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ARTICLE

# Ruthenium-containing P450 inhibitors for dual enzyme inhibition and DNA damage

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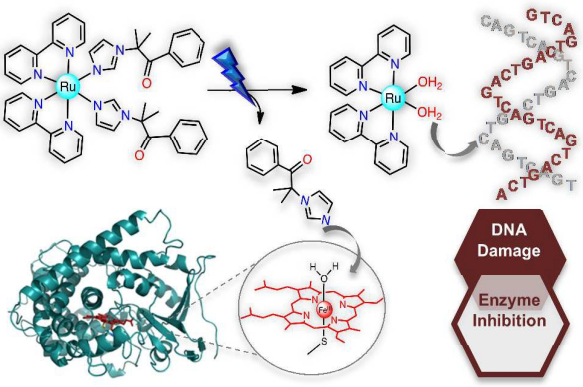
Ana Zamora,[†]b Catherine A. Denning,[†]a David K. Heidary,[a] Erin Wachter,[a] Leona A. Nease,[a] José Ruiz,[b] and Edith C. Glazer*[a]

Cytochrome P450s are key players in drug metabolism, and overexpression in tumors is associated with significant resistance to many medicinal agents. Consequently, inhibition of P450s could serve as a strategy to restore drug efficacy. However, the widespread expression of P450s throughout the human body and the critical roles they play in various biosynthetic pathways motivates the development of P450 inhibitors capable of controlled local administration. Ruthenium complexes containing P450 inhibitors as ligands were synthesized in order to develop pro-drugs that can be triggered to release the inhibitors in a spatially and temporally controlled fashion. Upon light activation the compounds release ligands that directly bind and inhibit P450 enzymes, while the ruthenium center is able to directly damage DNA.

## Introduction

Cytochrome P450s are essential enzymes that catalyze challenging organic transformations for the biosynthesis and metabolism of various key molecules, including steroids, retinoic acid, and vitamin D. In addition, hepatic P450s are responsible for the degradation of xenobiotics, and thus, play a central role in drug metabolism and deactivation. One problematic issue is that their substrates include many anticancer agents, decreasing the effective drug concentration in the body. Compounding this problem, some P450s are found specifically in tumors, where they are over expressed and play a direct role in cancer initiation, progression, and drug resistance. For example, CYP1B1 has been shown to metabolize procarcinogens to carcinogens to initiate DNA damage, and subsequently induce resistance to DNA damaging chemotherapeutics.[1-3] Alternatively, CYP19A (aromatase) converts androgens to estrogens, and is an important target in the treatment of estrogen driven cancers.[4-8] CYP17A1 is responsible for androgen synthesis, and abiraterone is a first-in-class steroidal inhibitor of this enzyme used in late-stage prostate cancer.[9-12]




**Scheme 1.** Design of dual action inhibitors.

To date, P450 inhibitors have been used as treatments for the inhibition of steroid biosynthesis, as for breast and prostate cancer, and for other indications, such as Cushing's disease.[13-15] The dangers associated with clinical use of P450 inhibitors is that they are generally not isoform selective (<$10^3$ difference in $K_d$), and long-term systemic inhibition of P450s can result in adverse drug interactions and altered hormone levels.[16, 17] An alternative approach to avoid these consequences would be to develop agents that can be activated to selectively inhibit desired P450 enzymes in a spatially and temporally regulated manner. In the context of anti-cancer agents, inhibition of P450s in cancerous tissues is also a rational strategy to sensitize the cells to DNA damaging agents,[1] while reducing the bioactivation of procarcinogens as a cancer driver. Furthermore, if the inhibition of the P450 could occur concurrently with the local administration of a cytotoxic agent only within the tumor, deactivation of the drug

[a] Department of Chemistry
University of Kentucky
505 Rose Street, Lexington, KY 40506 (USA)
[b] Departamento de Química Inorgánica and Regional Campus of International Excellence "Campus Mare Nostrum"
Universidad de Murcia, and Institute for Bio-Health Research of Murcia (IMIB-Arrixaca)
E-30071 Murcia (Spain)

† These authors contributed equally to this work.
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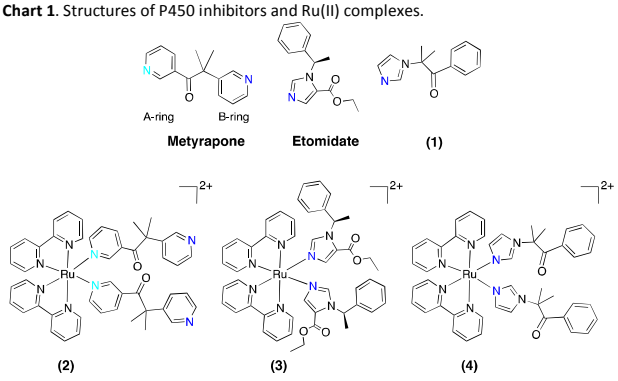
by hepatic P450s could be avoided. Accordingly, we have synthesized dual action Ru(II) complexes as pro-drugs that can be triggered with light to simultaneously release a P450 inhibitor and a DNA damaging metal center.

Photocaging is a well-established means to selectively release biologically active agents with temporal and spatial control.[18] Metal complexes have been used as photocaging groups with great success, with pioneering work by Etchenique,[19-21] Franz,[22-24] and Kodanko.[25-27] Our approach differed slightly from traditional photocaging, however, as the metal complex is intended to act as a caging group that would transform into an active biological effector in its own right, in addition to the ligand that it protected in the intact complex form.

