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New Ru(II) Complex for Dual Photochemotherapy: Release of Cathepsin K Inhibitor and $^1\text{O}_2$ Production

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Abstract

A new complex, $[\text{Ru}(\text{tpy})(\text{dppn})(\text{Cbz-Leu-NHCH}_2\text{CN})]^{2+}$ (**1**, tpy = 2,2':6',2''-terpyridine, dppn = benzo[*l*]dipyrido[3,2-*a*:2',3'-*c*]phenazine) was synthesized and its photochemical properties were investigated. This complex undergoes photorelease of the Cbz-Leu-NHCH₂CN ligand, a known cathepsin K inhibitor, with a quantum yield, Φ_{450} , of 0.0012(4) in water ($\lambda_{\text{irr}} = 450$ nm). In addition, **1** sensitizes the production of singlet oxygen upon visible light irradiation with quantum yield, Φ , of 0.64(3) in CH₃OH. The photophysical properties of **1** were compared with those of $[\text{Ru}(\text{tpy})(\text{bpy})(\text{Cbz-Leu-NHCH}_2\text{CN})]^{2+}$ (**2**, bpy = 2,2'-bipyridine), $[\text{Ru}(\text{tpy})(\text{dppn})(\text{CH}_3\text{CN})]^{2+}$ (**3**), and $[\text{Ru}(\text{tpy})(\text{bpy})(\text{CH}_3\text{CN})]^{2+}$ (**4**) to evaluate the effect of the release of the Cbz-Leu-NHCH₂CN inhibitor relative to the CH₃CN ligand, as well as the role of dppn as the bidentate ligand for $^1\text{O}_2$ production instead of bpy. Nanosecond transient absorption spectroscopy confirms the formation of the long-lived dppn-centered $^3\pi\pi^*$ state in **1** and **3** with a maximum at ~540 nm and $\tau \sim 20$ μs in deaerated acetonitrile. Complexes **1** and **3** are able to cause photoinduced damage to DNA ($\lambda_{\text{irr}} = 395$ nm), whereas **2** and **4** do not cause DNA photocleavage under similar experimental conditions. These results suggest that **1** is a promising agent for dual activity, both releasing a drug and producing singlet oxygen, and is poised to exhibit enhanced biological activity in phototochemotherapy upon irradiation with visible light.

Graphical abstract

Synopsis. A new Ru(II) complex releases a cysteine protease inhibitor and produces cytotoxic $^1\text{O}_2$ upon irradiation with visible light, making it potentially useful as a dual-action PDT agent.

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Introduction

The spatiotemporal control over therapeutic activity that photodynamic therapy (PDT) provides is attractive in the development of cancer treatment methods that are less invasive and present fewer side effects than surgical resection or chemotherapy.^{1–5} Traditional PDT requires ground state $^3\text{O}_2$, a photosensitizer with a long excited state lifetime, and activation using low energy visible light, preferably in the therapeutic window of 600 – 900 nm.^{6–8} For optimal performance, the photosensitizer should be inactive in the dark, but able to transfer energy from its excited state to $^3\text{O}_2$ to form cytotoxic $^1\text{O}_2$ upon irradiation, such that the therapeutic activity of PDT agents occurs only in tissue that is exposed to light.^{1–8} Photofrin, a mixture of hematoporphyrin and its oligomers, was approved in the U.S. as a PDT agent in 1995 for the treatment of head, neck, and esophageal cancers.⁹ Photofrin absorbs strongly in the therapeutic window due to the Q-band porphyrin transitions, leading to the population of the long-lived $^3\pi\pi^*$ excited state with a quantum yield of 0.83; the latter sensitizes the production of $^1\text{O}_2$ with quantum yields, Φ , of 0.65 in methanol and 0.74 in water at pH 7.4.⁹ One drawback of Photofrin is its inability to enter cells, such that $^1\text{O}_2$ production results in necrosis, making treatment painful to the patient.¹⁰ Instead, small cationic transition metal complexes possessing ligands with extended π -systems were shown to produce $^1\text{O}_2$ in high yield, enter cancer cells, and promote apoptosis upon irradiation.¹¹

Ru(II) diimine complexes represent one class of molecules with extremely high quantum yields of $^1\text{O}_2$ production owing to their $\sim 100\%$ yield of intersystem crossing to long-lived triplet metal-to-ligand charge transfer ($^3\text{MLCT}$) excited states.^{12,13} For example, $[\text{Ru}(\text{bpy})_3]^{2+}$ (bpy = 2,2'-bipyridine), $[\text{Ru}(\text{bpy})_2(\text{dppn})]^{2+}$ (dppn = benzo[*l*]-dipyrido[3,2-*a*:2',3'-*c'*]phenazine), and $[\text{Ru}(\text{bpy})_2(\text{ippy})]^{2+}$ (ippy = 2-(1-pyrenyl)-1*H*-imidazo[4,5-*f*][1,10]-phenanthroline) produce $^1\text{O}_2$ with $\Phi = 0.81$, 0.88, and 0.73, respectively.^{14,15,16} Although these complexes produce $^1\text{O}_2$ in high yields, the reliance of the action of the drug on the presence of O_2 represents a limitation for treatment for hypoxic tumors.^{9,17}

To overcome the limitations of traditional PDT agents in the treatment of hypoxic tumors, Ru(II)-diimine complexes that undergo light-induced ligand exchange to deliver a biologically active compound have been a subject of intense investigation.^{18–22} These potential PDT agents can function either by labilization of a metal-ligand bond to allow for covalent binding of the metal center to biomolecules, such as DNA, or by the release of a caged drug coordinated to the metal center.^{18–22,23} Photoinduced ligand exchange in Ru(II) complexes is generally believed to take place via the thermal population of metal-centered

