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Phototoxicity against Tumor Cells and Sindbis Virus by an Octahedral Rhodium Bisbipyridyl Complex and Evidence for the Genome as a Target in Viral Photoinactivation

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An octahedral rhodium complex (*cis*-dichloro(dipyrido[3,2*a*-2′,3′*c*]phenazine)(1,10-phenanthroline)rhodium(III) chloride; DPPZPHEN) has been prepared that can penetrate tumor cell membranes and the Sindbis viral capsid. The compound is phototoxic to these entities when irradiated with UVA light. Model studies with calf thymus and supercoiled plasmid DNA indicate that the complex can both bind with, and nick, nucleic acid. Analysis of Sindbis virus, following irradiation with the metal complex, confirmed that the viral genome was rendered noninfectious by this treatment.

Introduction

The use of light-activated drugs is a very attractive option for the development of therapeutic methodologies that minimize damage to normal tissue by allowing spatial and temporal control over toxicity. Some current applications have emphasized photodynamic therapy as a treatment for several forms of cancer[1] and the use of light-activated drugs for blood sterilization.[2] These methodologies rely heavily on the efficacy of the photosensitized formation of reactive oxygen species (ROS) by the light-absorbing agent.[3−6] However, the concomitant need to overcome tumor cell resistance due to hypoxia,[7] and to avoid the indiscriminate damage to biological blood components by ROS,[8] makes the development of photoactivated agents that utilize oxygen-independent mechanisms for toxicity a worthy goal. The specific targeting of (e.g., viral or bacterial) DNA and/or RNA is especially attractive for blood photosterilization since neither erythrocytes nor leucocytes contain genomic nucleic acid.[2,9]

Recently, there has been increasing interest in the potential use of photoactivated organometallic complexes as a source of nucleic acid inactivation.[10−12] When irradiated with UV light, a number of such complexes exhibit powerful nuclease activity and/or the capability to form covalent bonds with DNA and RNA bases.[13−18] Our own efforts have concentrated on bis(bipyridyl)rhodium(III) complexes, an example

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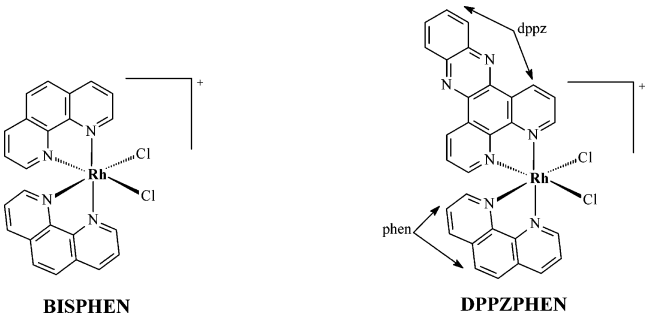

Figure 1. Structures of BISPHEN and DPPZPHEN.

of which is *cis*-dichlorobis(1,10-phenanathroline)rhodium-(III) chloride [*cis*-Rh(phen)$_2$Cl$_2^+$; BISPHEN; Figure 1]. Upon irradiation, this complex covalently binds to nucleic acid, primarily to guanosine,[13] cross-links RNA,[13f] and inactivates naked viral DNA.[13d] However, the complex shows minimal dark association with DNA, due to the absence of a ligand capable of intercalation, and lacks the hydrophobicity needed for uptake by mammalian cells. We have thus prepared an analogue in which one of the phen ligands has been replaced by the dipyrido[3,2*a*-2′,3′*c*]phenazine (dppz) moiety, that is, *cis*-Rh(dppz)(phen)Cl$_2^+$ (DPPZPHEN; Figure 1).

We herein demonstrate that DPPZPHEN (a) has a higher quantum yield of aquation as compared with BISPHEN, (b) can act as a photonuclease and as a photobinding agent with nucleic acid in an hypoxic environment, (c) is phototoxic to tumor cells and to the alphavirus Sindbis (SINV), and (d) renders the viral genome noninfectious. The structure of SINV is closely related to flaviviruses that include important pathogens such as the West Nile virus, yellow fever virus, and dengue virus.[19] Observation d requires that DPPZPHEN be capable of migrating through the viral glycoprotein, lipid, and capsid layers before inactivating the viral RNA genome. This is particularly noteworthy in light of the recent report that *N*-acetylaziridine reacts with the nucleic acid of flock house virus, human rhinovirus-14, and foot and mouth disease virus by permeating their protein capsids.[20] Our results provide a proof of principle that transition metal complexes have potential in the design of photoactivated antitumor and antiviral agents.

Experimental Section

Only the most salient information is presented here. Further details may be found in the doctoral dissertation of E.M.[21]

Materials. Rhodium(III) chloride ($RhCl_3 \cdot xH_2O$) was from Johnson Matthey or Pressure Chemical Co. 1,10-Phenanthroline and bromophenol blue were from Fisher. 9,10-Diaminophenanthrene, hydrazine monohydrochloride, glycerol, ethidium bromide, and 1,2-phenylenediamine (99% pure) were from Aldrich. Calf thymus DNA (type I, no. D-1501), cellulose dialysis sacks (250-7U), amphotericin B, Triton X-100, MTT, AA rhodium standard solution, minimum essential amino acids solution, Trisma hydrochloride, Trisma base, boric acid, superoxide dismutase, and bovine serum albumin were from Sigma. Dulbecco's modified Eagle's medium (without L-glutamine and without phenol red), 10× trypsin-EDTA (phenol red-free), penicillin−streptomycin, heat-inactivated fetal bovine serum, L-glutamine 200 mM solution, and Hank's salt solution were from Life Technologies. HPLC solvents, HCl, phosphate salts, acetic acid, sodium bicarbonate, and ammonium acetate were from Mallinckrodt. EDTA was from ICN Biomedical Inc. Agarose was from Bethesda Research Laboratories. ϕX-174 was from New England Biolabs. HPLC inserts (50 and 250 μL) were from Alltech. Conjugated goat anti-rabbit secondary antibody was from Kirkegaard and Perry. Fluorosave reagent was from Calbiochem.