In order to test this strategy, three P450 inhibitors were chosen that could be coordinated to Ru(II) complexes. These compounds all contain nitrogen heterocycles, and thus are able to directly ligate both the Ru(II) center and, after photorelease, the iron heme in P450s. Metyrapone and etomidate have been primarily used to inhibit P450 11B1, also known as steroid 11-beta-monooxygenase.[28] Both compounds appear to bind similarly in the binding site of CYP11B1, where the N-heterocycle is capable of ligating the catalytic heme iron while the other ring interacts with Arg110 and Phe130 via π-stacking.[29] A third, novel small molecule, compound **1**, was synthesized;[30] it has the metyrapone molecular skeleton with etomidate features: imidazole and benzene rings (Chart 1).

**Chart 1**. Structures of P450 inhibitors and Ru(II) complexes.




## Results and discussion

Ruthenium complexes **2–4** (Chart 1) were synthesized by refluxing the respective inhibitors with $Ru(bpy)_2Cl_2$ (bpy = 2,2'-bipyridine) or the corresponding bis-aqua $Ru(bpy)_2(OH_2)_2$ in $EtOH/H_2O$ (1:1) while protected from light (see the Experimental section). Abstraction of the chlorides was carried out with a silver salt in order to diminish the percentage of the undesired monocoordinate complexes, $[Ru(bpy)_2LCl]^+$, which complicated the purification. All complexes were formed in good yields (38–73% yield) and exhibited moderately intense metal-to-ligand charge transfer (MLCT) bands centered around 450–490 nm. Not surprisingly, compound **2** was isolated as a mixture of the possible coordination isomers, as either pyridyl ring in the asymmetric ligand (the A- or B-ring, as shown in Chart 1) can coordinate to the Ru(II) center.

Light-triggered release of the coordinated ligands was monitored under different solvent conditions by UV/Vis absorption spectroscopy, HPLC, and mass spectrometry. All complexes were able to cleanly release both monodentate ligands in acetonitrile (MeCN) after irradiation with blue light (470 nm). A biphasic blue-shift was observed in their spectra (Figures S6-S8), and a common final 425 nm band was found for **2–4**, which indicated the formation of a unique product, $[Ru(bpy)_2(MeCN)_2]^{2+}$, after the release of the corresponding inhibitors.

The photoproducts formed in aqueous solution were identified using HPLC and mass spectrometry analysis (Figures S9-S14). The chromatograms showed the appearance of new signals in irradiated samples, which corresponded to the free inhibitor and the mono-aqua Ru(II) complex. This was confirmed by the mass spectrum of the solution, with peaks corresponding to $[Ru(bpy)_2L(H_2O)]^{2+}$, $[Ru(bpy)_2L]^2$ and $[L+H]^+$ (Figures S10, S12, S14). It is to be noted that the UV/Vis profile of the products for **3** and **4** did not differ significantly from that of the complex protected from light. The extent of the photoejection reaction varied from 40 to 65%. This observation is consistent with other $[Ru(bpy)_2L_2]^{2+}$ and $[Ru(phen)_2L_2]^{2+}$ complexes, where substitution of the second N-monodentate ligand, L, requires much longer irradiation times or does not occur.[19,25, 31-34] Finally, in the dark, all complexes were stable at room temperature and at 37 °C when measured over 48 hrs (Figure S5).

In order to directly investigate the interactions of the metal complexes with a P450, cytochrome $P450_{BM3}$ (CYP102A1), a soluble bacterial P450, was chosen as a model system. A mutant form of $P450_{BM3}$ has been shown to recapitulate the activity of mammalian drug metabolizing P450s,[35] which has made $P450_{BM3}$ a commonly used experimental system.[36]

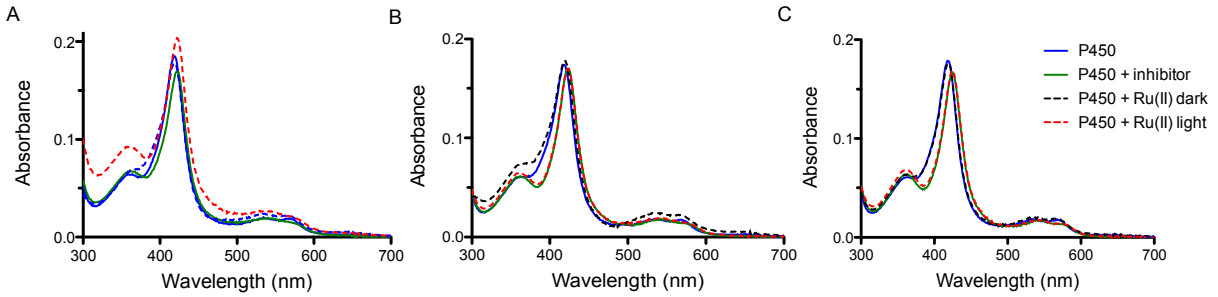



**Figure 1.** Absorption spectra of $P450_{BM3}$ inhibitor saturated and Ru(II) dark and light systems: **2** (A), **3** (B), **4** (C). The ratio used was P450: Complex (1:10) and P450: Ligand (1:4) for each of the respective ligands used to generate the complexes.

