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Inhibition of Transcription in Vitro by Anticancer Active Dirhodium(II) Complexes

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The DNA binding and inhibition of transcription in vitro by neutral $Rh_2(\mu\text{-}O_2CCH_3)_4$ and cationic *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ complexes were investigated. The binding constants of the two complexes to calf-thymus DNA were estimated from absorption titrations to be 4.6×10^2 M^{-1} and 1.7×10^4 M^{-1} for $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$, respectively. The shift to higher energies of the low-energy absorption of the complexes upon addition of DNA is consistent with axial binding of the complexes to duplex DNA. The relative concentrations, [complex]/[DNA], of $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ at which 50% of the transcription is inhibited (R_{inh}^{50}), are 0.0031 and 0.0011, respectively. These concentrations are significantly lower than that required for activated cisplatin, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, with $R_{inh}^{50} = 0.0085$ under similar experimental conditions. Upon incubation of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ with the template DNA prior to the addition of the enzyme and nucleobases necessary for the transcription reaction for 30 min at 37 °C, significantly lower concentrations of the complex were required to attain 50% inhibition. In contrast, similar incubation of the DNA with the dirhodium complexes did not result in better transcription inhibition. Experiments designed to elucidate the mechanism of the observed inhibition indicate that, unlike *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ appear to interact directly with the enzyme T7-RNA polymerase as their mode of inhibition.

Introduction

Agents that inhibit transcription are potentially useful in the treatment of various cancers and as antiviral agents.[1−5] Such drugs currently include actinomycin, anthracycline antibiotics, and cisplatin, among others.[6−8] Although many of these drugs exhibit strong binding to duplex DNA which ultimately results in the disruption of the transcription process, others bind directly to the polymerase enzyme, thus hindering its action.

It has long been known that $Rh_2(\mu\text{-}O_2CCH_3)_4$ (structure shown in Figure 1) binds to duplex DNA (ds-DNA), single-stranded DNA (ss-DNA), and various nucleic acids.[9,10] However, its mode of binding to duplex DNA remains

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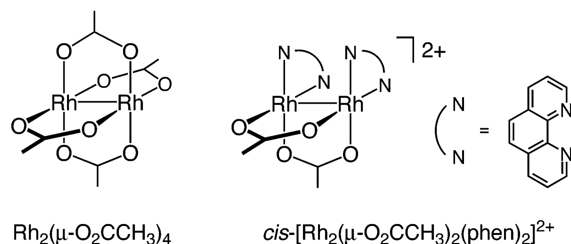

Figure 1. Molecular structures of $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$.

unknown. $Rh_2(\mu\text{-}O_2CCH_3)_4$ and other dirhodium(II) tetracarboxylates were shown to possess antitumor properties[11] and to inhibit DNA replication[9] and more recently were shown to act as antibacterial agents and to exhibit cytostatic activity against human oral carcinoma.[12] It has also been demonstrated that the cationic complex *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ (structure shown in Figure 1) is a better antibacterial agent than $Rh_2(\mu\text{-}O_2CCH_3)_4$.[12] Various related complexes have recently been shown to possess antitumor activity, in some cases higher than that of cisplatin, *cis*-$PtCl_2(NH_3)_2$.[13] The role of the complexes in the inhibition of DNA replication, as antibacterial agents, and their antitumor activity is still unclear. In addition, the DNA photocleavage by $Rh_2(\mu\text{-}O_2CCH_3)_4$ in the presence of electron acceptors was recently reported, although the mechanism of the scission has not been elucidated.[14]

In the present work, we explore the properties of two biologically active dirhodium(II/II) carboxylate compounds, namely $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]Cl_2$, as potential agents in RNA transcription inhibition. The results are compared to those obtained with activated cisplatin, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$. In addition, spectroscopic information that provides insight into the binding of the dirhodium complexes to ds-DNA is presented.