^3LF (ligand field) state(s) from the lowest energy $^3\text{MLCT}$ (metal-to-ligand charge transfer) state, which places electron density on Ru–L orbitals with σ^* antibonding character.^{25,26} Complexes of the type $[\text{Ru}(\text{bpy})_2(\text{L})_2]^{2+}$ ($\text{L} = \text{CH}_3\text{CN}$, pyridine, NH_3) and $[\text{Ru}(\text{bpy})_2(\text{L}')_2]^{2+}$ ($\text{L}' = 6,6'$ -dimethyl-2,2'-bipyridine, 7,10-dimethylpyrazino[2,3-*f*][1,10]phenanthroline) have been reported to exchange the monodentate L or bidentate L' ligands with solvent molecules upon visible light irradiation,²⁷ and the resulting $[\text{Ru}(\text{bpy})_2(\text{OH}_2)_2]^{2+}$ moiety was shown to covalently bind to DNA in a manner akin to cisplatin.^{18c,28}

In addition to these systems, photoinduced ligand release can be used to achieve spatiotemporal control over caged drug delivery. For example, $[\text{Ru}(\text{bpy})_2(5\text{CNU})_2]^{2+}$ ($5\text{CNU} = 5\text{-cyanouracil}$)^{28a} and $[\text{Ru}(\text{tpy})(5\text{CNU})_3]^{2+}$ were shown to release the cytotoxic 5CNU ligand upon irradiation,²⁹ and Ru(II) polypyridyl complexes have also been used to photorelease peptides that inhibit enzymes that promote metastasis and are overexpressed in cancer cells.^{20,30,31} Recently, $[\text{Ru}(\text{bpy})_2(\text{PPh}_3)(\text{RGH})]^{2+}$ ($\text{RGH} = \text{tripeptide Arg-Gly-His}$) was reported to release the bioactive RGH tripeptide upon irradiation.³¹ Similarly, complexes of the $[\text{Ru}(\text{bpy})_2(\text{peptide-CN})_2]^{2+}$ architecture, where $\text{peptide-CN} = \text{Ac-Phe-NHCH}_2\text{CN}$, $\text{Cbz-Leu-NHCH}_2\text{CN}$, $\text{Cbz-Leu-Ser}(\text{OBn})\text{-CN}$, were reported to release enzyme inhibiting peptides upon irradiation, leading to cell death.³⁰ Other examples of bioactive molecules released from Ru(II) cages upon irradiation include cytochrome p450 inhibitors, such as metyrapone and etomidate.^{18a,20b}

In order to increase the efficiency of PDT agents, complexes that are able to achieve cell death via multiple modes of action are being investigated. For example, $[\text{Ru}(\text{bpy})(\text{dppn})(\text{CH}_3\text{CN})_2]^{2+}$ combines the production of $^1\text{O}_2$ with photorelease of the CH_3CN ligands, and the metal-containing product is able to covalently bind to DNA.³² The dual activity by this complex results in ~ 1100 fold increase in toxicity upon irradiation as compared to dark activity, which represents an ~ 170 -fold increase in reactivity compared to that of the related complex $[\text{Ru}(\text{bpy})_2(\text{CH}_3\text{CN})_2]^{2+}$, which only exchanges ligands, and ~ 4 fold greater than $[\text{Ru}(\text{bpy})_2(\text{dppn})]^{2+}$ which is only able to produce $^1\text{O}_2$. Additionally, $[\text{Ru}(\text{tpy})(\text{Me}_2\text{dppn})(\text{py})]^{2+}$ ($\text{Me}_2\text{dppn} = 3,6\text{-dimethylbenzo}[i]\text{dipyrido}[3,2\text{-}a:2',3'\text{-}c]\text{phenazine}$) and $[\text{Ru}(\text{pydppn})(\text{biq})(\text{py})]^{2+}$ ($\text{pydppn} = 3\text{-(pyridin-2-yl)benzo}[i]\text{dipyrido}[3,2\text{-}a:2',3'\text{-}c]\text{phenazine}$, $\text{biq} = 2,2'\text{-biquinoline}$) have shown potential as dual action PDT agents due to the ability to produce $^1\text{O}_2$ and undergo photoinduced exchange of the pyridine ligand with a solvent molecule.³³

The work reported herein focuses on the combination of $^1\text{O}_2$ production and the release of a caged bioactive molecule upon irradiation with visible light by complexes with a $[\text{Ru}(\text{tpy})(\text{L}')(\text{L})]^{2+}$ architecture, where $\text{L}' = \text{bpy}$ or dppn and $\text{L} = \text{CH}_3\text{CN}$ or $\text{Cbz-Leu-NHCH}_2\text{CN}$ (Figure 1). $[\text{Ru}(\text{tpy})(\text{dppn})(\text{Cbz-Leu-NHCH}_2\text{CN})]^{2+}$ (**1**) produces $^1\text{O}_2$ and releases $\text{Cbz-Leu-NHCH}_2\text{CN}$, a known cathepsin K inhibitor, whereas $[\text{Ru}(\text{tpy})(\text{bpy})(\text{Cbz-Leu-NHCH}_2\text{CN})]^{2+}$ (**2**) is only able to undergo the release of the $\text{Cbz-Leu-NHCH}_2\text{CN}$ ligand. Two additional control complexes were also investigated, $[\text{Ru}(\text{tpy})(\text{dppn})(\text{CH}_3\text{CN})]^{2+}$ (**3**), which only produces $^1\text{O}_2$, and $[\text{Ru}(\text{tpy})(\text{bpy})(\text{CH}_3\text{CN})]^{2+}$ (**4**), which neither sensitizes the production of $^1\text{O}_2$ nor releases a nitrile drug upon irradiation. These controls were designed to gain further understanding of the impact of the $[\text{Ru}(\text{tpy})(\text{L}')(\text{OH}_2)]^{2+}$ complex after release of $\text{Cbz-Leu-NHCH}_2\text{CN}$, since complexes **1** and **3** result in the same photoproduct,