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Importantly, while mammalian P450s are generally membrane associated, which complicates analysis, $P450_{BM3}$ is soluble and thus amenable to various spectroscopic investigations as well as enzyme turnover assays. Furthermore, the *in vitro* system was chosen due to the intrinsic complications of studying P450 inhibition in cells or cell lysates, due to the need to create cell lines that overexpress the enzyme and reductase partners, and to provide exogenous reductants such as NADPH.

As all three free P450 inhibitors directly ligate the heme iron, either through a pyridyl or imidazole ring, a type II spectra shift was observed upon inhibitor binding. A 7 nm red-shift in the heme soret was observed for metyrapone, etomidate, and compound **1**, with the appearance of a second minor peak around 360 nm, where a shoulder is observed in the free enzyme (Figure 1). Difference spectra for all compounds are shown in Figure S17, with trough maxima around 410 nm. Significant shoulders are observed on the troughs at 390 nm, consistent with a mixture of type IIa and type IIb spectra[37-39] (type IIa spectra are observed when the enzyme is in the high-spin state in the absence of the ligand; type IIb is seen when the enzyme is in the low-spin state).[37] The $\Delta A_{max}$ between the peak and trough and the intensity of the shoulder varied as a function of the nitrogen containing coordinating ligand, as previously reported.[38]

Upon exposure to an Indigo LED array (28 J/cm$^2$), each complex induced the same mixed type IIa and IIb spectral shift observed with the free inhibitors. Conversely, when the Ru(II) complexes **3** and **4** were kept in the dark there as little or no observed change the $P450_{BM3}$ absorption spectra, indicating that the complexes function as pro-drugs and do not directly affect the active site of the enzyme. Complex **2**, containing the pendant metyrapone, did exhibit a slight change in absorption profile, suggesting the intact complex containing the pendant pyridyl ligand was capable of interacting with the enzyme enough to perturb the absorption spectra. While unexpected, previous structural studies have shown that small molecules that are tethered to large fluorophores[40-42] or even metal complexes[43-47] can bind P450s and induce opening of the substrate channel. Interestingly, the difference spectrum (Figure S17A) indicates a different binding mode than either free metyrapone or the light-activated **2**, and appears to be more a type I spectrum, consistent with displacement with water from the heme, but with no direct ligation of a coordinating nitrogen.[37]

Enzyme inhibition was tested using resorufin ethyl ether as a substrate in a fluorescence based assay.[48, 49] Each of the free ligands

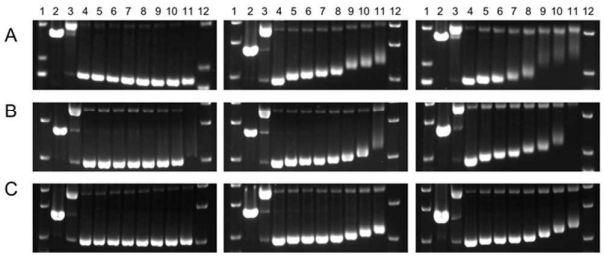


**Figure 3.** Agarose gels showing the dose response of **2** (A), **3** (B), and **4** (C) with 40 μg/mL pUC19 plasmid with and without irradiation (λ>470 nm). Dark, left; 1 hr irradiation, middle; 3 hr irradiation, right. Lane 1 and 12: DNA ladder; Lane 2: EcoRI; Lane 3: $Cu(OP)_2$; Lanes 4–11: 0, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 μM (500 μM corresponds to a metal center:base ratio of 4:1). EcoRI and $Cu(OP)_2$ are used as controls for linear and relaxed circular DNA. EtBr was used to visualize the DNA.

exhibited inhibition of enzyme activity (Figure 2 and Figure S18). As anticipated, the three Ru(II) complexes all demonstrated triggerable enzyme inhibition. Compound **4** gave the largest window for dark vs. light activity (Figure 2), with $IC_{50}$ values for enzyme inhibition of 6.8 and 0.05 μM, respectively. This compound thus provides a 136-fold difference between activity in the dark and the light. The $IC_{50}$ value for the free ligand **1** was 0.06 μM, in excellent agreement with the activity of the complex in the light. Each light activated system displayed very similar activity to the free inhibitor (Figure 2B; compound **2** $IC_{50}$ = 0.19 μM vs. 0.75 μM for metyrapone; compound **3** $IC_{50}$ = 0.04 μM vs. 0.02 μM for etomidate).

To extend the investigation to a medically validated, commonly used experimental system, pooled human liver microsomes (HLMs) were used to study the light-triggered inhibition of enzyme turnover. HLMs contain membrane-bound human CYPs which play major roles in first pass metabolism of xenobiotics.[50, 51] Incubation of HLMs with compound allows study of how these compounds impact the activity of multiple CYPs at once, and in a membrane environment. This provides a good model for human CYPs. Compound **4** exhibited the largest window for light-mediated enzyme inhibition for $P450_{BM3}$, so it was tested along with the free ligand **1**. In the dark, at 100 μM, compound **4** was found to minimally inhibit CYPs in HLMs, but complete inhibition was observed after irradiation (Figure 2C). A similar trend was seen with free ligand **1**, with nearly complete inhibition of enzyme turnover.