Synthesis of *cis*-dichloro(dipyrido[3,2*a*-2′,3′*c*]phenazine)(1,-10-phenanthroline)rhodium(III) Chloride [*cis*-Rh(dppz)(phen)-Cl$_2^+$; DPPZPHEN]. The ligand dppz[22] (30 mg; 0.106 mmol) (prepared from 1,10-phenanthroline-5,6-dione and high-purity 1,2-phenylenediamine; high purity is critical for the yield and quality of the ultimate complex) and $N_2H_4 \cdot HCl$ (2 mg; 0.03 mmol) were placed in a 50 mL three-neck round-bottom flask fitted with a condenser. Dimethylformamide (DMF; 5 mL) was added and the resultant slurry was degassed with nitrogen for 20 min. K[Rh(phen)-Cl$_4$].H$_2$O (51.3 mg, 0.106 mmol) in DMF (10 mL) was degassed for 20 min with N_2 under constant stirring to form an orange suspension that was transferred to the dppz slurry via a stream of N_2. The temperature was slowly increased to 110 °C, while the slurry was gently bubbled with N_2, until all the solids were dissolved and a bright orange solution was formed. The temperature was further increased and the solution allowed to reflux for 3.5 h, after which it had turned a gasoline-yellow color. The solution was cooled to room temperature with stirring and transferred to a 250 mL Erlenmeyer flask. Ether (ca. 60 mL) was added and the resultant beige precipitate was collected by vacuum filtration. This was washed immediately with copious amounts of ethanol, dissolved in 50 mL of boiling water, and filtered. A saturated solution of KCl was added until a precipitate start to form, and the mixture was then left overnight in a refrigerator. A beige precipitate of DPPZPHEN (55.8 mg, 76.5%) was collected by vacuum filtration that was >98% pure by HPLC. Electronic absorption in 50 mM phosphate buffer, pH 7.0 [nanometers (ϵ, liters per mole per centimeter]: 277 (77 798); 362 (13 864); 380 (14 400). ^{1}H NMR in deuterated dimethyl sulfoxide (DMSO-d_6), 500 MHz (δ, ppm): 10.13 (d, J = 5.25 Hz, 1H); 10.05−10.02 (dd, 2H); 9.65 (d, J = 8.16 Hz, 1H); 9.31 (d, J = 8.15 Hz, 1H); 8.92 (d, J = 8.09 Hz, 1H); 8.69−8.66 (dd, 1H); 8.57−8.53 (m, 3H); 8.47−8.42 (m, 2H); 8.22−8.15 (m, 4H); 7.93−7.90 (dd, 1H); 7.82−7.79 (dd, 1H). FAB MS (m/z) = 634.7, [Rh(dppz)(phen)Cl$_2^+$]; 599.7, [Rh(dppz)(phen)-Cl$^+$]; 564.7, [Rh(dppz)(phen)$^+$]. HRMS (m/z): found 635.0019, calcd 635.0025.

Instrumentation. High-performance liquid chromatography (HPLC) analyses were done on a Varian 5000 liquid chromatograph (LC) fitted with a 7125 Rheodyne injection valve, a 200 μL injection loop, a Varian 2050 variable-wavelength detector, and a Perkin-Elmer LCI-100 integrator. Phosphorescence emission was measured on an SPF-500C spectrofluorometer from SLM Instruments with interference filters placed on the emission side having transmission at >380 or >385 nm. Atomic absorption (AA) analyses were carried out on a Perkin-Elmer 3110 flame/furnace spectrophotom-

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eter. UV analyses utilized a Varian (Cary 100) UV−vis spectrophotometer.

Methods. HPLC analyses were carried out on an Alltech C8 4.6 × 250 mm column at a flow rate of 1 mL/min with the detector at 272 nm. Isocratic conditions, with 40% acetonitrile and 60% 100 mM ammonium acetate, pH 4.9, were used for DPPZPHEN (method A). DPPZPHEN eluted at 12.8 min. The monoaquo complexes appear at ca. 4 and 5 min by method A but they are poorly detected on aged (>100 injections) columns. HPLC was also used to monitor urocanic acid actinometry on the C8 column with a 1 mL/min flow rate but with 100% 50 mM ammonium acetate, pH 4.9, at 254 nm (method B). The *E* and *Z* isomers of urocanic acid eluted at 5 and 9 min, respectively. Area counts of the *Z* isomer were multiplied by 1.4 to compensate for its lower extinction coefficient at 254 nm.[23]

The quantity of DNA was determined by the Burton assay[24a] and its rhodium content was quantitated by AA.[13b] Cell viability was determined by the MTT assay.[25] SINV was purified on sucrose gradients as previously described.[26] The titer was generally 7−8 log plaque-forming units (pfu)/mL at the beginning of each experiment.

For phosphorescence measurements, samples (1 mL) were prepared in a 4/1 methanol/water mixture at concentrations such that the absorbance at the excitation wavelength was ca. 0.1. The samples were placed in electron spin resonance (ESR) tubes and slowly immersed in a liquid nitrogen-filled Dewar flask to form a glass. The phosphorescence excitation spectra for DPPZPHEN were obtained by monitoring the emission at 556 and 710 nm.