[Ru(tpy)(dppn)(OH₂)]²⁺ (**5**), and complexes **2** and **4** result in the same photoproduct, [Ru(tpy)(bpy)(OH₂)]²⁺ (**6**), upon irradiation with visible light in water. Cathepsin K is a protease that is overexpressed in various cancers and is associated with promoting metastasis.³⁴ Dual mode complexes that release protease inhibitors and generate ¹O₂ have potential applications in cancer treatment because they generate ¹O₂ to kill tumor cells and reduce metastasis through cathepsin K inhibition.

Experimental Section

Materials

All materials were used as received without further purification unless otherwise noted. Ethanol (200 proof) was purchased from Decon Laboratories, diethyl ether, acetone, acetonitrile, and methanol were procured from Fisher Scientific, and dichloromethane and hexanes were obtained from Macron Fine Chemicals. *N,N*-dimethylformamide was purchased from Sigma-Aldrich and was vacuumed distilled to dryness. Ammonium hexafluorophosphate, lithium chloride, potassium chloride, silver tetrafluoroborate, silver trifluoromethanesulfonate, 2,2':6',2''-terpyridine (tpy), 1,3-diphenylisobenzofuran (DPBF), methylene chloride-*d*₂ and acetone-*d*₆ were purchased from Sigma-Aldrich, and 2,2'-bipyridine was obtained from Alfa Aesar. Triethylamine and sodium bicarbonate were received from Fisher Scientific, and potassium ferrioxalate was purchased from Strem Chemicals. Neutral alumina, purchased from Fisher Scientific, was deactivated with methanol. The pUC19 plasmid was purchased from Bayou Biolabs and purified via the QIAprep Spin Miniprep Spin system purchased from Qiagen, and the *Sma*I restriction enzyme was purchased from Invitrogen Life Technology. The complexes Ru(tpy)Cl₃, [Ru(tpy)(bpy)Cl](PF₆), [Ru(tpy)(bpy)(CH₃CN)](PF₆)₂, [Ru(tpy)(dppn)(py)](PF₆)₂, [Ru(bpy)₂(dppn)](PF₆)₂, dppn, and Cbz-Leu-NHCH₂CN were synthesized according to published procedures.^{33a,35–39} The aqua complexes [Ru(tpy)(dppn)(OH₂)](PF₆)₂ (**5**) and [Ru(tpy)(bpy)(OH₂)](PF₆)₂ (**6**) were obtained by irradiating solutions of 0.5 mM [Ru(tpy)(dppn)(CH₃CN)](PF₆)₂ and 1.0 mM [Ru(tpy)(bpy)(CH₃CN)](PF₆)₂, respectively, in water (<5% acetone) under a nitrogen atmosphere with λ_{irr} > 395 nm until conversion was completed.

[Ru(tpy)(dppn)Cl](PF₆) was prepared by refluxing a mixture of Ru(tpy)Cl₃ (100 mg, 0.227 mmol), dppn (110 mg, 0.331 mmol), LiCl (120 mg, 2.83 mmol) and triethylamine (250 μL, 1.79 mmol) in 25 mL of EtOH for 4 hours under a nitrogen atmosphere. The purple reaction mixture was cooled to room temperature and stirred with aqueous NH₄PF₆. The EtOH was removed under a gentle stream of N₂, and the precipitate was collected by vacuum filtration and washed with H₂O, acetone, and toluene. Purification was achieved by first dissolving the resulting solid in CH₂Cl₂ and removing the remaining solid by centrifugation. The complex was isolated by column chromatography using a deactivated alumina stationary phase with a CH₂Cl₂ (1% MeOH) mobile phase. A yellow band containing excess dppn eluted first, followed by a purple band containing the desired product. Solvent was removed by rotary evaporation resulting in 80 mg of dark purple product (0.094 mmol, 42% yield). ¹H NMR (CD₂Cl₂) δ ppm 7.23 (t, 1H), 7.23 (dd, 1H), 7.52 (dd, 1H), 7.71 (m, 3H), 7.78 (m, 2H), 7.90 (td, 2H), 8.20 (t, 1H), 8.34 (m, 4H), 8.45 (dd, 1H), 8.48 (d, 2H), 9.11 (s, 1H), 9.21 (s, 1H),

9.43 (dd, 1H), 9.98 (dd, 1H), 10.68 (dd, 1H). ESI MS: $[M-PF_6]^+$ experimental $m/z = 702.11$, calculated $m/z = 702.07$.