Having confirmed the utility of the light active compounds for inhibition of cytochrome P450 activity, DNA damage was investigated using agarose gel electrophoresis. Dose responses were performed with compound **2**–**4** with pUC19 plasmid, and the solutions were either protected from light or irradiated for 1 or 3 hrs (60.4 or 181.4 J/cm$^2$) before incubation at 37 °C for 12 hrs (Figure 3). No effect was seen for any of the intact pro-drug forms of the complexes, but all induced DNA damage upon light activation. DNA damage was visualized by reduced mobility of DNA,

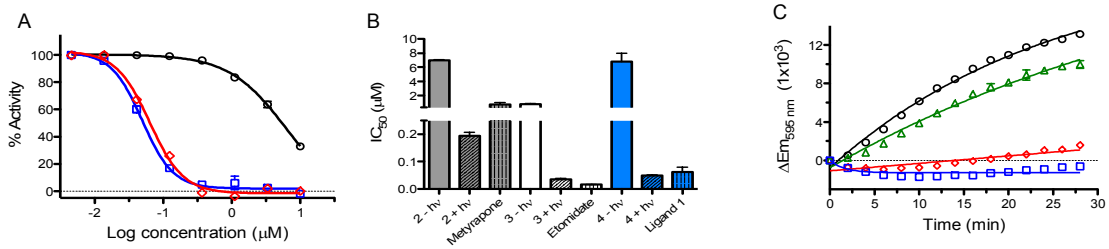


**Figure 2**. (A) Relative activity of $P450_{BM3}$ in the presence of **1** (red diamonds) and **4** in the dark (black circles) and following irradiation (blue squares). (B) $IC_{50}$ values for all three complexes and the respective free ligands. (C) Inhibition of CYPs in HLMs by free ligand **1** (red circles) and complex **4** (blue squares) after irradiation compared to no compound (black circles) and **4** in the dark (green triangles).

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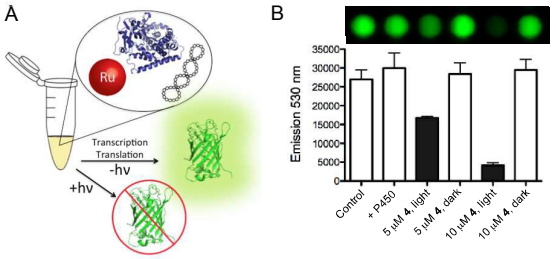


**Figure 4**. (A) An *in vitro* transcription and translation experiment allows for detection of DNA damage that results in inhibition of GFP production. Compound **4** was incubated in the presence of both plasmid and $P450_{BM3}$ at equal concentrations. (B) GFP production was unaffected by the addition of $P450_{BM3}$ (+ P450; lane 2) and the protein had no impact on the ability of compound **4** to damage DNA upon light activation (lanes 3–6).

consistent with direct adduct formation, as demonstrated in classical experiments on cisplatin[52-54] and more recently with analogous light-activated ruthenium compounds.[55, 56] Very little relaxed circular DNA was observed, but what was formed likely resulted from single strand breaks induced by singlet oxygen ($^1O_2$). Ligand ejection proceeds from an excited state that is populated from the $^3$MLCT state, which also generates $^1O_2$; thus, there is a competition in relaxation pathways, and complexes that have longer $t_{1/2}$ values have been found to induce more single strand breaks. We[57, 58] and others[59] have thus observed that the combination of the different relaxation pathways makes these agents capable of "dual photoreactivity" independent of the biological activity of the released ligands. However, little $^1O_2$ was detected, as shown in Figure S19, consistent with the DNA damage study and relaxation primarily through ligand ejection. Finally, all of the free ligands were tested as controls, and as anticipated, they had no impact on the plasmid DNA (Figure S20).

The ultimate experiment was to investigate if the light activated metal complexes were able to inhibit DNA function in the presence of the P450 enzyme. Accordingly, an *in vitro* transcription and translation experiment [60] was performed, as shown in Figure 4A. A plasmid coding for green fluorescent protein (GFP) was incubated with compound **4** in the dark or following activation with light. The experiment was also performed in the presence of $P450_{BM3}$. No impact on GFP production was observed for **4** in the dark, while a dose dependent inhibition of protein production was found following activation with light, as shown in Figure 4B. The addition of $P450_{BM3}$ had no impact on GFP transcription or translation in the absence of the Ru(II) complex, or in its presence. Thus, the $P450_{BM3}$ enzyme does not serve as a "sink" for the activated metal center, and does not interfere with the DNA damage mechanism. This validates that the Ru(II) center can target DNA while the released ligands target the P450 enzyme.

## Conclusions

The three complexes described in this report serve as proof-of-concept systems for single agent "drug cocktails" and demonstrate that, upon light activation, the metal center is able to damage DNA while the liberated ligand acts as a cytochrome P450 inhibitor. The best system, compound **4**, exhibited a 136-fold difference in protein inhibition when irritated with light as compared to in the dark, with an $IC_{50}$ of 0.05 μM upon activation. In the absence of irradiation, the complexes did not damage DNA or interfere with its function, as indicated by gel electrophoresis and activity in a transcription and translation assay. Upon light activation, protein production was inhibited with an $IC_{50}$ between 5 and 10 μM in the light. Furthermore, the metal center damages the DNA even in the presence of protein, indicating that DNA is the preferred target.

