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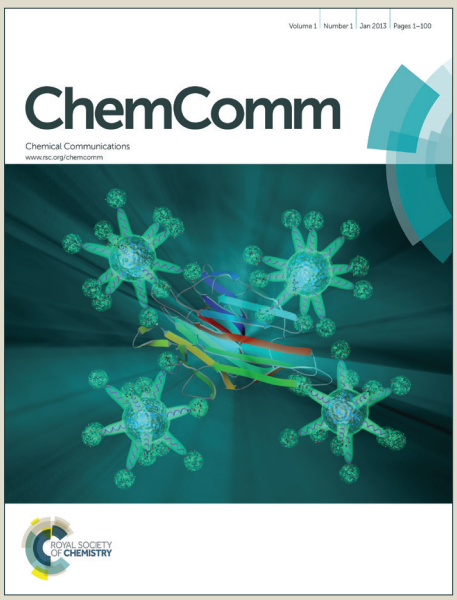





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# A luminescent ruthenium(II) complex for light-triggered drug release and live cell imaging

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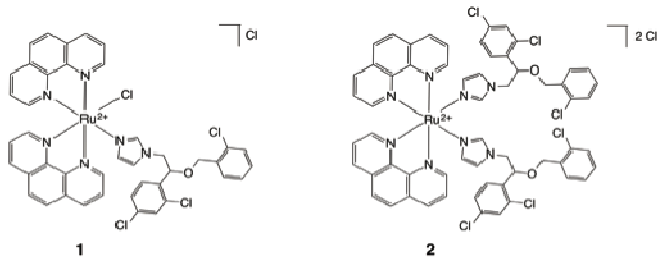
**We report a novel ruthenium(II) complex for selective release of the imidazole-based drug econazole. While the complex is highly stable and luminescent in the dark, irradiation with green light induces release of one of the econazole ligands, which is accompanied by a turn-off luminescence response and up to a 34-fold increase in cytotoxicity towards tumour cells.**

Over 50% of all anticancer drug candidates that enter clinical trials fail due to problems such as poor pharmacokinetics, limited accumulation in tumour cells, and low selectivity, with a significant proportion of therapeutics currently in clinical use also suffering problems of this nature.[1, 2] A viable strategy to overcome these limitations is to reversibly modify the physiochemical properties of a drug through coordination to a metal complex.[3, 4] This is a synthetically simple approach that can improve both the pharmacokinetic and pharmacodynamic properties of the parent drug. Furthermore, complexes can be designed where the metal-drug bond is selectively cleaved in the tumour environment. A number of rationally designed metal prodrug complexes have been reported in recent years that are activated by an intrinsic feature of tumour cells, including bioreductive drug chaperones,[5-7] enzyme-activated cobalamin conjugates,[8] and metallo-cages that can exploit the EPR effect for the transport of small hydrophobic molecules.[9, 10] An alternative approach is the use of localised light to activate a prodrug through photodynamic therapy (PDT), allowing both spatial and temporal control over the release of the active drug.[11] This approach has been explored in detail for the delivery of the gas molecules, CO[12-15] and NO,[16-18] and is receiving increasing attention as a means of selectively releasing small molecules.

Ruthenium(II) polypyridyl complexes are a promising platform for light-activated drug delivery with photolabile bonds that can be activated by visible[19-22] and even infrared light,[23] the optimum region for photodynamic therapy. Pioneering work by Etchenique et al. demonstrated the use of the complex $[Ru(bpy)_2(4\text{-aminopyridine})_2]^{2+}$ (bpy = 2,2'-bipyridine) for the delivery of the neurochemical 4-aminopyridine[24] and later other biologically active amines.[25] More recently, Turro and Kodanko have used Rubpy, Ruterpy (terpy = 2,2′:6′,2″-terpyridine) and Rutpa (tpa = tris(2-pyridlymethyl)amine) complexes to cage and release two nitrile-based potential anticancer agents, 5-cyanouracil and a cathepsin K inhibitor.[26-28] Similarly, Bonnet and coworkers used Ruterpy complexes to cage and release the bioactive thioethers N-acetylmethionine and biotin.[29] Ruthenium(II) complexes have also been investigated for the reduction of inert cobalt(III) complexes through photoelectron transfer.[30, 31]