[Ru(tpy)(dppn)(Cbz-Leu-NHCH₂CN)](PF₆)₂ ([1](PF₆)₂)

[Ru(tpy)(dppn)(Cbz-Leu-NHCH₂CN)](PF₆)₂ was prepared by refluxing a mixture of [Ru(tpy)(dppn)Cl](PF₆) (55 mg, 0.065 mmol), Cbz-Leu-NHCH₂CN (165 mg, 0.544 mmol) and AgBF₄ (62 mg, 0.32 mmol) in 20 mL of EtOH for 20 hours under a nitrogen atmosphere and shielded from room light with aluminum foil. The reaction mixture was cooled to room temperature and filtered through Celite to remove insoluble impurities. The filtrate was added dropwise to a solution of aqueous NH₄PF₆ and the resulting orange precipitate was collected by vacuum filtration. The solid was dissolved in a minimal amount of acetone then added dropwise to stirring diethyl ether; the precipitate was collected by vacuum filtration resulting in 65 mg of product (0.052 mmol, 79% yield). ¹H NMR ((CD₃)₂CO) δ 1.25 ppm (s, 1H), 1.51 (m, 3H), 1.66 (m, 1H), 2.66 (m, 1H), 2.80 (m, 4H), 4.08 (m, 1H), 4.33 (d, 2H), 5.00 (dd, 2H), 6.62 (d, 1H), 7.30 (s, 5H), 7.40 (t, 2H), 7.68 (m, 3H), 7.97 (t, 1H), 8.01 (t, 2H), 8.07 (dd, 1H), 8.11 (t, 2H), 8.33 (m, 2H), 8.50 (t, 1H), 8.57 (dd, 1H), 8.72 (d, 2H), 8.88 (d, 2H), 9.04 (s, 1H), 9.13 (s, 1H), 9.40 (dd, 1H), 9.92 (dd, 1H), 10.28 (dd, 1H). ESI MS: $[M-2PF_6]^{2+}$ experimental $m/z = 485.12$, calculated $m/z = 485.13$. Anal. Calc for C₅₃H₄₄F₁₂N₁₀O₃P₂Ru•(CH₃)₂CO•H₂O: C, 50.34; H, 3.92; N, 10.48. Found C, 49.92; H, 3.87; N, 10.48.

[Ru(tpy)(bpy)(Cbz-Leu-NHCH₂CN)](PF₆)₂ ([2](PF₆)₂)

[Ru(tpy)(bpy)(Cbz-Leu-NHCH₂CN)](PF₆)₂ was prepared by combining [Ru(tpy)(bpy)Cl](PF₆) (32 mg, 0.048 mmol), Cbz-Leu-NHCH₂CN (80 mg, 0.26 mmol), and AgBF₄ (28 mg, 0.14 mmol) in 15 mL of EtOH bubbled with N₂. The solution was refluxed overnight under a nitrogen atmosphere, while shielded from light with aluminum foil. The reaction mixture was cooled to room temperature then further cooled in an ice bath and filtered through Celite to remove solid impurities. The filtrate was added dropwise to a solution of aqueous NH₄PF₆ and the resulting orange precipitate was collected by vacuum filtration. Excess ligand was removed by dissolving solid in acetone followed by centrifugation. The acetone solution was then added dropwise into stirring diethyl ether, and the precipitate was collected by vacuum filtration yielding 28 mg (0.026 mmol, 54% yield). ¹H NMR ((CD₃)₂CO) δ 0.23 ppm (t, 2H), 0.40 (s, 2H), 0.58 (m, 2H), 0.73 (m, 2H), 2.68 (q, 1H), 3.16 (m, 1H), 3.32 (d, 1H), 3.38 (d, 1H), 4.14 (q, 2H), 5.73 (d, 1H), 6.36 (td, 1H), 6.46 (s, 5H), 6.62 (td, 3H), 6.76 (dt, 1H), 7.15 (m, 6H), 7.56 (m, 2H), 7.79 (t, 2H), 7.97 (d, 2H), 8.03 (d, 1H), 8.98 (d, 1H). ESI MS: $[M-2PF_6]^{2+}$ experimental $m/z = 397.11$, calculated $m/z = 397.12$. Anal. Calc for C₄₁H₄₀F₁₂N₈O₃P₂Ru•2(CH₃)₂CO: C, 47.04; H, 4.37; N, 9.34. Found C, 47.00; H, 4.43; N, 9.30.

[Ru(tpy)(dppn)(CH₃CN)](PF₆)₂ ([3](PF₆)₂)

[Ru(tpy)(dppn)(CH₃CN)](PF₆)₂ was prepared by refluxing a mixture of [Ru(tpy)(dppn)Cl](PF₆) (30 mg, 0.035 mmol) in 15 mL of 1:1 CH₃CN:H₂O (v/v) overnight under a nitrogen atmosphere, while shielded from light with aluminum foil. The reaction was cooled to room temperature, excess NH₄PF₆ was added, and the solution was stirred for 30 minutes. The orange precipitate was collected by vacuum filtration and washed with H₂O resulting in 15

mg (0.015 mmol, 43% yield). ^1H NMR ($(\text{CD}_3)_2\text{CO}$) δ 2.43 ppm (s, 3H), 7.45 (m, 2H), 7.76 (m, 1H), 7.80 (m, 2H), 8.09 (d, 2H), 8.16 (m, 3H), 8.44 (m, 2H), 8.58 (t, 1H), 8.63 (q, 1H), 8.81 (dt, 2H), 8.97 (dd, 2H), 9.15 (s, 1H), 9.25 (s, 1H), 9.52 (dd, 1H), 10.03 (dd, 1H), 10.35 (dd, 1H). ESI MS: $[\text{M}-2\text{PF}_6]^{2+}$ experimental $m/z = 354.06$, calculated $m/z = 354.07$. Anal. Calc for $\text{C}_{39}\text{H}_{26}\text{F}_{12}\text{N}_8\text{P}_2\text{Ru}\cdot\text{H}_2\text{O}$: C, 46.12; H, 2.78; N, 11.03. Found C, 46.29; H, 2.72; N, 10.80.