The choice to inhibit P450 enzymes is a key feature in the design of these pro-drugs. The targeting of enzymes implicated in drug resistance could result in synergistic activity for DNA damaging agents in cancer cells and tissues, though more involved studies in cancer cell lines engineered and optimized to detect P450 activity and inhibition will be required for full validation of this potential therapeutic approach. However, the clear inhibition of P450 activity and the DNA damage and suppression of transcription and translation *in vitro*, combined with the well-established cytotoxicity of light activated, ligand deficient Ru(II) complexes in cells are strongly promising. This is also, to the best of our knowledge, the first report of photocaged P450 inhibitors. These compounds may be useful for basic research applications as tools that provide spatial and temporal control over P450 inhibition, and could answer several open questions in the role that P450s play in malignant cell transformation and drug resistance. Single mode of action photocaged systems, which do not damage DNA, are also under development to allow for the triggered control of P450 activity without complications from the activity of the metal center.

## Experimental Section

### Materials and instrumentation

Chemicals used for synthesis were purchased from VWR or Fisher Scientific and used without further purification. *cis*-Dichlorobis(2,2'-bipyridine)ruthenium(II) dihydrate was purchased from Strem chemicals. Human liver microsomes were purchased from Sekisui Xenotech. The Singlet Oxygen Sensor Green was from Molecular Probes.

A Varian Mercury spectrometer was used to obtain $^1$H NMR (400 MHz) and $^{13}$C (100 MHz) spectra. Chemical shifts are reported relative to the solvent peak ($CD_3CN$ – δ 1.94 or $CDCl_3$ – δ 7.24 for $^1$H NMR; $CD_3CN$ – δ 1.39 for $^{13}$C NMR). Electrospray ionization (ESI) mass spectra were obtained using a Varian 1200L mass spectrometer at the University of Kentucky Environmental Research Training Laboratory (ERTL). Absorption spectra for the extinction coefficient determination, acetonitrile photoejection and binding constant ($K_d$) determination for ligands were obtained on a Cary 60 UV/Vis spectrophotometer. Full spectrum absorbance readings to study the photoejection for complexes **1**–**3** in different aqueous media were obtained using a BMG Labtech FLUOstar Omerga

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microplate reader. Binding saturation studies for each compound as well as $K_d$ determination for irradiated complexes **1–3** were completed using an Agilent 8453 UV/Vis spectrometer. Compound purity was determine with an Agilent 1100 Series HPLC using a previously reported method.[61] Light activation for photoejection experiments was achieved using a 470 nm LED array (16.7 mW/cm$^2$) from Elixa. An Indigo LED Flood Array (466 mW/cm$^2$) from Loctite was used for light activation for the enzyme assays. A Tecan SPECTRAFluorPlus Plate Reader was used to determine change in fluorescence for the enzyme activity assay and IVTT assay. Agarose gels were digitally imaged using a BioRad ChemiDoc System.

**Compound synthesis, characterization, and ion exchange**

2-(1-Imidazolyl)-2-methyl-1-phenyl-2−1-propanone) (**1**) was synthesized following a previously published procedure.[52] All metal complexes were synthesized under low ambient light and were protected using aluminum foil throughout each step of synthesis, isolation, and characterization. Silver salts were used to facilitate ligand exchange; the choice of the specific salts in the different reactions was due only to reagent availability.

$[Ru(bpy)_2(Met)_2](PF_6)_2$ (**2**):

$[Ru(bpy)_2Cl_2]\cdot 2\ H_2O$ (125 mg, 0.240 mmol) was dissolved in water (7 mL) under $N_2$ at 80 °C. To this 2-methyl-1,2-di-3-pyridil-1-propanone (136 mg, 0.6 mmol) was added, and the red solution was stirred overnight at 80 °C. The resulting solution was cooled to room temperature (RT) and extracted into $CH_2Cl_2$ (3x10 mL) to remove the excess free ligand. The complex was precipitated out of the aqueous phase with 1–2 mL of a saturated aqueous $KPF_6$ solution and extracted with $CH_2Cl_2$/MeCN (3x10 mL). The crude complex was purified by column chromatography using $H_2O$:MeCN:$KNO_3$ as eluent (from 0:100:0 to 12:87.2:0.8). The product was obtained in 38% yield (104 mg) as an orange solid. ESI MS $C_{48}H_{44}N_8O_2Ru$: m/z calcd $[M]^{2+}$ 433.13, found 433.2 $[M]^{2+}$. Purity by HPLC: 99.3 % by area; UV/Vis in $CH_3CN$, $\lambda_{max}$ ($\varepsilon$ M$^{-1}$ cm$^{-1}$) = 290 (43200), 345 (12100), 445 (8400). Note: NMR was not completed due to the compound containing a mixture of isomers.

$[Ru(bpy)_2(Eto)_2](PF_6)_2$ (**3**):

