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Live Cell Cytotoxicity Studies: Documentation of the Interactions of Antitumor Active Dirhodium Compounds with Nuclear DNA

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Abstract: The promising antitumor activity of dirhodium complexes has been known for over 30 years. There remains, however, a general lack of understanding of their activity *in cellulo*. In this study, we report the DNA interactions and activity in living cells of six monosubstituted dirhodium(II,II) complexes of general formula $[Rh_2(\mu\text{-}O_2CCH_3)_2(\eta^1\text{-}O_2CCH_3)(L)(CH_3OH)]^+$, where L = bpy (2,2′-bipyridine) (**1**), phen (1,10-phenanthroline) (**2**), dpq (dipyrido[3,2-*f*:2′,3′-*h*]quinoxaline) (**3**), dppz (dipyrido[3,2-*a*:2′,3′-*c*]phenazine) (**4**), dppn (benzo[*i*]dipyrido[3,2-*a*:2′,3′-*c*]phenazine) (**5**), and dap (4,7-dihydrodibenzo[*de*,*gh*][1,10]phenanthroline) (**6**). DNA interactions were investigated by UV/visible spectroscopy, relative viscosity measurements, and electrophoretic mobility shift assay. These measurements indicate that compound **5** exhibits the strongest interaction with DNA. Compound **5** also causes the most damage to DNA after cellular internalization, as evaluated by the alkaline comet assay. Compound **5**, however, is not the most effective at inhibiting cell viability of the human cancer cells HeLa and COLO-316. The greater hydrophobicity of **5** as compared to that of **4**, which is the most effective compound in the series, hinders its ability to reach its cellular target(s). Data from modulation studies of glutathione using *N*-acetylcysteine and L-buthionine-sulfoximine indicate that changes in glutathione levels do not affect the activity of these particular dirhodium complexes. These results suggest that glutathione is not the only agent involved in the deactivation of these dirhodium complexes.

Introduction

The finding by Rosenberg in 1965 that bacterial cell division is inhibited by cisplatin[1] led to the development of one of the most successful antitumor agents in the history of chemotherapy.[2] Mechanistic studies conducted over the past four decades have established that the activity of cisplatin is related primarily to its ability to form 1,2- and 1,3-intrastrand DNA cross-links.[3] Significantly, these DNA adducts interfere with important cellular processes that ultimately result in cell death.[3] In efforts to improve the efficacy and overcome the side effects associated with the use of cisplatin, compounds of numerous other transition metals have also been investigated over the years.[4,5]

Of direct relevance to the present study is the finding that certain dirhodium(II,II) compounds exhibit antitumor activity against a variety of cancer cell lines.[6−9] Dinuclear metal−metal bonded compounds of rhodium have attracted attention since the 1970s for their cytostatic properties[7,8,10,11] and are among the most promising non-platinum anticancer complexes.[9] It was first shown by Bear and collaborators that dirhodium tetraacetate exhibits appreciable cytostatic activity against a variety of cell lines, including sarcoma 180, Ehrlich ascites tumor, and P388 lymphocytic leukemia.[6−8,10,12] Later, compounds of general formula $Rh_2(\mu\text{-}O_2CCH_3)_2(DTolF)_2L_2$ (DTolF = *N*,*N*′-ditolylformamidinate and L= solvent molecules)[13] and the cationic diimine complexes of dirhodium(II,II), namely $Rh_2(\mu\text{-}O_2CCH_3)_2(diimine)_2^{2+}$ (diimine = 2,2′-bipyridine and 1,10-

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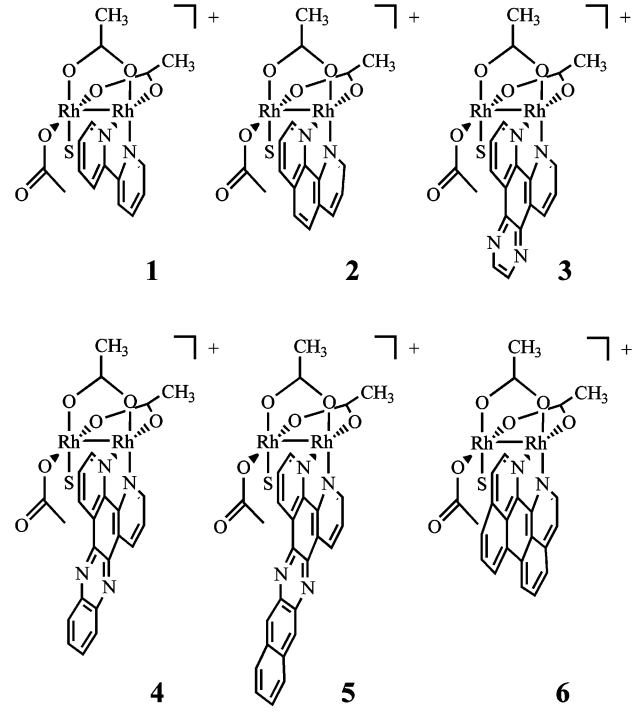
phenanthroline),[14] were also shown to exhibit antitumor activity, in some cases higher than that of cisplatin. More recently, Esposito and co-workers demonstrated that $Rh_2(\mu\text{-}ONHCCF_3)_4$ is active against U937 and K562 human leukemia cells as well as Ehrlich ascites tumor cells both *in vitro* and *in vivo*.[15,16]

Although the cellular target(s) of these compounds remains to be elucidated, we have established that dirhodium carboxylate compounds bind *in vitro* to nucleotides and form intrastrand DNA cross-links.[9] Despite earlier claims that $Rh_2(\mu\text{-}O_2CCH_3)_4$ did not react with double-stranded DNA (dsDNA),[7] work in our laboratories has provided evidence that dirhodium tetraacetate and its derivatives do indeed bind to dsDNA. A study aimed at investigating the interactions of dsDNA and dirhodium carboxylate compounds revealed that $Rh_2(\mu\text{-}O_2CCH_3)_4$, $Rh_2(\mu\text{-}O_2CCF_3)_4$, and $[Rh_2(\mu\text{-}O_2CCH_3)_2(CH_3CN)_6]^{2+}$ are capable of forming DNA interstrand cross-links.[17] Additionally, it was observed that other adducts, namely monofunctional and intrastrand adducts, are also formed during the course of the reaction. Adducts of DNA with dirhodium complexes most likely involve the full variety of coordination modes observed in model complexes, including *axial/axial* (*ax/ax*), *axial/equatorial* (*ax/eq*), and *equatorial/equatorial* (*eq/eq*).[17,18]