Experimental Section

Materials. $RhCl_3$, sodium acetate, *cis*-$PtCl_2(NH_3)_2$, and $AgBF_4$ were purchased from Aldrich and used without further purification. Agarose, ethidium bromide, RNA loading buffer, and 1,10-phenanthroline were purchased from Sigma and were used as received. Calf-thymus DNA was purchased from Sigma and was three times dialyzed against 5 mM Tris (50 mM NaCl, pH = 7.5) over a period of 48 h prior to use. The compound $Rh_2(\mu\text{-}O_2CCH_3)_4(H_2O)_2$ was synthesized by previously reported methods and was purified by elution with methanol from a silica gel column.[15−17]

Preparation of *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]Cl_2$. The synthesis was modified from a reported literature procedure and was carried out under an inert atmosphere.[18] A purple solution of $Rh_2(\mu\text{-}O_2CCH_3)_4$ (0.21 mmol) and NaCl (0.42 mmol) in 15 mL of CH_3CN was slowly added to an acetonitrile solution (10 mL) of 1,10-phenanthroline (0.42 mmol). The solution mixture was refluxed for 15 h, after which time the color of the solution was red-brown. The solution was concentrated in vacuo to ca. 5 mL. A copious amount of diethyl ether was added to precipitate an orange-brown solid which was dried in vacuo and redissolved in 3 mL of CH_3CN. The solution was layered with diethyl ether, and microcrystals were harvested. Yield = 0.11 g (70%). ^1H NMR, D_2O, δ/ppm (multiplicity): 2.66 (s), 7.59 (dd), 7.70 (s), 8.25 (d), 8.48 (d). IR, KBr, ν/cm^{-1}: 3094, 2936, 1630, 1557, 1518, 1445, 1431, 1414, 1343, 1065, 847, 716. UV−vis, H_2O, λ/nm ($\epsilon/M^{-1}\ cm^{-1}$): 341 (3991), 411 (1704), 562 (141).

Preparation of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$. The tetrafluoroborate salt of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ was synthesized by stirring *cis*-$[PtCl_2(NH_3)_2]$ in the presence of 1.9 equiv of $AgBF_4$ for 15 h in deionized water (18 MΩ). The AgCl precipitate was removed by filtration, and a yellow solution was collected for further dilution in the RNA transcription inhibition studies.

Methods. The transcription assay and the methods used for the determination of binding constants from absorption titration data have been previously reported.[19−21] The DNA binding studies were conducted in 2 mM Tris buffer, pH = 7, and 4.73 μM $Rh_2(\mu\text{-}O_2CCH_3)_4(H_2O)_2$ and 23.8 μM *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]Cl_2$ were used. The in vitro transcription experiment used the pGEM linear DNA template (3995 bp, 129 μM bases), the Ribomax large scale RNA production system was purchased from Promega, and the T7 RNA polymerase (80 units/μL) and 5× RNA loading buffer were purchased from OSU Molecular Biology Center. The transcription reaction was allowed to proceed for 1 h at 37 °C (40 mM Tris/HCl, 10 mM NaCl, pH = 7.5) in nuclease-free deionized water (18 MΩ) in the presence of 6 mM $MgCl_2$, 2 mM spermidine, and 250 μM of each nucleotide. The inhibition of mRNA production in vitro was examined through the measurement of mRNA produced upon addition of increasing amounts of metal complex relative to template DNA bases, R_{inh} = [complex]/[bases], to the assay.

Instrumentation. Absorption measurements were performed either on a Hewlett-Packard diode array spectrometer (HP8453) with HP8453 Win System software or on a Schimadzu UVPC-3001 spectrophotometer. The ethidium bromide stained agarose gels (1%) were imaged using a GelDoc 2000 transilluminator (BioRad) connected to a desktop computer. The digital images were analyzed using the Quantity One software package (Bio-Rad), which includes intensity integration. ^1H NMR measurements were performed on a Mercury 300 MHz spectrometer, and infrared spectra were collected on a Nicolet Nexus 470 FTIR spectrometer on samples pressed in KBr pellets.