For absorption titration studies, a 2.5 mL solution of 30 μM DPPZPHEN in 100 mM phosphate buffer, pH 7, was placed in a quartz cuvette, and its UV−vis absorption spectrum was scanned against a reference cell containing only buffer. Aliquots of 20 μL of a 4 mg/mL CT DNA solution in distilled water were added sequentially (to a total of 100 μL) to the DPPZPHEN solution, and the UV−vis spectra were recorded. The equilibrium constant was calculated from the data obtained at 380 nm by use of the relation $D/\Delta\epsilon_{ap} = (D/\Delta\epsilon) + (\Delta\epsilon K_{eq})^{-1}$, where D is the molar concentration of DNA in base pairs and $\Delta\epsilon_{ap} = (\epsilon_a - \epsilon_f)$.[27,28] The apparent absorption coefficient, ϵ_a, was obtained by calculating A_{obs}/[Rh], where A_{obs} is the absorption at 380 nm at each titration point and [Rh] is the molar concentration of DPPZPHEN at each titration point, corrected for dilution from titration. The term ϵ_f is the absorption coefficient of the free form of the rhodium(III) complex. $D/\Delta\epsilon_{ap}$ was plotted as a function of D and the slope of the best-fit curve was divided by the y intercept to yield the intrinsic binding constant.[27,28]

Quantum Efficiency of Aquation at 311 nm. Irradiations were carried in a photoreactor chamber with two TL/01 Philips 311 nm lamps wrapped in carbon paper containing a 5 mm slit at the center of each lamp. Aliquots of 2 mL of the following aqueous solutions were placed in quartz phototubes, saturated with argon, and irradiated in duplicate: 5 mM E-UCA, 0.265 mM DPPZPHEN, and 0.4 mM BISPHEN. At these concentrations there is complete absorption of light at 311 nm. The DPPZPHEN samples were irradiated for 10 min and the E-UCA and BISPHEN solutions were irradiated for 30 min. The metal complexes were analyzed by HPLC method A and the urocanic acid was analyzed by method B. The photon flux was calculated by use of the formula:

$$\text{photons/second} = \frac{(\text{moles of Z-UCA})N}{\Phi_{311\text{nm}}t}$$

N is Avogrado's number, Φ_{311nm} is the quantum yield of E → Z UCA isomerization (assuming the value of 0.52 previously determined at 308 nm),[23] and t is the time of irradiation in seconds. Correction for back reaction (Z → E) was done by irradiation of E-UCA until a photostationary state (51% E-UCA/ 49% Z-UCA) was achieved (ca. 30 min with four 311 nm lamps). The corrected fraction of isomerized UCA, f', was calculated from the following expression, in which f_{pss} is the fraction of the isomer present at the photostationary state, f_0 is the fraction of isomer initially present, and f is the observed fraction of isomer formed:

$$f' = (f_{pss} - f_0)\ln\left(\frac{f_{pss} - f_0}{f_{pss} - f}\right)$$

Under these conditions, the photon flux was found to be 1.74 × 10^{14} photons/s.

Photolysis with Calf Thymus DNA. For qualitative studies, buffered solutions consisting of 1 mL aliquots of 0.58 mM DPPZPHEN and 2.7 mg/mL CT DNA were irradiated with $\lambda >$ 330 nm light. After irradiation, the solutions were purified by exhaustive dialysis and three cycles of precipitation and resuspension. The latter involved the use of a 2 M NaCl solution (0.1 volume) and 95% ethanol (2 volumes), refrigeration for 8 h, and centrifugation to obtain a purified DNA pellet. The DNA and Rh content were determined and/or the DNA was utilized in size-exclusion chromatography experiments.[13b] Relative quantum efficiency measurements for covalent binding to the DNA were carried out with monochromatic 311 nm light. Stock solutions of CT DNA (1 mg/mL) and either BISPHEN or DPPZPHEN (0.3 mM) were prepared in a 50 mM sodium phosphate buffer. Solutions (6 mL) in quartz tubes were degassed with argon for 1 h and then irradiated, in duplicate, in a turntable for ca. 50 min. At these concentrations, both metal complexes absorb all the 311 nm light. The amount of recovered DNA was measured by a modification of the Burton assay[24b] after exhaustive dialysis through a 12 000 D cellulose membrane and also after one precipitation with the procedure described above. The pellets were also assayed for rhodium. The relative quantum efficiencies for binding were calculated by dividing the amounts of metal found in the pellets by the amounts of DNA isolated after dialysis and comparing these values for BISPHEN vs DPPZPHEN.

Photolysis with ϕX-174 Plasmid DNA. Metal complex solutions (with or without quenchers) were saturated with oxygen or argon prior to irradiation. The solutions were further degassed after being mixed with the plasmid. The final solution concentrations were as follows: DPPZPHEN (12 μM), ϕX-174 plasmid DNA (205 μM in base pairs), superoxide dismutase (25 μg/mL), 2-propanol (0.5 M), mannitol (6.25 mM), and histidine (8 mM). The samples were irradiated with 311 nm light and the solutions were subjected to gel electrophoresis.[29]

Photolysis with Tumor Cells. Cells were grown as monolayers in 75 cm^2 vented cell culture flask at 37 °C with 5% CO_2 and 90% humidity in phenol red-free Dulbecco's modified Eagle's medium. Before experiments the cells were trypsinized, plated in 20 35- × 10-mm tissue culture dishes, and incubated some 16−20 h before

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photolysis. At the beginning of an experiment, medium was removed from all plates and the cells were washed with 1 mL of PBS or Hank's salt solution. Metal complex solution (2 mL) or buffer solution (2 mL) was added to each dish. Controls included cell studies in the absence of metal complex and cells with complex in the absence of light. Cells were incubated, with or without metal complex, for 4 h. After incubation, the solutions were removed from the dishes and the dishes were washed with 1 mL of PBS or Hank's salt solution. A fresh 1 mL of PBS or Hank's salt solution was added to each dish and these were placed on a turntable that sat on the top part of the photolysis chamber. The dishes were irradiated from below with 311 nm light, the drug or control solutions were removed from the dishes, and 2 mL of fresh medium was added to each dish. The plates were then incubated for 40−48 h before a determination of the extent of cell toxicity by MTT assay.

