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Light Activation of a Cysteine Protease Inhibitor: Caging of a Peptidomimetic Nitrile with Ru^{II}(bpy)₂

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Abstract

A novel method for caging protease inhibitors is described. The complex [Ru^{II}(bpy)₂(**1**)₂](PF₆)₂ (**2**) was prepared from the nitrile-based peptidomimetic inhibitor Ac-Phe-NHCH₂CN (**1**). ¹H NMR, UV-vis and IR spectroscopic and mass spectrometric data confirm that two equiv of inhibitor **1** bind to Ru^{II} through the nitrile functional group. Complex **2** shows excellent stability in aqueous solution in the dark and fast release of **1** upon irradiation with visible light. Due to binding to the Ru^{II} center, the nitriles of complex **2** are caged, and **2** does not act as a potent enzyme inhibitor. However, when **2** is irradiated, it releases **1** that inhibits the cysteine proteases papain and cathepsins B, K and L, up to two times more potently than **1** alone. Ratios for IC₅₀ values for **2** range from 6:1 to 33:1 under dark vs. light conditions, against isolated enzymes and in human cell lysates, confirming a high level of photoinduced enzyme inhibition is obtained with this method.

Cysteine cathepsins are overexpressed in a variety of cancers.¹ Downregulation and gene knockout studies in mice support a causal role for cysteine proteases in tumor growth, migration, invasion, angiogenesis and metastasis.² Because of their broad protumorigenic activities, these enzymes are considered viable targets for chemotherapy.³ However, cathepsins are necessary for normal cell function,^{4,5} so selective inhibition within cancerous tissue would be beneficial to achieve high levels of therapeutic selectivity and avoid systemic toxicity issues found with cysteine cathepsin inhibitors. Towards this end, caging bioactive molecules and triggering their release with light⁶ provides a powerful method that yields spatial and kinetic control over compound activation. Light activated compounds are well poised to expand their role as drugs in photodynamic therapy, because lasers and fiber optics now make it possible for light to reach almost any tissue in the human body.⁷ In this

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Supporting Information. Experimental procedure for preparation of **1** and **2**, characterization data for **1** and **2**, a more detailed discussion of stereochemical properties of **2**, experimental procedures for photo-chemical studies and enzyme inhibition studies, IC₅₀ plots, complete Ref. 10 and 14. This material is available free of charge via the Internet at <http://pubs.acs.org>.

communication, we report a novel method for caging cysteine protease inhibitors, wherein a peptidomimetic nitrile-based inhibitor is rendered inert through binding to a ruthenium center. Upon photolysis, the nitrile-based inhibitor is unleashed, providing high levels of selectivity for enzyme inhibition under light vs. dark conditions. This strategy was proven effective against purified enzymes and in lysates.

Most cysteine protease inhibitors contain electrophilic groups that react with nucleophilic thiolates of active site cysteines and anchor the inhibitor to the target enzyme, including epoxides, ketones, alkyl halides and nitriles.⁸ From this class, nitriles are attractive because they are biologically robust and not readily metabolized.⁹ A series of potent and selective peptidomimetic inhibitors were developed against cysteine cathepsins that contain C-terminal nitriles,^{10–13} including analogs targeting cathepsin K that moved into Phase II clinical trials.^{14,15} Interaction between a nitrile and the active site cysteine of cathepsin B was confirmed through X-ray crystallographic analysis to generate a thioimide,¹⁰ which forms in a reversible fashion upon inhibitor binding. We recognized that if the nitrile functional group of a protease inhibitor could be bound in a stable fashion to a metal center, it would likely be inert towards attack by active site cysteines. Thus, metal binding would cage the inhibitor, which could be released upon photolysis to interact with the target enzyme (Figure 1).

