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Photobiological Impact of $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$ [bpy = 2,2′-Bipyridine; dpp = 2,3-Bis(2-pyridyl)pyrazine] on Vero Cells

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The in vitro photobiology of the supramolecular complexes $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$ [bpy = 2,2′-bipyridine; dpp = 2,3-bis(2-pyridyl)pyrazine] with African green monkey kidney epithelial (Vero) cells was investigated. Previously, the complexes have been shown to photocleave DNA in the presence or absence of O_2. Vero cell replication was uninhibited for cells exposed to the metal complex but protected from light. Vero cells that were exposed to metal complex, rinsed, and illuminated with >460 nm light showed a replication response that was metal complex concentration-dependent. Vero cells exposed to 3.0–120 μM $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and illuminated showed inhibition of cell growth, with evidence of cell death seen for complex concentrations $\geq$10 μM. Cells exposed to $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$ at concentrations of 5.5–110 μM, rinsed, and illuminated showed only inhibition of cell growth. The impact of $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$ on cell growth following illumination shows the promise of this new structural motif as a photodynamic therapy agent.

Molecules possessing reactive, electronic excited states have found clinical use as photodynamic therapies (PDTs) for the treatment of cancer.[1] Oscar Raab in 1900 described photodynamic action as the photosensitization of oxygen within a cell, leading to cell death.[2] Thirty years ago, researchers began using hematoporphyrin and light to kill cancer cells, directly leading to current clinical applications of PDT (Photofrin).[3] Organic photosensitizers like porphyrins, chlorins, and phthalocyanines garner much of the attention in PDT research.[1] Although many metal complexes have light-activated reactions with DNA, few have been investigated as PDT agents in vitro or in vivo.

Ruthenium polypyridine complexes have rich photochemistry that makes them good candidates for PDT.[4,5] The complex $[Ru(bpy)_3]^{2+}$ (bpy = 2,2′-bipyridine) has electronic excited-state dynamics dominated by the emissive, lowest-lying Ru(dπ) to bpy(π*) (metal-to-ligand) charge-transfer (MLCT) excited state with a lifetime of $\sim$1 μs.[5,6] The triplet MLCT (^{3}MLCT) state undergoes efficient excited-state energy and electron transfer. Like the $^3\pi-\pi^*$ states of organic photosensitizers used for PDT, the ^{3}MLCT state of $[Ru(bpy)_3]^{2+}$ is quenched by molecular oxygen to generate singlet oxygen ($^1O_2$).[7] Cell studies using $[Ru(bpy)_3]^{2+}$ and $[Ru(phen)_3]^{2+}$ (phen = 1,10-phenanthroline) showed that $^1O_2$ caused apoptosis of cells treated with these complexes and light.[8] The ruthenium complexes can damage the cell membrane upon illumination, allowing complex diffusion into the cytoplasm.[8] Barton demonstrated that the lipophilic 4,7-diphenyl-1,10-phenanthroline ligand enhances cellular uptake through passive diffusion.[9]

Phototoxic molecules that do not rely on molecular oxygen would have special clinical applications. PDTs in clinical use today all function by generating $^1O_2$ or other reactive oxygen species.[10,11] Reactive oxygen species are thought to cleave DNA, destroy proteins, and cause lysis of cell and organelle membranes.[12] Tumor cells are often hypoxic, so

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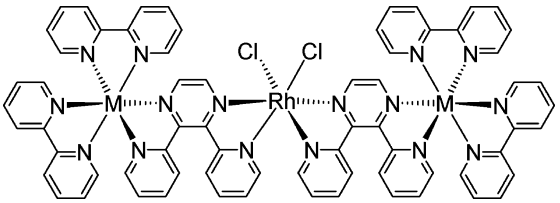

Figure 1. Mixed-metal supramolecules $[\{(bpy)_2M(dpp)\}_2RhCl_2]Cl_5$ used for photobiology studies, where M = Ru^{II} or Os^{II}, bpy = 2,2′-bipyridine, and dpp = 2,3-bis(2-pyridyl)pyrazine.

the efficacy of PDT on imbedded, large, and mature tumors is limited. PDT causes oxygen depletion within the cell, further limiting efficacy of traditional PDT agents.