Photolysis with SINV. DPPZPHEN/SINV solutions were prepared by adding a 0.3 mL aliquot of a 600 μM DPPZPHEN stock solution in PBS to 3 mL of purified virus at a concentration of 7−8 log pfu/mL and bringing the total volume to 4 mL. The DPPZPHEN/SINV solutions were incubated for 2 h prior to irradiation, following which 3 mL of the solution was bubbled with oxygen (or, for hypoxic conditions, nitrogen) for ca. 20 min. The solution was then irradiated in quartz cuvettes with 355 nm light from an Nd:YAG laser under a constant stream of oxygen or nitrogen with stirring. Controls included studies of the virus in the absence of metal complex and in the presence of complex but in the absence of light. Aliquots of 100 μL were withdrawn over time and analyzed for virus infectivity. The inactivation rate constant was calculated by single-hit theory.[30,31] For the studies measuring infectivity of genome RNA, a 6.9 μL aliquot of 0.623 M DPPZPHEN stock solution in DMSO was added to a 100 μL of TE12 wild-type SINV solution (10 log pfu/mL) to obtain a 40 mM DPPZPHEN/virus solution. Aliquots of 50 μL were placed into 250 μL inserts, incubated for 2 h, and either kept in the dark or irradiated for 60 min with light > 330 nm. At the completion of the experiment, all the samples were diluted with 6 mL of 1× PBS buffer and the virus was precipitated for 2 h at 4 °C with 10% poly(ethylene glycol) (PEG) + NaCl (200 mM). The PEG-precipitated virus was centrifuged at 4 °C for 30 min at 14 000 rpm in a microfuge, and the resulting pellet was resuspended in 200 μL of 1× PBS buffer and extracted with equal volumes of phenol and chloroform. The virion RNA was precipitated with ethanol, resuspended in 100 μL of 1× PBS, and electroporated into ca. 2×10^7 baby hamster kidney (BHK) cells by use of 0.2 cm gap cuvettes. The electroporation utilized 200 Ω, 1.5 kV, and 25 μF. The cells were incubated at 37 °C for 12 h in MEM supplemented with 10% fetal calf serum, and the viral supernatants were harvested and assayed for the presence of infectious virus by plaque assay.

An immunofluorescence assay was also employed to detect the presence of SINV capsid protein newly translated from the irradiated viral genomic RNA. SINV RNA from virus that had been either incubated in the dark or irradiated with DPPZPHEN was isolated and transfected into baby hamster kidney cells as described above. Following electroporation, the cells were plated on coverslips and incubated at 37 °C for 12 h. The cells on the coverslips were then fixed with methanol for 15 min and washed twice with 1× PBS buffer. They were then incubated with anti-capsid (SINV) polyclonal antibody[32] for 45 min at 37 °C. After being washed three times with 1× PBS buffer, the cells were incubated with fluorescein 5-isothiocyanate- (FITC-) conjugated goat anti-rabbit secondary antibody at 37 °C for 45 min. The control and experimental samples were also stained with DAPI (4,6-diamidino-2-phenylindole), which was used as a counterstain to highlight the nuclei of BHK cells. The cover slips were washed three times with 1× PBS and mounted on microscope slides with Fluorosave reagent. They were viewed under a Nikon Eclipse TE200 with a TE-FM Epi-fluorescence attachment. Relatively low levels of transfected cells were observed in these experiments due to the difficulty of isolating sufficient amounts of RNA from purified virions. Furthermore, for these experiments the virus solutions had to be 10 log pfu/mL, which is a virus concentration 3 orders of magnitude higher than that used in the previous experiments. Also, the DPPZPHEN solutions had to be prepared in 10% DMSO to maintain the ratio of drug to virus as in the previous experiments. Preliminary results demonstrated that solutions with up to 10% DMSO (v/v) had no effect on the virus or on the rate of photoinactivation by DPPZPHEN.

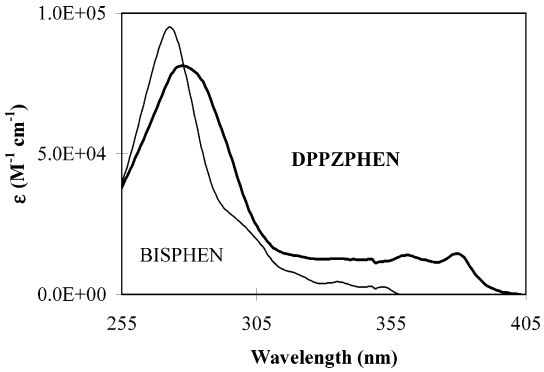

Figure 2. UV−Vis absorption spectra for BISPHEN and DPPZPHEN in phosphate buffer, pH 7.

Results and Discussion

Spectral Properties of the DPPZPHEN Complex. The DPPZPHEN UV−vis absorption spectrum, taken in 50 mM phosphate buffer, pH 7, is shown in Figure 2. The absorption is markedly red-shifted from that of BISPHEN (Figure 2) and slightly red-shifted relative to the spectrum of the free dppz ligand. The absorption in the free ligand consists of $\pi \rightarrow \pi^*$ and/or $n \rightarrow \pi^*$ dppz intraligand transitions. Superimposed on these transitions in the complex will be a metal-centered transition, which is anticipated to fall in the 380 nm region, on the basis of observations made for BISPHEN.[33] Thus, irradiation at these wavelengths should directly populate both the ^{1}d,d and the π,π^* or n,π^* excited states. Similar absorption spectral properties have been observed for the rhenium(I) dipyridophenazine metal complex, *fac*-(dppz)Re(CO$_3$)(4-Mepy)Cl (*fac* = facial; 4-Mepy = 4 methylpyridine), and its phenanthroline analogue, *fac*-(phen)Re(CO$_3$)(4-Mepy)Cl.[34]

DPPZPHEN is nonemissive at room temperature in either protic or aprotic solvents. However, it does phosphoresce when cooled in a rigid matrix at 77 K (Figure 3). Deconvolution by use of a chopper makes evident that the

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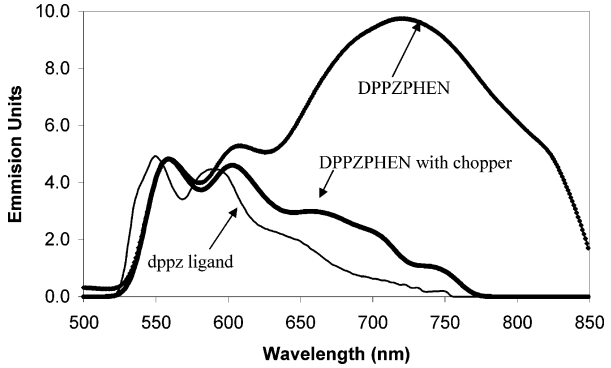

Figure 3. Emission spectra for the dppz ligand and DPPZPHEN at 77 K in a 4:1 methanol/water mixture.

phosphorescence is a composite of two components. The complexity of the spectrum is not due to contamination by an adventitious impurity since excitation spectra, obtained by monitoring either the metal-centered transition at 710 nm or the ligand-centered transition at 554 nm, were identical to one another and in good agreement with the compound's absorption spectrum. It is also obvious from Figure 3 that traces of the free ligand cannot be a source of the complex's structured emission.