Silver triflate (99 mg, 0.384 mmol) was added to a suspension of $[Ru(bpy)_2Cl_2]\cdot 2\ H_2O$ (100 mg, 0.192 mmol) in water (15 mL) and the mixture was stirred overnight at RT. The solution was filtered under $N_2$. Etomidate (94 mg, 0.384 mmol) and 15 mL of EtOH were added to the solution, which was then stirred at 85 °C under $N_2$ for 24 hr. After cooling the reaction, the solution was concentrated, 1-2 mL of a saturated aqueous $KPF_6$ solution was added, and the precipitate was extracted into $CH_2Cl_2$ (3x15 mL). The crude was purified by column chromatography using $H_2O$:MeCN:$KNO_3$ as eluent (from 0:100:0 to 20:80:0.4). The product was obtained in 47% yield (108 mg) as a crystalline red solid. $^1$H NMR ($CD_3CN$, 400 MHz): δ 9.03 (d, *J* = 5.6 Hz, 1H), 8.97 (d, *J* = 5.2 Hz, 1H), 8.35 (d, *J* = 8.4 Hz, 2H), 8.27 (d, *J* = 8.0 Hz, 1H), 8.24 (d, *J* = 8.4 Hz, 1H), 8.12 (q, *J* = 8.4 Hz, 2H), 8.00 (d, *J* = 5.2 Hz, 1H), 7.87 (m, 3H), 7.70 (m, 3H), 7.49 (s, 1H), 7.37-7.19 (m, 10H), 6.88 (d, *J* = 6.4 Hz, 2H), 6.72 (d, *J* = 7.2 Hz, 2H), 6.29 (q, *J* = 7.0 Hz, 1H), 6.19 (q, *J* = 7.2 Hz, 1H), 4.15 (m, 4H), 1.75 (d, *J* = 7.2 Hz, 3H), 1.71 (d, *J* = 7.2 Hz, 3H), 1.19 (m, 6H); $^{13}$C NMR ($CD_3CN$, 100 MHz): δ 159.57, 159.48, 159.01, 158.96, 158.47, 158.37, 154.10, 154.00, 153.96, 153.83, 142.93, 142.82, 141.88, 141.52, 138.42, 138.35, 138.16, 138.10, 129.90, 129.23, 129.09, 128.46, 128.10, 128.03, 126.95, 126.63, 126.10, 126.05, 124.70, 124.48, 62.41, 57.86, 57.62, 22.35, 22.12, 14.46, 14.42 ppm; ESI MS $C_{48}H_{48}N_8O_4Ru$: m/z calcd $[M]^+$ $PF_6^-$ 1047.25, $[M]^{2+}$ 451.14, found 1047.1 $[M]^+$ $PF_6^-$, 451.1 $[M]^{2+}$. Purity by HPLC: 98.3 % by area; UV/Vis in $CH_3CN$, $\lambda_{max}$ ($\varepsilon$ M$^{-1}$ cm$^{-1}$) = 235 (50900), 290 (53900), 325 (8900), 475 (8200).

$[Ru(bpy)_2(\mathbf{1})_2](PF_6)_2$ (**4**):

Silver nitrate (65.2 mg, 0.384 mmol) was added to a suspension of $[Ru(bpy)_2Cl_2]\cdot 2\ H_2O$ (100 mg, 0.192 mmol) in water (15 mL), and the mixture was stirred overnight at RT. The solution was filtered under $N_2$. 2-(1-Imidazolyl)-2-methyl-1-phenyl-2−1-propanone; compound **1**) (102.8 mg, 0.480 mmol) and 15 mL of EtOH were added to the solution, which was stirred at 85 °C under $N_2$ for 24 hr. After cooling the reaction, the solution was concentrated, 1–2 mL of a saturated aqueous $KPF_6$ solution was added and the precipitate was extracted into $CH_2Cl_2$ (3x15 mL). The crude was purified by column chromatography using $H_2O$:MeCN:$KNO_3$ as eluent (from 0:100:0 to 10:90:0.2). The product was obtained in 73% yield (158 mg) as a crystalline red solid. $^1$H NMR ($CD_3CN$, 400 MHz): δ 8.66 (d, *J* = 5.2, 2H), 8.22 (d, *J* = 8.0 Hz, 2H), 8.08 (d, *J* = 8.0 Hz, 2H), 7.99 (t, *J* = 8.0 Hz, 2H), 7.75 (m, 4H), 7.53 (m, 4H), 7.29 (s, 2H), 7.25 (s, 2H), 7.17 (m, 6H), 7.06 (d, *J* = 7.6 Hz, 4H), 6.53 (s, 2H), 1.78 (s, 12H); $^{13}$C NMR ($CD_3CN$, 100 MHz): δ 158.67, 158.16, 153.58, 153.25, 139.57, 137.97, 137.54, 135.15, 134.23, 130.68, 129.61, 128.80, 128.12, 127.76, 124.54, 124.26, 120.71, 67.75, 30.99, 27.54, 27.26 ppm; ESI MS $C_{46}H_{44}N_8O_2Ru$: m/z calcd $[M]^+$ $PF_6^-$ 987.23, $[M]^{2+}$ 421.13, found 987.4 $[M]^+$ $PF_6^-$, 421.1 $[M]^{2+}$. Purity by HPLC: 97.5 % by area; UV/Vis in $CH_3CN$, $\lambda_{max}$ ($\varepsilon$ M$^{-1}$ cm$^{-1}$) = 245 (41200), 290 (50400), 335 (8200), 485 (8300).

Compounds **2–4** were converted to $Cl^-$ salts by dissolving 5–20 mg of product in 1–2 mL methanol. The dissolved product was loaded onto an Amberlite IRA-410 chloride ion exchange column, eluted with methanol, and the solvent was removed *in vacuo*.

The purity of each Ru(II) complex was analyzed using the method in Table S1 (mobile phases of 0.1% formic acid in d$H_2O$ and 0.1% formic acid in HPLC grade $CH_3CN$). Samples of each Ru(II) complex were prepared in d$H_2O$ and protected from light before injection on the HPLC.

