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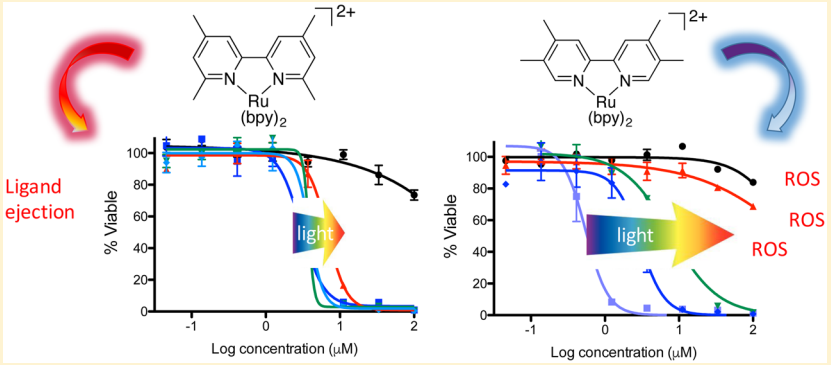
Photochemical and Photobiological Activity of Ru(II) Homoleptic and Heteroleptic Complexes Containing Methylated Bipyridyl-type Ligands

Lars Kohler,[†,§] Leona Nease,[‡,§] Pascal Vo,[†] Jenna Garofolo,[‡] David K. Heidary,[†] Randolph P. Thummel,*[,†] and Edith C. Glazer*[,‡]

[†]Department of Chemistry, University of Houston, 112 Fleming Building, Houston, Texas 77204-5003, United States

[‡]Department of Chemistry, University of Kentucky, Lexington, Kentucky 40506, United States

Supporting Information



ABSTRACT: Light-activated compounds are powerful tools and potential agents for medical applications, as biological effects can be controlled in space and time. Ruthenium polypyridyl complexes can induce cytotoxic effects through multiple mechanisms, including acting as photosensitizers for singlet oxygen ($^1O_2$) production, generating other reactive oxygen species (ROS), releasing biologically active ligands, and creating reactive intermediates that form covalent bonds to biological molecules. A structure−activity relationship (SAR) study was performed on a series of Ru(II) complexes containing isomeric tetramethyl-substituted bipyridyl-type ligands. Three of the ligand systems studied contained strain-inducing methyl groups and created photolabile metal complexes, which can form covalent bonds to biomolecules upon light activation, while the fourth was unstrained and resulted in photostable complexes, which can generate $^1O_2$. The compounds studied included both bis-heteroleptic complexes containing two bipyridine ligands and a third, substituted ligand and tris-homoleptic complexes containing only the substituted ligand. The photophysics, electrochemistry, photochemistry, and photobiology were assessed. Strained heteroleptic complexes were found to be more photoactive and cytotoxic then tris-homoleptic complexes, and bipyridine ligands were superior to bipyrimidine. However, the homoleptic complexes exhibited an enhanced ability to inhibit protein production in live cells. Specific methylation patterns were associated with improved activation with red light, and photolabile complexes were generally more potent cytotoxic agents than the photostable $^1O_2$-generating compounds.

INTRODUCTION

Research in the area of medicinal inorganic chemistry generally involves the generation and testing of metal complexes that function as prodrugs, undergoing chemical reactions as a result of thermal ligand exchange, electron transfer reactions, or photophysical or photochemical reactions. There is an added benefit of potential selectivity for tumor tissues when working with compounds which are triggered by electrons, photons, or other species that can be controlled externally.[1] Alternatively, environmental features, such as hypoxia, which are associated with the tumor microenvironment, can also provide for selectivity.[2,3] In the context of controlling reactivity through photons, ruthenium polypyridyl complexes have been the subject of extensive study, as the complexes absorb visible light and have multiple excited state relaxation pathways that can be harnessed to induce cytotoxicity. This has been a very productive area of research, with contributions from many groups applying a variety of photochemical, photophysical, and biological approaches and mechanisms; a limited selection of references closely related to this current work is provided.[4−26]

Three main excited state reaction pathways are currently utilized. The first is type II photoreactivity, where the triplet metal to ligand charge transfer (^{3}MLCT) excited state sensitizes tissue oxygen, producing singlet oxygen ($^1O_2$).[7] The second pathway is electron transfer reactions, which can be oxidative or

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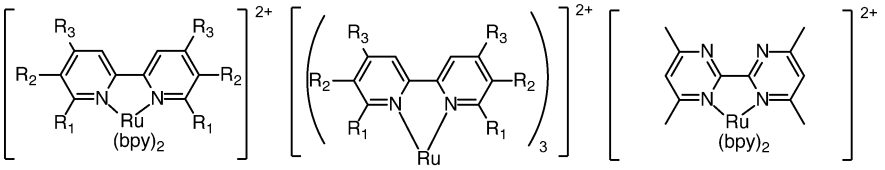
Chart 1. Structures of Compounds Included in This Study



1: $R_1 = R_3 = CH_3$, $R_2 = H$
2: $R_1 = R_2 = CH_3$, $R_3 = H$
3: $R_2 = R_3 = CH_3$, $R_1 = H$
4: $R_1 = R_3 = CH_3$, $R_2 = H$
5: $R_1 = R_2 = CH_3$, $R_3 = H$
6: $R_2 = R_3 = CH_3$, $R_1 = H$
7

Table 1. Electronic Absorption, Emission, and Ground State Electrochemical Potential Data for All Ru Complexes

compound	λ_{max} (log ε)[a]	λ_{em} (20 °C)[b]	$E_{1/2}^{ox}$ (ΔE)[c]	$E_{1/2}^{red}$ (ΔE)[c]
1	244 (4.36), 288 (4.86), 451 (4.05)	nonemissive	1.23 (81)	−1.37 (80), −1.59 (97), −1.87 (108)
2	245 (4.09), 289 (4.57), 452 (3.80)	nonemissive	1.25 (80)	−1.37 (74), −1.59 (98), −1.95 (124)
3	254 (4.41), 288 (4.94), 451 (4.12)	625	1.15 (78)	−1.41 (64), −1.61 (84), −1.97 (112)
4	257 (4.25), 297 (4.79), 455 (4.02)	nonemissive	1.18 (76)	−1.48 (69), −1.70 (74), −1.96 (99)
5	270 (4.41), 307 (4.67), 445 (3.89)	nonemissive	1.26 (83)	−1.57 ir
6	266 (4.48), 291 (4.90), 450 (4.13)	599	0.99 (73)	−1.65 (83), −1.83 (89), −2.07 (107)
7	246 (4.49), 286 (4.82), 444 (4.12)	nonemissive	1.33 (76)	−1.20 (71), −1.51 (76), −1.75 (80)