Rhodium complexes are known to intercalate, photobind, and photocleave DNA in an aerated and oxygen-free solution, with recent studies providing promising in vitro properties. Barton pioneered the use of rhodium complexes in DNA interaction studies.[13] The complexes *cis*-$[Rh(NN)_2Cl_2]^+$, where NN = phen, bpy, dipyrido[3,2-*a*:2′,3′-*c*]phenazine (dppz), or 3,4,7,8-tetramethyl-1,10-phenanthroline, inhibit tumor cell replication when illuminated with UV light.[14,15] The complex *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(dppz)_2]^{2+}$ is phototoxic to human skin cancer cells (Hs-27) when illuminated with 400−700 nm light.[16] *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(bpy)(dppz)]^{2+}$ has improved phototoxicity, similar to that of hematoporphyrin.[17] The photoactivated cisplatin analogue *cis*-$[Rh_2(\mu\text{-}O_2CCH_3)_2(CH_3CN)_6]^{2+}$ was recently reported to have a 34-fold increase in toxicity toward Hs-27 when treated with visible light.[18]

Recently, Brewer showed that the mixed-metal supramolecules $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$ [dpp = 2,3-bis(2-pyridyl)pyrazine] photocleave supercoiled circular plasmid DNA when irradiated with light >450 nm in the presence or absence of molecular oxygen (Figure 1).[19,20] The mixed-metal supramolecules are efficient light absorbers with high absorptivity throughout the visible region (Figure 2).[21] The ^{3}MLCT state undergoes intramolecular electron transfer to the rhodium(III) center, generating the metal-to-metal charge-transfer state (^{3}MMCT)

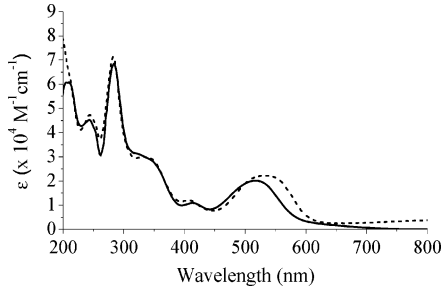

Figure 2. Electronic absorption spectra of the mixed-metal supramolecules $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ (—) and $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$ (- - -) [bpy = 2,2′-bipyridine; dpp = 2,3-bis(2-pyridyl)pyrazine].

and leading to DNA cleavage.[22] Each complex has coincident absorptions in the UV region corresponding to ligand-centered $\pi \rightarrow \pi^*$ transitions. In the visible region, $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ has MLCT transitions centered at 514 nm that are shifted to 525 nm for $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$. The osmium-containing complex has a more intense low-energy tail from direct population of the ^{3}MLCT state (Figure 2). Both systems possess a Rh(dσ^*) lowest uoccupied molecular orbital, leading to the lowest-lying ^{3}MMCT excited state.

Reported herein is a photochemical study of its impact on African green monkey (*Chlorocebus sabaceus*) kidney epithelial (Vero) cell replication when photolyzed after exposure to $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$. Vero cells are adherent mammalian cells that double in population over 24 h, making it a convenient probe of PDT action.[23] The complexes $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$ were added to a growth medium with Vero cells and incubated overnight. The cells were rinsed to remove free metal complex and counted, and a series of grids were exposed to focused microscope light for 4 min. Rinsing the cells prior to illumination means that any impact on the replication either was produced by the metal complex already entered in the cell or was preassociated with the cell surface. Cells were then incubated for 48 h and counted to assess the impact of exposure to light and the complexes on cell growth. Dark controls without exposure to light and light controls without metal complex exposure were conducted. Both dark and light controls showed no inhibition of cell replication, with cell counts 4 times those prior to 48 h of incubation.

The Vero cell cultures exposed to the metal complex and light were examined using transmission and fluorescence microscopy. Light exposure was for a period of 4 min in a circular pattern centered inside four grids (Figure 3A). Phase contrast photographs of the plates were taken before and 48 h after light treatment and used for initial and final cell counting (Figure 3B). Staining of the cells with calcein AM following the 48 h growth period allowed visualization of live cells (Figure 3C). Staining with ethidium homodimer-1 revealed dead cells (Figure 3D). Images of the cells showed that inhibition of replication or cell death was limited to the area of spot illumination of cells pretreated with $[\{(bpy)_2Os$-

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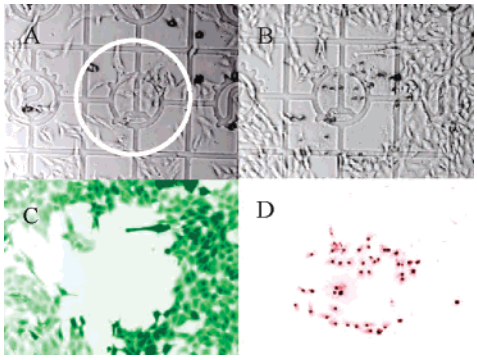

Figure 3. Representative micrographs of Vero cells after exposure to 122 μM $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$, rinsing with a clean medium, and 4 min of illumination by focused light (>460 nm): (A) immediately after photolysis (the circle represents the border of the illumination spot); (B) after a 48 h growth period in the dark; (C) after a 48 h growth period with live cell (green) visualization with calcein AM fluorescence; (D) after a 48 h growth period with dead cell (red) visualization with ethidium homodimer-1 fluorescence.