We assign the two components to emission from two excited states,that is, (1) a ligand-centered state having a long-lived structured emission on the blue edge of the spectrum that appears to be a modestly red-shifted analogue of the π,π^* emission seen from the free ligand and (2) a metal-centered transition giving structureless, short-lived emission on the red edge of the spectrum that is quite similar to the structureless emission observed from BISPHEN. Multiple emission from two distinct states is well-precedented for Rh(III) tris(bipyridyl) complexes.[35,36] An example is $[Rh(phen)_3]^{3+}$, which shows both $^3\pi,\pi^*$ and ^{3}dd emission at room temperature.[37] The two emissions have the same lifetime and the authors propose that the lowest-lying ^{3}dd state and the $^3\pi,\pi^*$ state are in thermal equilibrium at room temperature. The tris complex $Rh(3,3'\text{-}Me_2bpy)_3^{3+}$ also shows dual-state emission.[36] At 77 K the spectrum consists of a dominant, broad, long-lived emission from the ^{3}d,d state, superimposed on which is a weak, shorter-lived emission from the $^3\pi,\pi^*$. Though the two states do not interconvert at 77 K, they thermally equilibrate at $T > 231$ K,. The authors suggest that there may be "large, viscosity-dependent activation barriers" that inhibit $T_2 \rightarrow T_1$ internal conversion at low temperature.

There is one report of dual-state emission for a Rh(III) bis(bipyridyl) complex but only at higher temperatures.[38] $[Rh(phen)_2(NH_3)_2]^{3+}$ shows only $^3\pi,\pi^*$ emission at 77 K but shows dual emission in the range of 170−200 K. Interestingly, this complex is nonemissive at room temperature. The authors propose that the two states are in thermal equilibrium, as suggested for $[Rh(phen)_3]^{3+}$ (see above).

In sum, our observation of dual-state emission for DPPZPHEN is, to our knowledge, the first example of multiple-state emission from a Rh(III) bis(bipyridyl) complex at 77 K.[39,40] The properties of the complex are most analogous to those of $Rh(3,3'\text{-}Me_2bpy)_3^{3+}$ outlined above and are consistent with inhibited internal conversion within the triplet manifold in the low-temperature glass.

Chemical Properties of DPPZPHEN. Solutions of DPPZPHEN in 50 mM phosphate buffer were stable in the dark for 7 h at 40 °C and for 3 months at 4 °C. Solid samples were stable indefinitely when kept in a desiccator in the dark at room temperature. By contrast with such typical dark stability, the photochemistry of octahedral bis(bipyridyl)rhodium(III) complexes is characterized by the facile replacement of the chloride ligands by water. It was this well-known property that led to our initial studies with BISPHEN.[13a,b] The irradiation of 50 mM phosphate buffer solutions of DPPZPHEN (100 μM), with either monochromatic 311 nm light or broad-band light of $\lambda > 330$ nm, produced two products detectable by HPLC. HPLC-MS analysis indicated that both products had molecular weights equivalent to that of monoaquated DPPZPHEN ($-$ Cl + H_2O; $m/z = 617$). Because of the chirality of the complex, a pair of diastereomeric monoaquo complexes is to be expected. In addition, irradiation of a DPPZPHEN solution under argon with 311 nm light to 50% disappearance, followed by ESI mass spectrometric analysis of the photoproduct mixture, showed formation of both mono- and bis-aquated product, with molecular ions at 617 and 600 m/z, respectively. The aquo complexes are expected to have absorption spectral properties similar to those of the dichloride. This was confirmed by noting that the spectra of an extensively irradiated DPPZPHEN solution and of a pure solution of starting material were virtually identical.

Because of the extensive literature on this photoaquation chemistry, no further structural studies were carried out on the products, but the quantum efficiency of the process was determined in order to compare the DPPZPHEN complex with the parent BISPHEN. The quantum efficiency for disappearance of the complex, measured at 311 nm, was found to be $\Phi_{311nm} = 0.090$, by comparison with a value of 0.024_5 for BISPHEN.[41,42] The enhancement of photoaquation by the DPPZ ligand mirrors that which we have observed with methylated phenanthroline ligands.[43] The blue-shifted phosphorescence of DPPZPHEN relative to BISPHEN indicates that the ^{3}d,d triplet state is raised in energy in the DPPZ complex. This is a known consequence of increased ligand strength, and increased ligand strength correlates with

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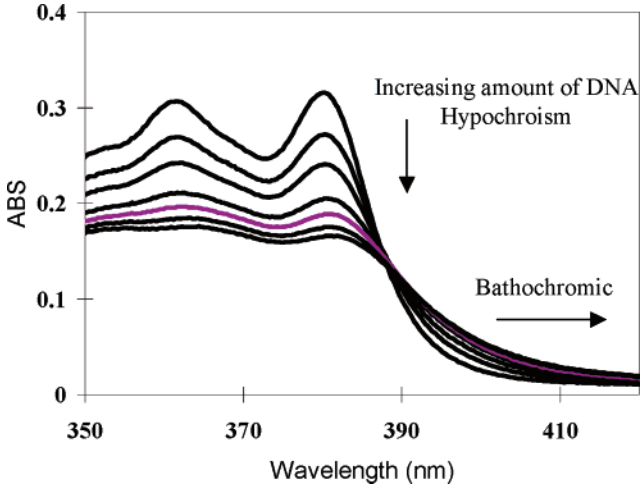