[a]Measured in CH_3CN (1.0×10^{-5} M) at 20 °C; λ in nm and ε in L mol^{-1} cm^{-1}. [b]Measured in CH_3CN at 20 °C, excitation at absorption maxima; λ in nm. [c]Measured with a glassy-carbon electrode at 100 mV/s in CH_3CN containing 0.1 M NBu_4PF_6 with $E_{1/2}$ reported in volts relative to SCE; $E_{1/2} = (E_{pa} + E_{pc})/2$ in V and $\Delta E = E_{pa} - E_{pc}$ in mV. ir = irreversible.

reductive, as Ru(II) polypyridyl complexes are both strong oxidizing and reducing agents in the excited state.[27] The third is a photodissociative pathway, where the ^{3}MLCT can relax to a formally antibonding metal-centered (^{3}MC) state, which results in ligand loss. While this is a possible relaxation pathway in all Ru(II) polypyridyl complexes, the efficiency can be radically enhanced and controlled by the incorporation of sterically hindered ligands, as elegantly demonstrated by Sauvage.[28,29] We have previously utilized this photochemical reactivity to develop potent cytotoxic agents.[30−33]

In the present study, we systematically investigated the effect of methylation patterns on bidentate ligands with regard to electrochemistry, photophysics, photochemistry, and photobiological activity of the corresponding Ru(II) complexes. In addition to the bipyridine ligand, bipyrimidine was also explored. Five of the complexes contained strain-inducing ligands and were photolabile. Subsequently the coordinatively unsaturated complex can form covalent bonds to biomolecules after light activation. Two of the studied complexes were unstrained and can generate $^1O_2$ under irradiation. Surprising trends emerged that resulted in the identification of specific methylation patterns and symmetry features that correlated with reactivity and specific biological effects.

RESULTS

Synthesis and Characterization. The Ru(II) complexes discussed in this paper are depicted in Chart 1. The symmetrical tetramethyl derivatives of 2,2′-bipyridine (bpy) used in complexes **1**−**6** have been reported (4,4′,6,6′-tetramethyl-2,2′-bipyridine,[34−37] 5,5′,6,6′-tetramethyl-2,2′-bipyridine,[38] and 4,4′,5,5′-tetramethyl-2,2′-bipyridine[39]) and were prepared by the oxidative homocoupling of the corresponding dimethylpyridine under the influence of Pd/C at elevated temperature. The 4,4′,6,6′-tetramethyl-2,2′-bipyrimidine ligand used in complex **7** was prepared by reported procedures.[40] All complexes were synthesized as racemic mixtures of the Δ and Λ enantiomers. The synthesis, isolation, and characterization of the complexes was performed under low-light conditions. The heteroleptic complexes **1**−**3** and **7** were generated in good yields by treating $[Ru(bpy)_2Cl_2]\cdot 2H_2O$ with 1 equiv of the appropriate ligand and precipitation of the complex with NH_4PF_6. The preparation of the homoleptic Ru complexes proved to be more challenging. Complexes **4**−**6** were prepared by treating $RuCl_3\cdot 3H_2O$ with 3.1 equiv of the appropriate ligand under microwave irradiation followed by precipitation of the desired complex with NH_4PF_6. The reactions involving tetramethyl-bpy (tmbpy) ligands carrying methyl groups ortho to the nitrogens proceeded in low yields of 17% for **4** and 3% for **5**, probably due to steric hindrance by the

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methyl groups. Indeed, structural studies have revealed significant distortions in homoleptic complexes containing related strain-inducing ligands.[41]

The Ru complexes were characterized by their ^{1}H NMR spectra, which showed very clear first-order behavior (see the Supporting Information). The electrochemical redox potentials were measured in acetonitrile, and all complexes exhibited one reversible oxidation wave and, with the exception of complex **6**, two reversible and one quasi-reversible reduction wave (Table 1). The UV−visible absorption spectra of the complexes were measured for 10^{-5} M acetonitrile solutions at 20 °C, and the data are recorded in Table 1. All of the complexes showed a characteristic long-wavelength absorption band typically associated with a metal to ligand charge transfer (MLCT) involving the promotion of an electron from a metal-based HOMO to a ligand-based LUMO. For all of the complexes, this absorption occurs over a narrow range of 444−455 nm with very comparable molar extinction constants.

The complexes containing methyl groups ortho to the coordinating nitrogen were anticipated to be susceptible to ligand loss upon irradiation, as these methyl groups introduce steric interference near the metal center. This interference activates a photodissociative pathway from a triplet metal-centered (^{3}MC) excited state.[42,43] The ^{3}MC is populated from the ^{3}MLCT state and provides an additional, irreversible nonradiative decay pathway that overrides the radiative relaxation pathway. As a result, none of the complexes containing methyl groups at the 6,6′-positions showed any emission. In contrast, the unstrained heteroleptic and homoleptic complexes of 4,4′:5,5′-tetramethyl-bpy (**3** and **6**) were emissive; they also did not show any significant photodissociation even after 6 h of irradiation.

Photochemical Characterization. All of the complexes containing strain-inducing ligands were photoactive and underwent light-induced ligand exchange. The behavior was similar to that of $[Ru(bpy)_2dmbpy]^{2+}$ (dmbpy = 6,6′-dimethyl-2,2′-bipyridine), which was used as a control for all studies; we have previously demonstrated strain-induced photochemical ligand loss with this complex.[30] The different complexes exhibited half-life ($t_{1/2}$) values that were dependent on the wavelength of light and power used for the photochemical reaction. Several different light sources and wavelengths were evaluated, as shown in Table 2. The photochemical ligand release was always fastest with the indigo LED (Loctite), which gave the highest power (485 mW/cm^2). Next was the blue long-pass filter (80 mW/cm^2), followed by green (68 mW/cm^2) and then red (79 mW/cm^2). While only small changes in the $t_{1/2}$ values were observed on moving from the blue to the green cutoff filter, the red filter resulted in significant reductions in the rates of the photochemical reactions. This is likely related to the very low absorptivity of the complexes at wavelengths >600 nm.

Table 2. Photoejection Half-Life Analysis Using Different Color Light Sources[a]

compound	indigo	blue	green	red
1	<2 s	14.4 s	35 s	37 min
2	<2 s	10.5 s	26 s	49 min
3				
4	10.3 s	4.5 min	8.7 min	250 min
5	4.4 s	2.9 min	7.8 min	200 min
6				
7	<2 s	32 s	78 s	44 min
$Ru(bpy)_2dmbpy$	<2 s	9.1 s	29 s	72.3 min

[a]Measured using the Loctite indigo LED or a slide projector equipped with different long-pass filters; see the Experimental Section.