To investigate the caging of nitrile-based inhibitors, the moiety $\text{Ru}^{\text{II}}(\text{bpy})_2$ was chosen, which displays excellent caging and photoreactive properties. In support, efficient caging with $\text{Ru}^{\text{II}}(\text{bpy})_2$ was already demonstrated with bioactive amines.^{16–18} Furthermore, the complex $[\text{Ru}^{\text{II}}(\text{bpy})_2(\text{MeCN})_2]^{2+}$ was known to release two equiv of MeCN and $[\text{Ru}^{\text{II}}(\text{bpy})_2(\text{H}_2\text{O})_2]^{2+}$ upon photolysis in aqueous solution.¹⁹ Importantly, if this strategy was effective, it would have the potential added benefit of unleashing multiple biologically active agents upon photoactivation from a single precursor, including two equiv of nitrile-based inhibitor and 1 equiv of $[\text{Ru}^{\text{II}}(\text{bpy})_2(\text{H}_2\text{O})_2]^{2+}$. Possessing a dual mode of action could make this class of compounds useful for targeting cancer cells, because previous work has shown that *cis*- $[\text{Ru}(\text{L})_2(\text{H}_2\text{O})_2]^{2+}$ (L = bpy, phen) and *cis*- $\text{Ru}(\text{phen})_2\text{Cl}_2$ covalently bind to DNA.^{20–22}

Synthesis of the Ru^{II} inhibitor complex started from the known nitrile-based inhibitor **1** (Scheme 1).¹³ Reaction of $\text{Ru}^{\text{II}}(\text{bpy})_2\text{Cl}_2$ with 5 equiv of **1** and excess AgBF_4 in EtOH for 12 h resulted in a color change from dark violet to orange, consistent with displacement of the chloride groups on $\text{Ru}^{\text{II}}(\text{bpy})_2$ by nitrile **1**. After filtration, concentration and precipitation from acetone and ether, the residue was dissolved in H_2O and the aqueous layer was washed with EtOAc to remove excess **1**. Subsequent anion exchange, by treatment of the aqueous solution with excess NH_4PF_6 , resulted in formation of an orange precipitate. The compound $[\text{Ru}^{\text{II}}(\text{bpy})_2(\textbf{1})_2](\text{PF}_6)_2$ (**2**) was obtained as a microcrystalline yellow solid in analytically pure form from this material by slow crystallization from a cold acetone and dichloromethane mixture.

Complex **2** was characterized by ^1H NMR, UV-vis and IR spectroscopies, mass spectrometry, and elemental analysis. ^1H NMR spectroscopic analysis confirmed **2** was obtained as a 1:1 mixture of diastereoisomers. This was expected because **1** is chiral and

enantioenriched, prepared from L-phenylalanine (*S* configuration), and $\text{Ru}^{\text{II}}(\text{bpy})_2\text{Cl}_2$ is a racemic mixture of Λ and Δ stereoisomers. Thus a mixture of (Λ , *S*, *S*) and (Δ , *S*, *S*) are isolated (see Figure S7 for more details). Obtaining a mixture of stereoisomers does not affect enzyme inhibition, because **1** is released from **2** during photolysis and $[\text{Ru}^{\text{II}}(\text{bpy})_2(\text{H}_2\text{O})_2]^{2+}$ does not act as a potent inhibitor (*vide infra*). The ^1H NMR spectrum of **2** in acetone- d_6 shows two acetyl peaks, one for each diastereomer of **2** (Figure S3). In turn, each diastereomer possesses two nitrile-based inhibitors that appear as one resonance because they are magnetically equivalent due to C_2 symmetry. Further analysis by ^1H NMR spectroscopy verified that the methylene protons adjacent to the nitrile are shifted by about 0.6 ppm in Ru^{II} complex **2** relative to **1**, consistent with binding of the nitrile to the Ru^{II} center. The IR spectrum of **2** shows a resonance at 2280 cm^{-1} (Figure S4), shifted by 30 cm^{-1} relative to **1** ($\nu_{\text{CN}} = 2250\text{ cm}^{-1}$), again consistent with nitrile binding to the Ru^{II} center.²³ The UV-vis spectrum of **2** in DMSO (Figure 2) agrees well with data for the related complex $[\text{Ru}^{\text{II}}(\text{bpy})_2(\text{MeCN})_2](\text{PF}_6)_2$, showing λ_{max} at 281 nm ($\epsilon = 60,000\text{ M}^{-1}\text{cm}^{-1}$) and 422 nm ($\epsilon = 10,700\text{ M}^{-1}\text{cm}^{-1}$).¹⁹ The mass spectrum of **2** (ESI) shows a prominent peak at $m/z = 452.1366$, along with a suitable isotope pattern, consistent with a dication of the formula $[\text{Ru}^{\text{II}}(\text{bpy})_2(\text{1})_2]^{2+}$ (Figures S5 and S6).