Systematic studies of structure−activity relationships among dirhodium complexes have provided insight into the molecular characteristics that control their activity. A study performed on the series of dirhodium tetracarboxylate complexes $Rh_2(\mu\text{-}O_2CR)_4$ ($R = CH_3, C_2H_5, C_3H_7$), which exhibit cytostatic activity against Ehrlich ascites tumor, Leukemia L1210, and Sarcoma 180 cells, revealed that the activity in this series increases with the lipophilicity of the R group, but that a further lengthening of the carboxylate group beyond the pentanoate group reduces their therapeutic efficacy.[19] The increased activity of the compounds with lipophilicity underscores the importance of diffusion across lipid bilayers for the biological activity of dirhodium complexes. More recently, new results from our laboratories have provided evidence that the ligand lability and the accessibility of the equatorial sites also play a critical role in the activity of these complexes.[18] The role of the axial position has also recently been documented. A study of three dirhodium complexes containing two, one, or zero available axial positions for substitution chemistry revealed that their ability to react with DNA and to inhibit transcription is hindered when these positions are blocked.[20]

In the present work, the series of complexes $[Rh_2(\mu\text{-}O_2CCH_3)_2(\eta^1\text{-}O_2CCH_3)(L)]^+$, where L = bpy (2,2′-bipyridine) (**1**), phen (1,10-phenanthroline) (**2**), dpq (dipyrido[3,2-*f*:2′,3′-*h*]quinoxaline) (**3**), dppz (dipyrido[3,2-*a*:2′,3′-*c*]phenazine) (**4**), dppn (benzo[*i*]dipyrido[3,2-*a*:2′,3′-*c*]phenazine) (**5**), and dap (4,7-dihydrodibenzo[*de*,*gh*][1,10]phenanthroline) (**6**) (Chart 1), was studied *vis-à-vis* the effect of the diimine ligand on the biological properties of the compounds. The impact of the hydrophobicity and the effect of glutathione on the *in cellulo* activity of these complexes were also explored. The results are an important addition to our ongoing efforts to understand the mechanism of action of this promising class of metal complexes and are expected to aid in the design of more effective therapeutic dirhodium agents.

Chart 1. Structures of Compounds **1**−**6** (S = CH_3OH)



Experimental Section

Materials. The reagents 2,2′-bipyridine (bpy), 1,10-phenanthroline (phen), diaminoethylene, and 2,3-diaminonaphthalene were purchased from Acros. Calf thymus DNA, ethidium bromide, Hoechst 33258, *n*-octanol, 1,10-phenantroline-5,6-dione, L-buthionine-sulfoximine (BSO), and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich and used as received. The circular plasmid pUC18 was purchased from Fermentas. The DNA oligonucleotide (5′-ATCACCTAAAATGGCG-3′) and its complementary strand were purchased from The Midland Certified Reagent Co. (Midland, TX) as pure materials. The starting material $RhCl_3 \cdot H_2O$ was purchased from Pressure Chemicals and used as received.

The ligands pyrazino[2,3-*f*][1,10]phenanthroline (dpq),[21] dipyrido[3,2-*a*:2′,3′-*c*]phenazine (dppz),[22] benzodipyrido[3,2-*a*:2′,3′-*c*]phenazine (dppn),[23] and 4,7-dihydrodibenzo[*de*,*gh*][1,10]-phenanthroline (dap)[24] were synthesized according to reported procedures.[21−24] The dirhodium complex $Rh_2(\mu\text{-}O_2CCH_3)_4$ was prepared according to published literature procedures.[25] The following monosubstituted dirhodium complexes were all prepared according to previously described procedures: *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(\eta^1\text{-}O_2CCH_3)(bpy)(CH_3OH)](O_2CCH_3)$ (**1**),[26] *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(\eta^1\text{-}O_2CCH_3)(phen)(CH_3OH)](O_2CCH_3)$ (**2**),[26] *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(\eta^1$-

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O_2CCH_3)(dppz)(CH_3OH)](O_2CCH_3) (**4**),[27,28] *cis*-[Rh_2(μ-O_2CCH_3)$_2$(η^1-O_2CCH_3)(dppn)(CH_3OH)](O_2CCH_3) (**5**),[29] and *cis*-[Rh_2(μ-O_2CCH_3)$_2$-(η^1-O_2CCH_3)(dap)(CH_3OH)](O_2CCH_3) (**6**).[30]

Synthesis of *cis*-[Rh_2(μ-O_2CCH_3)$_2$(η^1-O_2CCH_3)(dpq)(CH_3OH)]-[O_2CCH_3] (3). A suspension of dpq (200 mg, 0.86 mmol) and Rh_2(μ-O_2CCH_3)$_4$(CH_3OH)$_2$ (381 mg, 0.86 mmol) in acetone (30 mL) was stirred at room temperature under N_2 for 48 h. The resulting green precipitate was filtered and washed with acetone (3 × 5 mL). The solid was suspended in CH_3OH (50 mL) and stirred at room temperature for 24 h. The resulting green solution was filtered and concentrated under reduced pressure to 2 mL, and the product was precipitated by addition of Et_2O. The green solid was filtered, washed with Et_2O, and dried *in vacuo* (260 mg, 47%). ESI-MS: *m*/*z* 646.93 ([Rh_2(μ-O_2CCH_3)$_2$(η^1-O_2CCH_3)(dpq)(CH_3OH)]$^+$), 614.85 ([Rh_2(μ-O_2CCH_3)$_2$(η^1-O_2CCH_3)(dpq)]$^+$). ^{1}H NMR (CD_3OD): δ (ppm) 1.07 (s, 3H, CH_3CO_2), 1.88 (s, 3H, CH_3CO_2), 2.34 (s, 3H, CH_3CO_2), 2.40 (s, 3H, CH_3CO_2), 3.31 (s, 3H, CH_3OH), 8.14 (m, 2H, dpq), 8.86 (dd, 2H, dpq), 9.22 (s, 2H, dpq), 9.643 (m, 2H, dpq). Anal. Calcd: C, 38.95; H, 3.27; N, 8.66. Found: C, 39.27; H, 4.09; N, 8.41.