The quantum yield of photolysis was determined for the compounds using the indigo LED, assumed to be 450 nm monochromatic light.[44] The measured values were 0.08. 0.09, 0.004, 0.01, and 0.05 for compounds **1**, **2**, **4**, **5**, and **7**, respectively. As a control, $[Ru(bpy)_2dmbpy]^{2+}$ was analyzed and gave a value of 0.10. This is somewhat dissimilar to a previously reported value of 0.19 for this compound.[45] The discrepancy may be due to inaccuracies in the measurement stemming from the rapid photoejection with the Indigo LED.

Cell Cytotoxicity. Given the strain-mediated photochemical reactivity of the family of compounds, light-induced cytotoxicity was investigated using the HL60 leukemia cell line. The complex $[Ru(bpy)_2dmbpy]^{2+}$ was used as a control, as this compound is a potent light-activated cytotoxic agent that has minimal toxicity in the dark.[30] All compounds were incubated for 1 h in the dark with the cells before irradiation. Cells were then exposed to light or, alternatively, kept in the dark. Cell survival was quantified 72 h later. As shown in Table 3, cell death was observed following light irradiation, with six of the seven compounds exhibiting IC_{50} values below 10 μM, though compound **6** only exhibited this activity upon irradiation with the indigo LED. Only one compound exhibited significant cytotoxicity in the dark, with IC_{50} values below 100 μM. Notably, compound **7**, which contained the bipyrimidine ligand, was one of the least potent of the light-activated complexes.

Given the need to use longer wavelengths of light to activate compounds in deeper tissues, the ability of these complexes to be activated by red light was investigated. Following an established procedure, the cells were treated with compounds and then exposed to red light (using a >600 nm cutoff filter).[31] Despite the fact that none of the compounds have strong absorption features in this region of the spectrum, compounds **1** and **2** maintained the ability to kill cells using this low-energy light. The efficacy is particularly notable, as less than 10% of the compound was activated with this light dose, but the IC_{50} value only increased by 2−3-fold.

Recent reports have demonstrated significant cytotoxicity for bipyridine-type ligands containing methyl substituents at the positions ortho to the nitrogen.[46,47] The cytotoxicity of the strained, ejecting ligands for compounds **1** and **4**, **2** and **5**, and **7** were tested, and no cytotoxicity was observed at concentrations up to 30 μM. Accordingly, the activity of the complexes was attributed to the biological interactions of the Ru(II) fragment.[48]

Singlet Oxygen Production. While compounds that photoeject ligands are likely to induce cell cytotoxicity through formation of covalent adducts, compounds **3** and **6** do not photoeject and thus are anticipated to work through an alternative mechanism. As unstrained Ru(II) complexes possess long-lived ^{3}MLCT excited states, they are able to generate reactive oxygen species. Accordingly, Singlet Oxygen Sensor Green, a fluorescent reporter for the production of singlet oxygen ($^1O_2$), was used to determine the ability of the different compounds to create this reactive oxygen species. Tris(bathophenanthroline)ruthenium(II) ($[Ru(dpp)_3]^{2+}$) was used as a positive control, as this compound has a quantum yield for $^1O_2$ production (Δ_O) of 0.42.[49] In addition,

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Table 3. Cellular Cytotoxicity Values in HL60 Cells Using Different Light Sources[a]

compound	dark IC_{50} (μM)	IC_{50} (μM) (% photoejected)[a]			
		indigo	blue	green	red
1	>100	3.0 ± 0.2 (100)	3.9 ± 0.2 (100)	3.8 ± 0.4 (98)	6.8 ± 0.7 (9.3)
2	>100	1.2 ± 0.2 (100)	1.8 ± 0.1 (100)	2.9 ± 0.1 (99.5)	3.9 ± 0.9 (7.4)
3	>100	0.5 ± 0.2 (0)	3.0 ± 0.5 (0)	7.7 ± 1.3 (0)	>100 (0)
4	~100	5.8 ± 1.2 (100)	7.4 ± 1.2 (55)	14.0 ± 2 (38)	60 ± 5[b] (2.8)
5	42.2 ± 9.3	3.1 ± 1.2 (100)	4.7 ± 1.1 (72)	6.0 ± 1.3 (40)	20.4 ± 2.3 (2.5)
6	>100	2.9 ± 0.9 (0)	32 ± 3.5 (0)	~36 ± 3.9 (0)	~43 ± 4.2 (0)
7	>100	4.9 ± 0.8 (100)	ND (100)	ND (100)	>100 (7.3)
$Ru(bpy)_2dmbpy$	>100	3.4 ± 1.1 (100)	3.6 ± 1.1 (100)	3.7 ± 1.1 (99.5)	11.7 ± 1.4 (4)
cisplatin	3.1 ± 0.3	nd[c]	nd	nd	nd
ALA	>100	nd	16.2 ± 3.2	nd	nd

[a]Measured using the Loctite indigo LED or a slide projector equipped with different long-pass filters; see the Experimental Section. [b]Only 60% cell death achieved at highest dose point; therefore, the IC_{50} value represents 30% cell death. [c]nd = Not determined.