Instrumentation and Methods

^1H NMR spectra were collected with a Bruker 400 MHz DPX spectrometer. The sample was dissolved in acetone- d_6 or methylene chloride- d_2 , syringe filtered to remove any solid, and the chemical shifts were referenced the residual solvent peak (2.05 ppm or 5.32 ppm). Electrospray ionization mass spectrometry (ESI-MS) was performed with a Bruker micrOTOF instrument dissolved in methanol. Elemental analysis was performed at the CENTC Elemental Analysis Facility at the University of Rochester (Rochester, NY). Electronic absorption spectra were obtained using a Hewlett-Packard 8453 diode array spectrophotometer and luminescence was measured using a Horiba Fluoromax-4 spectrometer in 1×1 cm quartz cuvettes. Extinction coefficients were determined in triplicate and reported as the average value with the error as one standard deviation. Sample irradiation was performed using a 150 W Xe arc lamp (USHIO) in a MilliArc lamp housing unit with an LPS-220 power supply and an LPS-221 igniter (PTI). The desired irradiation wavelengths were controlled by selecting the appropriate long pass (CVI Melles Griot) or bandpass (Thorlabs) filters. In general, the samples were irradiated in water (<5% acetone) in a quartz cuvette, and a 395 nm long pass filter placed between the arc lamp and the sample holder. Electronic absorption spectra were recorded at various time points throughout the photolysis. The ligand exchange quantum yields (Φ_L) were determined by irradiating the samples (concentration ranging from 35 μM to 70 μM) in water using a 450 nm band pass filter together with a 395 nm long pass filter to ensure that no high energy light reached the sample. The electronic absorption spectra were recorded at early irradiation times (<10% conversion), and the rate of consumption of the reactant was determined from the slope of the line of a plot of the moles of reactant vs irradiation time. The photon flux of the lamp was determined to be $2.4(1) \times 10^{-7}$ Einsteins/min using potassium ferrioxalate as the chemical actinometer.⁴⁰ The quantum yield for ligand dissociation using 450 nm light (Φ_L) was calculated as the rate of moles of reactant consumed divided by the photon flux and corrected for the mean fraction of light (f_m) absorbed by the sample, see Equation S1 and Equation S2. The quantum yields for $^1\text{O}_2$ production (Φ) were measured using $[\text{Ru}(\text{bpy})_3]^{2+}$ as a standard, with $\Phi = 0.81$ in CH_3OH ,⁴¹ and 1,3-diphenylisobenzofuran (DPBF) as a $^1\text{O}_2$ trap. DPBF is emissive in CH_3OH , and the resulting product from the reaction of DPBF with $^1\text{O}_2$ is nonemissive. The absorbance values of samples in methanol were matched at the irradiation wavelength ($A = 0.01$ at 460 nm) in a 1×1 cm quartz cuvette. The samples were irradiated in the sample compartment of the fluorimeter ($\lambda_{\text{irr}} = 460$ nm) in the presence of 1.0 μM DPBF, and the quenching of the DPBF emission ($\lambda_{\text{exc}} = 405$ nm; $\lambda_{\text{em}} = 479$ nm) was monitored as a function of irradiation time, resulting in a linear plot of the emission intensity vs irradiation time, and the quantum yield was determined by comparing the slope for the sample with that obtained for the $[\text{Ru}(\text{bpy})_3]^{2+}$ standard. DNA photocleavage experiments were carried out with 20 μL total volume in transparent 0.5 mL

Eppendorf tubes containing 100 μM pUC19 plasmid (concentration in bases), 10 or 20 μM of each complex, and 5 mM Tris buffer ($\text{pH} = 7.5$, 50 mM NaCl) in water. After incubation for 30 minutes in the dark at room temperature, the samples were irradiated with a 150 W Xe arc lamp ($\lambda_{\text{irr}} = 395 \text{ nm}$). After irradiation, 4 μL of loading dye buffer was added to each sample and the solutions were loaded into a 1% agarose gel (stained with 0.5 $\mu\text{g/mL}$ ethidium bromide) in a $1\times$ TAE buffer (40 mM Tris-acetate, 1 mM EDTA, $\text{pH} \sim 8.5$). Gel electrophoresis was performed at a voltage of 60 V for ~ 1.5 hours; different conditions are described as needed. The gels were imaged using a BioRad Gel Doc 2000 transilluminator. For nanosecond transient absorption (nsTA) experiments, the excitation source was an optical parametric oscillator (basiScan, Spectra-Physics), pumped by a Nd:YAG laser (Quanta-Ray INDI, Spectra-Physics), with ca. 6 ns at a repetition frequency of 10 Hz. The spectrometer (LP980, Edinburgh Instruments) used a continuous 150 W xenon arc lamp as probe source. The spectral measurements were collected with an ICCD camera while the kinetic traces were measured with a PMT and a digital oscilloscope. Samples for nsTA were prepared in a $1\times 1 \text{ cm}$ quartz cuvette with an absorbance value of 0.5 OD at the excitation wavelength.

Results and Discussion

Synthesis and Electronic Absorption Spectroscopy

Complex **1** was designed to both release the active Cbz-Leu-NHCH₂CN cathepsin K inhibitor and to produce cytotoxic ¹O₂ with high efficiency upon irradiation with visible light, generating [Ru(tpy)(dppn)(OH₂)]²⁺ (**5**) upon photoinduced ligand exchange in aqueous media. The sensitized production of ¹O₂ is not expected to take place in **2** because it lacks the extended π -system of dppn, but it should be able to release the caged inhibitor when irradiated resulting the formation of [Ru(tpy)(bpy)(OH₂)]²⁺. Additionally, the two corresponding CH₃CN complexes were prepared, **3** and **4**, as analogs that cannot release the caged drugs, however, their photoinduced ligand exchange in water also results in the formation of **5** and [Ru(tpy)(bpy)(OH₂)]²⁺ (**6**), respectively. Moreover, **3** possesses the capability to produce ¹O₂, but will serve as a control for **1** because it does not release a biologically active molecule.