In addition to applications in drug delivery, a number of luminescent ruthenium(II) polypyridyl complexes have been evaluated as cellular probes and organelle stains.[32] Here we investigate the possibility of combining these properties to develop a luminescent and photolabile ruthenium complex for dual applications in cell imaging and light-activated drug delivery. Towards this aim, we began our investigations using Ru(II) polypyridyl complexes with imidazole-based ligands, as several recent papers have highlighted the application of ruthenium(II) polypyridyl imidazole complexes, both as luminescent and cytotoxic agents,[33] and for the photouncaging of histamine and histidine.[34, 35]

As a prototype complex, we began with the imidazole-based




1 2

Figure 1: Chemical structures of complexes **1** and **2**.

antifungal agent econazole. Econazole is currently being investigated for oral and intravenous applications in the treatment of cancer,[36, 37] mycobacterium tuberculosis,[38] and leishmania.[39] Despite showing promising activity *in vitro* against each of these diseases, oral and intravenous administration have proven ineffective due to the poor pharmacokinetics of the drug, which has no gastrointestinal absorption, and rapidly undergoes metabolism and protein binding in the blood stream.[36, 40] As such, a prodrug system capable of increasing the effective dose of econazole could be beneficial to the treatment of a number of conditions.

Ruthenium complexes with 1 or 2 econazole ligands were prepared in a single step in moderate yield from reaction of econazole nitrate with $[Ru(phen)_2Cl_2]$ (phen = 1,10-phenanthroline). Complexes **1** and **2** were characterised by $^1$H NMR, UV-visible absorption and fluorescence spectroscopy, mass spectrometry and elemental analysis. The electroionisation spray mass spectrum of **1** shows a single peak at 878.53, corresponding to the monocation $[Ru(phen)_2(Ec)Cl]^+$, while that of **2** shows a single peak at 611.93, corresponding to the dication $[Ru(phen)_2Ec_2]^{2+}$ (Fig. S3, ESI†). The $^1$H NMR spectra of both complexes show significant shifting up field of the imidazole peaks, consistent with binding at the immine imidazole nitrogen (Fig. S2, ESI†).[33] The spectra of **1** and **2** both have two sets of signals indicative of diastereomers at a ratio of approximately 1:1 and 4:5 respectively, resulting from the combination of a racemic mixture of $[Ru(phen)_2Cl_2]$ with a racemic mixture of econazole nitrate.

The UV-visible absorption spectra of **1** and **2** in water show a broad and intense band at 454 nm ($\varepsilon$ = 8981 mol$^{-1}$ L cm$^{-1}$) and 486 nm ($\varepsilon$ = 9570 mol$^{-1}$ L cm$^{-1}$) respectively, which can tentatively be assigned to the metal-ligand charge transfer (MLCT) transition (Fig. 2a, Table S1) The emission spectra of **2** has an intense emission peak, attributed to a $^3$MLCT excited state, at 636 nm (Fig. 2b) with a luminescence quantum yield ($\phi_{Lum.}$) in aerated water of 0.067. **1** displays no emission under the same conditions. Both the absorbance and emission spectra of **2** closely resemble those of $[Ru(phen)_2(imidazole)_2](PF_6)_2$.[33]

The stability of **1** and **2** in water was evaluated by monitoring the UV-visible absorbance and ESI mass spectra of the complexes over 24 h in the dark. The absorbance spectrum of **1** shows a significant blue shift of the MLCT band from 454 nm to 422 nm after 24 h (Fig. S4a, ESI†) and the mass spectrum of **1** showed that the original complex had been completely aquated to $[Ru(phen)_2ec(H_2O)]^{2+}$. In contrast, **2** demonstrated excellent stability in water with only a 2% decrease in its absorbance maximum after 24 h and no additional peaks in the mass spectrum (Fig. S4b, ESI†). This is encouraging for the development of a prodrug of econazole as the free drug is rapidly metabolized to inactive products *in vivo*.[36, 40]