Results and Discussion

DNA Binding Studies. We conducted spectroscopic titrations of a given concentration of each complex with calf-

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thymus DNA in order to obtain their binding constants and to gain some insight into their mode of binding to duplex DNA. The binding constant, K_b, of $Rh_2(\mu\text{-}O_2CCH_3)_4$ to DNA was determined to be 4.6×10^2 M^{-1} (2 mM Tris buffer, pH = 7), a value which is similar in magnitude to the data previously reported for the complex with nucleotides,[9b] and is not affected by the additional buffer and salt present under the conditions of the transcription reaction (40 mM Tris, pH = 7, 10 mM NaCl). The cationic complex *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ exhibits significantly stronger binding to ds-DNA, with $K_b = 1.7 \times 10^4$ M^{-1} (2 mM Tris buffer, pH = 7). The positive charge of *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ plays a significant role in the binding of the complex to the polyanionic DNA, because the spectral changes upon addition of DNA at higher salt concentration (40 mM Tris, pH = 7, 10 mM NaCl) were negligible.[21,22]

The shifts of the electronic absorption spectra of both $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ upon addition of increasing amounts of calf-thymus DNA were measured. The lowest energy transition of $Rh_2(O_2CCH_3)_4$ at 585 nm in H_2O (ϵ = 241 M^{-1} cm^{-1}), assigned as arising from π^*(Rh−Rh) to σ^*(Rh−Rh), shifts to higher energy upon addition of DNA.[13,23−25] Similar shifts are observed upon axial coordination of $Rh_2(\mu\text{-}O_2CCH_3)_4$ by strong σ-donor ligands, whose antisymmetric linear combination interacts with σ^*(Rh−Rh), thus raising the energy of that orbital.[26,27] Therefore, it appears that there is an initial axial interaction between $Rh_2(\mu\text{-}O_2CCH_3)_4$ and ds-DNA. The lowest energy transition observed in the electronic absorption spectrum of *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ at 562 nm (ϵ = 141 M^{-1} cm^{-1}), attributed to π^*(Rh−Rh) to σ^*(Rh−Rh), also shifts to higher energy upon addition of DNA.

Transcription Inhibition. In the transcription reaction, single-stranded RNA is produced with linear ds-DNA as the template in the presence of T7-RNA polymerase enzyme and each of the nucleotide monophosphates. The RNA produced in the transcription reaction decreases as the concentration of each metal complex is increased. This effect is shown for *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ in Figure 2a. Integration and interpolation of the imaged signal from the RNA produced in the presence of different concentrations of the metal complexes can be used to calculate the relative complex concentration ([complex]/[DNA bases]) required to inhibit 50% of the transcription, R_{inh}^{50}. The values of R_{inh}^{50} obtained in this manner for $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ are 0.0031 and 0.0011, respectively. For comparison, the transcription inhibition assay was also conducted in the presence of various amounts of activated cisplatin, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, which resulted in R_{inh}^{50} = 0.0085 (Figure 2b).

