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Conformation of DNA Modified by Monofunctional Ru(II) Arene Complexes: Recognition by DNA Binding Proteins and Repair. Relationship to Cytotoxicity

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Summary

We analyzed DNA duplexes modified at central guanine residues by monofunctional Ru(II) arene complexes [(η^6-arene)Ru(II)(en)(Cl)]$^+$ (arene = tetrahydroanthracene or *p*-cymene, Ru-THA or Ru-CYM, respectively). These two complexes were chosen as representatives of two different classes of Ru(II) arene compounds for which initial studies revealed different binding modes: one that may involve DNA intercalation (tricyclic-ring Ru-THA) and the other (mono-ring Ru-CYM) that may not. Ru-THA is ~20 times more toxic to cancer cells than Ru-CYM. The adducts of Ru-THA and Ru-CYM have contrasting effects on the conformation, thermodynamic stability, and polymerization of DNA in vitro. In addition, the adducts of Ru-CYM are removed from DNA more efficiently than those of Ru-THA. Interestingly, the mammalian nucleotide excision repair system has low efficiency for excision of ruthenium adducts compared to cisplatin intrastrand crosslinks.

Introduction

There is much current interest in the potential of ruthenium complexes as new metal-based antitumor drugs [1, 2]. Although the pharmacological target for antitumor ruthenium compounds has not been unequivocally identified, several ruthenium(III) compounds have been found to inhibit DNA replication, exhibit mutagenic activity, induce the SOS repair mechanism, bind to nuclear DNA, and reduce RNA synthesis, which is consistent with DNA binding of these compounds in vivo [2]. Thus, DNA interactions of antitumor ruthenium agents are of potential importance. Organometallic ruthenium(II) arene complexes of the type [(η^6-arene)Ru(II)(en)Cl][PF_6] (en = ethylenediamine) constitute a relatively new group of anticancer compounds [3, 4]. These monodentate complexes appear to be novel anticancer agents with a mechanism of action different from those of the ruthenium(III) complexes (ImH)[*trans*-$Ru(III)Cl_4$Im(Me_2SO)] (Im = imidazole, NAMI-A) and (IndH)[*trans*-$RuCl_4(Ind)_2$] (Ind = indazole, KP1019), which are currently in clinical trials [5]. The (η^6-arene)Ru(II) π bonds in the monofunctional [(η^6-arene)Ru(II)(en)(Cl)]$^+$ complexes are inert toward hydrolysis, but the chloride ligand is readily lost, and the complex is transformed into the corresponding more reactive, aquated species [6]. It has also been shown that in cell-free media ethylenediamine Ru(II) arene compounds, in which arene = biphenyl, dihydroanthracene, tetrahydroanthracene, *p*-cymene, or benzene, bind preferentially to guanine residues in natural double-helical DNA. In addition, DNA binding of the complexes containing biphenyl, dihydroanthracene, or tetrahydroanthracene ligands can involve combined coordination to G N7 and noncovalent, hydrophobic interactions between the arene ligand and DNA, which may include arene intercalation and minor groove binding [7, 8]. In contrast, the single hydrocarbon rings in the *p*-cymene and benzene ruthenium complexes cannot interact with double-helical DNA by intercalation [8]. Interestingly, adducts of the complex containing the *p*-cymene ligand, which has methyl and isopropyl substituents, distort the conformation and thermally destabilize double-helical DNA distinctly more than the adducts of the tricyclic-ring Ru(II) arene compounds. It has been suggested that the different character of conformational alterations induced in DNA as a consequence of its global modification, and the resulting thermal destabilization, may affect differently further "downstream" effects of damaged DNA [8] and consequently may result in different biological effects of this new class of metal-based antitumor compounds.

To achieve a rational design of novel antitumor Ru(II) arene compounds capable of circumventing inherent or acquired resistance to metal-based drugs already used in the clinic, it is important to understand in detail the differences in DNA binding properties of these new ruthenium complexes and their possible relationship to cytotoxicities in different tumor cell lines. This may provide grounds for establishing new structure-pharmacological activity relationships for this class of metal-based complexes. In this work, we have considered the activity of two Ru(II) arene complexes from the [(η^6-arene)Ru(II)(en)(Cl)]$^+$ family (arene = tetrahydroanthracene and *p*-cymene, Ru-THA and Ru-CYM, respectively, Figure 1A) in two tumor cell lines. These two complexes were chosen as representatives of two different classes of Ru(II) arene compounds for which initial studies of global modification of natural DNA revealed [8] different binding modes: one that may involve DNA intercalation (tricyclic-ring Ru-THA) and the other (mono-ring Ru-CYM) that cannot interact with double-helical DNA by intercalation. We compare the cytotoxicity data with those for DNA binding obtained previously [8], new data obtained in the present work relating to conformational distortions induced by single, site-specific monofunctional adducts of the Ru(II) arene complexes in short oligodeoxyribonucleotide duplexes, and the recognition of these DNA adducts by specific proteins and their repair, i.e., the most important factors that

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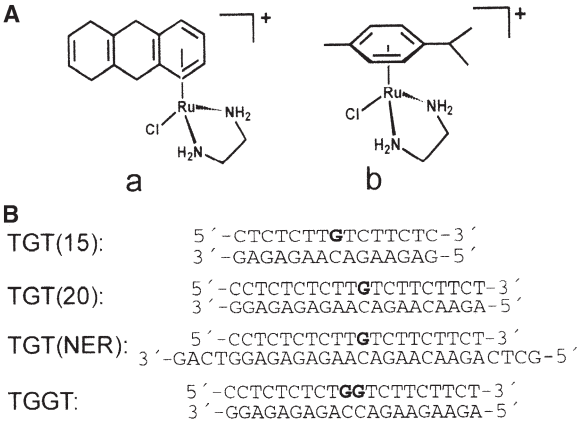

Figure 1. Structures of the Ruthenium Complexes and the Nucleotide Sequences of the Synthetic Oligodeoxyribonucleotide Duplexes with the Abbreviations Used in the Present Study

(A) Structures. (a) Ru-THA; (b) Ru-CYM.