Instrumentation. The ^{1}H NMR spectrum of the new complex was recorded on a Varian spectrometer at 300 MHz and referenced to the residual proton impurities in the deuterated solvents. Mass spectra were acquired on a PE SCIEX QSTAR Pulsar electrospray ionization mass spectrometer at Texas A&M University. Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA). The UV−visible measurements were performed on a Shimadzu UV-1601PC spectrophotometer or on a Cary 100 Bio Thermal UV/vis spectrometer equipped with a Cary temperature controller for thermal denaturation studies. The ethidium bromide-stained agarose gels were imaged on an Alpha Imager 2000 transilluminator (Alpha Innotech Corp., San Leandro, CA). Confocal microscopy was performed using an Olympus IX81 confocal microscope.

Methods. Binding Constants. Binding titration experiments were performed using a fixed concentration of metal complex (120 μM) with increasing concentrations of calf thymus DNA (0 to 200 μM) in 5 mM Tris-HCl buffer, pH 7.5, and 20 mM NaCl. The dilution of metal complex concentration at the end of each titration was negligible. The DNA binding constant, K_b, was obtained from fits of the titration data to eq 1,[31]

$$\frac{\varepsilon_a - \varepsilon_f}{\varepsilon_b - \varepsilon_f} = \frac{b - (b^2 - 2K_b{}^2C_t[\mathrm{DNA}]_t/s)^{1/2}}{2K_bC_t} \qquad (1)$$

where $b = 1 + K_bC_t + K_b[\mathrm{DNA}]_t/2s$, C_t and $[\mathrm{DNA}]_t$ represent the total complex and DNA concentrations, respectively, s is the base pair binding site size, and ϵ_a, ϵ_f, and ϵ_b represent the apparent, free complex, and bound complex molar extinction coefficients, respectively. The value of ϵ_b was determined from the plateau of the DNA titration, where addition of DNA did not result in further changes to the absorption spectrum.

Viscosity. The relative change in viscosity was measured using an Ubbelohde viscometer maintained at constant temperature (27 °C) in a thermostatic bath. Sonicated herring sperm DNA (200 μM), 5 mM Tris-HCl, pH 7.5, 20 mM NaCl, and increasing concentrations of complexes were used.[32] Data are presented as

$$\left(\frac{\eta}{\eta_0}\right)^{1/3} \quad \text{vs} \quad \frac{[\mathrm{M}]}{[\mathrm{DNA}]}$$
$$\eta = t_1 - t_2$$
$$\eta_0 = t_n - t_0$$

where η is the viscosity of DNA in the presence of the complex, η_0 is the viscosity of DNA in the absence of the complex, t_0 is the time of buffer alone, and t_n is the time of DNA and complex.

Melting Temperature. Melting temperature experiments were recorded by measuring the absorbance at 260 nm. The experiment was performed using 20 μM complex and a 100 μM solution of DNA (5′-ATCACCTAAAATGGCG-3′) in 1 mM phosphate buffer, pH 7.2, and 2 mM NaCl. The value of T_m was determined as the temperature corresponding to a maximum on the first-derivative profile of the melting curves,[31,32] using the Cary WinUV Bio software.

Electrophoretic Mobility Shift Assay. Aliquots of 50 μM (in base pairs) native pUC18 were incubated in the dark with different compound concentrations (5, 10, and 25 μM) at room temperature using 10 mM phosphate buffer, pH 7.2. After incubation for 24 h, electrophoresis was carried out using 1% agarose gel, 1X TAE buffer (40 mM Tris−acetate and 1 mM EDTA, pH ∼8.2). The applied voltage was 40 V, and the gels were run for a period of 16 h. After electrophoresis, the gels were stained with 0.5 mg/L ethidium bromide and imaged under UV light.[33−36]

Partition Coefficient Determination. The lipophilicity of the complexes was determined by the "shake flask" method using a pH 7.4 phosphate buffer (0.129 M NaCl) and *n*-octanol as solvents.[37] Each compound was dissolved in the phase in which it is most soluble, resulting in typical concentrations of 50−350 μM. Duplicate determinations using three different solvent ratios were performed for each complex. Following mixing and phase separation according to literature methods,[37] each phase was analyzed for solute content, and the concentration was determined using spectrophotometric methods. All the *n*-octanol/water partition coefficients were determined by UV/vis spectroscopy. Octanol and buffer solutions were presaturated with each other prior to use. Fifty rotations were performed by hand, followed by 1 h of settling time. Equilibration and absorption measurements were made at 20 °C.[29]

Cell Culture. HeLa cells were obtained from the American Type Culture Collection, cell line CCL-2. COLO-316 cell line was kindly provided by Robert Burghardt (Texas A&M, Department of Veterinary Anatomy and Public Health). Both cells lines were cultured in Dulbecco's modified Eagle medium, containing 10% fetal bovine serum (Invitrogen), 50 μg/mL gentamicin, 4.5 mg/mL glucose, and 4 mM L-glutamine (Invitrogen). Cell cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

***In Vitro* Cytotoxicity.** The viability of COLO-316 and HeLa cells in the presence of the compounds under investigation was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[38] (Invitrogen). Subconfluent (50−80% confluent) monolayers of cells at a concentration of 5000−10000 cells/μL were used. Cells were plated in 96-well sterile plates at a density of 20−30 cells/μL (volume of 100 μL/well) and preincu-

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bated for 48 h. After the cells reached 100% confluency, the medium was replaced by 100 μL of L-15 medium (HyClone Laboratories Inc.) containing different complex concentrations. The plates were incubated for 24 h, after which time 10 μL of fresh MTT solution (12 mM) was added, followed by incubation for 4 h. One hundred microliters of fresh SDS solution in 0.01 M HCl was added, and after 16 h of incubation absorbance at 570 nm was measured using a Bio-Rad plate reader.