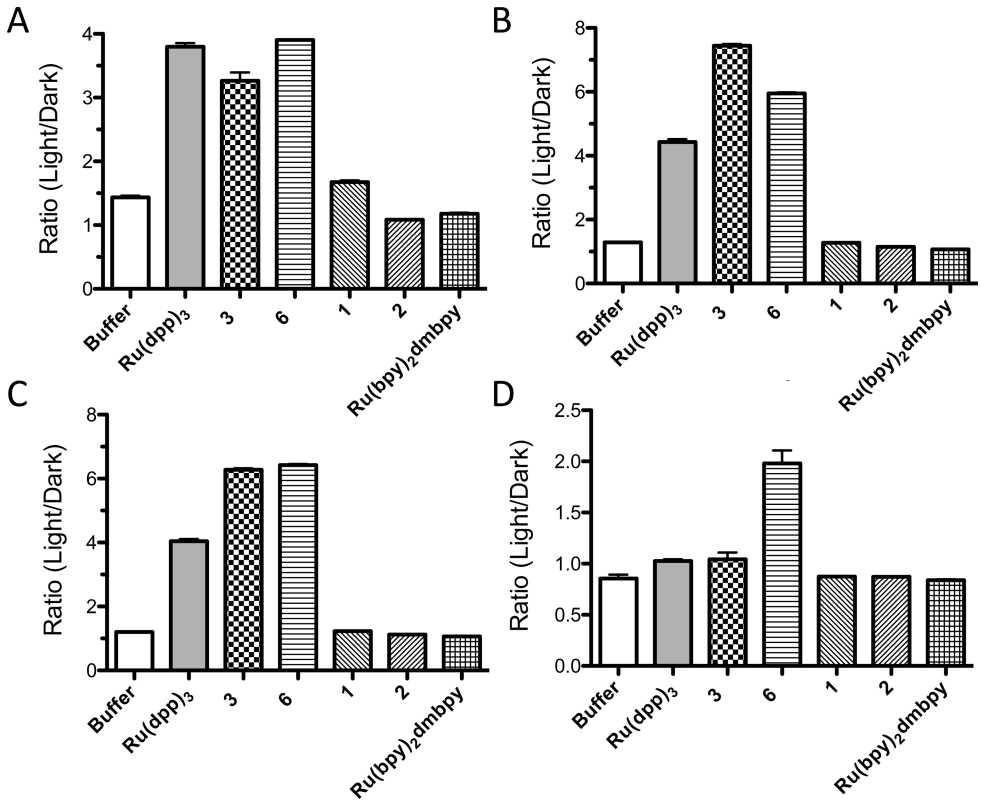

Figure 1. Irradiation-induced $^1O_2$ production as determined by the Singlet Oxygen Sensor Green assay as a function of light source (compound concentrations 20 μM): (A) indigo LED; (B) blue cutoff filter; (C) green cutoff filter; (D) red cutoff filter.

$[Ru(bpy)_2dmbpy]^{2+}$ was investigated as a negative control, and it was also used to determine if photoejection of a ligand had any impact on $^1O_2$ levels.

As shown in Figure 1, all unstrained compounds are able to produce $^1O_2$ upon illumination. Somewhat surprisingly, even excitation with wavelengths >600 nm produced significant levels of this reactive oxygen species with compound **6**, though the indigo LED was more efficient. $[Ru(dpp)_3]^{2+}$, compound **3**, and compound **6** all produced $^1O_2$ in a concentration-dependent manner. Compounds **3** and **6** were both more potent than $[Ru(dpp)_3]^{2+}$ with the indigo LED, but only compound **6** was able to produce an appreciable amount of $^1O_2$ at a concentration of 10 μM using red light.

Unexpectedly, both the photoejecting control compound $[Ru(bpy)_2dmbpy]^{2+}$ and compound **2** slightly *quenched* the $^1O_2$ in the buffer on irradiation with the indigo LED. As these compounds photoeject rapidly, it appears that the quenching may be related to the presence of the photoejected product in solution.

Inhibition of Transcription and Translation. Ru(II) complexes that become ligand deficient upon irradiation have been demonstrated to directly damage DNA[30,31,50] and RNA.[51] Rather than probing damage to nucleic acids through detection of structural changes in the biomolecules induced by metal complexes, functional assays can provide a more sensitive and meaningful report for types of biological interactions that inhibit functions essential for cell health and survival. DNA replication[52] and transcription[53] and translation assays have been used, along with in-cell transcription and translation assays.[54] The in vitro transcription and translation (IVTT)

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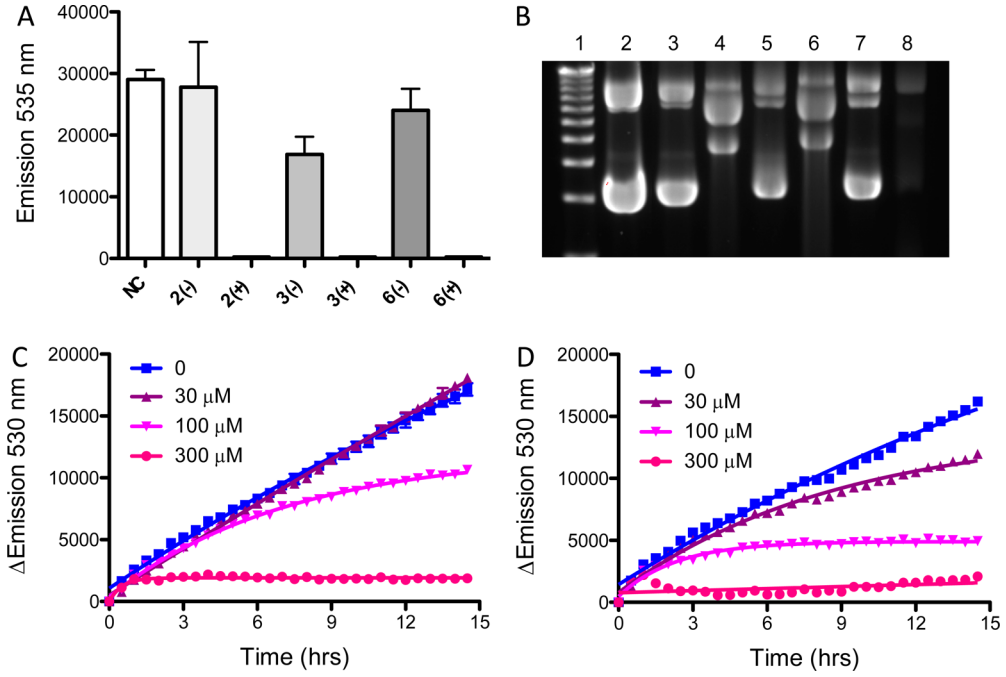

Figure 2. Irradiation-induced DNA damage and inhibition of transcription and translation. (A) Inhibition of GFP synthesis following treatment of the pCFE-GFP plasmid with various compounds at 5 μM concentration. (B) Agarose gels showing the DNA damage with 40 μg mL^{-1} pCFE-GPF plasmid used in the IVTT reactions: lane 1, DNA Ladder; lane 2, pCFE-GFP control; lane 3, compound **3**, no light; lane 4, compound **3**, light; lane 5, compound **6**, no light; lane 6, compound **6**, light; lane 7, compound **2**, no light; lane 8, compound **2**, light. (C) Time-dependent inhibition of production of Dendra2 by compound **1** following irradiation in HEK293 T-REx cells. (D) Time-dependent inhibition of production of Dendra2 by compound **4** following irradiation in HEK293 T-REx cells. The compounds were dosed at the indicated concentrations. The indigo LED was used for all sample irradiation for both in vitro and in cell experiments.

assay assesses nucleic acid damage via a readout of production of green fluorescent protein (GFP), providing both ease of compound analysis and high sensitivity.[51] All compounds were incubated with the plasmid DNA containing the gene for GFP (pCFE-GFP) for 12 h at 22 °C, either in the dark or after activation with light using the indigo LED. After this, the plasmid DNA solution was added to the IVTT reaction solution and GFP emission was quantified after a 2 h incubation at 30 °C to allow sufficient time for transcription and translation of GFP.