Complex **2** shows excellent stability in solution in the dark and fast release of **1** upon irradiation with visible light. Rates of decomposition for **2** were determined spectrophotometrically in the dark in aqueous phosphate buffer and DMSO solutions. Plots of $\ln A$ vs t were linear and provided rate constants of $2.3 \times 10^{-7}\text{ s}^{-1}$ and $2.8 \times 10^{-7}\text{ s}^{-1}$ in buffer and DMSO, respectively (Figures S8 and S9). These values prove that **2** has a $t_{1/2} > 28$ days in solution. The changes in the electronic absorption spectrum of a 1% DMSO aqueous solution of **2** ($30\text{ }\mu\text{M}$) upon irradiation with visible light ($\lambda_{\text{irr}} > 395\text{ nm}$) were used to monitor the progress of the photochemical reaction, as shown in Figure 3. A decrease in the MLCT absorption of the reactant at 414 nm is observed with a concomitant appearance of a new peak at 444 nm within 1 min of irradiation (Figure 3 inset). This peak is attributed to the $\text{Ru} \rightarrow \text{bpy}$ MLCT absorption of the mono-aqua complex, $\text{cis-}[\text{Ru}(\text{bpy})_2(\text{1})(\text{H}_2\text{O})]^{2+}$. With continued irradiation the absorption at 444 nm decreases with an increase in the intensity of the peak at 490 nm ($\epsilon = 9,300\text{ M}^{-1}\text{cm}^{-1}$) known to correspond to $\text{cis-}[\text{Ru}(\text{bpy})_2(\text{H}_2\text{O})_2]^{2+}$. Although not measured directly, facile release of **1** from **2** was implied in enzyme studies (*vide infra*). The photochemistry of **2** is similar to that of numerous related complexes, including $\text{cis-}[\text{Ru}(\text{bpy})_2(\text{MeCN})_2]^{2+}$ and $\text{cis-}[\text{Ru}(\text{bpy})_2(5\text{-cyanouracil})_2]^{2+}$.^{19,24,25} The quantum yield for the disappearance of the reactant **2** (R) to form the mono-aqua intermediate (I), $\Phi_{\text{R} \rightarrow \text{I}}$, was measured at early reaction times to be 0.080(4), whereas that determined for the formation of the product $\text{cis-}[\text{Ru}(\text{bpy})_2(\text{H}_2\text{O})_2]^{2+}$ (P) from **2** ($\Phi_{\text{R} \rightarrow \text{P}}$) was 0.00091(7). From these values, the quantum yield of the second step of the reaction, $\Phi_{\text{I} \rightarrow \text{P}}$, can be calculated to be 0.011(1). The overall photoaquation quantum yield, $\Phi_{\text{R} \rightarrow \text{P}}$, is significantly smaller than that reported for $\text{cis-}[\text{Ru}(\text{bpy})_2(\text{MeCN})_2]\text{Cl}_2$,¹⁹ 0.21 ($\lambda_{\text{irr}} = 400\text{ nm}$), but similar to that measured for the formation of the mono-aqua species, $\Phi_{\text{R} \rightarrow \text{I}}$, upon irradiation of $\text{cis-}[\text{Ru}(\text{bpy})_2(5\text{-cyanouracil})_2]\text{Cl}_2$, 0.16(4).²⁵