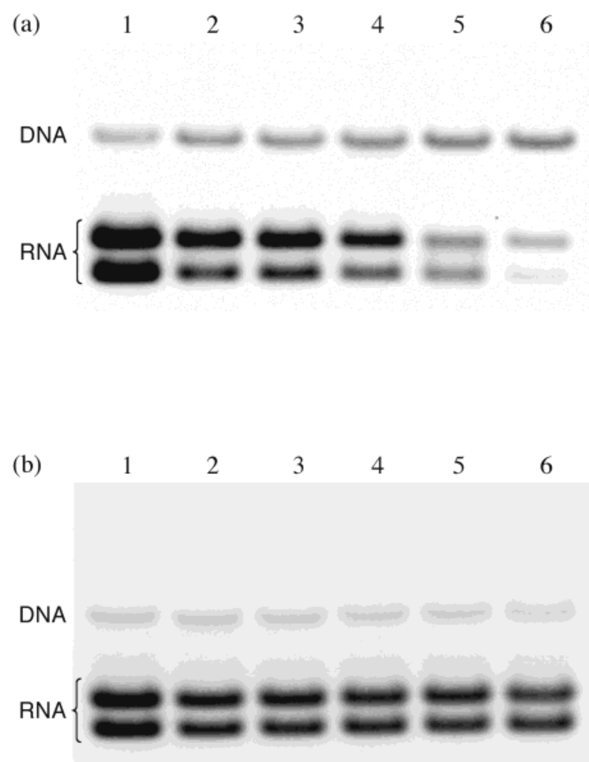

Figure 2. Ethidium bromide stained agarose gel (1%) of transcribed mRNA in the presence of (a) *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ and (b) *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ at various [complex]/[template DNA base] ratios, *R*. Both the DNA template (120 μM) and mRNA are imaged on the gels (labeled). (a) Lanes 1−6, *R* = 0.0000, 0.0005, 0.0010, 0.0015, 0.0020, 0.0025 and (b) lanes 1−6, *R* = 0.000, 0.002, 0.004, 0.006, 0.008, 0.010.

Experiments where the transcription reaction was allowed to run in the absence of metal complex followed by addition of 0.37 μM $Rh_2(\mu\text{-}O_2CCH_3)_4$ (*R* = 0.0031) and 0.15 μM *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ (*R* = 0.0011) prior to loading the gel resulted in the imaging of 100% of the RNA produced relative to the lane to which no complex had been added. These control experiments show that the addition of metal complexes did not degrade (hydrolyze) the RNA once formed or interfere with the ethidium bromide imaging of the RNA. Binding of the complexes to the NTPs can be ruled out as a mechanism of transcription inhibition, because each NTP is present in large excess (250 μM) relative to the concentration of metal complexes (<1 μM).

In order to differentiate between an inhibition mechanism involving binding of the complex to DNA or to the enzyme, an experiment was performed in which the concentration of all components with the exception of that of the enzyme was 10-fold lower. In this experiment, the ratio of the complex concentration to that of the template DNA was kept constant. Therefore, if the mechanism of transcription inhibition involves binding of the complex to DNA, then similar relative inhibition interpreted from R_{inh}^{50} is expected. Alternatively, because the relative concentration of the enzyme

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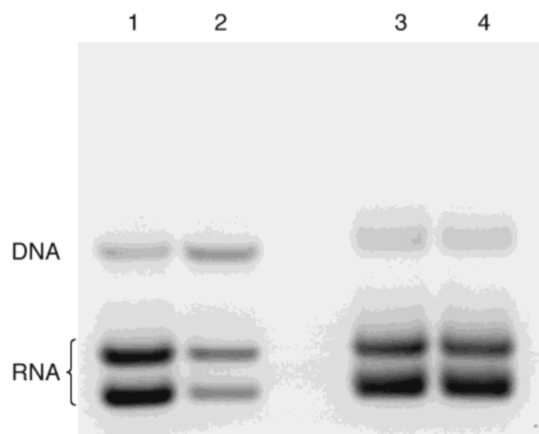

Figure 3. Ethidium bromide stained agarose gel (1%) of transcribed mRNA in the absence (lanes 1 and 3) and presence of *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ (lane 2, 225 nM complex, $R = 0.0015$; lane 4, 22.5 nM complex, $R = 0.0015$). Lanes 1 and 2: 150 μM template DNA, 50 units polymerase, 1 mM nucleotides (250 μM each), 15 min reaction time. Lanes 3 and 4: 15 μM template DNA, 50 units polymerase, 100 μM nucleotides (25 μM each), 150 min reaction time.

to the complex was increased by 10-fold, it would be predicted that, if binding of the complex to the enzyme results in the inhibition of transcription, greater RNA production is expected under these conditions. The results shown in Figure 3 for *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ clearly show that a 10-fold increase of the enzyme concentration relative to that of the complex, whose concentration was held at [complex]/[DNA bases] = 0.0015, results in significantly less or negligible inhibition of transcription. Similar results were obtained for $Rh_2(\mu\text{-}O_2CCH_3)_4$. In contrast, the decrease in RNA production remained unchanged as the relative concentration of the enzyme was increased by a factor of 10 in the case of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$.