(B) Sequences. The top and bottom strands of the pair of oligonucleotides are designated "top" and "bottom," respectively. The boldface letter in the top strand of the duplexes indicates the ruthenated residues.

modulate the antitumor effects of platinum antitumor drugs already used in the clinic.

Results

Chemical Probes of DNA Conformation

We demonstrated in our preceding paper [8] that Ru(II) arene compounds bind preferentially to guanine residues in natural double-helical DNA forming monofunctional adducts. In order to obtain information on how these adducts affect DNA conformation, the oligonucleotide duplexes containing a site-specific monofunctional adduct of Ru-THA or Ru-CYM (Figure 1A), [(η^6-tetrahydroanthracene)Ru(II)(en)(Cl)]$^+$ and [(η^6-*p*-cymene)Ru(II)(en)(Cl)]$^+$, respectively, at the G residue were further analyzed by chemical probes of DNA conformation. The ruthenated duplex TGT(20) (Figure 1B) was treated with several chemical agents that are used as tools for monitoring the existence of conformations other than canonical B DNA. These agents include $KMnO_4$ and diethyl pyrocarbonate (DEPC). They react preferentially with single-stranded DNA and distorted double-stranded DNA [9, 10]. We used for this analysis exactly the same methodology as described in detail in our recent papers in which we studied DNA adducts of various antitumor platinum drugs [9, 10], and, therefore, these experiments are described in more detail in the Supplemental Data (see the Supplemental Data available with this article online). The results demonstrated in Figure 2 indicate that the distortion induced by the nonintercalating Ru-CYM extended over at least 7 base pairs (bp), whereas the distortion induced by Ru-THA was less extensive.

Isothermal Titration Calorimetry

A calorimetric technique was employed to characterize the influence of the monofunctional adduct of Ru-THA

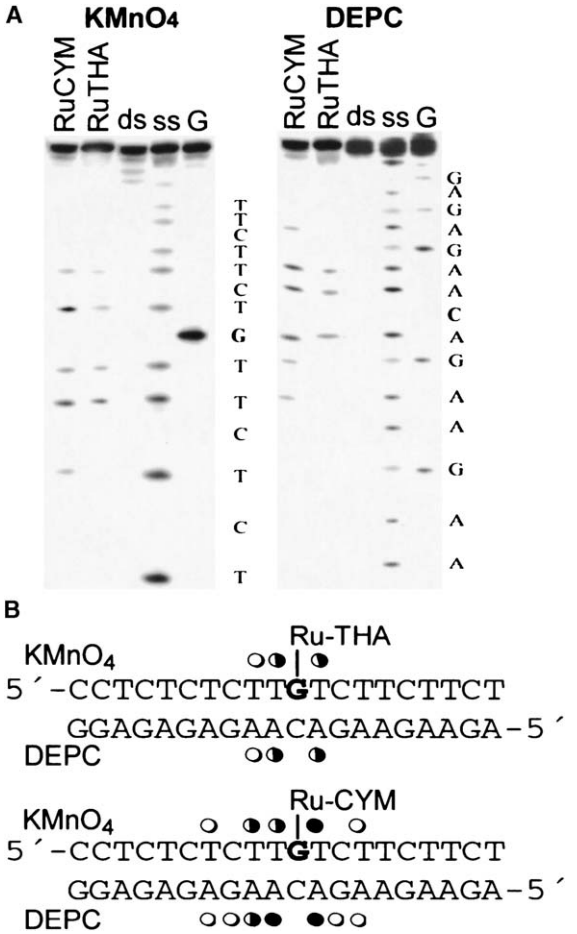

Figure 2. Chemical Probes of DNA Conformation

(A) Piperidine-induced specific strand cleavage at $KMnO_4$-modified (lanes marked with $KMnO_4$) and DEPC-modified (lanes marked with DEPC) bases in the nonmodified TGT(20) duplex or that containing the single, monofunctional adduct at the central G in the top strand. The oligomer was 5′-end labeled at its top ($KMnO_4$) or bottom (DEPC) strand. Lanes: ss, the nonmodified single strand; ds, the nonmodified duplex; RuTHA, the duplex containing an adduct of Ru-THA; RuCYM, the duplex containing an adduct of Ru-CYM; G, a Maxam-Gilbert-specific reaction for the unplatinated duplex. The boldface letters in the sequences indicate the ruthenated G and complementary C residues.

(B) Summary of the reactivity of chemical probes of DNA conformation. Upper panel, adduct of Ru-THA; lower panel, adduct of Ru-CYM. Filled circle, strong reactivity; half-filled circle, medium reactivity; open circle, weak reactivity. The boldface letter in the top strand of the duplexes indicates the ruthenated G residue.

and Ru-CYM on the thermal stability and energetics of the site-specifically ruthenated 15 bp DNA duplex. Such thermodynamic data can reveal how the ruthenium adduct influences duplex stability, a property that has been shown to play a significant role in cellular processes such as recognition of DNA damage by DNA binding proteins and repair of this damage, i.e., the processes that may modulate potency of antitumor drugs, including metal-based cytostatics. Recently, differential scanning calorimetry (DSC) was employed to characterize the influence of different crosslinks of platinum

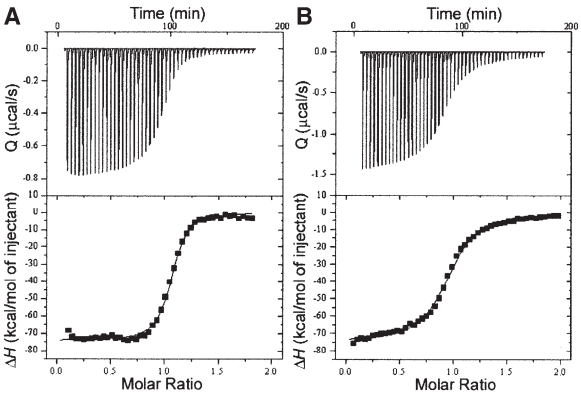

Figure 3. Isothermal Titration Calorimetry

(A and B) ITC binding isotherm for the association of the top strand of the 15 bp TGT duplex containing a single, monofunctional adduct of (A) Ru-THA or (B) Ru-CYM with the complementary (nonmodified) strand (bottom strand of the duplex TGT(15)) at 25°C in 10 mM phosphate buffer (pH 7.0) containing 50 mM NaCl. The upper panels show total heat released upon injecting 5 μl aliquots of the 50 μM bottom strand into a 1.4 ml reaction cell containing the 5 μM top strand. The lower panels show the resultant binding isotherm (full squares) obtained by integrating the peak areas of each injection. The continuous line represents the nonlinear least squares fit of the affinity (*K*), enthalpy (Δ*H*), and stoichiometry (*n*) to a single-site binding model. For other details, see the text.