Complexes **1** – **4** were prepared by reacting the desired bidentate L' ligand, bpy or dppn, with Ru(tpy)Cl₃ in the presence of excess chloride and the reducing agent triethylamine to produce the corresponding [Ru(tpy)(L')Cl]⁺ complexes. The inner-sphere chloride anion was replaced with CH₃CN by refluxing [Ru(tpy)(L')Cl]⁺ (L' = bpy, dppn) in CH₃CN/H₂O (1:1, v:v) to generate the corresponding [Ru(tpy)(L')(CH₃CN)]²⁺ complex, while refluxing each reactant in EtOH with excess Cbz-Leu-NHCH₂CN resulted in [Ru(tpy)(L')(Cbz-Leu-NHCH₂CN)]²⁺ (L' = bpy, dppn). The samples were characterized by ¹H NMR, and the ESI-MS for each complex exhibits molecular ion peaks corresponding to the divalent [M] – 2PF₆ parent ion, where [M] = **1** – **4** (Figure S1 and S2).

The Ru(II) complexes **1** – **4** absorb ultraviolet and visible light efficiently, extending to $\sim 600 \text{ nm}$ at low energies. The electronic absorption spectra of **1** – **4** collected in CH₃CN are shown in Figure S3, and the ¹MLCT absorption maxima and extinction coefficients are listed in Table 1. The Ru $\rightarrow$ tpy/bpy ¹MLCT transitions are observed at 455 nm ($\epsilon = 9,700 \text{ M}$

$^{-1}\text{cm}^{-1}$) and 454 nm ($\epsilon = 10,400 \text{ M}^{-1}\text{cm}^{-1}$) for **2** and **4** in acetone, respectively. The similarity in absorption maxima and extinction coefficients demonstrates that the substituents on the nitrile ligand have a negligible impact on the $^1\text{MLCT}$ transition. Similarly to the analogous bpy complexes, the $\text{Ru} \rightarrow \text{tpy/dppn } ^1\text{MLCT}$ transitions of **1** and **3** are observed at 455 nm with $\epsilon = 16,800 \text{ M}^{-1}\text{cm}^{-1}$ and $15,700 \text{ M}^{-1}\text{cm}^{-1}$ respectively. The enhanced extinction coefficients of the dppn complexes as compared to their bpy counterparts are likely due to contributions from overlapping dppn-centered $^1\pi\pi^*$ transitions with maxima at 385 nm and 407 nm; these peaks exhibit ϵ values of $14,000 \text{ M}^{-1}\text{cm}^{-1}$ and $16,400 \text{ M}^{-1}\text{cm}^{-1}$ in **1**, respectively, and of $12,600 \text{ M}^{-1}\text{cm}^{-1}$ and $14,700 \text{ M}^{-1}\text{cm}^{-1}$ in **3**, respectively. The enhancement of the extinction coefficient is similar to that reported in related systems such as $[\text{Ru}(\text{tpy})(\text{dppn})(\text{py})]^{2+}$ as compared to $[\text{Ru}(\text{tpy})(\text{bpy})(\text{py})]^{2+}$, with molar absorptivities of $12,900 \text{ M}^{-1}\text{cm}^{-1}$ at 474 nm for the former and $8,120 \text{ M}^{-1}\text{cm}^{-1}$ at 468 nm for the latter.^{33a}

Photochemistry in Solution

Upon irradiation with visible light, the monodentate nitrile ligand, Cbz-Leu-NHCH₂CN in **1** and **2** or CH₃CN in **3** and **4**, is replaced by a solvent H₂O molecule to produce the corresponding $[\text{Ru}(\text{tpy})(\text{L}')(\text{OH}_2)]^{2+}$ ($\text{L}' = \text{bpy}, \text{dppn}$) photoproduct. For example, the changes in the electronic absorption spectrum of **2** as a function of irradiation time (0 – 80 min) are shown in Figure 2a ($\lambda_{\text{irr}} = 395 \text{ nm}$), which result in a red shift in the $^1\text{MLCT}$ transition from 455 nm to 478 nm with isosbestic points at 376 and 459 nm. The absorption of the photoproduct is consistent with that previously reported for $[\text{Ru}(\text{tpy})(\text{bpy})(\text{OH}_2)]^{2+}$, formed following the exchange of Cbz-Leu-NHCH₂CN for a solvent H₂O molecule.^{35b} In addition, the spectral changes observed upon irradiation are in agreement with those of **4** (Figure S4a), which generates the same product upon solvolysis. This process for **2** occurs with a quantum yield ($\Phi_{\text{L}}, \lambda_{\text{irr}} = 450 \text{ nm}$) of 0.0012(4), a value lower than that measured for **4**, $\Phi_{\text{L}} = 0.0026(2)$. The difference in efficiency is attributed to the lower solubility and size of the peptide ligand compared to CH₃CN in water, making the escape from the solvent cage less probable in **4** than in **2**, such that the regeneration of the starting material is more likely in the former.⁴²