The photolytic stability of the complexes was monitored by UV-visible absorbance and emission spectroscopy, and mass spectrometry. An aqueous solution of **2** was irradiated with green light (520 nm) for 1 h (53 J cm$^{-2}$) and changes in its spectra monitored over time. Continued irradiation resulted in a blue shift of the MLCT band from 486 to 444 nm with a clear isosbestic point at 398 nm (Fig. 3a), suggesting that **2** is converted to a single product rather than a combination of the mono and bis aqua complexes. This was confirmed by the mass spectrum of the end point solution, which shows peaks corresponding to $[Ru(phen)_2ec(H_2O)\text{-}H]^+$ and $[econazole+H]^+$ (Fig. S3c, ESI†). This observation is consistent with other $[Ru(phen)_2L_2]^{2+}$ and $[Ru(bpy)_2L_2]^{2+}$complexes, where substitution of the second ligand from $[Ru(X)_2L(H_2O)]^{2+}$ requires much longer irradiation times or does not occur.[24, 26, 41, 42] Accordingly, no evidence of econazole release was observed when a solution of **1** was irradiated under the same conditions.

The quantum yield of photolysis of **2** to the monoaqua product is 0.005. The low efficiency of photosubstitution for complex **2** in comparison to other $[Ru(phen)_2L_2]$ and $[Ru(bpy)_2L_2]$ complexes is consistent with its comparatively high luminescence quantum yield, as both pathways occur from the $^3$MLCT state. While quantum yields of photoaquation in the range 0.01-0.4 have been reported for other $[Ru(phen)_2L_2]^{2+}$ and $[Ru(bpy)_2L_2]^{2+}$ complexes, these are non or weakly emissive at room temperature.[24, 26, 41, 42] Hence **2** offers a compromise between the two pathways, with moderate quantum yields of both luminescence and photosubstitution. A steady decrease in the emission intensity of **2** was also observed in response to increasing light irradiation (Fig. 3b). This is consistent with the formation of the non-luminescent complex $[Ru(phen)_2ec(H_2O)]^{2+}$. This turn-off luminescence response provides a convenient means of observing the photouncaging of econazole from **2**.

The cellular uptake of the complexes was evaluated by confocal fluorescence microscopy and inductively coupled plasma mass spectrometry (ICP-MS). Intracellular ruthenium concentrations in DLD-1 colon carcinoma cells were determined for **2**, and for the analogous imidazole complexes $Ru(phen)_2(imidazole)Cl]Cl$ (**3**) and $[Ru(phen)_2(imidazole)_2]Cl_2$ (**4**), using ICP-MS, and are reported in Fig. S7. **2** was found to accumulate in significantly higher concentrations than the imidazole analogue (**4**), (176.6 vs. 38.1 ng / mg cellular protein) after incubation for 4 h, likely due to the lipophilicity afforded

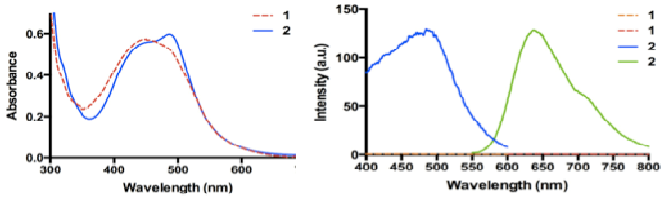



Figure 2: UV-visible absorbance (left) and emission spectra (right) of **1** and **2** in water.