antitumor drugs on the thermal stability and energetics of 15–20 bp DNA duplexes site-specifically modified by these drugs [11–13]. We decided to expand these studies to oligodeoxyribonucleotide duplexes containing unique monofunctional adducts of the Ru-THA or Ru-CYM complexes. DSC makes it possible to measure excess heat capacity versus temperature profiles for the thermally induced transitions of nonmodified DNA duplexes and those containing a unique adduct of the metal-based drug. Such thermograms are usually recorded with the heating rate of 60°C/hr, and after reaching the maximum temperature of 95°C, the samples are cooled at the same rate to the starting temperature of 25°C [11–13]. This implies that the duplexes containing the unique adduct are exposed to higher temperatures for a relatively long period of time. Therefore, we verified first the stability of the Ru(II) arene adducts at various temperatures and found that the adducts formed in the TGT(15) duplex by Ru-THA and Ru-CYM are stable for more than 2 hr only at temperatures lower than 50°C. Hence, it is apparent that DSC cannot be used to analyze the duplexes containing the adducts of these two Ru(II) arene complexes. A suitable alternative is isothermal titration calorimetry (ITC), which makes it possible to study the thermodynamic parameters of the duplex formation from its two complementary single strands over a range of temperatures including those at which the DNA adducts of Ru(II) arene compounds were stable for a long period of time [14].

Figure 3 shows ITC profiles of duplex formation from the nonmodified bottom strand of the duplex TGT(15) titrated into the complementary nonmodified top strand of the duplex TGT(15) or the same strand containing a single monofunctional adduct of Ru-THA or Ru-CYM at 25°C. It was verified that the melting temperature of the duplexes was significantly higher than this temperature and that the adducts of Ru-THA and Ru-CYM formed in the TGT(15) duplex or in its single-stranded top strand were stable for more than 24 hr. The ITC profiles were analyzed as described in the Experimental Procedures to obtain the results listed in Table 1. Inspection of these thermodynamic parameters reveals that the exothermic formation of the single monofunctional adduct in the duplex TGT(15) by Ru-THA or Ru-CYM resulted in a large decrease of the change in the enthalpy of duplex formation by 4.4 and 7.4 kcal/mol, respectively. In other words, the monofunctional adduct of Ru-THA or Ru-CYM enthalpically destabilizes the duplex relative to its nonmodified counterpart. In addition, the formation of the monofunctional adducts by Ru-THA or Ru-CYM resulted in a substantial increase in the entropy of the duplex TGT(15) of 11.6 or 18.2 cal/K.mol ($T\Delta\Delta S$ = 3.6 and 5.4 kcal/mol at 25°C), respectively. In other words, the monofunctional adduct of Ru-THA or Ru-CYM increases the entropy of the ruthenated duplexes and, in this way, entropically stabilizes the duplex. Thus, the 4.4 or 7.4 kcal/mol enthalpic destabilization of the TGT(15) duplex due to the monofunctional adduct of Ru-THA or Ru-CYM is partially, but not completely, compensated by the entropic stabilization of the duplex induced by these adducts of 3.5 or 5.4 kcal/mol at 25°C, respectively. The net result of these enthalpic and entropic effects is that the formation of the monofunctional adducts of Ru-THA and Ru-CYM with the duplex TGT(15) at 25°C induces a decrease in duplex thermodynamic stability ($\Delta\Delta G_{25}$) of 0.8 or 2.0 kcal/mol, respectively, with this destabilization being enthalpic in origin. In this respect, the monofunctional adduct of Ru-CYM was considerably more effective than that of Ru-THA.

Probing by DNA Polymerase

It has been demonstrated that various DNA secondary structures have significant effects on the processivity of a number of prokaryotic, eukaryotic, and viral DNA polymerases. Interestingly, with DNA templates containing site-specifically placed adducts of various platinum compounds, a number of prokaryotic and eukaryotic DNA polymerases were blocked but could also traverse through the adducts depending on their character and conformational alterations induced in DNA. Inhibition of prokaryotic DNA-dependent RNA polymerase by the adducts on DNA globally modified by Ru arene compounds, including Ru-THA and Ry-CYM, has already been demonstrated in in vitro transcription mapping experiments [8]. Interestingly, monofunctional adducts of cisplatin or transplatin and those of the monodentate compounds such as chlorodiethylenetriamineplatinum(II) chloride ([PtCl(dien)]Cl) or $[PtCl(NH_3)_3]Cl$ terminate DNA synthesis by DNA polymerases in vitro markedly less efficiently than crosslinks of platinum complexes [15, 16]. It is, therefore, interesting to examine whether a DNA polymerase, which processes DNA substrates containing monofunctional adducts of Ru-THA or Ru-CYM, can reveal potential specific features of conformational alterations imposed on DNA by the monofunctional adducts of these two Ru(II) arene compounds.