**Photoejection studies**

MeCN photoejection studies:

Photoejection studies were performed on the $PF_6^-$ salts of **2–4** (30 μM) in 3 mL of acetonitrile in a 1 cm pathlength quartz cuvette placed 12 inches below a 470 nm LED array in duplicate. Each sample was prepared from the dissolution of the pure solid in acetonitrile and diluting it to the above final concentration. The samples were protected from ambient light until irradiated with the LED array. Ligand ejection was monitored by taking absorption spectra after specific time points until the spectra ceased to evolve. The half-life ($t_{1/2}$) of photoejection was determined by plotting the difference in absorbance between two points around the isosbestic point versus time using Graphpad Prism software.

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Aqueous photoejection studies:

Photoejection studies using the $Cl^-$ salts of **2–4** in aqueous media (water, 1X PBS and Opti-MEM with 1% FBS) were performed in triplicate using a Greiner UV clear half-area 96-well plate. The kinetics for ligand ejection were determined for **2–4** (40 μM) with a final volume of 200 μL. The well plate was positioned 12 inches below a 470 nm LED array, and full spectra were collected after set time points of light exposure for a total of 5 hrs. The change in absorbance was plotted using the same method as described for acetonitrile.

Photoejection reactions were followed by HPLC using 40 μL injections of 200 μM solution of each Ru(II) complex prior to irradiation and after 1 min irradiation with 470 nm light. The free ligands were used as controls. Complexes **2** and **4** were run using the method described in Table S1, and complex **3** was run using the method described in Table S2. Samples of each Ru(II) complex were prepared in $dH_2O$ and protected from light before injection when necessary. Ligands were fully dissolved in DMSO and diluted in $dH_2O$, so that the final solution contained 1% DMSO. Products were characterized by the retention time ($t_R$) as well as the UV/Vis profile corresponding to the HPLC peak. The extension of the reaction was calculated by integration of the area corresponding to the free and photoejected ligand. Finally, the irradiated sample was also analyzed by ESI MS.

**Expression and purification of $P450_{BM3}$**

The pCWori vector containing the gene for the heme domain (Thr 1–Thr 463) of $P450_{BM3}$ with five mutations incorporated (R47L, F81I, F87V, L188Q, E267V, "PM BM3") was transformed into BL21(DE3) cells. After transformation, cells were grown overnight on 50 μg/ml carbenicillin plates at 37 °C.

Small 5 mL growths in Luria Broth (LB) with 100 μg/mL ampicillin were grown overnight and then added to 1 L of Terrific Broth (TB) with 100 μg/mL ampicillin and 0.4% glycerol. Cells were grown at 180 rpm and 37 °C until an $OD_{600}$ of 0.6–0.8 was reached. Protein production was induced by addition of 0.5 mM isopropyl β-D-1 thiogalactopyranoside (IPTG). Temperature and shaking were decreased to 30 °C and 150 rpm, respectively. After 16-20 hrs, the cells were harvested by centrifugation at 4,000 rpm for 15 min at 4 °C. The supernatant was decanted and the cell pellet was resuspended in lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, 0.1 mM EDTA, and 0.1 μM phenylmethylsulfonyl fluoride (PMSF), pH 8.0). The resuspended pellet was sonicated on ice using a Branson Sonifer 250 microtip, for 15 min with output control of 3 and duty cycle of 50%. The lysate was then centrifuged for 1 hr at 17,000 x g and 4 °C.

The supernatant was decanted and syringe filtered with a 0.45 μm polytetraflurorethylene filter prior to addition to a His-Trap column (GE Healthcare) equilibrated in buffer A (50 mM $NaH_2PO_4$, 300 mM NaCl, and 20 mM imidazole, pH 8.0). PM BM3 was eluted using a linear gradient of 20 mM to 200 mM imidazole with buffer B (50 mM $NaH_2PO_4$, 300 mM NaCl, and 200 mM imidazole, pH 8.0). Fractions were collected based on color and absorbance at 420 nm and 280 nm. PM BM3 containing fractions were then concentrated using Ultracel-30K Millipore centrifugal units at 4500 x g and 4 °C. Protein was further purified by loading onto a Hi-Prep 26/60 Sephacryl S200 HR (GE Healthcare) sizing column equilibrated with gel filtration buffer (20 mM Tris, 150 mM NaCl, pH 8.0).

All fractions with a 420/280 nm ratio above 1.2 were concentrated using Ultracel-30K Millipore centrifugal units. For storage, glycerol was added to give a final concentration of 50%, the protein was aliquoted, snap frozen, and stored at –80 °C.

Prior to using PM BM3, glycerol was removed and the buffer was exchanged to assay buffer (20 mM Tris, 20 mM NaCl, 10 mM $CaCl_2$, pH 7.4) using a PD-10 desalting column (GE Healthcare). To determine protein concentration, a CO binding assay was used as previously described.[62]

**$P450_{BM3}$ binding affinity**

To determine if the inhibitors could saturate PM BM3, the protein was added to a 3 mL 1 cm pathlength quartz cuvette at a final concentration of 2.5 μM. UV/Vis spectra were taken before and after the addition of compound. Ligands were tested at 10 μM, whereas Ru(II) complexes were tested at 25 μM in the dark and after 1 min irradiation. After the addition of compound the samples were allowed to incubate at RT for 30 sec before data collection.