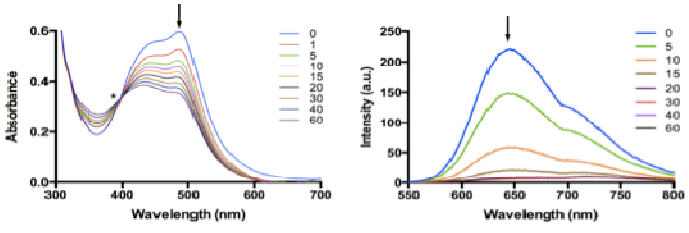



Figure 3: Changes in the UV-visible absorbance (left) and emission (right) spectra of an aqueous solution of **2** irradiated with light for 0-60 minutes.

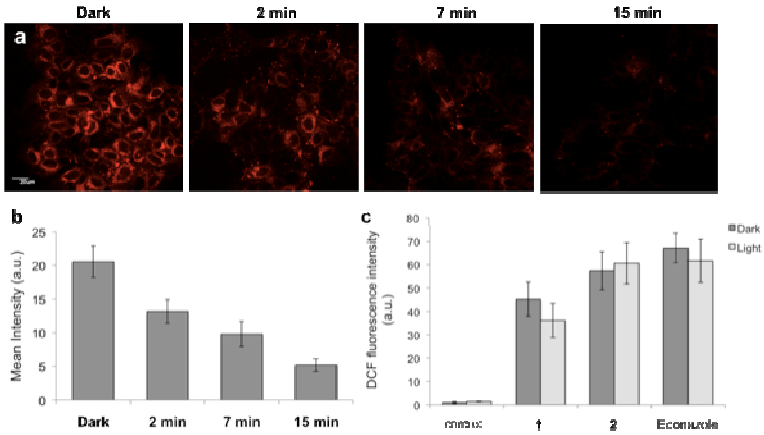



Figure 4: a) Confocal fluorescence images of DLD-1 cells treated with **2** and irradiated with light for different time periods (scale bar = 20 um). b) Mean luminescence intensities of cells samples after light treatment. c) Mean fluorescence intensities of MCF-7 cells treated with DCFDA.

by the econazole ligands. The intracellular concentration of cells dosed with **1** could not be accurately determined due to a small degree of precipitation of **1** in the cellular media.

Confocal fluorescence microscopy of accumulates in DLD-1 cells treated with **2** showed red luminescence in the cytoplasm of both live and fixed cells treated after 4 h, with no evidence of nuclear accumulation (Fig. S5, ESI†). As ligand exchange would result in a non-luminescent complex, this observation suggests that **2** is stable under physiological conditions in the dark and is accumulating in cells with both econazole ligands coordinated. This is encouraging for the use of **2** as a light-activated prodrug, being able to transport econazole into cells at high concentrations in an inert form.

To establish whether **2** could release econazole when irradiated with light, the emission intensity of cells treated with **2** was monitored in response to increasing doses of light. DLD-1 cells were treated with **2** for 4 h then exposed to various doses of green light and imaged directly (Fig 4.) A clear decrease in emission intensity was observed with increasing irradiation times, indicating the photoactivation of **2** to the non-luminescent products $[Ru(phen)_2ec(H_2O)]^{2+}$ and econazole.

The cytotoxicity and photocytotoxicity of complexes **1** – **4**, $[Ru(phen)_2ec(H_2O)]^{2+}$, and econazole nitrate was evaluated against a small panel of tumour cell lines: MCF-7 breast carcinoma, LNCaP and PC-3 prostate carcinoma, and DLD-1 colon carcinoma, as econazole nitrate has been reported to induce apoptosis and delay tumour growth in xenografts of these cell lines.[36, 37, 43] Cells were dosed with the compounds for 24 h then irradiated with light for 15 minutes (final dose = 13.3 J $cm^{-2}$, $\lambda_{irr}$ =520 nm) or protected from light over the same time period. Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. $IC_{50}$ values and phototoxicity indexes (PI) are given in Tables 1 and S2, and dose response curves in Fig. S6, ESI†. Complex **2** showed moderate to low cytotoxicity in the dark against each of the cell lines. Notably, $IC_{50}$ values were comparable to or lower than those of econazole nitrate. Irradiation of cells dosed with **2** resulted in a significant decrease in cell viability in each of the 4 cell lines. This effect was most pronounced in the prostate cancer lines, PC-3 and LNCaP, with phototoxicity indexes of 19 and 34 respectively. These PIs are comparable or superior to many clinically used photosensitisers,[44] though it should be noted that ruthenium complexes with PIs of up to 1800 have been reported.[45]