Table 1. Calorimetrically Derived Thermodynamic Parameters for the Formation of the 15 bp Nonmodified Duplexes or Those Containing Single, Site-Specific Monofunctional Adducts of Ru-THA or Ru-CYM at 25°C

Duplex	ΔH^a (kcal/mol)	ΔS^a (cal/K.mol)	$\Delta G_{25}{}^a$ (kcal/mol)	K^b (M^{-1})	N^b
TGT(15)	–79.3	–228	–11.3	1.98×10^8	1.08
TGT(15)·Ru-THA	–74.9	–216	–10.5	3.8×10^7	1.06
TGT(15)·Ru-CYM	–71.9	–210	–9.3	6×10^6	0.94

The ΔH and ΔS values are averages derived from three independent experiments.
[a] ΔH, ΔS, and ΔG_{25} denote, respectively, the enthalpy, entropy, and free energy (at 25°C) of duplex formation.
[b] K and n denote, respectively, association constant and binding stoichiometry for strand association.

We constructed 8-mer/23-mer primer/template nonmodified duplexes or those containing the monofunctional adduct of Ru-THA, Ru-CYM or [PtCl(dien)]Cl in the central TGT sequence (for sequences, see Figure 4). The first eight nucleotides on the 3′ terminus of the 23-mer template strand were complementary to the nucleotides of the 8-mer primer, and the guanine involved in the monofunctional adduct of Ru-THA, Ru-CYM, or [PtCl(dien)]Cl on the template strand was located at the 13th position from the 3′ terminus (Figure 4). After annealing the 8-mer primer to the 3′ terminus of the nonmodified or metallated template strand, positioning the 3′ end of the primer five bases before the adduct in the template strand, we examined DNA polymerization through the single, monofunctional adducts of Ru-THA, Ru-CYM, or [PtCl(dien)]Cl by a Klenow fragment of DNA polymerase I (KF) in the presence of all four deoxyribonucleoside 5′ triphosphates. The reaction was stopped at various time intervals, and the products were analyzed by using a sequencing 24% polyacrylamide (PAA)/8 M urea gel (shown for the monofunctional adducts of Ru-THA and Ru-CYM in Figure 4). Polymerization with the 23-mer template containing the adduct of Ru-THA proceeded rapidly up to the nucleotide preceding and at the sites opposite the adduct, such that the 12 and 13 nucleotide products accumulated to a significant extent (shown in Figure 4, lanes 6–10). The larger DNA intermediates were not observed to a considerable extent, whereas no intermediate products were seen with the 23-mer control template or the template containing the monofunctional adduct of [PtCl(dien)]Cl as the full-length products were formed (shown in Figure 4 for control template, lanes 1–5). The full-length products were also noticed with the 23-mer template containing the adduct of Ru-THA (Figure 4, lanes 6–10). This result demonstrates that the monofunctional adduct of Ru-THA effectively inhibits DNA synthesis, but translesion synthesis may occur. Under the same experimental conditions, DNA polymerization by KF with the template containing the monofunctional adduct of Ru-CYM proceeded up to the nucleotide preceding the site opposite the ruthenated G involved in the adduct and to the following nucleotide residue (Figure 4, lanes 11–15). There was no accumulation of shorter intermediates, but larger DNA intermediates (corresponding to 14 and 15 nucleotide products) and the full-length products were noticed. The amount of the full-length products increased with reaction time, but with a somewhat lower rate compared to the polymerization with the template containing the adduct of Ru-THA.

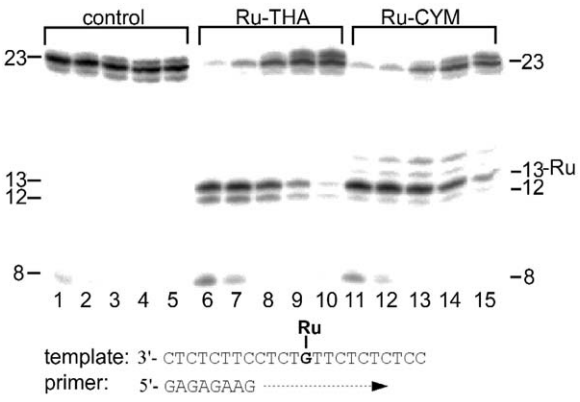

Figure 4. Primer Extension Activity of Klenow Fragment of DNA Polymerase I

Experiments were conducted by incubating 8-mer/23-mer primer/template duplex for various times (lanes 1–5), or the template containing a monofunctional adduct of Ru-THA (lanes 6–10) or of Ru-CYM (lanes 11–15). Timings were as follows: 1 min, lanes 1, 6, and 11; 3 min, lanes 2, 7, and 12; 15 min, lanes 3, 8, and 13; 30 min, lanes 4, 9, and 14; 60 min, lanes 5, 10, and 15. The pause sites opposite the ruthenated guanine and the preceding residues are marked 13 and 12, respectively (the site opposite the ruthenated residue is still marked "Ru"). The nucleotide sequences of the template and the primer are shown beneath the gels.

Repair

Figure 5A illustrates an experiment that measures DNA repair synthesis by a repair-proficient HeLa cell-free extract (CFE) in pUC19 plasmid modified at $r_b = 0.05$ by Ru-THA or Ru-CYM, and for comparative purposes, also by cisplatin. Repair activity was monitored by measuring the amount of incorporated radiolabeled nucleotide. A similar amount of undamaged pBR322 of a slightly different size is included in the reactions to show the background incorporation into undamaged plasmid. This background incorporation was subtracted from that found for metallated pUC19 plasmid. Considerably different levels of damage-induced DNA repair synthesis were detected in the plasmid modified by Ru-THA, Ru-CYM, and cisplatin (Figures 5A and 5B). The level of the synthesis detected in the plasmid modified by Ru-THA was ~6 times lower than that in the plasmid modified by Ru-CYM.