Inhibitor **1** and complex **2** were evaluated for their ability to inhibit the cysteine protease papain. IC_{50} values were determined for **1** and **2** (with and without irradiation) with enzyme

(650 nM) in phosphate buffer at pH 6.5 (Figure 4). Studies with **1** agree well with literature data,¹³ proving that **1** is a potent inhibitor of papain under these conditions ($IC_{50} = 638$ nM). Ruthenium complex **2** showed more potent inhibition than **1** ($IC_{50} = 295$ nM) upon irradiation with visible light for 10 min, consistent with its release of > 1 equiv of **1**. Complex **2** in the dark was significantly less potent than **1**, with an $IC_{50} = 9.5$ μ M, indicating that inhibition by **2** was enhanced 32 fold between light vs. dark conditions. Inhibition by **2** in the dark may indicate that a small amount of **1** ($< 5\%$) is released from **2** under the reaction conditions. Alternatively, these data may indicate that **2** acts as a weak inhibitor of papain using non-bonding interactions between the peptide and/or $Ru^{II}(bpy)_2$ groups and the enzyme. Control experiments proved that $[Ru^{II}(bpy)_2(MeCN)_2](PF_6)_2$ is not a potent inhibitor ($IC_{50} > 500$ μ M) and shows the same profile under light and dark conditions, which is consistent with released **1**, rather than the ruthenium byproduct, being most responsible for the inhibition observed by **2**. Enzyme inhibition or inactivation due to 1O_2 is not likely due to the short lifetime of the excited state and the similar level of inhibition observed for $[Ru^{II}(bpy)_2(MeCN)_2](PF_6)_2$ under light and dark conditions. In conclusion, the level of enhancement observed under light and dark conditions supports the hypothesis that the nitrile-based inhibitors are efficiently caged by the Ru^{II} center in **2**, and are not susceptible to attack by the active site cysteine thiolate of papain.

Cathepsins B, K and L were examined for photoinduced inhibition with **2**. IC_{50} values for **1** and **2** (with and without irradiation) were determined in aqueous buffer at pH 5.5 for cathepsins L and K, and pH 6.0 for cathepsin B using fluorogenic substrates selective for each enzyme (Table 1). These data reveal that although inhibitor **1** is significantly less potent against cathepsins B, K and L compared to papain, in all cases a significant enhancement of inhibition was observed for **2** under light vs. dark conditions. Control experiments show some level of inhibition by $[Ru^{II}(bpy)_2(MeCN)_2](PF_6)_2$, which may explain the lower ratios for dark vs. light conditions observed with cathepsins vs. papain. Nonetheless, light-activated compound **2** was more potent than **1** under all conditions, which is again consistent with **2** releasing two equiv of inhibitor **1**.

IC_{50} values represent the average from three independent experiments. Standard deviations for these assays were typically within 40% of the IC_{50} values. Activities were plotted against $\log$ [inhibitor] and fit to a sigmoidal curve to calculate the IC_{50} values, with 100% activity equal to the activity of the control reaction in the absence of inhibitor. Activities were determined with the fluorogenic substrates Z-Arg-Arg-AMC (cathepsin B), Z-Gly-Pro-Arg-AMC (cathepsin K) and Z-Phe-Arg-AMC (cathepsin L). Substrate concentrations were 100 μ M. Reactions were conducted in the dark or after 10 min irradiation for **2** with a tungsten halogen lamp (H_2O filter, 250 W). Cathepsin B conditions: 0.4 M acetate buffer, pH 6.0, 4 mM EDTA, 8 mM DTT, [cat B] = 8 nM, Cathepsin K and L conditions: 0.4 M acetate buffer, pH 5.5, 4 mM EDTA, 8 mM DTT, [cat K or L] = 20 nM. Final solutions contain 1% DMSO. See Supporting Information for more details.