Alkaline Comet Assay. Single-cell gel electrophoresis was performed using a commercially available kit (R&D Systems). HeLa cells were incubated with metal complexes at a concentration that allows for 75% viability. After 4 h, cells were harvested and embedded in 0.75% low-melting-point agarose at a volume of 1:10 on microscope slides. Cells were lysed in the dark for a minimum of 1 h at 4 °C. Cells were incubated in an alkaline solution containing NaOH (0.3 M) for a period of 1 h. Electrophoresis was performed for 30 min at 28 V in TBE buffer (pH 13). After electrophoresis, the cells were stained with SYBR green dye and imaged using an inverted microscope. A total of 100 cells were scored per sample, using the CometScore software.[39]

SYTOX Blue Assay. HeLa cells at a concentration of 5000−10000 cell/μL were harvested, 75 μL samples of cells were plated in an 8-well sterile plate, and 125 μL of fresh medium was added to give a total volume of 250 μL. Cells were preincubated at 37 °C. After 24 h, cells were washed with sterile PBS and the medium was replaced by 250 μL of L-15 medium containing the different complexes at their LC_{50} concentration. Plates were incubated for 4 h, after which time they were treated with 5 μL of a 5 mM SYTOX Blue solution and incubated for 5 min before imaging.

Glutathione Modulation. COLO-316 cells were plated in 96-well sterile plates at a density of 20−30 cells/μL (volume of 100 μL/well) and preincubated for 48 h. After the cells reached 100% confluency, the medium was replaced by 100 μL of fresh medium containing either 500 μM BSO or 5 mM NAC. Incubation with NAC was performed for a period of 2 h, whereas BSO-containing plates were incubated overnight. After the respective incubation period, cells were washed twice with sterile PBS, L-15 medium containing a concentration of the dirhodium complex corresponding to its LC_{50} was added, and cells were incubated overnight. To assess cell viability, an MTT assay was performed as described above.

Results

Synthesis and Characterization of the Monosubstituted Dirhodium Complexes. Compounds **1**, **2**, **4**, **5**, and **6** have all been reported previously.[26−30] The *cis*-[$Rh_2(\mu$-$O_2CCH_3)_2(\eta^1$-$O_2CCH_3)$-(dpq)(CH_3OH)](O_2CCH_3) (**3**) compound was synthesized in ~50% yield by the reaction of $Rh_2(\mu$-$O_2C_2H_3)_4(CH_3OH)_2$ with 1 equiv of the dpq ligand. The reaction is performed in two steps, and solvent, temperature, and time are very important factors for the first step in order to obtain a good yield. The mixture should not be heated in order to avoid the formation of the bis-substitued derivative or other undesired byproduct. The synthesis of monosubstituted compounds using CH_2Cl_2 has also been reported, but in this case acetone was used because the reaction is less temperature sensitive with this solvent. The reaction mixture was stirred for 48 h, which is necessary for the achievement of the maximum yield. During the course of the reaction, a green precipitate is obtained, which is the intermediate $Rh_2(\mu$-$O_2CCH_3)_2(\eta^2$-$O_2CCH_3)(\eta^1$-$O_2CCH_3)$(dpq) with an acetate ion that chelates via *ax* and *eq* positions of one rhodium atom. After the intermediate is stirred in methanol for 24 h, the chelating acetate group is displaced by methanol molecules and the diimine ligand rearranges to an *eq*/*eq* binding mode, as found in the final product.

(39) *CometScore*, v. 1.5; TriTek Corp.: Sumerduck, VA, 2006.

Table 1. DNA Binding Constants and ΔT_m of Compounds **1**−**6**

compd	K_b (M^{-1})[a]	ΔT_m (°C)[b]
1	3.2×10^4	2 ± 1
2	3.2×10^4	4 ± 1
3	3.5×10^5	16 ± 1
4	4.4×10^5	18 ± 1
5	9.7×10^5	24 ± 1
6	3.6×10^5	10 ± 1

[a] Binding constants were measured using 1 mM phosphate buffer, pH 7.2, and 2 mM NaCl. [b] Melting temperature experiments were performed using 1 mM phosphate buffer, pH 7.2, and 2 mM NaCl.

Binding Affinities for Calf Thymus DNA. The binding constants (K_b) of this family of dirhodium complexes were measured by titrating a fixed concentration of complex with increasing concentrations of DNA. The K_b values (Table 1) were determined from fits of the change in the absorption of each complex as a function of the DNA concentration. As expected, compounds **4** and **5**, *viz*., those with the largest intercalating ligands, interact with DNA to the greatest degree, with binding constants $K_b = 4.4 \times 10^5$ M^{-1} ($s = 1.4$) and 9.7×10^5 M^{-1} ($s = 1.6$), respectively. These values are typical for metal complexes that bind to DNA via intercalation. For example, values of $K_b = 1.24 \times 10^5$ and 2.0×10^5 M^{-1} were reported for $[Ru(NH_3)_4(dppz)]^{2+}$ [40] and $[(\eta^6$-$C_6Me_6)RuCl(dppz)]^+$,[41] respectively. Similarly, **3** (3.5×10^5 M^{-1}, $s = 1.2$) and **6** (3.6×10^5 M^{-1}, $s = 1.3$) exhibit binding constants of the same order of magnitude. The values of K_b for **1** (3.2×10^4 M^{-1}, $s = 0.02$) and **2** (3.2×10^4 M^{-1}, $s = 0.01$) are 1 order of magnitude lower than those for the other members of the series. Metal complexes that exhibit binding constants of the same order of magnitude as **1** and **2** have been identified in the literature as intercalating agents,[42] but an inspection of the structures of the two compounds clearly reveals that it would be impossible for these compounds to intercalate without a major change in the structure of the complex.