Figure 4. Titration of DPPZPHEN with CT DNA.

increased electron donation by the ligand.[44] It is quite reasonable that increased electron donation by the ligand facilitates photosolvolysis.[43]

It is also interesting that oxygen partially (36%) quenches the photoaquation of DPPZPHEN. BISPHEN photoaquation shows no such quenching.[45] The quantum efficiencies of singlet oxygen formation by these complexes have been measured.[46] They are far too low to explain the quenching of DPPZPHEN photoaquation, and in fact, that for DPPZPHEN ($\Phi_{355nm} = 0.068$) is actually lower than that for BISPHEN ($\Phi_{355nm} = 0.087$). An alternative possibility is electron transfer from the ligand-based triplet to oxygen. Using the simplified form of the Rehm−Weller equation[47] ($-\Delta G_{ET} = E_T - E^\circ_{ox} + E^\circ_{red}$), with E°_{ox} as the oxidation potential of the dppz ligand (1.80 eV),[48] E_T as the energy of the metal complex ligand-based triplet transition (2.3 eV) estimated from the DPPZPHEN phosphorescence spectrum, and E°_{red} as the reduction potential of oxygen (−0.076 V),[49] one can estimate $\Delta G_{ET} \cong -10$ kcal/mol. This possibility becomes additionally relevant in light of the plasmid studies discussed below.

Interaction of DPPZPHEN with CT-DNA in the Dark. As noted in the Introduction, a primary objective in preparing DPPZPHEN was to create a complex that would show a much greater level of association with DNA than the minimal amount seen with BISPHEN. In fact, titration of a DPPZPHEN solution with increasing amounts of CT-DNA produced a bathochromic shift and hypochromism of the DPPZPHEN UV absorption bands that occur between 350 and 400 nm (i.e., transitions primarily due to the dppz ligand; see Figure 4). The data were analyzed by a procedure[27] that is based on the Schemel and Crothers[28] modification of the classic Benesi and Hildebrand analysis.[50] The intrinsic binding constant of $K_{eq} = 3.4 \times 10^5$ M^{-1} is in good agreement with other binding constants found for monocationic metal complexes containing the dppz ligand. For example, the binding constant for *fac*-(dppz)$Re^I(CO)_3$-(4-MePy)$^+$ is reported[34] as $K_{eq} = 6.0 \times 10^5$ M^{-1}. There is general agreement that the binding of metal complexes with a dppz ligand involves intercalation of the complex into the DNA.[34,51]

In designing DNA-targeting molecules, one needs to always be cognizant of potential association of the "drug" with protein. We therefore used competitive dialysis experiments wherein a 150 μM DPPZPHEN solution was simultaneously dialyzed against a 50 mg/mL solution of CT-DNA (1.5 mM in base) and a 50 mg/mL solution of bovine serum albumin (BSA; 3.6 mM in amino acid) in a three-chamber dialysis vessel. After equilibrium was established, 94% of the complex was found associated with the DNA.

Photolysis of DPPZPHEN with Nucleic Acid. Irradiation of a mixture of CT DNA and DPPZPHEN with $\lambda > 330$ nm light, under argon or oxygen, led to the recovery of nucleic acid containing 165 and 150 nmol of rhodium bound/mg of DNA, respectively. These levels correspond to ca. 1 atom of Rh per 18 bases. Size-exclusion chromatography was used to confirm that the metal was indeed covalently bound to the DNA. The complex and the nucleic acid coeluted at a volume between 18 and 36 mL from a 40 mL wet-bed Sephadex column (as evidenced by concomitant analysis of eluents at 260 and 380 nm). A control run with DNA mixed with the metal complex in the dark showed the complex eluting much later in the chromatogram (between 78 and 93 mL), well resolved from the DNA. Photoaquation occurs concomitantly with covalent binding to the DNA in these experiments. It was shown earlier that irradiation of the bisaquo product from BISPHEN leads to covalent binding to DNA at a level comparable to that observed for the parent dichloro complex.[13b] We have not explored this point for the DPPZPHEN complexes but expect the same is likely to be the case. A minimal (ca. 10 nmol of Rh/mg of DNA) level of covalent binding is found when the DPPZPHEN bisaquo complex is incubated with DNA in the dark. The BISPHEN photoproducts showed no detectable covalent binding in the dark when tested.[13b]

The efficiency of binding of DPPZPHEN was measured relative to that for BISPHEN by use of 311 nm light. At the concentrations employed, the metal complexes in both samples absorbed all of the incident light. The amounts of metal bound to DNA for DPPZPHEN and BISPHEN were within experimental error of one another (22 and 21 nmol of Rh/mg of DNA, respectively). However, only 62% of the DNA was recovered from the dialysis of the DPPZPHEN sample whereas 97% was recovered from the BISPHEN sample. This suggested that DPPZPHEN was photolytically nicking the DNA into relatively small (<36 base pairs)

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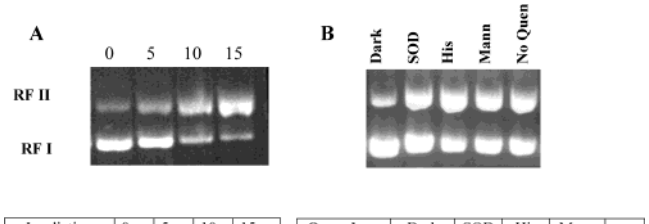

Irradiation (min)	0	5	10	15
%RF I	88	75	45	24

Quenchers	Dark Control	SOD	His	Mann	—
%RF I	80	56	50	52	53

Figure 5. (A) Nicking of ϕX-174 supercoiled plasmid DNA (form I; 205 μM in base pairs) by 12 μM DPPZPHEN with 311 nm light as a function of irradiation time. Lanes: 1, plasmid with DPPZPHEN in the dark; 2, irradiation with DPPZPHEN for 5 min; 3, irradiation with DPPZPHEN for 10 min; 4, irradiation with DPPZPHEN for 15 min. (B) Nicking of ϕX-174 supercoiled plasmid DNA (form I) by DPPZPHEN with 311 nm light for 10 min in the presence of qunechers. Lanes: 1, plasmid with DPPZPHEN in the dark; 2, irradiation with DPPZPHEN plus 25 μg/mL SOD; 3, irradiation with DPPZPHEN plus 8 mM histidine; 4, irradiation with DPPZPHEN plus 12 μM mannitol; 5, irradiation with DPPZPHEN in the absence of any quenchers.

fragments. That conclusion was confirmed by irradiating DPPZPHEN with ϕX-174 supercoiled plasmid DNA.