DNA repair synthesis can be due to various DNA repair mechanisms. Bulky, helix-distorting DNA adducts, such as those generated by various chemotherapeutics, including cisplatin, are removed from DNA by nucleotide excision repair (NER), which is an important

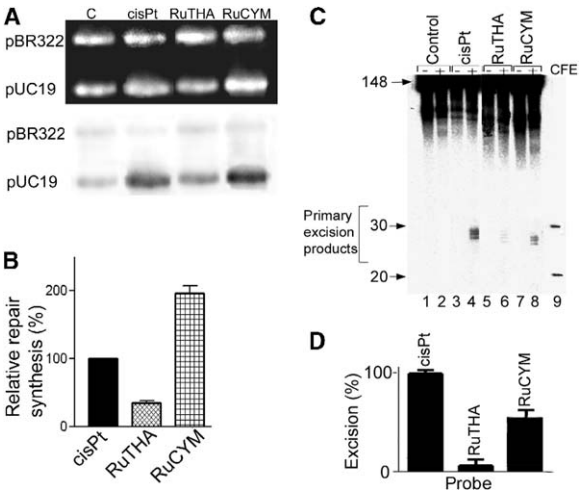

Figure 5. Repair DNA Synthesis and Nucleotide Excision Repair

(A and B) In vitro repair synthesis assay of the extract prepared from the repair-proficient HeLa cell line. Repair synthesis used as substrates nonmodified pBR322 plasmid and nonmodified pUC19 plasmid (lane C) or pUC19 plasmid modified at r_b = 0.05 by cisplatin, Ru-THA, or Ru-CYM (lanes cisPt, RuTHA, and RuCYM, respectively). (A) Results of a typical experiment. The top panel is a photograph of the EtBr-stained gel, and the bottom panel is the autoradiogram of the gel showing incorporation of [α-^{32}P]dCMP. (B) Incorporation of dCMP into nonmodified, platinated, or ruthenated plasmids. For all quantifications representing mean values of three separate experiments, incorporation of radioactive material is corrected for the relative DNA content in each band. The radioactivity associated with the incorporation of [α-^{32}P]dCMP into DNA modified by cisplatin was taken as 100%.

(C and D) Excision of the adducts of ruthenium complexes by rodent excinuclease. (C) The 148 bp substrates were incubated with CHO AA8 CFE and subsequently treated overnight with NaCN prior to analysis in 10% PAA/8 M urea denaturing gel; lanes: 1 and 2, control, nonmodified substrate; 3 and 4, the substrate containing the 1,2-GG intrastrand crosslink of cisplatin; 5 and 6, monofunctional adduct of Ru-THA; 7 and 8, the monofunctional adduct of Ru-CYM. Lanes 1, 3, 5, and 7, no extract added; lanes 2, 4, 6, and 8, the substrates were incubated with CHO AA8 CFE for 40 min at 30°C. Lane M, the 20- and 30-mer markers. (D) Quantitative analysis of removal of the adducts. The columns marked cisPt, RuTHA, and RuCYM represent 1,2-GG intrastrand crosslink of cisplatin, the monofunctional adduct of Ru-THA, and the monofunctional adduct of Ru-CYM, respectively. The radioactivity associated with the fragments excised from the duplex containing the 1,2-GG intrastrand CL of cisplatin was taken as 100%. Data are the average of two independent experiments performed under the same conditions.

component of the mechanism underlying the biological effects of these agents. Efficient removal of crosslinks formed in DNA by platinum antitumor compounds has already been reported for various NER systems, including human and rodent excinucleases [17–20]. The result presented in Figure 5C, lane 4 is consistent with these reports. The major excision fragment contains 28 nucleotides, and other primary excision fragments are 24–29 nucleotides in length [17–20]. In contrast, the monofunctional adducts of Ru-THA and Ru-CYM were also excised by both human and rodent excinucleases (shown for rodent excinuclease in Figures 5C and 5D), although with a noticeably lower efficiency than the major intrastrand crosslink of cisplatin; the adduct of Ru-CYM was excised slightly more than that of Ru-THA. Consistent with this observation were the results of the gel mobility shift assay analysis (Supplemental Figure S1) employing replication protein A (RPA) (which belongs to the initial damage-sensing factors of eukaryotic excision nuclease initiating repair) and DNA probes containing the adducts of Ru-THA, Ru-CYM, or cisplatin. The analysis performed with the substrate containing the major intrastrand crosslink of cisplatin revealed considerably higher binding than that performed with the substrate containing the adduct of Ru-THA or Ru-CYM (Supplemental Figure S1). In addition, a lower binding of RPA to the substrate containing the adduct of Ru-THA than to the substrate with the adduct of Ru-CYM was observed (Supplemental Figure S1). Thus, these results (described in detail in the Supplemental Data) corroborate the findings in Figures 5C and 5D and demonstrate the low efficiency of the mammalian NER systems employed in the present work to excise Ru(II) arene adducts and especially that of Ru-THA.

Cytotoxicity

The cytotoxic activity of the Ru(II) arene compounds tested in the present work was evaluated as described previously [3, 4] and has been determined in two cancer cell lines, A2780 and HT29. The compounds were incubated for 24 hr with the tumor cell lines [3, 4]. IC_{50} values (compound concentration that produces 50% of cell killing) of 0.4 and 10 μM were obtained in A2780 cells, and values of 3 and >100 μM were obtained in HT29 cells for Ru-THA and Ru-CYM, respectively (D.I. Jodrell and R. Aird, personal communication). Hence, the tricyclic-ring complex Ru-THA was considerably more potent than the Ru-CYM complex. Thus, the capability of the Ru(II) arene complex to intercalate DNA correlates with the noticeably enhanced activity of this class of Ru(II) arene compounds in several cancer cell lines.

Discussion

Our initial studies [8] suggested that Ru(II) arene compounds containing multi-ring biphenyl, dihydroanthracene, or tetrahydroanthracene ligands bind to DNA differently in comparison to the complexes containing single hydrocarbon rings, such as *p*-cymene or benzene. DNA binding of the multiring Ru(II) arene complexes involves not only coordination to G N7 together with C6O··(en) H–bonding, but also noncovalent, hydrophobic interactions between the arene ligand and DNA, which may include arene intercalation and minor groove binding. In contrast, the Ru arene compounds containing single hydrocarbon rings cannot interact with double-helical DNA by intercalation. Therefore, we tried first to find out whether this different DNA binding mode correlates with the cytotoxicity of the Ru(II) arene compounds in different tumor cell lines. Interestingly, the results of the previous [3, 4] and present work (vide supra) support the view that the presence of an arene ligand in these classes of ruthenium complexes that is capable of noncovalent, hydrophobic interaction with DNA (presumably intercalation) considerably enhances the cytotoxicity in a number of tumor cell lines.