The photoinduced ligand exchange of **1** was monitored in water deoxygenated by bubbling with N₂ for 10 min to prevent the formation of $^1\text{O}_2$ (Figure 2b). As the sample was irradiated, the $^1\text{MLCT}$ transition red shifted from 448 nm to 477 nm with an isosbestic point at 460 nm. A small decrease of the dppn $^1\pi\pi^*$ absorption bands at 389 and 410 nm was observed attributed to the bathochromic shift of the overlapping $^1\text{MLCT}$ band, thus providing a lower contribution to the absorption in this region. The $^1\text{MLCT}$ absorption maximum of the photoproduct is consistent with that of the reported spectrum for $[\text{Ru}(\text{tpy})(\text{dppn})(\text{OH}_2)]^{2+}$,^{19b} indicative of the exchange of Cbz-Leu-NHCH₂CN for a solvent water molecule upon irradiation. These results parallel those obtained for **3** shown in Figure S4b. The quantum yields of photoinduced ligand exchange in water were measured to be 0.00014(3) and 0.00023(1) for **1** and **3**, respectively ($\lambda_{\text{irr}} = 450 \text{ nm}$). The observation that the quantum yields for the two dppn complexes are lower than those measured for the corresponding bpy compounds can be explained by the competitive population of the long-lived dppn $^3\pi\pi^*$ state in the former, reducing that of the dissociative ^3LF state. It should be

noted that each complex is stable in solution in the dark for at least 30 minutes (Figures S5-S8), which represents an important feature in the design of light activated chemotherapeutic agents.

The incorporation of the dppn ligand in **1** renders the complex in its excited state capable of $^1\text{O}_2$ generation, and a quantum yield Φ , of 0.64(3) was measured for the process in air-saturated methanol ($\lambda_{\text{irr}} = 460 \text{ nm}$). As expected, a similar Φ value was measured for **3**, 0.67(6), under similar experimental conditions. It should also be noted that the photoproduct of ligand exchange for complexes **1** and **3**, $[\text{Ru}(\text{tpy})(\text{dppn})(\text{OH}_2)]^{2+}$, is capable of $^1\text{O}_2$ generation with a quantum yield, Φ , of 0.52(1). These values are in agreement with that reported for the related complex $[\text{Ru}(\text{tpy})(\text{Me}_2\text{dppn})(\text{py})]^{2+}$, $\Phi = 0.69(9)$.^{33a} It is believed that the Φ values for these complexes are lower than unity because of the competitive population of the dppn/ Me_2dppn -centered $^3\pi\pi^*$ state and the dissociative ^3LF state from the $^3\text{MLCT}$ state. In contrast, $[\text{Ru}(\text{tpy})(\text{dppn})(\text{py})]^{2+}$, which does not undergo ligand exchange upon excitation, produces $^1\text{O}_2$ with $\Phi = 0.98(6)$,^{33a} explained by the absence of competitive population of the ^3LF and $^3\pi\pi^*$ states in this complex. The production of $^1\text{O}_2$ is not observed for **2** or **4**, since these complexes do not possess long-lived excited states able to transfer energy to $^3\text{O}_2$ in solution.

The long lived excited state of **1**, **3**, and **5** were investigated by nanosecond transient absorption spectroscopy (nsTA). Figure 3 shows the nsTA spectra of **1** and **3** in deaerated acetonitrile and both feature a strong positive signal centered at $\sim 540 \text{ nm}$ that decays monoexponentially with lifetimes of 22 μs and 20 μs respectively ($\lambda_{\text{exc}} = 480 \text{ nm}$, 5 mJ/pluse). The features of the excited state absorption and their lifetimes are consistent with the reported nsTA spectra of complexes containing dppn ligands, such as $[\text{Ru}(\text{bpy})_2(\text{dppn})]^{2+}$ ($\tau = 33 \mu\text{s}$) and $[\text{Ru}(\text{tpy})(\text{dppn})(\text{py})]^{2+}$ ($\tau = 50 \mu\text{s}$), and assigned to the dppn-centered $^3\pi\pi^*$ state.^{15,33a} The nsTA spectrum of **5** could not be obtained in acetonitrile due its rapid conversion to the acetonitrile bound complex **3**. The nsTA experiments were also performed in deaerated methanol (<5% acetone), where **1**, **3**, and **5** exhibit a strong positive feature centered at $\sim 540 \text{ nm}$ that decays monoexponentially with lifetimes of 34 μs , 36 μs , and 3.1 μs respectively ($\lambda_{\text{exc}} = 480 \text{ nm}$, 5 mJ/pluse). Once exposed to air, the lifetime of each complex decreases dramatically to 260 ns, 397 ns and 338 ns for **1**, **3**, and **5**, respectively, in methanol due to the quenching of the excited state by dissolved oxygen, resulting in respective calculated Stern-Volmer quenching rate constants, k_q , of $3.7 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, $2.4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, and $2.6 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. These values are on the same order of magnitude as other bimolecular quenching constants by oxygen, for example, $2.8 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for $[\text{Ir}(\text{phen})_3]^{3+}$ (phen = 1,10-phenanthroline) and $3.4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for $[\text{Ir}(\text{bpy})_3]^{3+}$ in methanol.^{40,41}