Irradiation with 311 nm light of ϕX-174 supercoiled plasmid DNA (form I) with the DPPZPHEN produced an appreciable amount of frank nicks in a dose-dependent manner (Figure 5A). Comparable experiments with BISPHEN did not generate such frank nicks.

Others have also observed that metal complexes containing the dppz ligand nick DNA when irradiated, but no mechanism has yet been described for this phenomenon.[34,52] It is evident, however, that diffusible reactive oxygen species do not seem to be required. To confirm this, the plasmid was irradiated with DPPZPHEN for 10 min in an oxygen-saturated solution in the presence of various quenchers: superoxide dismutase (SOD) to probe for superoxide, histidine to probe for singlet oxygen, and mannitol to probe for alkoxy radicals. There was no significant level of protection of the DNA, relative to a simple buffered control, by any of these additivies (see Figure 5B). A plausible, alternative mechanism for the photonicking would involve the formation of a dppz radical by ILCT (i.e., n $\rightarrow$ π^*) excitation or by ligand to metal electron transfer. Both photophysical options have been proposed to explain the nicking of plasmid DNA by $Rh(phen)_2(phi)^{3+}$.[10,53] The $\Delta G°$ for the ligand to metal electron transfer can be estimated by using values of 1.80 eV for the oxidation potential of the dppz ligand,[48] 2.3 eV for E_T, and -0.57 V for the Rh^{III} reduction potential.[54] Estimated in this manner, this process is essentially thermoneutral.

Tumor Cell Phototoxicity. DPPZPHEN proved to be effective as a phototoxic reagent against three different tumor cell lines: GN4, M109, and KB. The levels of toxicity, in each case, were relatively constant between 10 and 40 μM metal complex. This suggests that the three cell lines are saturated at a common intracellular concentration of the metal

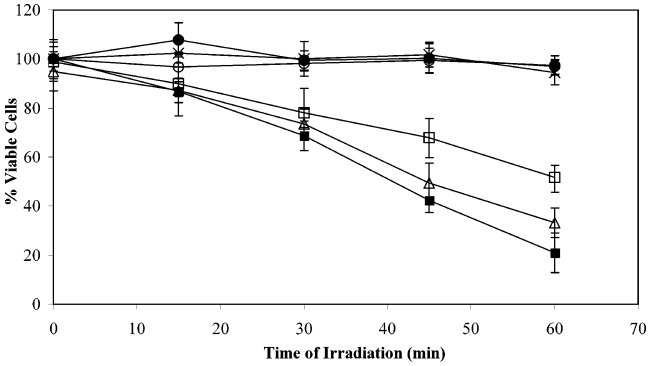

Figure 6. Irradiation of 40 μM DPPZPHEN with tumor cell lines by 311 nm light. Series without metal complex: (○) GN4; (●) M109; (×) KB. Irradiation with metal complex: (□) GN4; (△) M109; (■) KB. The time = 0 points correspond to samples from series △, □, and ■ that were incubated with DPPZPHEN in the dark.

complex. The phototoxicity was dose-dependent for all three cell lines; the results for 40 μM DPPZPHEN, with 311 nm light, are presented in Figure 6. As seen in this figure, as much as 80% of the KB cells were rendered nonviable in 60 min. The complex was slightly less effective on the M109 line and least effective (ca. 50% toxicity) on GN4 cells. There was no appreciable degree of cell toxicity in the absence of light (zero time point in Figure 6), nor were any of the cell lines affected by light in the absence of the metal complex.

Though we have shown that DPPZPHEN is photoreactive with DNA in vitro and that this complex is phototoxic to tumor cells, clearly these results do not allow one to conclude that nucleic acid is a cellular target. Further studies with the RNA virus SINV provided evidence that nucleic acid can indeed be targeted in a biological system.

Photoinactivation of SINV. SINV is a member of the Togaviridae family of viruses, which contains several members that cause significant disease in domesticated animals and humans. It has a complex structure consisting of an outer glycoprotein shell, a lipid bilayer, and an inner nucleocapsid shell that surrounds the single-strand RNA genome. As noted in the Introduction, its structure is closely related to another family of RNA viruses, the Flaviviridae, that are also significant human pathogens.[19] Using 45 μM DPPZPHEN and 355 nm light, we observed that the metal complex is quite potent toward the infectivity of the virus, with a 6 log reduction in viral titer over the course of the experiment (see Figure 7A). The virus was unaffected by irradiation in the absence of the metal complex or by the metal complex in the dark (Figure 7A). Simple hit theory analysis of the data provides an inactivation rate constant $k = 2.4 \times 10^{-19}$ photons^{-1} (Figure 7B). Additionally, the rate of photoinactivation was observed to be the same under oxygen or nitrogen. Though one cannot eliminate the possible presence of trace oxygen in the degassed solutions, the data strongly suggest that the metal complex is photoactive in a hypoxic environment.