Light-triggered cathepsin inhibition with **2** was further extended to a series of human cell lysates. Specifically examined were lysates from DU145 prostate carcinoma cells derived from brain metastases,²⁶ which exhibit significant cathepsin B activity,²⁷ and lysates from primary human bone marrow stromal cells (hBMSC), an important source of cathepsin

activity that modulates progression of metastatic cancer in bone.^{27,28} Activities associated with cathepsin K and L were significantly lower than those associated with cathepsin B. Therefore the cathepsin B selective substrate Z-Arg-Arg-AMC was used in all subsequent experiments, and cathepsin B activity was determined for DU145 and hBMSC lysates with **1** and **2** under light and dark conditions (Table 2). The IC₅₀ values observed for human cathepsin B in DU145 lysates were close to those observed for hMBSCs, suggesting similar levels of cathepsin B activity. In addition, these values were in agreement with results observed for isolated enzymes (Table 1), confirming that inhibition with **2** can be activated efficiently with light in lysates.

See Table 1 footnote for experimental method. Activities were determined in DU145 and hBMSC lysates using the fluorogenic substrate Z-Arg-Arg-AMC. Reactions were conducted in the dark or after 10 min irradiation for **2** with a tungsten halogen lamp (>390 nm and H₂O filter, 250 W). See Supporting Information for more details.

In conclusion, caging and light-activated release of a nitrile-based cysteine protease inhibitor with a ruthenium complex was described. This method allows inhibitor activation with high levels of selectivity between light and dark conditions. Extension of this method to other, more potent nitrile-based inhibitors is now underway. Importantly this method provides a novel way to achieve kinetic control over protease activity that may be useful for chemical biology and anticancer applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AMC	aminomethylcoumarin
bpy	2,2'-bipyridine
phen	1,10-phenanthroline

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Figure 1.
Caging strategy for nitrile-based cysteine protease inhibitors

Figure 2.
UV-vis spectrum of **2** (black) and $[Ru^{II}(bpy)_2(MeCN)_2](PF_6)_2$ (red) in DMSO

Figure 3.

Changes to the electronic absorption spectrum of $30\text{ }\mu\text{M}$ $\text{cis-}[\text{Ru}(\text{bpy})_2(\mathbf{1})_2](\text{PF}_6)_2$ ($\mathbf{2}$) in a 1% DMSO aqueous solution upon irradiation ($\lambda_{\text{irr}} > 395\text{ nm}$) at $t_{\text{irr}} = 0, 2, 3, 4, 5, 6,$ and 7 min ; inset: $0, 1\text{ min}$.

Figure 4.

IC₅₀ curves for **1** (red) and **2** (with irradiation, blue and without, black) with the cysteine protease papain. Enzyme activity was determined with the chromogenic substrate BAPNA and is expressed as a percentage, with 100% equal to activity in the absence of inhibitor. Data points represent an average of three reactions and error bars are standard deviations. Data are representative of three independent experiments. Conditions: 0.1 M phosphate buffer, pH 6.5, 11% DMSO, [papain] = 650 nM, [BAPNA] = 1.0 mM, 10 min irradiation for **2** with a tungsten halogen lamp (>395 nm and H₂O filter, 250 W), see Supporting Information for more details.

Scheme 1.

Synthesis of the ruthenium-based, caged protease inhibitor $[\text{Ru}^{\text{II}}(\text{bpy})_2(\mathbf{1})_2](\text{PF}_6)_2$ (**2**)

Table 1

IC₅₀ values (μM) for **1** and **2** (with and without irradiation) for human cathepsins B, L and K with ratios under light vs. dark conditions

human cathepsin	1	2 (dark)	2 (light)	dark/light ratio
B	133	892	63	14
K	12	176	5.4	33
L	72	225	40	5.6

Table 2

IC₅₀ values (μM) for **1** and **2** (with and without irradiation) for human cathepsins B in lysates with ratios under light vs. dark conditions

Lysate	1	2 (dark)	2(light)	dark/light ratio
DU145	182	658	82	8.0
hBMSC	183	580	88	6.6