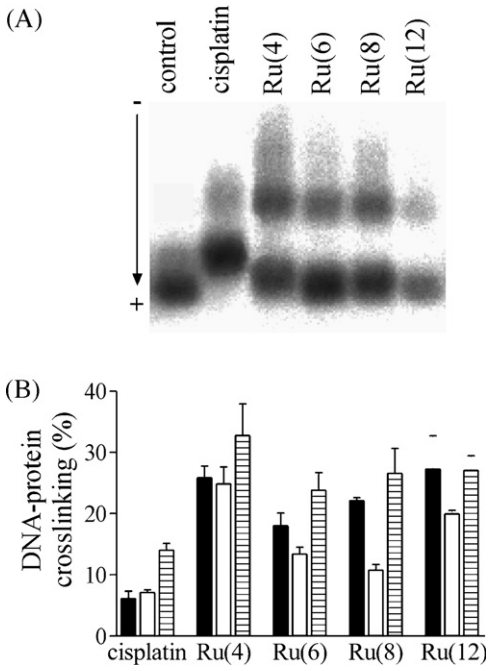

Fig. 8 – Formation of DNA–protein cross-links of unmodified and ruthenated DNA fragment globally modified by cisplatin, Ru(4), Ru(6), Ru(8), or Ru(12) with KF^- assessed by agarose gel electrophoresis; the fragment was incubated with the protein for 20 h. (A) Electrophoretogram. Lanes: control, the non-modified 221-bp fragment incubated with KF^-. cisPt, Ru(4), Ru(6), Ru(8), Ru(12), the 221-bp fragment modified at $r_b = 0.03$ by cisplatin, Ru(4), Ru(6), Ru(8), Ru(12), respectively, incubated with KF^-. (B) Percentual evaluation of formation of DNA cross-linked to proteins. The 107-bp DNA fragment globally modified by cisplatin, Ru(4), Ru(6), Ru(8), or Ru(12) ($r_b = 0.05$) with KF^- (solid bars) and histone H1 (open bars); the 221-bp DNA fragment globally modified by cisplatin, Ru(4), Ru(6), Ru(8), or Ru(12) ($r_b = 0.03$) with KF^- (striped bars). See the text for other details.

4. Discussion

Recently, dinuclear Ru^{II} arene complexes based on pyridinone ligands, such as those shown in Fig. 1, were found to exert promising cytotoxic effects in human tumor cell lines and an intriguing feature of biological effects of these compounds is that their two Ru centers act synergistically [24]. Also interestingly, their activity against human tumor cells increases with the length of the linker. Previous studies with platinum compounds have shown that bifunctional dinuclear complexes can lead to novel DNA lesions, which can be important in the avoidance of cellular cross-resistance [22,41–48]. We report in the present work the detailed DNA-binding study of the bifunctional dinuclear Ru^{II} arene complexes Ru(4), Ru(6), Ru(8), and Ru(12) (Fig. 1). In addition, we investigated the effects and extent of changes induced in the DNA upon binding of the dinuclear Ru^{II} arene complexes, and these observations are compared with other metal-based anticancer agents.

It is notable that all these complexes bind polymeric DNA. Binding to DNA has often been associated with the cytotoxic action of metal-based anticancer agents [1,5,10] and therefore DNA may be a possible biological target for this class of dinuclear Ru^{II} arene complexes.

Our studies of the binding of the dinuclear Ru^{II} arene complexes Ru(4), Ru(6), Ru(8), and Ru(12) to natural double-helical CT DNA show that the reactions are markedly faster than that for cisplatin. The DNA adducts of complexes Ru(4), Ru(6), Ru(8), and Ru(12) are relatively stable, with little loss of bound Ru after extensive dialysis (against 1.0 M NaCl).

Transcription mapping experiments (Fig. 2) have shown that guanine residues are the preferential binding sites when polymeric DNA is modified with dinuclear Ru^{II} arene complexes in a random fashion. The selectivity for G bases was also found previously in our studies of the monofunctional and mononuclear Ru^{II} arene ethylenediamine complexes [11].

It is reasonable to expect that DNA-binding mode of monomeric Ru units constituting the dinuclear complexes tested in the present work is identical. However, the binding of the dinuclear complex Ru(12) to DNA results in some degree of unwinding (7°; Fig. 4) whereas the binding of the other compounds does not. The DNA unwinding efficiency of Ru(12) can be explained by the contribution to unwinding associated with the interaction of the arene ligands in complex Ru(12) with the duplex upon binding of Ru central atom(s). In other words, the arene moiety(ies) in DNA adducts of the dinuclear Ru^{II} arene compound Ru(12) could be geometrically positioned favorably to interact with the double helix. In contrast, the complexes Ru(4), Ru(6), and Ru(8) do not unwind the DNA significantly (Fig. 4). The explanation behind this phenomenon is unclear, nevertheless it may be hypothesized that the length of the linkers is not favorable for the interaction of the arene rings in the Ru(4), Ru(6), and Ru(8) complexes with the double helix. In summary, it seems reasonable to suggest that the ligands in Ru(4), Ru(6), and Ru(8) do not interact with the double helix in a way similar to Ru(12), thus also supporting a different DNA-binding mode for this compound in comparison with the other three complexes.