Melting Temperature. The melting temperature, T_m, of 100 μM DNA in the presence of a 20 μM concentration of each complex was measured and compared to that of DNA alone (Table 1). The DNA used was a 16-mer DNA sequence (5′-ATCACCTAAAATGGCG-3′) with $T_m = 53$ °C. When a compound intercalates into DNA, it stabilizes the base stacking, which leads to an increase in the DNA melting temperature. The largest difference in melting temperature was recorded for compound **5** ($\Delta T_m = 24$ °C), which has the largest planar surface area among the members of the series. Compounds **1** and **2**, with less expanded planar ligands, exhibit modest shifts of +2 and +4 °C, respectively. These shifts are most likely due to the ionic character of the compounds. These values are similar to values reported for species such as [Ru(tpy)-(bpy)OH_2]$^{2+}$ ($\Delta T_m = 2$ °C) and [Ru(tpy)(phen)OH_2]$^{2+}$ ($\Delta T_m =$ 7.2 °C), which are known to interact with DNA solely through electrostatic interactions.[43] Compounds **3**, **4**, and **6** also show large ΔT_m values (>10 °C) that are comparable to data in the

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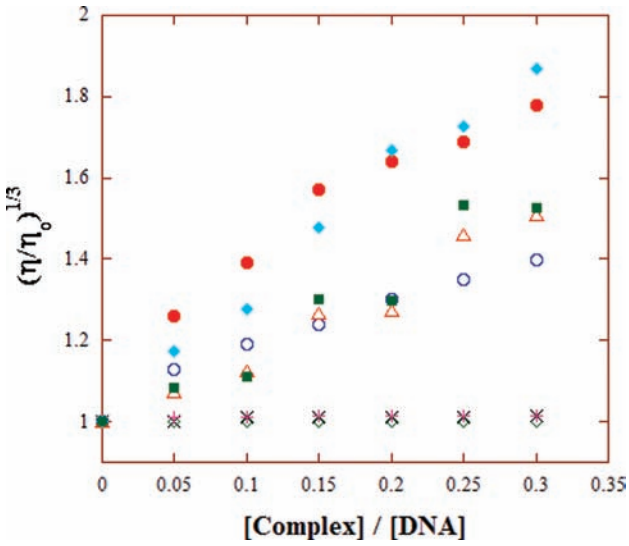

Figure 1. Relative viscosity changes of solutions containing 200 μM sonicated herring sperm DNA as the concentration of EtBr (red ●), **1** (×), **2** (red +), **3** (red △), **4** (blue ○), **5** (light blue ◆), **6** (green ■), and Hoechst 33258 (◇) is increased.

literature for intercalators.[44] For example, the known metallointercalators $[Rh(phi)_2phen]^{3+}$ and $[Ru(phi)_2phen]^{2+}$ (phi = 9,10-phenanthrenequinone diimine) increase the melting temperature of a 15-mer duplex by 21 and 15 °C, respectively.[44] In the context of this discussion, it is important to note that compound **6** was reported previously to exhibit a change of $\Delta T_m = -6$ °C, but, in that particular study, compound **6** was first covalently bound to one of the strands of the DNA sequence and then the product was annealed to its complementary strand.[30]

Viscosity. The relative change in viscosity was measured using 200 μM sonicated herring sperm DNA with increasing concentrations of the dirhodium complexes. Relative viscosity measurements have proven to be a reliable method for the assignment of the mode of binding of compounds to DNA.[45] Intercalation of molecules between DNA bases causes a change in the relative viscosity of solutions due to the unwinding and elongation of the double helix.[45] For compounds **1** and **2**, no change in the relative viscosity is observed after their addition. This behavior is similar to what one observes with the minor groove binder Hoescht 33258.[46] The addition of compounds **3**, **4**, and **6** increases the relative viscosity of the solution, although to a lesser extent than the changes observed with ethidium bromide. Similar increases in relative viscosity have been documented for other intercalating metal complexes, for example $[(\eta^6\text{-}C_6Me_6)RuCl(dpq)](CF_3SO_3)$ and $[(\eta^6\text{-}C_6Me_6)RuCl(dppz)](CF_3SO_3)$.[41] Finally, compound **5** produces a more pronounced change in the relative viscosity of DNA as compared to other members of the series, and its behavior is similar to that observed with the intercalator EtBr (Figure 1).

Electrophoretic Mobility Shift Assay. To further evaluate the formation of adducts with DNA, an electrophoretic mobility shift assay (EMSA) was performed.[33,34] Binding of molecules such as alkylating[47,48] or intercalating agents[33,34] to the covalent

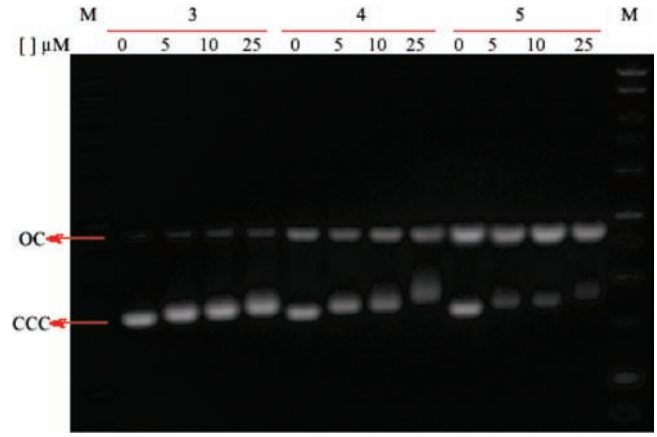

Figure 2. Ethidium bromide agarose gel (1%) and 100 μM pUC18 incubated with compounds **3**−**5** at different concentrations. Lanes 1 and 14, standard 1 kb leader marker; lanes 2, 6, and 10, native pUC 18, control; lanes 3−5, increasing amounts of compound **3**; lanes 7−9, increasing amounts of compound **4**; and lanes 11−13, increasing amounts of compound **5**.