Since the SINV genomic RNA is infectious, RNA isolated from virions can be used to probe whether DPPZPHEN directly targets the viral genome. For this purpose, RNA from virus that had been irradiated with >330 nm light in the presence of the metal complex was isolated and transfected

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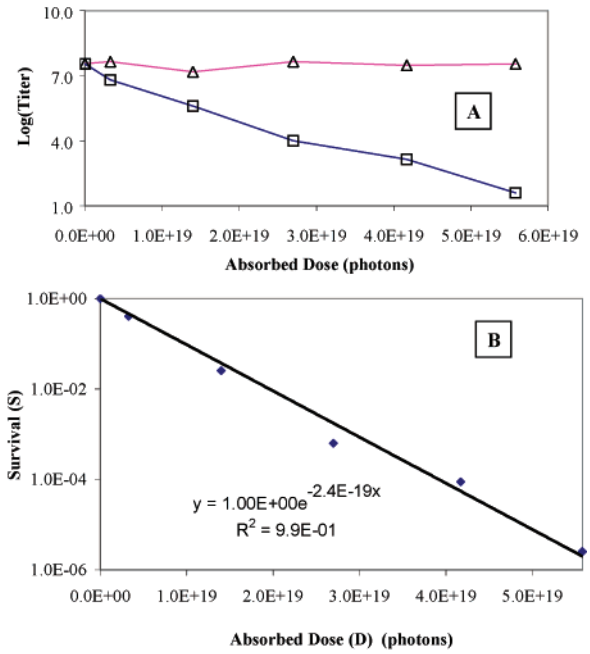

Figure 7. Irradiation of 45 μM DPPZPHEN with SINV by 355 nm light. (A) (△) irradiation of virus without complex; (□) irradiation of virus with complex. The data point for □ at 0 dose corresponds to SINV incubated with DPPZPHEN in the dark. (B) Simple hit theory analysis of data from panel A.

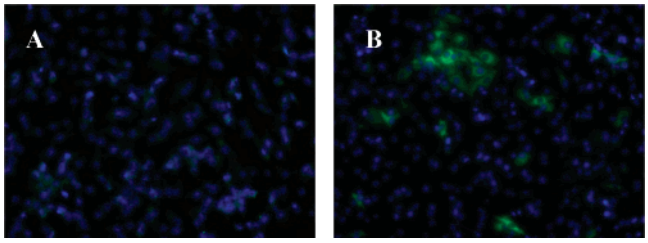

Figure 8. Immunofluorescence images of BHK cells transfected with RNA from (A) SINV photoinactivated by irradiation with DPPZPHEN by >330 nm light and (B) SINV irradiated with >330 nm light in the absence of DPPZPHEN.

into susceptible cells to investigate whether DPPZPHEN inactivated SINV by damaging its viral genome. Virus irradiated without the metal complex, and a DPPZPHEN/SINV solution kept in the dark, were used as controls. Cells that were transfected with RNA from these controls showed a complete cytopathic effect in 48 h posttransfection. However, cells that received RNA isolated from virus irradiated with DPPZPHEN showed no plaque formation.

An immunofluorescence assay was utilized to confirm that the DPPZPHEN had indeed caused inactivation of the SINV RNA. RNA isolated from virus irradiated with or without DPPZPHEN was transfected into BHK cells as previously described, and the presence of newly translated SINV capsid protein was analyzed by immunofluorescence assays. Since SINV replicates in the cytoplasm of BHK cells, the capsid protein was expected to localize in the cytoplasm. DAPI was used as a counterstain to highlight the nuclei of individual BHK cells (blue fluorescence). In this assay, capsid protein in cells transfected with viral control RNA exhibits green fluorescence. As shown in Figure 8A, capsid protein is not observed in cells transfected with RNA isolated from virus irradiated with DPPZPHEN (no green fluorescence), but it is observed in the control (Figure 8B).

Summary and Conclusions

The new metal complex, DPPZPHEN, has interesting photophysical and photochemical properties. The latter include the capacity to form covalent adducts with, and to nick, DNA. The complex is phototoxic toward tumor cells and an enveloped animal viral particle. For the latter, we have also shown that the SINV genome is a primary target for the photoactivated complex. This observation is significant. The ability of the metal complex to directly target the viral genome indicates that it is capable of penetrating two protein layers and the lipid bilayer of the virus. Furthermore, by producing an extensive amount of damage to the pathogen genome, DPPZPHEN "kills" the infectious agent rather than inhibiting one of its biological functions, thus curbing the pathogen's ability to develop resistance. The ability to "kill" pathogens rather than inhibit their biological functions is thought to be of paramount importance for the effectiveness of photoactive drugs. A similar observation has been reported for methylene blue, which is a photoactive drug known to "kill" methicillin- or vancomycin-resistant strains of *Staphylococcus aureus*.[55,56]

Since DPPZPHEN has demonstrated a dual mode of reaction with nucleic acid, i.e., covalent binding as well as nicking, it is not possible to provide specific details for a mechanism of viral and cellular toxicity. It is noteworthy, however, that neither photonicking of, nor photobinding to, nucleic acid was found to be affected by the presence of oxygen, thus eliminating the involvement of diffusible reactive oxygen species in the phototoxic sequence. These observations, and the lack of a dependence of DPPZPHEN's viral phototoxicity on the presence of oxygen, suggest the possibility that such complexes and their analogues might be effective against hypoxic tumor cells.

Additionally, DPPZPHEN's lack of production of ROS, its high association constant with DNA ($K_{eq} = 3.4 \times 10^5$ M^{-1}), and its ability to target the viral genome are all attractive characteristics for the ultimate design of phototoxic agents that might be free of indiscriminate photosensitized damage of blood components. The latter represents one of the major drawbacks of currently employed photodecontaminating drugs[8] that rely on the formation of singlet oxygen for their effectiveness.[2] Thus, these results provide a proof of principle for the use of rhodium metal complexes as phototoxic agents that appear to have a universal targeting mechanism for both the DNA and RNA genomes. With the ongoing discovery of new infectious agents transmitted through blood products, the further development of DPPZPHEN analogues may lead to efficacious and cost-effective strategies for securing the blood supply against unforeseen pathogens, since the use of such compounds does not require prior knowledge of the presence of infectious agents.

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Supporting Information Available: One additional figure (PDF). This information is available free of charge via the Internet at http://pubs.acs.org.

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