As shown in Figure 2A, neither compound **2** nor compound **6** inhibited protein production on incubation with the DNA in the dark. In marked contrast, protein synthesis was inhibited for all compounds on exposure to light. Thus, at concentrations of 5 μM the compounds are able to cause the complete cessation of protein synthesis. Interestingly, the only compound that affected protein synthesis in the dark, compound **3**, inhibited ca. 40% of the control level of GFP production.

When the plasmids used in the IVTT experiment were analyzed for structural damage, the different mechanisms could be visualized by differences in DNA migration and EtBr staining. Both compounds **3** and **6** induced strand breaks in the DNA upon irradiation, as evidenced by the loss of the primary, supercoiled band for the pCFE-GFP plasmid and the appearance of new bands. In marked contrast, compound **2** induced covalent adducts, as indicated by the smearing and reduced mobility on the gel, and the loss of the EtBr signal, which has been previously demonstrated is not due to a loss of the DNA but rather that the presence of the Ru(II) adduct either inhibits EtBr binding or quenches its emission.[31,53]

The ability of the compounds to inhibit protein production was also assessed in live cells using an assay that reports on the production of a photoconvertable protein, Dendra2.[55] Dendra2 is a fluorescent protein that is expressed with a chromophore that can be excited at 485 nm to induce emission in the green region of the spectrum at 530 nm, similar to the case for GFP. However, the chromophore can be subjected to a photochemical reaction by exposure to 405 nm light, resulting in emission at 595 nm. This creates a stable pool of protein with emission in the red region of the spectrum, while newly synthesized protein will emit at 530 nm. The analysis of the ratio of the two emission features allows for correction for cell number and cell health over the time course of the experiment.[55]

As shown in Figure 2C, D, photoejecting compounds were able to completely abrogate new protein synthesis at the highest dose point of 300 μM. Inhibition was observed for compound **4** at concentrations down to 30 μM, in contrast to compound **1**, where inhibition was only observed at 100 μM and higher doses. Compound **4** also inhibited protein production slightly at 300 μM in the dark (see Figure S30 in the Supporting Information). Similar behavior was observed for compound **5**. Compound **2** was similar to compound **1**, though less potent (Figure S31 in the Supporting Information). Unfortunately, compounds **3** and **6** gave inconclusive data due to the fact that the compounds are emissive.

DISCUSSION

The series of compounds studied involved bis-heteroleptic complexes incorporating two bpy ligands and a third, tetramethyl-substituted ligand and homoleptic complexes incorporating only the tetramethyl-substituted bidentate ligand. The two compounds with the smallest differences in activity under irradiation vs in the dark were compounds **4** and **5**.

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These are both homoleptic complexes containing strained 4,4′,6,6′-tetramethyl-2,2′-bipyridyl and 5,5′,6,6′-tetramethyl-2,2′-bipyridyl ligands. Interestingly, while these ligands are able to induce intramolecular strain, the homoleptic complexes eject far less efficiently than the corresponding bis-heteroleptic complexes **1** and **2**, which was associated with a ca. 20-fold difference in the $t_{1/2}$ values. This is qualitatively consistent with a report from Hauser, who compared compound **4** to a tris complex containing the asymmetric ligand 6-methyl-2,2′-bipyridyl.[56] The ^{3}MC state was stabilized for compound **4** in comparison to the complex containing fewer strain-inducing methyl groups, and there was a concomitant decrease in the lifetime of the dissociative excited state, which correlated with a decrease in the photodissociative quantum yield. Presumably, the same effect would occur in complexes that contain one or more unstrained ligands.

The heteroleptic complexes **1** and **2** provided better PI values than the associated tris complexes, with greater than 33- and 83-fold differences in toxicity in the dark and with the indigo light. In addition, both **1** and **2** could be activated with red light, with only a 2−3-fold reduction in potency from the values obtained with the indigo light. From an analysis of these four compounds, it appears that heteroleptic compounds containing the 5,5′,6,6′-tetramethyl-2,2′-bipyridine ligand provide improved biological properties for compounds with larger therapeutic windows.

Of the compounds that do not undergo light-induced ligand dissociation, the bis-heteroleptic complex **3** and the tris-homoleptic complex **6** had IC_{50} values that increased ca. 10-fold upon irradiation with indigo light, which is similar to the values observed for compounds such as $[Ru(bpy)_3]^{2+}$, which have a moderate ability to produce singlet oxygen. Compound **6** maintained the ability to generate $^1O_2$ even under red light irradiation, in contrast to the control compound $[Ru(dpp)_3]^{2+}$, though this did not translate to cytotoxicity under irradiation with the same light. Finally, compound **7** is an outlier, as it exhibited essentially poor toxicity upon irradiation with red light, despite the fact that the compound photoejects to give the same amount of the activated species as for compound **2**. We are not able to explain this finding, but it is possible that photoejection is reduced in the biological environment of the cell.

A specific mechanism of DNA damage is not required to inhibit the processes of transcription and translation using an in vitro assay. Both the covalent adducts formed by compound **2**, visualized by the loss of signal of the EtBr stained DNA, and ROS mediated DNA damage produced by compounds **3** and **6**, which led to strand scission, were equally effective in eliminating the production of GFP (Figure 2A). However, the nature of the IVTT assay can bias the results toward the observation of DNA damage, as the compounds are incubated with the nucleic acid with no other competing biomolecules in solution.

An in cell assay we recently developed to monitor effects on protein production[55] gave intriguing results for the different compounds as a function of compound structure. The bis-heteroleptic compound **1**, containing the 4,4′,6,6′-tetramethyl-2,2′-bipyridine ligand, was able to completely abrogate protein production at high concentrations on irradiation, but its tris-homoleptic analogue **4** was even more potent, with effects observed at the 30 μM concentration point (Figure 2C, D). This compound, however, also has some inhibitory activity at high concentrations in the dark. While the strain-inducing ligand is the same in the two complexes, compound **4** transforms into the "active" diaqua species that still contains two 4,4′,6,6′-tetramethyl-2,2′-bipyridyl ligands, in contrast to compound **1**, which becomes the less sterically encumbered $[Ru(bpy)_2(H_2O)_2]^{2+}$ upon light activation. Clearly, the presence of the 4,4′,6,6′-tetramethyl-2,2′-bipyridine ligands increases the potency of the aqua species for inhibition of protein production, and the presence of three of these ligands imparts some activity (or affinity for a biological target) before irradiation.

Also notable is the fact that compounds **1** and **4** have a rapid impact on protein production, with effects observed as soon as 2 h after treatment. This observation may not be fully consistent with a mechanism where the compounds only inhibit transcription, as the mRNA for the Dendra2 reporter protein is present in the cells for hours after its production and, thus, transcriptional inhibition is unlikely to be observed until several hours have passed. It is possible that this effect is indicative of either damage to the mRNA encoding the Dendra2 protein or direct inhibition of translation.