closed circular (CCC) plasmid DNA generates topoisomers that migrate at different rates. Moreover, it was observed for a family of platinum compounds that can potentially intercalate and bind covalently to DNA that the degree of migration increased as the binding mode changed from monofunctional (covalent) binding to bifunctional (covalent and intercalating) binding.[35] The EMSA was performed after a 24 h incubation period of the dirhodium complexes with pUC18 plasmid in the dark to avoid DNA damage by the compounds. This was particularly important for compound **4**, as it is known to be a DNA photocleavage agent.[31] After incubation, the solution was loaded into a 1% agarose gel, and electrophoresis was carried out for a period of 16 h. The electrophoresis voltage was maintained at a low value (40 V) to ensure observation of a visible shift of the formed adducts. As can be observed in Figure 2, the dirhodium complexes under study form DNA adducts that migrate at different rates. The most significant shifts were observed with compounds **4**−**6**, whereas the DNA adducts with compounds **1**−**3** migrate to a lesser extent (Figure 2 and Supporting Information). This difference in the migration pattern of the adducts points to a difference in the binding modes of the compounds containing extended aromatic ligands as compared to the other members of the series.

Partition Coefficient Determination. Partition coefficients, *P*, were measured to determine how easily the compounds are able to pass through a biological membrane.[37] The *P* measurements are based on the difference in solubility that a given compound exhibits in an aqueous versus a hydrophobic medium.[37] The correlation of the activity of a compound with its log *P* value depends on the solvent system used as a model for the membrane.[37,49] The "shake flask" method used during the course of these studies has been shown to work well for molecules with log *P* values that

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Table 2. Log *P* and Cytotoxicity Values of Compounds **1**−**6**

		$LC_{50} \pm SD$ (μM)[b]	
compd	log *P*[a]	HeLa	COLO-316
1	-1.90 ± 0.03	129 ± 4	70.8 ± 3
2	-1.98 ± 0.02	128 ± 6	73.6 ± 4
3	-1.53 ± 0.03	124 ± 5	71.2 ± 3
4	-0.30 ± 0.02	86 ± 4	54.0 ± 1
5	0.91 ± 0.01	118 ± 7	71.3 ± 3
6	0.63 ± 0.02	130 ± 3	54.1 ± 3

[a] Partition coefficient $P = C_o/C_w$ (C_o and C_w are the complex concentrations in *n*-octanol and water, respectively). [b] Determined using the MTT assay. LC_{50} values are concentrations of drug required to kill 50% of the cells.

range from −2 (most hydrophilic) to +4 (most hydrophobic).[49−52] The values obtained for the members of this series range from −1.98 to +0.91 (Table 2). By comparing the partition coefficients of these compounds (Table 2), it can be seen that the log *P* values become more positive as the π-system of the ligands in the complexes is extended.

The difference in the log *P* values of compounds **1** and **2** is small despite the fact that one more ring is added to the system. Subsequent ring additions to the system increases the log *P* values to a greater extent. Adding the fourth ring, as in the case of compound **3**, increases the log *P* value by 0.45, whereas the fifth and sixth rings in compounds **4** and **5** increase the log *P* value by 1.23 and 1.21, respectively. Extending the width of the system has a more pronounced effect, as can be observed in the log *P* difference (2.16) between compounds **3** and **6**.

***In Vitro* Cytotoxicity.** The compounds were tested in a cell proliferation assay on two human cell lines, *viz.*, HeLa and COLO-316. The LC_{50} values were calculated after 24 h of incubation with complexes **1**−**6** and are listed in Table 2. For this particular dirhodium family, it is clear that the human ovarian carcinoma cells COLO-316 are more sensitive than HeLa cells. Compound **4** exhibits the highest activity, $LC_{50} = 86 \pm 4$ and 54 ± 1 μM in HeLa and COLO-316, respectively. Any further modification of the ligand length or width decreases the activity of the dirhodium complex on HeLa cells. When the size of the intercalating moiety was reduced, as in the case of compounds **1** ($LC_{50} = 120 \pm 4$ μM), **2** ($LC_{50} = 129 \pm 6$ μM), and **3** ($LC_{50} = 128 \pm 5$ μM), the cytotoxicity toward HeLa cells was diminished by approximately 1.5-fold. A similar reduction of the activity was observed when the ligand was expanded either in length or in width, as in **5** ($LC_{50} = 120 \pm 7$ μM) and **6** ($LC_{50} = 130 \pm 3$ μM), respectively. In the case of COLO-316 cells, a decrease or increase in the length of the aromatic moiety results in lower activity, approximately 1.3-fold. Compound **6**, however, exhibits activity similar to that of compound **4** in this cell line.

Alkaline Comet Assay. An alkaline single-cell gel electrophoresis was performed to evaluate the ability of these complexes to form DNA adducts in a cellular environment.[53−56] The Alkaline Comet Assay is a sensitive technique that can be used to detect single- and double-strand breaks and cross-links, as well as alkali-labile sites.[53,54] Assays were performed at a concentration that allows for 75% cell viability using HeLa cells. For each compound, 100 cells were scored using the CometScore software.[39] This program allows quantification of the amount of DNA present in the tail of the comet. which is representative of the amount of DNA damage caused by the added agent.[39] As shown in Figure 3, all the compounds produce more damaged DNA (represented as a larger percent of DNA in the tail) than the control. Compounds **4** and **5** led to the highest DNA damage, with $72.4 \pm 7.7\%$ and $80.1 \pm 7.4\%$ of the DNA being found in the comet tail, respectively. This level of damage is similar to that observed when a comparable amount of cisplatin ($79.7 \pm 8.0\%$ DNA in tail) is used (Supporting Information). Compounds **1** ($44.4 \pm 10.3\%$), **2** ($48.5 \pm 9.6\%$), **3** ($41.7 \pm 10.8\%$), and **6** ($39.2 \pm 14.7\%$) did not cause as much nuclear DNA damage as compounds **4** and **5**. In fact, these values are quite similar to that observed with $Rh_2(\mu\text{-}O_2CCH_3)_4$, with a percentage of DNA in the tail equal to $37.9 \pm 5.8\%$ (Supporting Information).