It has been demonstrated that the biological activity of several transition metal-based complexes is modulated by the "downstream" effects of damaged DNA, such as recognition of damaged DNA by specific proteins and/or repair [21, 22]. For instance, recognition of DNA adducts of several antitumor metal-based drugs and removal of these adducts from DNA is dependent on the character of the distortion and thermodynamic destabilization induced in DNA by these adducts [11, 23]. A more detailed analysis of conformational distortions induced in DNA by Ru-THA and Ru-CYM carried out in the present work revealed substantial differences in the character of these distortions. Their analysis by chemical probes of DNA conformation demonstrated (Figure 2) that the distortion induced by the nonintercalating Ru-CYM extended over at least 7 bp, whereas the distortion induced by Ru-THA was less extensive. Consistent with this observation were the results of the ITC analysis (Figure 3 and Table 1). The association constants, *K*, for the formation of duplexes containing adducts of Ru-THA or Ru-CYM were 5 or 33 times lower, respectively, than the *K* value for the formation of the control (nonmodified) duplex (Table 1). Hence, the adducts of both Ru-THA and Ru-CYM thermodynamically destabilized DNA, with this destabilization being enthalpic in origin. The adduct of Ru-CYM destabilized DNA significantly more than the adduct of Ru-THA, whose DNA binding mode, additionally, involves noncovalent, hydrophobic interactions between the arene ligand and DNA, such as arene intercalation. Various intercalators thermodynamically stabilize DNA since they lengthen and unwind DNA, increasing the phosphate spacing along the helix axis [24, 25]. Hence, it is reasonable to suggest that the higher thermodynamic stability of DNA containing the adducts of Ru-THA observed in the present work is associated with this hydrophobic interaction.

Consistent with the different character of the adducts of Ru-THA and Ru-CYM and with the different impact of these adducts on DNA conformation and stability are also their different effects on primer extension activity of KF (Figure 4). The results of the present work suggest that the monofunctional adducts of Ru-THA and Ru-CYM efficiently inhibit DNA polymerization and in different ways. These studies demonstrate that the monofunctional adducts of Ru-THA and Ru-CYM constitute a fairly strong block to DNA synthesis catalyzed by KF; however, this block is not absolute, allowing translesion DNA synthesis with a limited efficiency. Hence, DNA polymerization appears to be inhibited by Ru(II) arene adducts markedly more strongly than by the adducts of simple monofunctional platinum(II) compounds. This provides a new dimension to the design of Ru(II) arene compounds for affecting processes in tumor cells, possibly including replication or DNA repair.

It has been suggested [21, 22] that HMG domain proteins play a role in sensitizing cells to cisplatin. It has been shown [26] that HMG domain proteins recognize and bind to DNA crosslinks formed by cisplatin. The details of how the binding of HMG domain proteins to cisplatin-modified DNA sensitize tumor cells to cisplatin are still not completely resolved, but possibilities such as shielding cisplatin-DNA adducts from repair or that these proteins could be recruited from their native transcriptional regulatory function have been suggested [21, 22] as clues for how these proteins are involved in the antitumor activity. In addition, an important structural motif recognized by HMG domain proteins on DNA modified by cisplatin is a directional bend of the helix axis toward the major groove [27]. No recognition of the DNA monofunctional adducts of Ru-THA or Ru-CYM by HMGB1 protein was observed in the present work (see the Supplemental Data). A plausible explanation of this observation may be that these adducts do not bend DNA, thus affording no structural motif recognized by HMG domain proteins. From these considerations, we could conclude that the mechanism of antitumor activity of Ru(II) arene compounds does not involve recognition of its DNA adducts by HMG domain proteins as a crucial step, in contrast to the proposals for cisplatin and its direct analogs [21, 22].

Another important feature of the mechanism underlying antitumor effects of DNA binding metal-based compounds is repair of their DNA adducts [21, 28]. A persistence of these DNA adducts may potentiate their antitumor effects in the cells sensitive to these compounds [21, 22, 29]. DNA repair synthesis was investigated in the present work by using the CFE from human tumor cells and DNA substrates randomly modified by the Ru(II) arene compounds (Figures 5A and 5B). Importantly, Ru-THA adducts induced a considerably lower level of repair synthesis than the adducts of Ru-CYM and also of cisplatin (Figure 5B), suggesting a less efficient removal from DNA and enhanced persistence of the adducts of more potent multi-ring and intercalating Ru(II) arene compounds in comparison with the adducts of less potent and nonintercalating Ru(II) arene compounds. Additionally, the level of DNA repair synthesis induced by the adducts of Ru-CYM was still markedly higher than that induced by cisplatin; this finding is consistent with the lower cytotoxicity of this ruthenium compound (vide supra).

There are several types of DNA repair, for instance base excision, NER, mismatch, and recombination repair. The assay based on the measurement of DNA repair synthesis may reflect the effectiveness of all of these repair mechanisms. Several reports have demonstrated [30–32] that NER is a major mechanism contributing to cisplatin resistance. The examination of excision of monofunctional adducts of Ru-THA and Ru-CYM has revealed that these adducts also can be removed from DNA by NER (Figures 5C and 5D), but considerably less efficiently than the adducts of cisplatin. This is in contrast to the results of DNA repair synthesis (Figures 5A and 5B) and implies a less significant role of NER in the mechanism underlying the antitumor effects of Ru(II) arene compounds than in the mechanism of cisplatin. In other words, the results of the present work indicate that the adducts of Ru(II) arene compounds are preferentially removed from DNA by repair mechanisms other than NER, which provides additional support for a mechanism of antitumor activity of Ru(II) arene compounds different from that of cisplatin. Nevertheless, the results of both repair assays employed in the present work (Figure 5) demonstrate clearly that the adducts of Ru-CYM, which distort and destabilize DNA more than the adducts of Ru-THA, are removed from

DNA more effectively, independent of the type of the repair mechanism.