It also is apparent that the cytotoxic potency of compounds **1** and **2** (IC_{50} values of 3 and 1.2 μM) is not matched by the potency for inhibition of protein synthesis, as no effects are observed for either compound at concentrations below 30 μM. The values for compound **4** are in slightly better agreement, which may suggest that inhibition of protein production is an important component of the mechanism of action of this compound. However, the different time scales for the two experiments (15 h for the protein production assay and 72 h for cytotoxicity) may result in an amplification of what appears to be a small effect over a short time period for the translation assay.

In conclusion, comparison of various complexes containing methylated bipyridine and bipyrimidine ligands revealed unanticipated structure−activity relationships. In all cases, bis-heteroleptic complexes were more potent in cytotoxicity assays, regardless of the nature of the reactive species produced ($^1O_2$ or ligand-deficient metal centers). The heteroleptic complexes ejected more rapidly and generally exhibited lower dark toxicities. Complexes containing bipyridine ligands were preferable to the bipyrimidine ligand containing the same methylation pattern (compound **1** vs compound **7**). Analysis of effects on transcription and translation potentially suggest that specific methylation patterns on the ligands increase potency, but the complexes may also have undesired activity in the dark, which increases with the number of methylated ligands. Finally, several complexes could be activated with red light, which was not anticipated on the basis of the absorption profile. This phenomenon has been described before, notably by the groups of Morrison,[57,58] Sadler,[59] and McFarland,[60−62] with the explanation of direct excitation to the triplet excited states despite the low oscillator strength. In such cases, the "action spectrum" does not recapitulate the absorbance spectrum. This appears to be the case for several of the methylated Ru(II) complexes in this report. The 5,5′,6,6′-tetramethyl-2,2′-bipyridine ligand is a lead compound for incorporation into heteroleptic complexes to provide good PI values and activation with red light, and the 4,4′,6,6′-tetramethyl-2,2′-bipyridine ligand results in the most efficacious inhibition of protein production in live cells. Complexes containing these ligands or their derivatives will be the subject of further research.

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EXPERIMENTAL SECTION

The symmetrical tetramethyl derivatives of 2,2′-bipyridine (bpy) 4,4′,6,6′-tetramethyl-2,2′-bipyridine,[34−37] 5,5′, 6,6′-tetramethyl-2,2′-bipyridine,[38] and 4,4′,5,5′-tetramethyl-2,2′-bipyridine[39] have been reported and were prepared by the oxidative homocoupling of the corresponding dimethylpyridine under the influence of Pd/C at elevated temperature. 4,4′,6,6′-Tetramethyl-2,2′-bipyrimidine was prepared by reported procedures.[40]

The ^{1}H NMR spectra were recorded on a JEOL ECX-400 or ECA-500 spectrometer operating at 400 or 500 MHz, respectively. The chemical shifts are reported in parts per million (ppm) and were referenced to the solvent residue peaks, which were referenced to tetramethylsilane. CV measurements were carried out on a Bioanalysis BAS Epsilon Electroanalytical System. The CV experiments were performed in a one-compartment cell equipped with a glassy-carbon working electrode, a SCE, and a platinum-wire auxiliary electrode. For compound synthesis, a household microwave oven (Samsung, Model MW 2000 U) was modified according to a previously published description.[63]

Absorption spectra were obtained on an Agilent 8453 diode array spectrometer. Samples were read at a concentration of 20 μM in a quartz cuvette at room temperature; the absorption was corrected for the background absorption of the solvent. For indigo light, samples were irradiated with a LOCTITE LED Flood System Indigo Array Light. For the red, green, and blue light samples, the compounds were irradiated in a 410 W 3 M 955 overhead projector with the stage glass removed. Edmund Optics filters (item numbers NT43-941, NF49-935, NT43-947, and NT43-954) were used to cut off the appropriate sections of the UV and visible spectrum for the respective colors. Prism software was used to analyze data, and the kinetic half-lives were measured from fitting the data points to a one-phase association curve.

Quantum yields of photolysis were determined as described in the literature.[13] Briefly, ferrioxalate was used as an actinometer to determine the photon flux of the indigo LED, and as Φ_λ (the quantum yield of the actinometer at 450 nm, the wavelength of the LED lamp) has not been reported, Φ_{436} was used with a reported value of 1.11.[64] The decrease in absorption of each complex was determined as a function of irradiation time, data being taken every 1 s. The value at each time point for moles of compound was then plotted against the moles of photons, and the initial linear region was used to determine the slope of the line, which provides the quantum yield. Significant uncertainty is associated with compounds with $t_{1/2}$ values of less than 10 s.

All synthesized compounds were isolated in >94% purity, as determined by analytical HPLC. For HPLC analysis, the ruthenium complexes were injected into an Agilent 1100 series HPLC equipped with a model G1311 quaternary pump, G1315B UV diode array detector, and ChemStation software version B.01.03. Chromatographic conditions were optimized on a Phenomenex C18(2), 100 A (250 mm × 4.6 mm inner diameter, 5 μM) fitted with a Phenomenex C18 (4 mm × 3 mm) guard column. Injection volumes of 20 μL of 100 μM solutions of the complex were used. The detection wavelength was 280 nm. Mobile phases were as follows: mobile phase A, formic acid (0.1%) in distilled water (dH_2O); mobile phase B, formic acid (0.1%) in HPLC grade acetonitrile. The mobile phase flow rate was 1.0 mL min^{-1}. The following mobile phase gradient was used: 98−95% A (containing 2−5% B) from 0 to 5 min; 95−70% A (5−30% B) from 5 to 15 min; 70−40% A (30−60% B) from 15 to 20 min; 40−5% A (60−95% B) from 20 to 30 min; 5−98% A (95−2% B) from 30 to 35 min; re-equilibration at 98% A (2% B) from 35 to 40 min.

Before photochemical and biological experiments were performed, all compounds were converted to Cl^- salts by dissolving 5−20 mg of product in 1−2 mL of methanol. The dissolved product was loaded onto an Amberlite IRA-410 chloride ion exchange column and eluted with methanol, and the solvent was removed in vacuo.