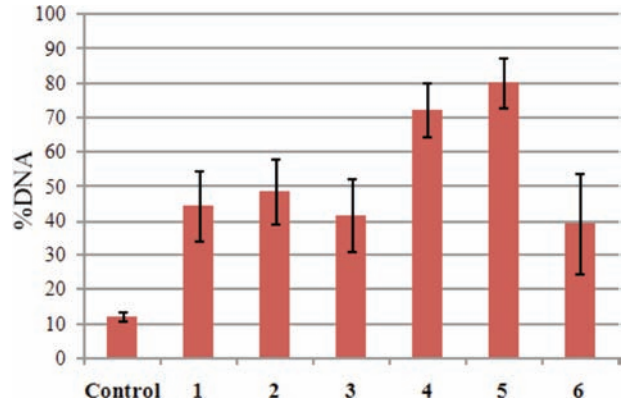

Figure 3. Percentage of nuclear DNA damaged after treatment with compounds **1**−**6**, represented as percent of DNA in comet tail. Error bars represent standard deviation values.

SYTOX Blue Assay. SYTOX Blue is a cell-impermeable agent that can enter cells only when their membranes have been damaged.[57] Upon entering the cell, SYTOX Blue binds nuclear DNA and undergoes a 100-fold increase in fluorescence. After 4 h of incubation of HeLa cells with the dirhodium compound of interest at a concentration equal to their calculated LC_{50} values, it is possible to see ~60% of the cells with blue stain in their nuclei. This implies that the cells treated with compounds **1**−**6** have been severely compromised or are no longer viable. In Figure 4, it is also possible to see that the morphology of the cells differs from that of a viable HeLa cell.

Glutathione Modulation. Glutathione is the most abundant thiol found in cells, present at concentrations of 0.5−10 mM depending on the cell type.[58] Some of the functions of glutathione include protection of the cell integrity from reactive oxygen species (ROS) and heavy metal detoxification.[59] It has been observed in some cisplatin-resistant cell lines that their glutathione levels are higher than normal.[60] Given this fact, it is of considerable interest to determine the effects of glutathione modulation on the cytotoxicity of this family of dirhodium complexes.

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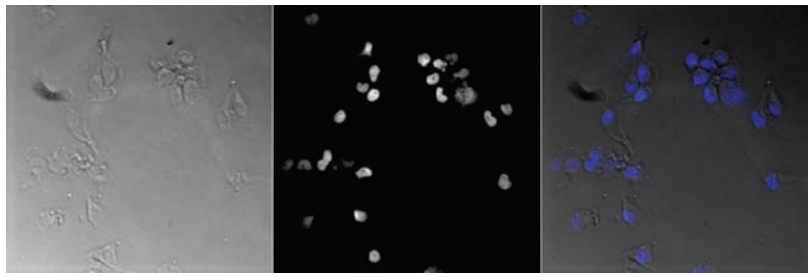

Figure 4. Phase contrast and fluorescent image of HeLa cells treated with compound **4**: left, phase contrast; center, SYTOX Blue fluorescence emission; and right, overlay of the phase contrast and SYTOX Blue fluorescence emission (pseudocolored blue) images.

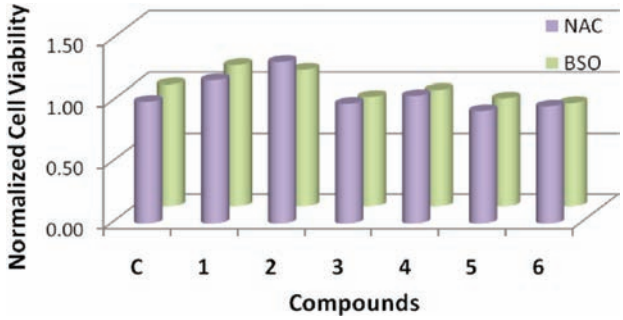

Figure 5. Effect of compounds **1**−**6** on COLO-316 cells after glutathione modulation by NAC (increase of cellular glutathione levels) and BSO (decrease of glutathione levels).

The molecule NAC is known to increase levels of glutathione and radical scavengers in cells.[61] COLO-316 cells were pretreated with NAC and then incubated with the dirhodium complexes **1**−**6**, and the change in the cytotoxicity of these complexes was measured (Figure 5). The cells treated with compound **2** showed a slight decrease in activity, as observed by the increase in cell viability (+32%), whereas compounds **3** (−2%) and **5** (−8%) showed the largest activity increases in the series. These variations in activity can be considered minimal and correspond to a small effect of the increase of glutathione and radical scavenger levels on the cytotoxicity of this family of dirhodium complexes.

The levels of glutathione were depleted with BSO, a selective inhibitor of γ-glutamylcysteine synthase, a key enzyme in the glutathione biosynthetic pathway.[58,62] Upon exposure of the cells to BSO for 20−28 h, levels of glutathione in COLO-316 cells have been shown to decrease to ~13% of the initial value.[63,64] In COLO-316, depletion of glutathione did not have a major effect on the cytotoxicity of cisplatin,[63] although increase in cisplatin cytotoxicity has been observed in other cell lines.[65] In the case of the dirhodium complexes used in this study, the depletion of glutathione by BSO did not lead to large variations in their cytotoxicity (Figure 5).