Hence, the character and extent of DNA distortion induced in DNA by the adducts of Ru(II) arene complexes and resulting thermodynamic destabilization of DNA control the biological effects of this class of ruthenium complexes. The results of the present work afford further details, which allow for improving the structure-pharmacological relationship of Ru(II) arene compounds, and should provide a more rational basis for the design of new antitumor ruthenium drugs and chemotherapeutic strategies.

Significance

Organometallic ruthenium(II) arene complexes of the type [(η^6-arene)Ru(II)(en)Cl][PF_6] (en = ethylenediamine) constitute a new group of anticancer compounds. To achieve a rational design of novel antitumor Ru(II) arene compounds, it is important to understand the differences in DNA binding properties of these complexes and their possible relationship to cytotoxicities in different tumor cell lines. In this work, we studied the activity of two Ru(II) arene complexes from the [(η^6-arene)Ru(II)(en)(Cl)]$^+$ family (arene = tetrahydroanthracene and p-cymene, Ru-THA, and Ru-CYM, respectively) in two tumor cell lines, conformational distortions induced by monofunctional adducts of these complexes, and their recognition by DNA binding proteins and repair, i.e., the most important factors that modulate the antitumor effects of related platinum drugs. These two ruthenium complexes were chosen as representatives of two different classes of Ru(II) arene compounds that modify DNA differently: one that may interact with DNA by intercalation (tricyclic-ring Ru-THA), and the other (mono-ring Ru-CYM) that cannot.

The presence of the arene ligand in this class of ruthenium complexes capable of noncovalent, hydrophobic interaction with DNA considerably enhances cytotoxicity in several tumor cell lines. An analysis of DNA duplexes modified by Ru-THA and Ru-CYM revealed substantial differences in the impact of their monofunctional adducts on the conformation and thermodynamic stability of DNA and DNA polymerization in vitro. In addition, the adducts of Ru-CYM are removed from DNA more efficiently than those of Ru-THA. Interestingly, the adducts of Ru(II) arene compounds are preferentially removed from DNA by mechanisms other than nucleotide excision repair. This provides additional support for a mechanism underlying antitumor activity of Ru(II) arene compounds different from that of cisplatin. Hence, the character of DNA distortion induced in DNA by the adducts of Ru(II) arene complexes and the resulting thermodynamic destabilization of DNA control the biological effects of this class of ruthenium complexes.

Experimental Procedures

Starting Materials

The complexes Ru-THA and Ru-CYM (Figure 1A) were prepared by the methods described in detail previously [3, 7]. Cisplatin, glycogen, and dimethyl sulfate (DMS) were obtained from Sigma. [PtCl(dien)]Cl was kindly provided by G. Natile. The stock solutions of the ruthenium and platinum complexes at the concentration of 5×10^{-4} M in H_2O were prepared in the dark at 25°C. Plasmids pUC19 (2686 bp) and pBR322 (4363 bp) were isolated according to standard procedures. The synthetic oligodeoxyribonucleotides were purchased from VBC-Genomics (Vienna, Austria) and were purified as described previously [33]. Restriction endonucleases, T4 polynucleotide kinase, KF, and bovine serum albumin were purchased from New England Biolabs. A CFE was prepared from the HeLa S3 cell line as described [18]. This extract was kindly provided by J.T. Reardon and A. Sancar from the University of North Carolina. Acrylamide, agarose, bis(acrylamide), ethidium bromide (EtBr), urea, and NaCN were purchased from Merck KgaA. Creatine phosphokinase and creatine phosphate were purchased from ICN Biomedicals, Inc. The radioactive products were purchased from Amersham.

Metallation of Oligonucleotides

The single-stranded oligonucleotides (the top, pyrimidine-rich strands containing a single central G of the TGT(15), TGT(20), or TGT(NER) duplexes; Figure 1B) were reacted in stoichiometric amounts with either Ru-THA, Ru-CYM, or [PtCl(dien)]Cl. The ruthenated or platinated oligonucleotides were purified by ion-exchange fast protein liquid chromatography (FPLC). It was verified by ruthenium or platinum flameless atomic absorption spectrophotometry (FAAS) and by optical density measurements that the modified oligonucleotides contained one ruthenium or platinum atom. It was also verified by using DMS footprinting of ruthenium or platinum on DNA [34] that one molecule of ruthenium or platinum complex was coordinated to the N7 atom of the single G in the top strand of each duplex. FPLC purification and FAAS measurements were carried out on a Pharmacia Biotech FPLC System with a MonoQ HR 5/5 column and a Unicam 939 AA spectrometer equipped with a graphite furnace, respectively. The duplexes containing a single, central 1,2-GG intrastrand crosslink of cisplatin in the pyrimidine-rich top strand were prepared as described [12]. The nonmodified, ruthenated, or platinated duplexes used in the studies of recognition by RPA protein were purified by electrophoresis on native 15% PAA gels (mono:bis[acrylamide] ratio = 29:1).

Isothermal Titration Calorimetry

The standard isothermal titration calorimetry (ITC) buffer for these studies contained 50 mM NaCl with 10 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4 [pH 7.0]). Sufficient quantities of ITC solutions were prepared to perform a set of titrations of the 50 μM solution of the bottom strand of the duplex TGT(15) (for its sequence, see Figure 1B) into the 5 μM solution of the top strand of nonmodified TGT(15) or that containing the single, site-specific monofunctional adduct of Ru-THA or Ru-CYM at 25°C. Molar extinction coefficients for the single-stranded oligonucleotides (related to the strands that were 15 nucleotides long) used in ITC experiments were determined by phosphate analysis [35]. The following extinction coefficients at 260 nm and 25°C were obtained: 108,000 and 128,000 $M^{-1}\cdot cm^{-1}$ for the upper and bottom strands of the nonmodified TGT(15) duplex, respectively; 113,000 and 111,000 $M^{-1}\cdot cm^{-1}$ for the upper strand of the TGT(15) containing the single monofunctional adduct of Ru-THA and Ru-CYM, respectively. Stock solutions of the strands for ITC studies were prepared in the ITC buffer and were exhaustively dialyzed against this buffer. It was verified that enthalpies of ITC injections of each individual oligomer into buffer, of buffer into buffer, and of excess oligomer into a solution of duplex were all the same as water into water injections, within error. From these data, it was concluded that effects of any solvent mismatching are negligible. Titrations were carried out on a VP-ITC instrument (MicroCal LLC, Northampton, MA). For each titration, the top strand of the nonmodified TGT(15) duplex or that containing the single, site-specific adduct of Ru-THA or RuCYM was loaded into the 1.4 ml sample cell, and the complementary oligomer (bottom strand of the duplex TGT(15)) was loaded into the 300 μl injection syringe. The stirring rate of the injection syringe was 400 rpm, and samples were equilibrated thermally prior to a titration until the baseline had leveled off and the rms noise was less than 0.015 $\mu cal\cdot s^{-1}$. A typical titration consisted of 50 injections of 5 μl each,