Compound 1, [Ru(bpy)$_2$(4,4′,6,6′-tetramethyl-2,2′-bipyridine)](PF$_6$)$_2$. [Ru(bpy)$_2$Cl$_2$]·2H$_2$O (258 mg, 0.496 mmol) and 4,4′,6,6′-tetramethyl-2,2′-bipyridine (119 mg, 0.559 mmol) were suspended in ethylene glycol (10 mL), and the reaction mixture was irradiated with microwaves for 30 min (2 × 5 min, 2 × 10 min). The reaction mixture was cooled to room temperature, and NH_4PF_6 (400 mg) dissolved in water (20 mL) was added. The precipitate was filtered, washed with water and diethyl ether, and dried. It was purified by chromatography on alumina, with CH_2Cl_2/acetone as eluent, followed by recrystallization from acetone/diethyl ether to provide the desired complex as an orange solid (300 mg, 66%). ^{1}H NMR (acetone-d_6): δ 8.83 (2H, d, J = 7.8 Hz), 8.76 (2H, d, J = 7.8 Hz), 8.51 (2H, s), 8.29−8.23 (4H, m), 8.13 (2H, dt, J = 7.8, 1.4 Hz), 8.00 (2H, d, J = 5.5 Hz), 7.65 (2H, dt, J = 6.6, 1.4 Hz), 7.43 (2H, dt, J = 6.6, 1.4 Hz), 7.33 (2H, s), 2.50 (6H, s), 1.80 (6H, s). ESI MS: calcd for $C_{34}H_{32}N_6Ru$ $[M]^{2+}$, 313.1; found, 313.0 $[M]^{2+}$. Purity by HPLC: 95.1% by area.

Compound 2, [Ru(bpy)$_2$(5,5′,6,6′-tetramethyl-2,2′-bipyridine)](PF$_6$)$_2$. [Ru(bpy)$_2$Cl$_2$]·2H$_2$O (122.6 mg, 0.236 mmol) and 5,5′,6,6′-tetramethyl-2,2′-bipyridine (50.0 mg, 0.236 mmol) were treated as described above for **1** to provide a crude product that was filtered, washed with water and diethyl ether, and dried. The product was isolated as an orange solid (198 mg, 92%). ^{1}H NMR (CD_3CN): δ 8.47 (2H, d, J = 8.2 Hz), 8.39 (2H, d, J = 8.2 Hz), 8.12−8.06 (4H, m), 7.96−7.91 (4H, m), 7.75 (2H, d, J = 8.2 Hz), 7.63 (2H, d, J = 5.0 Hz), 7.46 (2H, dt, J = 6.7, 1.4 Hz), 7.21 (2H, dt, J = 5.7, 1.4 Hz), 2.19 (6H, s), 1.59 (6H, s). ESI MS: calcd for $C_{34}H_{32}N_6Ru$ $[M]^{2+}$, 313.1; found, 313.1 $[M]^{2+}$. Purity by HPLC: 98.6% by area.

Compound 3, [Ru(bpy)$_2$(4,4′,5,5′-tetramethyl-2,2′-bipyridine)](PF$_6$)$_2$. [Ru(bpy)$_2$Cl$_2$]·2H$_2$O (122.6 mg, 0.236 mmol) and 4,4′,5,5′-tetramethyl-2,2′-bipyridine (50.0 mg, 0.236 mmol) were treated as described above for **1** to provide a crude product that was filtered, washed with water and diethyl ether, and dried. The product was isolated as an orange solid (189 mg, 88%). ^{1}H NMR (acetone-d_6): δ 8.81 (2H, d, J = 8.2 Hz), 8.80 (2H, d, J = 8.2 Hz), 8.59 (2H, s), 8.23−8.17 (4H, m), 8.05 (2H, d, J = 5.5 Hz), 8.03 (2H, d, J = 6.0 Hz), 7.68 (2H, s), 7.59−7.53 (4H, m), 2.49 (6H, s), 2.12 (6H, s). ESI MS: calcd for $C_{34}H_{32}N_6Ru$ $[M]^{2+}$, 313.1; found, 313.1 $[M]^{2+}$. Purity by HPLC: 97.0% by area.

Compound 4, [Ru(4,4′,6,6′-tetramethyl-2,2′-bipyridine)$_3$](PF$_6$)$_2$. $RuCl_3$·3H$_2$O (37.3 mg, 0.143 mmol) and 4,4′,6,6′-tetramethyl-2,2′-bipyridine (100.0 mg, 0.471 mmol) were suspended in ethylene glycol (10 mL), and the reaction mixture was irradiated with microwaves for 20 min (4 × 5 min). The reaction mixture was cooled to room temperature, and NH_4PF_6 (400 mg) dissolved in water (20 mL) was added. The precipitate was filtered, washed with water and diethyl ether, and dried. Chromatography on alumina, first with CH_2Cl_2/acetone (4/1) and then CH_2Cl_2/acetone (1/1) as eluent, followed by recrystallization from acetone/diethyl ether afforded the product as an orange solid (25 mg, 17%). ^{1}H NMR (acetone-d_6): δ 8.26 (6H, s), 7.30 (6H, s), 2.49 (18H, s), 1.91 (18H, s). ESI MS: calcd for $C_{42}H_{48}N_6Ru$ $[M]^{2+}$, 369.1; found, 369.2 $[M]^{2+}$. Purity by HPLC: 94.6% by area.

Compound 5, [Ru(5,5′,6,6′-tetramethyl-2,2′-bipyridine)$_3$](PF$_6$)$_2$. $RuCl_3$·3H$_2$O (37.3 mg, 0.143 mmol) and 5,5′,6,6′-tetramethyl-2,2′-bipyridine (100.0 mg, 0.471 mmol) were treated as described above for **1** to provide the product as an orange solid (5 mg, 3%). ^{1}H NMR (acetone-d_6): δ 8.26 (6H, d, J = 8.2 Hz), 7.88 (6H, d, J = 8.2 Hz), 2.22 (18H, s), 1.95 (18H, s). ESI MS: calcd for $C_{42}H_{48}N_6Ru$ $[M]^{2+}$, 369.1; found, 369.0 $[M]^{2+}$. Purity by HPLC: 92.5% by area.

Compound 6, [Ru(4,4′,5,5′-tetramethyl-2,2′-bipyridine)$_3$](PF$_6$)$_2$. $RuCl_3$·3H$_2$O (37.3 mg, 0.143 mmol) and 4,4′,5,5′-tetramethyl-2,2′-bipyridine (100.0 mg, 0.471 mmol) were treated as described above for **1** to provide the product as an orange solid (140 mg, 95%). ^{1}H NMR (acetone-d_6): δ 8.55 (6H, s), 7.63 (6H, s), 2.48 (18H, s), 2.10 (18H, s). ESI MS: calcd for $C_{42}H_{48}N_6Ru$ $[M]^{2+}$, 369.1; found, 369.1 $[M]^{2+}$. Purity by HPLC: 97.4% by area.