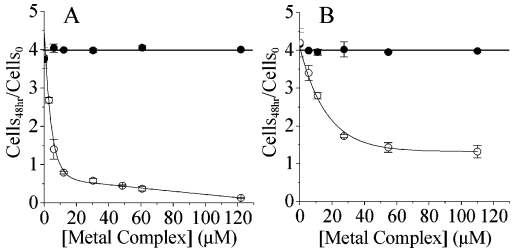

Figure 4. Plot of inhibition of cell replication after exposure to the metal complex, with (○) or without (●) 4 min of illumination with >460 nm light, as a function of the concentration of (A) $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and (B) $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$ [bpy = 2,2′-bipyridine; dpp = 2,3-bis(2-pyridyl)pyrazine].

$(dpp)\}_2RhCl_2]Cl_5$ or $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$. Grids not exposed to light revealed uninhibited replication of Vero cells. The localized impact of illumination suggests that these complexes are highly selectively light-active, making them a very promising new structural motif for PDT development.

Vero cell populations before and after the 48 h growth period were compared using fluorescence microscopy to quantify the photobiological impact of $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$ on Vero cells at a variety of metal concentrations, each conducted in triplicate. Vero cells were treated with the metal complex for 48 h, visualized and counted, exposed to light for 4 min, grown in the dark, and visualized and counted again. Dark and light controls were also conducted, visualized, and counted. The number of cells in a specific grid after the 48 h growth period was divided by the initial number of cells in that grid. Vero cells are adherent, so changes in number reflected cellular replication ($cells_{48h}/cells_0 > 1$) or cell death ($cells_{48h}/cells_0 < 1$). The $cells_{48h}/cells_0$ growth ratios were plotted as a function of the metal complex concentration, with graphical comparisons made between dark controls and illuminated samples (Figure 4). Remarkably, completely normal cell growth was seen for all dark controls. In marked contrast, cells exposed to the metal complexes, rinsed, and illuminated showed a metal complex concentration-dependent replication response. Samples exposed to $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ at 3.0 μM, rinsed, and then irradiated with >460 nm light had cell growth limited to 2.7 times the initial cell population. Growth decreased rapidly as the concentration of $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ was increased from 3.0 to 12 μM ($cells_{48h}/cells_0 = 2.7$ and 0.8, respectively). At concentrations of $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ >12 μM, cell death increased with the concentration. Cells previously exposed to aqueous 120 μM $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and illuminated had a population of 0.13 times that of the original culture. The osmium complex at 5.5 μM limited cell growth to 3.4 times the population immediately following illumination. Cell death was not observed for the cells treated with light and the osmium complex at the concentrations tested, which instead led to inhibition of cell replication at high concentrations. At the highest concentration examined, 110 μM $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$, severely limited cell growth is observed, $cells_{48h}/cells_0 = 1.3$. The deviation of the photobiological response between $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$ on Vero cells is surprising given their similar photoreactivities with DNA, which may suggest differing mechanisms of action.[18]

The results of the Vero cell assay show the high phototoxicity or light-activated inhibition of replication following exposure to $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$, respectively. With growth in the dark, no inhibition of replication occurs when cells have been exposed to micromolar concentrations of $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ or $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$. Replication of cells is retarded after exposure to the mixed-metal supramolecule, rinsing, and short illumination. Only cells that were illuminated were affected, while similar cells kept in the dark grew normally. The collective results of the cell photolysis assays show that $[\{(bpy)_2Ru(dpp)\}_2RhCl_2]Cl_5$ and $[\{(bpy)_2Os(dpp)\}_2RhCl_2]Cl_5$ are promising candidates for the development of new PDT agents, representing a molecular motif that has not been investigated previously. The interesting differences between the osmium- and ruthenium-based systems seen with these cell studies were not evident in the typically reported DNA photocleavage studies, and this illustrates the significance of this approach.

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Supporting Information Available: Detailed materials and methods section. This material is available free of charge via the Internet at http://pubs.acs.org.

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