with 3 min between injections. Data from individual titrations were analyzed by using the Origin 5.0 software package (Origin, Northampton, MA) to extract the relevant thermodynamic parameters (the enthalpy change [ΔH], the entropy change [ΔS], the stoichiometry [n], and the equilibrium constant [K] for strand association).

Inhibition of DNA Polymerization

We investigated DNA polymerization using the templates site-specifically modified by Ru-THA or Ru-CYM by KF. The DNA polymerase I class of enzymes has served as the prototype for studies on structural and biochemical mechanisms of DNA replication [36, 37]. The 23-mer templates containing a single monofunctional adduct of Ru-THA or Ru-CYM were prepared in the same way as described above. The eight-mer DNA primer was complementary to the 3′ terminus of the 23-mer template. The DNA substrates were formed by annealing templates and 5′-end-labeled primers at a molar ratio of 3:1. All experiments were performed at 25°C in a volume of 50 μl in a buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 50 μg/ml BSA, 25 μM dATP, 25 μM dCTP, 25 μM dGTP, 25 μM TTP and 0.5 U KF. Reactions were terminated by the addition of EDTA so that its resulting concentration was 20 μM and by heating at 100°C for 30 s. Products were resolved on a denaturing 24% PAA/8 M urea gel and then visualized and quantified by using the FUJIFILM bio-imaging analyzer and AIDA image analyzer software.

Repair Synthesis by Human Cell Extracts

Repair DNA synthesis of CFEs was assayed by using pUC19 and pBR322 plasmids. Each reaction of 50 μl contained 250 ng nonmodified pBR322 and 250 ng nonmodified or platinated pUC19; 2 mM ATP; 30 mM KCl; 0.5 mg/ml creatine phosphokinase (rabbit muscle); 20 mM of each dGMP, dCTP, and TTP; 8 mM dATP; 74 kBq [α-^{32}P]dAMP in the buffer composed of 40 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 22 mM creatine phosphate, 1.4 mg/ml bovine serum albumin, and 150 μg CFE. Reactions were incubated for 3 hr at 25°C and terminated by adding EDTA to a final concentration of 20 mM, SDS to 0.6%, and proteinase K to 250 μg/ml and then incubating for 30 min. The products were extracted with 1 volume 1:1 phenol:chloroform. The DNA was precipitated from the aqueous layer by the addition of 1/50 volume 5 M NaCl, 5 mg glycogen, and 2.5 volumes ethanol. After 20 min of incubation on dry ice and centrifugation at 12,000 × g for 30 min at 4°C, the pellet was washed with 0.5 ml 70% ethanol and dried in a vacuum centrifuge. DNA was finally linearized before electrophoresis on a 1% agarose gel containing 0.3 mg/ml EtBr. The basic principles of this assay are shown schematically in Supplemental Figure S2A.

Nucleotide Excision Assay

The 149 bp substrates containing a single monofunctional adduct of Ru-THA or Ru-CYM were assembled from three oligonucleotide duplexes. The central duplex was TGT(NER) duplex (shown in Figure 1B) to which two duplexes (arms) with random base pair sequences with overhangs partially overlapping those of the modified duplex were ligated (one to each side) by T4 DNA ligase. The top strand of the modified central duplexes were 5′-end labeled with ^{32}P before ligation. Substrates containing a single, central 1,2-GG intrastrand crosslink of cisplatin were prepared in a similar way to that described previously [38]. Full-length substrates (nonmodified, containing the monofunctional adduct of Ru-THA or Ru-CYM or the 1,2-intrastrand crosslink of cisplatin) were separated from unligated products on a denaturing 6% PAA gel, purified by electroelution, reannealed, and stored in annealing buffer (50 mM Tris-HCl [pH 7.9], 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol) at 20°C. In vitro repair of monofunctional adducts of Ru(II) arene complexes and of the 1,2-intrastrand crosslink of cisplatin was measured in an excision assay as described previously [38], with minor modifications. The reaction mixtures (25 μl) contained 10 fmol radiolabeled DNA, 50 μg CFE, 20 μM dATP, 20 μM dCTP, 20 μM dGTP, and 20 μM TTP in reaction buffer (23 mM HEPES [pH 7.9], 44 mM KCl, 4.8 mM $MgCl_2$, 0.16 mM EDTA, 0.52 mM dithiothreitol, 1.5 mM ATP, 5 μg bovine serum albumin, and 2.5% glycerol) and were incubated at 30°C for 40 min. DNA was deproteinized and precipitated by ethanol. Reaction products were treated overnight with 0.4 M NaCN (pH 10–11) at 45°C and precipitated by ethanol prior to resolution on the gels. The excision products were separated on denaturing 10% PAA gels and visualized by using the PhosphorImager. The basic principles of this assay are shown schematically in Supplemental Figure S2B.

Supplemental Data

A description of the experiments with chemical probes of DNA conformation, recognition by HMGB1 and RPA proteins, corresponding experimental procedures, and the basic principles of repair DNA synthesis and nucleotide excision repair assays are available at http://www.chembiol.com/cgi/content/full/12/1/121/DC1/.

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