Absorbance binding titrations of ligands and light activated complexes **2–4** were performed in a 1 cm pathlength quartz cuvette with 2.5 μM protein and a total volume of 3 mL. The absorbance was measured after each ligand or Ru(II) addition from 0–64.0 μM (metyrapone), 0–30.3 μM (etomidate and **1**), 0–64.7 μM (**2**), 0–54.2 μM (**3**) and 0–45.8 μM (**4**). The Ru(II) only absorbance was measured and blanked in parallel. Binding constants were determined by plotting the change in absorbance at 425 nm vs. concentration of ligand or Ru(II). Data was plotted using Graphpad Prism software and fit using a one site-total binding equation.

**$P450_{BM3}$ inhibition assay**

Inhibition assay with purified PM BM3:

An enzymatic turnover assay was utilized to determine the inhibition of resorufin ethyl ether metabolism by PM BM3 in the presence of added compound. Each compound was added to 250 nM PM BM3 in 1X PBS (phosphate buffered saline, pH 7.5) at varying concentrations between 0–10 μM in Greiner clear 96 well plates and incubated for 10 min. Ru(II) complexes were tested in the presence and absence of light, where stock solutions were irradiated for 1 min prior to incubation with PM BM3. Following incubation with compound, 5 μM resorufin ethyl ether was added and incubated at RT for 5 min. To initiate enzymatic turnover, 5 mM hydrogen peroxide was added, and changes in fluorescence of resorufin ethyl ether were monitored over 5 min using a Tecan spectrafluor plus microplate reader at excitation 535 nm and emission 595 nm.

Inhibition assay with Human liver microsomes (HLMs):

An enzymatic turnover assay was utilized to determine the inhibition of resorufin ethyl ether metabolism by enzymes responsible for first-pass metabolism in pooled human liver microsomes (HLMs) in the presence of added compound. 125 μM of **1** or **4** was added to 20 mg/mL HLM to a final concentration of 100 μM in 100 mM $KH_2PO_4$, 10 mM $MgCl_2$ buffer, pH 7.5. After

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incubation with compound for 10 min, 5 μM resorufin ethyl ether was added followed by 1.3 mM NADPH to initiate enzymatic turnover. Changes in fluorescence of resorufin ethyl ether were monitored over 30 min using a Tecan spectrafluor plus microplate reader at the same settings as above. Compound **4** was incubated with HLMs in the dark and after 1 min irradiation with the Indigio LED.

#### *In vitro* transcription and translation

A 1-Step Human Coupled IVT Kit–DNA (Thermo Scientific) was used to carry out the experiment.[60] For each reaction, 0.5 μg of the pCFE-GFP plasmid and 5–20 μM **4** was used. Complex **4** was either irradiated for 1 min with the Loctite Indigo LED or kept in the dark. Prior to carrying out the IVT reaction, **4** was incubated with the plasmid overnight in the presence or absence of PM BM3 (0.5 μg). All IVT reactions were scaled to 12.5 μL total volume. Following the completion of the IVT reaction, the GFP emission was read in a Greiner-Bio One 384-well small volume plate on a Tecan SPECTRAFluorPlus Plate Reader with 485 nm excitation and 535 nm emission filters.

#### DNA gel electrophoresis

Compounds were mixed with 40 μg/mL pUC19 plasmid in 10 mM potassium phosphate buffer, pH 7.4. To determine the effect of light, samples were irradiated with a 470 nm LED for 1 hr. Samples were then incubated for 12 hr at 37 °C in the dark. Single and double-strand DNA break controls were prepared, and the DNA samples were resolved on agarose gels, as described previously.[55]

In brief, samples were resolved on a 1% agarose gels prepared in tris-acetate buffer with 0.3 μg of plasmid/lane. The gels were stained with 0.5 μg/mL ethidium bromide in tris-acetate buffer at RT for 40 min, destained with tris-acetate buffer, and imaged on a ChemiDoc MP System (Bio-Rad).

#### Singlet oxygen assay

Compounds were serially diluted in 96 well plates in Extracellular Solution (10 mM HEPES pH 7.5, 145 mM NaCl, 10 mM Glucose, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 3.3 mM $KH_2PO_4$, 0.8 mM $K_2HPO_4$, 50 U/ml Penicillin and 50 mg/ml Streptomycin). To this was added Singlet Oxygen Sensor Green (SOSG) reagent to give a final concentration of 5 μM. The plates were read on a SpectraFluor Plus plate reader with an excitation filter of 485 nm and emission of 535 nm both pre- and post-irradiation with the Loctite Indigo LED for 1 min. The relative values of the SOSG emission were plotted as a function of compound concentration to give a dose response.

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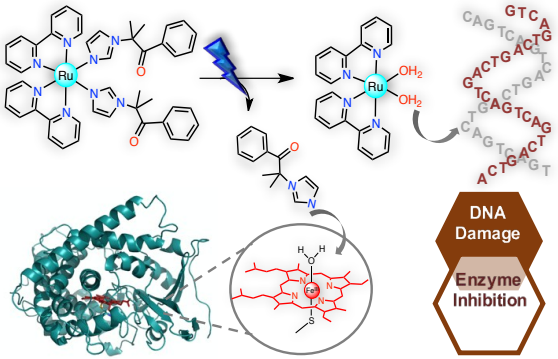


A light-activated prodrug delivers both a DNA damaging metal center and a cytochrome P450 inhibitor to prevent drug resistance.