Compound 7, [Ru(bpy)$_2$(4,4′,6,6′-tetramethyl-2,2′-bipyrimidine)](PF$_6$)$_2$. [Ru(bpy)$_2$Cl$_2$]·2H$_2$O (122.6 mg, 0.236 mmol) and 4,4′,6,6′-tetramethyl-2,2′-bipyrimidine (50 mg, 0.236 mmol) were treated as described above for **1** to provide the product as an orange solid (134 mg, 65%). ^{1}H NMR (acetone-d_6): δ 8.83 (2H, d, J = 8.2 Hz), 8.76 (2H, d, J = 8.2 Hz), 8.36 (2H, d, J = 6.0 Hz), 8.27

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(2H, t, J = 7.8 Hz), 8.15 (2H, t, J = 7.8 Hz), 8.02 (2H, d, J = 5.5 Hz), 7.64 (2H, t, J = 6.8 Hz), 7.51 (2H, s), 7.45 (2H, t, J = 6.9 Hz), 2.65 (6H, s), 1.83 (6H, s). ESI MS: calcd for $C_{32}H_{30}N_8Ru$ $[M]^{2+}$, 314.1; found, 314.0 $[M]^{2+}$. Purity by HPLC: 97.1% by area.

Cell Culture. HL60 human leukemic cells were obtained from ATCC, maintained in Iscove's media, and supplemented with 10% FBS and 1x penicillin−streptomycin. HEK293 T-Rex cells were maintained in DMEM media with 10% FBS. Cells were incubated at 37 °C and 5% CO_2. An extracellular solution was used for cell cytotoxicity studies in place of opi-MEM to prevent cellular damage from light irradiation. An extracellular solution was made with 10 mM HEPES, 10 mM glucose, 1.2 mM $CoCl_2$, 1.2 mM $MgCl_2$, 3.3 mM KH_2PO_4, 0.83 mM K_2HPO_4, and 145 mM NaCl in water.

Cell Cytotoxicity Assay. Cells were plated at a density of 30000 cells per well in 96-well plates in extracellular solution. Compounds were added to cells and incubated for 1 h before irradiating with light. The time used for irradiation was varied on the basis of the wavelength and intensity of the light source. Cells were irradiated under indigo light for 1 min (29.1 J/cm^2), blue light for 5 min (24 J/cm^2), green light for 5 min (20.4 J/cm^2), and red light for 6 min (28.4 J/cm^2). Cells were irradiated under projector light at 30 s intervals with light exposure never exceeding 6 min to ensure cell viability. Once the samples were irradiated with light, opti-MEM containing 2% FBS was added to the cells and incubated at 37 °C for 72 h. Cell viability was determined using resazurin, where the dye was added at a final concentration of 70 μM. Cell viability was quantified after 3 h using a SpectraFlour Plus (Tecan) Plate Reader with an excitation wavelength of 530 nm and emission measured at 590 nm. Background fluorescence was measured in wells that did not contain cells and subtracted from the rest of the values. Prism software was used to analyze data, and the IC_{50} values were measured from fitting the data points to a sigmoidal dose response curve.

Singlet Oxygen Assay. The Singlet Oxygen Sensor Green kit was obtained from Molecular Probes. The Singlet Oxygen Sensor Green (SOSG) was diluted in 33 μL of ethanol to create a 5 mM stock solution. Compounds were diluted in extracellular solution to mirror the cell cytotoxicity assay and added to a 96-well plate followed by the addition of SOSG. The SOSG in the assay was used at a concentration of 5 μM. To measure background fluorescence before the samples were subjected to light irradiation, the plate was read in a SpectraFluor plus plate reader with an excitation of 485 nm and an emission of 530 nm. The plate was then irradiated and read on the plate reader a second time. Compounds were irradiated under indigo light for 1 min (29.1 J/cm^2), blue light for 5 min (24 J/cm^2), green light for 5 min (20.4 J/cm^2), and red light for 6 min (28.4 J/cm^2). The singlet oxygen level was determined by calculating the ratio from the signal observed before and after light irradiation for each sample.

In Vitro Transcription/Translation Assay. A 1-Step Human Coupled IVT Kit−DNA (Thermo Scientific) was used to carry out experiments. A 0.5 μg portion of pCFE-GFP plasmid was used for all reactions, and compounds were used at a concentration of 5 μM. Compounds were irradiated with 1 min of indigo light (29.1 J/cm^2) then incubated with plasmid overnight. The reaction mix was made according to the procedure described by Thermo Scientific, and all reactions were scaled down to 12.5 μL. Reactions were incubated in a water bath for 2 h at 30 °C and then read in a Greiner-Bio One 384-well small volume plate. All samples were read on a SpectraFluor Plus (Tecan) Plate Reader with 485 nm excitation and 530 nm emission.

DNA Damage Assay. A 1% agarose gel was made with 1x Tris-Acetate buffer. An aliquot of the plasmid and compound solution prepared for IVTT was used for the assay after it was diluted with SDS-BME dye. A 12 μL sample was loaded on the agarose gel, and the samples were resolved by running the gel at 100 V for 1 h. The gel was stained with 150 mL of Tris-Acetate buffer with 0.5 μg/mL of ethidium bromide for 45 min. The gel was then destained in Tris-Acetate buffer for 1 h. The gel was imaged on a Chemi-Doc instrument (Bio-Rad) using the UV Trans illumination light and 605/50 nm filter.

In Cell Transcription and Translation Assay. A 96-well plate was coated with matrigel, followed by the addition of HEK293 T-Rex-dendra2 cells at a density of 30000 cells/well, and incubated with 1 μg/mL of tetracycline for 16 h. The medium was aspirated, and 50 μL of L-15 medium with 1 μg/mL of tetracycline was added to the cells. The plate was then irradiated with 1 min of 405 nm light to photoconvert the Dendra2 in the cells. After this, 50 μL of compound was added to each well and incubated with the cells for 2 h. The plate was then irradiated with 1 min of indigo light from the LED to activate compounds. The plate was set in the SpectraFluor Plus (Tecan) plate reader, and the green channel was read with an excitation of 485 nm and emission of 530 nm while the red channel was read with an excitation of 530 nm and emission of 595 nm. A scan was taken every 30 min for 15 h. Prism software was used to analyze the data.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.7b01642.

NMR spectra, dose response curves, and inhibition of production of Dendra2 (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail for R.P.T.: thummel@uh.edu.

*E-mail for E.C.G.: ec.glazer@uky.edu.

ORCID

Randolph P. Thummel: 0000-0001-9586-786X

Edith C. Glazer: 0000-0002-0190-7742

Author Contributions

§L.K. and L.N. contributed equally.

Notes

The authors declare no competing financial interest.

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