The observations for activated cisplatin are consistent with the covalent binding of the complex to duplex DNA, ultimately resulting in the inhibition of transcription.[28] These results are indicative of a mechanism of the inhibition of transcription by $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ which involves interaction of the complexes with the enzyme T7-RNA polymerase. These findings are in accord with the early results of Bear and co-workers who reported that dirhodium complexes bind to proteins and enzymes, particularly those with a sulfhydryl functional group.[29] Additional studies between dirhodium carboxylates and SH-containing compounds have also been carried out in our laboratories in an attempt to understand metabolic pathways of these complexes in cellular media.[30]

Furthermore, experiments in which the concentration of the enzyme was increased 4 times while those of all other components in the transcription reaction were kept the same as those used in the experiments shown in Figure 2 are also consistent with this interpretation. In these experiments, the ratio of complex to template DNA was held constant at $R = 0.0015$. For $[Pt(NH_3)_2(H_2O)_2]^{2+}$, the increase in the enzyme concentration does not result in an increase in transcription, as expected for a mechanism that involves the binding of the complex to the template duplex DNA. In contrast, for $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$, the reactions with additional enzyme resulted in a greater production of RNA, pointing at transcription inhibition resulting from interaction of the complexes with the T7-RNA polymerase.

In addition, experiments were conducted in which the dirhodium(II/II) complexes were incubated with the template DNA for 30 min at 37 °C prior to the addition of the enzyme and nucleotides, which is known to result in increased binding of the molecules to DNA. These reactions resulted in slightly higher concentrations of the complexes required to attain 50% transcription inhibition, with R_{inh}^{50} values of 0.0035 and 0.0017 for $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$, respectively. These results are also consistent with a mechanism inhibition that involves binding of complexes to the enzyme. The increased binding of the complexes to DNA upon incubation would be expected to result in a lower equilibrium concentration of each free complex in solution available to interact with the enzyme, thus requiring a greater quantity of the dirhodium(II/II) complexes to achieve the same inhibition.

Previous studies of inhibition of transcription in vitro utilizing a different enzyme/template system showed only small effects at much greater complex concentrations (20−200 μM).[9a,11a] In the assay presented here with T7-RNA polymerase, significantly smaller concentrations (1.1 and 3.0 μM of $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$, respectively) are required to inhibit 50% of the transcription. These concentrations are lower than those required for activated cisplatin under similar conditions. It is apparent that a mechanism where the T7-RNA polymerase enzyme action is blocked by the Rh_2(II/II) complexes is operative here. In addition to the binding of the complexes to protein or enzyme-SH groups, it was recently shown that both $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ bind to histidine (His) residues of human serum albumin (HSA).[31] A possible explanation of the results presented here is that the Rh_2(II/II) complexes bind to the active site of the enzyme, thus affecting its action. Residues that have been shown to play a role in the activity of T7-RNA polymerase are glycine Gly542 in nucleotide binding, aspartate Asp812 in catalysis, asparagine Asn748 in promoter interaction, and glutamate Glu148 in RNA binding.[32] The Asp812, Asn748, and Glu148 residues could interact with the dirhodium core through axial coordination. In addition, cysteine Cys540 and histidine His545 are present near Gly542, and His811 is next to Asp812, all of which could bind to the Rh_2(II/II) complexes.

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Conclusions

The present work supports the conclusion that complexes $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ inhibit transcription with R_{inh}^{50} values of 0.0031 and 0.0011, respectively. Spectroscopic titrations of the complexes with calf-thymus DNA yield binding constants, K_b, of 4.6×10^2 M^{-1} and 1.7×10^4 M^{-1} for $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$, respectively. In addition, the shift to higher energy of the lowest energy transition of the complexes is consistent with axial coordination to DNA at room temperature occurring instantaneously upon mixing. Unlike cisplatin, a plausible mechanism of the inhibition of transcription by $Rh_2(\mu\text{-}O_2CCH_3)_4$ and *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(phen)_2]^{2+}$ is the binding of the complexes to the enzyme T7-RNA polymerase rather than strong interaction with the DNA template. Nonetheless, the low concentrations of the dirhodium complexes required to inhibit transcription compared to activated cisplatin ($R_{inh}^{50} = 0.0085$) indicate that these dirhodium complexes may have promising potential applications as chemotherapeutic agents.

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