EtBr as a fluorescent probe can be used to characterize DNA binding of small molecules such as platinum and ruthenium antitumor drugs [11,33,49]. Binding of EtBr to DNA by intercalation is blocked in a stoichiometric manner by a wide spectrum of DNA-binding ligands. On the other hand, the modification of DNA by monofunctional ligands, such as [PtCl(dien)]Cl, results in only a slight decrease of EtBr fluorescence intensity as compared with that for the complex of non-metalated DNA with EtBr. Competitive binding of other intercalators leads to the loss of fluorescence because of depletion of the DNA–EtBr complex (free EtBr is poorly fluorescent).

The formation of adducts of the dinuclear complexes with DNA is accompanied by a release of the EtBr intercalator (Fig. 3). The adducts of compounds Ru(4), Ru(6), and Ru(8) do not unwind DNA (Fig. 4). Thus, the results of the unwinding experiments are consistent with the view that the arene ligands or other groups in Ru(4), Ru(6), and Ru(8) do not interact substantially with the double helix upon coordination of the dinuclear Ru^{II} complexes.

In contrast, Ru(12) unwinds the DNA (by ca. 7°, Fig. 4), but replaces the EtBr intercalator more efficiently than the other three compounds and even markedly more than cisplatin (Fig. 3). It seems reasonable to suggest that the arene ligands or other groups in Ru(12) do interact with the double helix, although not very strongly. It cannot be excluded that some DNA-binding properties of Ru(12) observed in the present work may be also associated with a higher lipophilicity of this complex [24]. Thus, the solution behavior of the DNA adducts of dinuclear Ru^{II} arene complexes appears interesting and merits further study.

In addition, comparison with bifunctional platinum polynuclear complexes [41,45,46] suggests that the conformational distortion induced in DNA by the adducts of Ru(12) is delocalized and spans more base pairs around the binding sites. Thus, the EtBr fluorescence results (Fig. 3) suggest formation of long-range cross-links or of other lesions that would extend over more than two base pairs.

The CD spectra of DNA modified by complexes Ru(4), Ru(6), Ru(8), and Ru(12) (Fig. 5A–D) indicate that the binding of these complexes results in conformational alterations in double-helical DNA. All complexes bound to CT DNA yield a positive ICD centered at around 335 nm (Fig. 5A–D). On the other hand, complex Ru(12) bound to CT DNA yields also a broad negative ICD centered at around 310 nm (Fig. 5D).

The results of our biophysical studies are consistent with intrastrand and interstrand cross-links formed by dinuclear Ru^{II} arene complexes within one DNA duplex. Intrastrand cross-linking was difficult to quantify, but interstrand cross-links were found to be formed in DNA by the dinuclear Ru^{II} arene complexes Ru(4), Ru(6), Ru(8), and Ru(12) in cell-free media and in absence of proteins with the same frequency (Fig. 6) as observed for the antitumor drug cisplatin [29]. Another interesting phenomenon specific for the DNA binding of these dinuclear Ru^{II} arene complexes is that they also form interduplex cross-links that are tethered by ruthenium–DNA bonds (Fig. 7). A plausible explanation of this observation might be that DNA that has been modified by Ru(4), Ru(6), Ru(8), and Ru(12) aggregates to an extent that allows interduplex contacts that are sufficient for the interduplex cross-linking.

Interestingly, in accordance with the ability of dinuclear Ru^{II} arene complexes to cross-link two DNA duplexes, the results of the present work convincingly demonstrate that these dinuclear complexes also form specific DNA lesions which can efficiently cross-link proteins to DNA (Fig. 8). If such a ternary complex is formed, it is reasonable to expect that in the first step relatively flexible DNA-binding proteins (such as those tested in the present work, i.e., KF^{-} and the linker histone H1) come into close contact with DNA at the site of ruthenation. Then one molecule of the dinuclear Ru^{II}-arene complex can be bound simultaneously to a base residue in DNA and a reactive group in the protein, but only if a non-covalent preassociation takes place first. The formation of DNA–protein ternary complexes mediated by dinuclear Ru^{II} arene complexes raises the possibility of ''suicide'' lesions, which may irreversibly sequester various DNA-binding proteins, such as transcription factors or repair proteins [15,50]. In addition, the cross-linking procedures involving these ruthenium agents may provide a tool for identification of proteins or protein domains closely positioned to DNA including mapping of protein-binding sites on DNA *in vivo* [51,52].

In conclusion, the concept for the design of interhelical and DNA–protein cross-linking agents based on dinuclear Ru^{II} arene complexes with sufficiently long linkers may result in new compounds which exhibit a variety of biological effects and can be also useful in nucleic acids research. The earlier observation [24] that complex Ru(12) is markedly more potent than cisplatin in human cancer cells, including colon adenocarcinoma cells with the IC_{50} of Ru(12) being more than one order of magnitude lower than that of cisplatin, is consistent with this idea. Such results indicate promising compounds with which to tackle the common problem of

developed cisplatin resistance, frequently occurring during chemotherapy.

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Authors' contributions: ON and VB designed research; ON performed research; AAN synthesized compounds; CGH and BKK supervised AAN; ON and VB analyzed data; and VB wrote the paper.

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