Discussion

The collective aforementioned data point to the conclusion that the size of the diimine ligands bound to the dirhodium core in compounds **1**−**6** controls both the *in vitro* affinity and interaction with ds-DNA and the *in cellulo* reactivity of the complexes. The progressively increasing ligand size for the series results in a larger affinity for DNA and a change in the binding mode from primarily electrostatic (compounds **1** and **2**) to primarily intercalative (compounds **3**−**6**) in the initial stages of their interaction. As we observed in previous studies, monosubstituted diimine complexes of this class are capable of simultaneously intercalating between DNA bases and binding covalently to DNA.[30] Compound **5** exhibits the strongest interactions of the group with the DNA double helix (Table 1 and Figure 1). These results are nicely supported by the values of the binding affinity toward DNA. Thus, hydrophobic interactions appear to be an important factor for the DNA binding affinity of this family of complexes. Compound **5**, however, is not the most cytotoxic of the series in either HeLa or COLO-316 cell lines. A feasible hypothesis is that this dichotomy is due to differences in the ability of the compounds to cross the cellular plasma membrane. Many studies over the years support the conclusion that the reactivity and affinity of a compound are not the only important factors in inhibiting a cellular process, but that the availability of the agent to interact with its target also plays an important role.[20,29] To address this issue, the values of the partition coefficient, log *P*, between *n*-octanol and water of compounds **1**−**6** were measured (Table 1). As expected, compounds **1** and **2** are the most hydrophilic compounds of the series. The hydrophobicity increases throughout the series as the planar aromatic region is expanded and reaches a maximum at compound **5**. It is reasonable to postulate that the hydrophobicity of **5** does not permit rapid internalization of the compound, which would account for the decreased cytotoxicity as compared to that of **4** in HeLa cells or **4** and **6** in COLO-316. Compounds **4** and **6** interact to a lesser degree with DNA but are less hydrophobic and may be capable of traversing the cellular membrane more effectively. It must be pointed out that in a previous study we did not find any correlation between the partition coefficient and cytotoxicity of the dirhodium complexes studied.[29] This outcome may be rationalized on the basis of the structural differences among the previous compounds, which, not unexpectedly, may lead to very different mechanisms of action in live cells. In the present study, however, we have focused on a homologous family of dirhodium complexes that, due to their similarity, would be expected to behave in a comparable manner in the intracellular space. Hence, differences in cellular uptake should be reflected in the cytotoxicity of the compounds (Figure 6).

In support of this contention are early data by Bear and coworkers who reported, for the family of dirhodium tetracarboxylate complexes $Rh_2(\mu\text{-}O_2CR)_4$ (R = CH_3, C_2H_5, C_3H_7, and C_4H_9), an increase in cytotoxicity against Ehrlich ascites tumor

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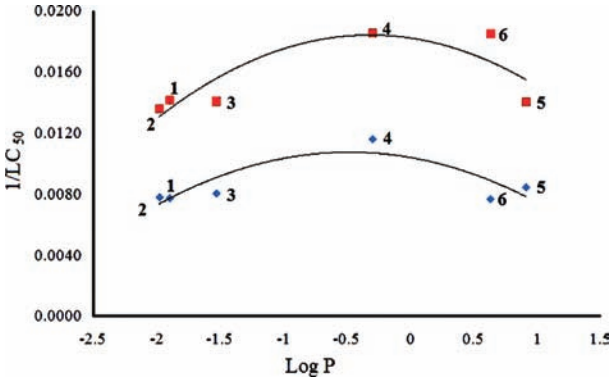

Figure 6. Correlation between cytotoxicity and partition coefficient: HeLa (blue ◆) and COLO-316 (red ■) cells.

cell lines on going from the acetate to the butyrate derivative, whereas a decrease was observed with a further lengthening of the R group.[19] Similarly, Sheldrick et al. found that the family of compounds $[(\eta^6\text{-}C_6Me_6)RuCl(pp)]^+$ (pp = dpq, dppz, and dppn) exhibited cytotoxicity increases that correlate with the hydrophobicity and the cellular uptake efficiency of the compounds.[41]

Importantly, the alkaline comet assay results indicate better activity for **5** once inside the cell; i.e., this compound causes the greatest amount of DNA damage (80.1 ± 7.4%). The next most potent compound is **4** (72.4 ± 7.7%). Compounds **1**, **2**, **3**, and **6** show less DNA damage, a fact that may be related to their lower affinity for DNA. The DNA damage observed in the comet assay could be caused by direct interaction of the compound with DNA, or it may be due to the formation of ROS by the metal complex. Evidence against the latter scenario is found in the experiments in which cells were pretreated with NAC. If the damage caused by the dirhodium complex originated from the presence of ROS, pretreatment with NAC should increase the cell viability, yet no changes in the cytotoxicity of the complexes upon pretreatment with NAC were observed (Figure 5). Therefore, it is reasonable to suggest that direct damage of the nuclear DNA occurs, and thus, the dirhodium complexes bind to nuclear DNA in living cells.

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Although glutathione, as in the case of cisplatin,[66] is likely to play a role in dirhodium deactivation, it does not seem to be the sole factor involved in the present case. The results obtained in the cell viability studies using BSO (Figure 5) demonstrate that, despite the decrease in glutathione levels, more dirhodium compound does not reach its cellular target. In this context, we note that there are other thiols that play a role in the deactivation of cisplatin. For example, the low-molecular-weight protein metallothionein contains 20 cysteine residues and is actively involved in the detoxification of heavy metal compounds in cells.[66] Certainly, it is reasonable to postulate that this protein may also be involved in the detoxification of cells from dirhodium complexes.

Conclusions

The results presented herein support the conclusion that DNA is a cellular target for the new dirhodium complexes tested. A clear relationship between the interaction of the compounds with DNA and their cytotoxicity is established. Moreover, the ability to cross the cellular plasma membrane appears to be another significant factor that affects activities of the compounds *in live cell* assays. In the field of drug design, it is well accepted that antitumor efficacy may be enhanced by the combination of more than one pharmacological property; therefore, metal complexes such as compounds **4**−**6** that can potentially intercalate as well as covalently bind to DNA are promising lead compounds that deserve further scrutiny. Studies aimed at evaluating the cellular uptake and distribution of these compounds as well as new generations of complexes with enhanced uptake are currently in progress.

Acknowledgment. This work was supported by the Welch Foundation (K.R.D.) and the National Science Foundation (CHE 0503666 joint grant between C.T. and K.R.D.).

Supporting Information Available: ^{1}H NMR spectrum of compound **3**; melting temperature curves; EMSA results for compounds **1**, **2**, and **6**; details of the alkaline single-cell gel electrophoresis assay for $Rh_2(\mu\text{-}O_2CCH_3)_4$; and absorption titration curves for compounds **1**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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