In contrast, light treatment had very little effect on the viability of cells treated with econazole nitrate, which exhibited moderate cytotoxicity in each of the cell lines tested. The effect of irradiation was also markedly less in cells treated with complex **1**, where a significant effect was only observed in the prostate cancer lines, PC-3 and LNCaP. **1** was notably more cytotoxic in the dark than both econazole nitrate and **2** in all cell lines tested. This may be due to the difference in stability of the two complexes: **1** is aquated in aqueous media to give a labile aqua complex, which would be capable of binding to cellular targets. It is likely that the low $IC_{50}$ values of **2** in combination with light, (in the nanomolar range for MCF-7 and LNCaP cells), are due to the combined release of econazole and the labile ruthenium complex $[Ru(phen)_2ec(H_2O)]^{2+}$. The aqua complex itself was less active than both **1** and **2** (Table S1), possibly due to lower cellular uptake as it is less lipophillic. The imidazole complexes **3** and **4** exhibited no cytotoxicity up to a concentration of 100 μM in either PC-3 or DLD-1 cells, suggesting that the econazole ligands are essential to the activity of **1** and **2** (Fig. S6). The formation of reactive oxygen species (ROS) was also investigated as both econazole and related ruthenium(II) complexes are known to induce apoptosis through ROS production in the mitochondria.[46, 47, 21, 48] **1**, **2**, and econazole nitrate exhibited significantly higher ROS levels than the untreated control cells, however no clear correlation between cell viability and ROS levels was observed (Fig. 4c).

In conclusion, **2** represents an inert and relatively nontoxic prodrug of econazole that can be efficiently activated using green light. **2** offers a number of key advantages over the parent drug, including improved aqueous solubility and stability, good intracellular accumulation, and photoselective toxicity. The complex exhibits low to moderate toxicity in the dark, but $IC_{50}$ values in the nanomolar range when combined with green light. Furthermore, the luminescence of **2** allows it to be visualised in live cells and drug release to be observed in real time by the turn-off luminescence response. To the best of our knowledge this is the first example of a ruthenium(II) complex with the dual properties of luminescence and photoselective drug release. Work is underway to expand this approach for the delivery of a range of imidazole-based drugs.

**Table 1: $IC_{50}$ values (μM) and photoselectivity indexes (PI) in tumour cells. PI = dark $IC_{50}$ value / light $IC_{50}$ value**

| Compound | MCF-7 | | | LNCaP | | | PC-3 | | | DLD-1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dark | Light | PI | Dark | Light | PI | Dark | Light | PI | Dark | Light | PI |
| **1** | 1.9±0.4 | 1.4±0.5 | **1.4** | 4.2±0.9 | 0.4 ±0.1 | **10** | 13.6±0.4 | 1.8±0.4 | **7.5** | 8.1±1.7 | 3.2±0.9 | **2.5** |
| **2** | 10.4±0.8 | 0.9±0.3 | **12** | 9.23±1.4 | 0.3±0.1 | **34** | 49.2±4.7 | 2.6±0.4 | **19** | 28.6 ±5.1 | 2.85±1.2 | **10** |
| Econazole nitrate | 12.5±3.1 | 15.3±4.5 | **0.8** | 8.6±2.4 | 10.4±3.1 | **0.8** | 18.4±2.3 | 15.1±3.7 | **1.2** | 42.5±6.2 | 43.4±1.5 | **1.0** |

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## Notes and references

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