DNA Photocleavage

The ability of the complexes containing the dppn ligand, **1** and **3**, and the dppn-containing photoproduct **5** were evaluated for their ability to photocleave DNA upon irradiation in air ($\lambda_{\text{irr}} = 395 \text{ nm}$). Lane 1 of Figure 4 shows supercoiled pUC19 plasmid (form I, 100 μM bases) in the absence of any complex, featuring a small amount of the nicked open circular impurities (form II), while lane 2 shows the position of the band corresponding to pUC19

plasmid linearized by the *Sma*I restriction enzyme (form III). In the absence of light, 10 μ M of the dppn-containing complexes **1**, **3**, and **5** do not result in the cleavage of DNA, as evidenced by lanes 3, 5, and 7, respectively. However, irradiation of each complex in air (λ_{irr} 395 nm), lanes 4, 6, and 8, respectively, results in an increase of the amount of nicked open circular DNA (form II). This DNA photocleavage is similar to that previously reported by us and can be attributed to the production of $^1\text{O}_2$, a species known to result in the generation of 8-oxo-guanine, a base modification that leads to DNA damage.^{43,44} These results are consistent to those previously reported for related complexes of dppn, $[\text{Ru}(\text{tpy})(\text{dppn})(\text{py})]^{2+}$ (**7**), reproduced in lanes 9 (dark) and 10 (irradiated) and $[\text{Ru}(\text{bpy})_2(\text{dppn})]^{2+}$ (**8**), reproduced in lanes 11 (dark) and 12 (irradiated), both of which are only able to produce singlet oxygen upon irradiation and do not undergo ligand exchange.^{15,33a} The photoproduct of **1** and **3**, $[\text{Ru}(\text{tpy})(\text{dppn})(\text{OH}_2)]^{2+}$ (**5**), is also able to cleave DNA upon irradiation, as compared in lanes 7 (dark) and 8 (irradiated). This finding suggests that after the inhibitor is delivered, the remaining ruthenium fragment is still able to produce singlet oxygen but not as efficiently as the parent complexes **1** and **3**. The bpy complexes **2**, **4** and $[\text{Ru}(\text{tpy})(\text{bpy})(\text{OH}_2)]^{2+}$ did not display DNA photocleavage under similar experimental conditions (Figure S9), consistent with their lack of ability to sensitize the production of $^1\text{O}_2$.

Conclusions

$[\text{Ru}(\text{tpy})(\text{dppn})(\text{Cbz-Leu-NHCH}_2\text{CN})]^{2+}$, **1**, represents a potential PDT agent with dual reactivity, undergoing ligand exchange to deliver a known cysteine protease inhibitor Cbz-Leu-NHCH₂CN, and producing $^1\text{O}_2$ upon irradiation with visible light. The presence of the dppn ligand in the complex results in a long-lived ligand-centered $^3\pi\pi^*$ state able to produce $^1\text{O}_2$ in good yield upon irradiation with visible light. Although the quantum yield for ligand exchange decreases with the introduction of the dppn ligand, the ability of **1** to undergo both processes with spatiotemporal control is promising to achieve anticancer activity via two different mechanisms. In addition, the resulting ligand exchange photoproduct of **1**, $[\text{Ru}(\text{tpy})(\text{dppn})(\text{OH}_2)]^{2+}$ (**5**), is also able to produce $^1\text{O}_2$ in good yield, such that the latter may continue to result in cell death after the inhibitor is delivered. As such, the dual activity of **1** upon exposure to visible light makes it an attractive candidate for further photochemotherapeutic investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Complex	L	L'
1	Cbz-Leu-NHCH ₂ CN	dppn
2	Cbz-Leu-NHCH ₂ CN	bpy
3	CH ₃ CN	dppn
4	CH ₃ CN	bpy

Figure 1.
Schematic representations of the structures of **1** – **4**.

Figure 2.

Changes to the electronic absorption spectra upon irradiation ($\lambda_{\text{irr}} = 395$ nm) of (a) **2** for 0 – 80 min and (b) **1** for 0 – 180 min in water (< 5% acetone; bubbled with N_2 for 10 min).

Figure 3. Transient absorption spectra of (a) **1** and (b) **3** in CH_3CN under N_2 atmosphere ($\lambda_{\text{exc}} = 480$ nm, irf = 5.5 ns, 5 mJ/pluse).

Figure 4.

Ethidium bromide-imaged agarose gel (1%) of 100 μ M pUC19 and 10 μ M of each complex in air (5 μ M Tris buffer, pH = 7.5, 50 μ M NaCl); lanes 1, 2, 3, 5, 7, 9, 11 were kept in the dark and lanes 4, 6, 8, 10, 12 were irradiated (λ_{irr} = 395 nm, 5 min); lane 1, plasmid only; lane 2 plasmid treated with *Sma*I; lanes 3 and 4, **1**; lanes 5 and 6, **3**; lanes 7 and 8, **5**; lanes 9 and 10, **7**; and lanes 11 and 12, **8**.

Table 1

¹MLCT Absorption Maxima, λ_{abs} , and Molar Extinction Coefficients, ϵ , in Acetone, Quantum Yield of Photoinduced Ligand Exchange in H₂O, Φ_{L} , and Quantum Yield of ¹O₂ Production, Φ , for **1** – **4**.

Complex	$\lambda_{\text{abs}}/\text{nm}$ ($\epsilon/\text{M}^{-1}\text{cm}^{-1}$)	Φ_{L}	Φ
[Ru(tpy)(dppn)(Cbz-Leu-NHCH ₂ CN)] ²⁺ (1)	455 (16,800)	0.00014(3)	0.64(3)
[Ru(tpy)(bpy)(Cbz-Leu-NHCH ₂ CN)] ²⁺ (2)	455 (9,700)	0.0012(4)	
[Ru(tpy)(dppn)(CH ₃ CN)] ²⁺ (3)	455 (15,700)	0.00023(1)	0.67(6)
[Ru(tpy)(bpy)(CH ₃ CN)] ²⁺ (4)	454 (10,400)	0.0026(2)	