The mitochondrial calcium uniporter (MCU) is a highly selective ion channel that is responsible for shuttling Ca\(^{2+}\) into mitochondria. Dysregulation of the MCU can trigger an overload of mitochondrial Ca\(^{2+}\), leading to deleterious conditions including neurodegeneration, ischemia-reperfusion injury, and cancer. Therefore, molecules that can inhibit the MCU are valuable for understanding the role of mCa\(^{2+}\) dynamics in humans. Among small-molecule MCU inhibitors, Ru coordination complexes are the most prominent, as evidenced by the widespread use of the established compounds ruthenium red and cancer. Thus, modification of the axial ligands of Ru265 can afford promising MCU inhibitor prodrugs with tunable properties.

Although Ru265 and its analogues possess useful biological properties, they are not amenable to non-invasive imaging due to their lack of appropriate spectroscopic or nuclear properties. To fill this gap, we sought to impart fluorescent properties upon Ru265 by functionalizing the axial ligands. In particular, the introduction of well-established coumarin-based fluorophores to the axial position of this complex would be a promising approach, because coumarin-containing metal complexes have been extensively investigated for their photophysical properties, cellular imaging applications, and antiproliferative effects. Specifically, 7-diethylaminocoumarin-3-carboxylate (OCou\(^-\)) was chosen as the axial ligand because its intrinsic mitochondrial-targeting properties have been extensively investigated for their photophysical properties, cellular imaging applications, and antiproliferative effects. The current mechanistic understanding is that Ru265 first aquates to the diaqua-capped [Ru(μ-N)Rh(NH\(_3\))\(_3\)]\(^{2+}\) (Ru265'), to be a potent MCU inhibitor that protects against in vitro hypoxia-reoxygenation and an in vivo model of ischemic stroke. The current mechanistic understanding is that Ru265 first aquates to the diaqua-capped [Ru(μ-N)Rh(NH\(_3\))\(_3\)]\(^{2+}\) (Ru265') with a half-life of several minutes under physiological conditions (Scheme 1), and Ru265' inhibits mCa\(^{2+}\) by binding to a highly conserved and exposed peptide sequence, known as the DIME region, of the MCU pore. The (NH\(_3\))\(_3\)Ru(μ-N)Ru(NH\(_3\))\(_3\), core is critical for the MCU-inhibitory activity of Ru265, but the axial chloride ligands can be altered to optimize the biological properties of this compound. For example, we recently reported that analogues of Ru265 bearing axial carboxylates instead of chlorides undergo aquation on the timescale of hours under physiological conditions, releasing the potent MCU inhibitor Ru265' [25]. Thus, modification of the axial ligands of Ru265 can afford promising MCU inhibitor prodrugs with tunable properties.

**Scheme 1.** Activation pathways of Ru265 and RuOCou.
Ru265 derivatives. In short, Ru265 was allowed to react with 5 equiv of AgOTf in water to remove both the outer- and inner-sphere chlorides as insoluble AgCl and produce the diaqua-capped synthon Ru265'. Treatment of Ru265' with OCou affords RuOCou, which is fully characterized using NMR and IR spectroscopy (Figures S2–S5) as well as X-ray crystallography (Figure 1 and Tables S1 and S2). The crystal structure of the complex reveals the expected geometry, comprising an almost linear Ru(μ-N)Ru core (172.9(1)°) with eight equatorial NH ligands and two axial OCou' ligands. Overall, the Ru–N and Ru–O interatomic distances agree well with previously reported carboxylate-capped Ru265 derivatives.

Next, the aquation kinetics of RuOCou in pH 7.4 MOPS buffer at 37 °C were determined by UV/Vis spectroscopy (Figure S6). The aquation half-life of RuOCou is 2.1 h (Table 1), which is within the range reported for other carboxylate derivatives of Ru265 (3.3–9.9 h). We also monitored this process under the same conditions by fluorescence spectroscopy. Over time, an increase in fluorescence intensity is observed (Figure 2). The rate of the emission increase ($t_{1/2} = 2.2$ h) is almost identical to the spectroscopic changes measured by UV/Vis spectroscopy, indicating that the enhancement of fluorescence is a consequence of aquation. In this case, a likely explanation for this emission increase is that the fluorescence of the coumarin ligand is quenched when coordinated to the Ru centers. Leveraging the sensitivity of fluorescence spectroscopy, we also measured the aquation kinetics of RuOCou in HeLa cell lysates, revealing a similar rate of activation ($t_{1/2} = 3.5$ h) (Figure S7). In consideration of the possibility of endocytosis of this complex, we also evaluated the aquation kinetics under the more acidic conditions found in the lysosomes. Notably, the aquation half-life at pH 5 ($t_{1/2} = 2.6$ h) as measured by UV/Vis spectroscopy (Figure S8) is not substantially different than that at pH 7.4, indicating that dissociation of the complex is not accelerated under acidic conditions.

To further understand the mechanism of fluorescence quenching of OCou upon coordination to Ru, we performed density functional theory (DFT) and time-dependent density functional theory (TD-DFT) calculations. The frontier molecular orbitals (FMOs) of the DFT-optimized structures of RuOCou and the free ligand OCou are shown in Figure 3. The highest occupied molecular orbital (HOMO) of OCou and HOMO-1 (H-1) and HOMO of RuOCou are coumarin-based π orbitals, as are the lowest unoccupied molecular orbital (LUMO) of OCou and LUMO + 2 (L-2) and L-3 of RuOCou. In comparing relative energies, these coumarin-based orbitals are lower in RuOCou, indicating that they are stabilized upon coordina-

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**Table 1:** Relevant biological properties of Ru265 and RuOCou.

<table>
<thead>
<tr>
<th>Property</th>
<th>Ru265</th>
<th>RuOCou</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquation half-life[a]</td>
<td>2.3 min</td>
<td>2.1 ± 0.05 h</td>
</tr>
<tr>
<td>mCa$^{2+}$ inhibition IC$_{50}$ [nM][b]</td>
<td>2.2 ± 0.6</td>
<td>16.1 ± 3.6</td>
</tr>
<tr>
<td>Cytotoxicity IC$_{50}$ [μM][c]</td>
<td>195 ± 8</td>
<td>55.5 ± 11.9</td>
</tr>
<tr>
<td>Cellular uptake [pg Ru/μg protein]</td>
<td>33 ± 7</td>
<td>395 ± 75</td>
</tr>
</tbody>
</table>

[a] pH 7.4 at 37°C. [b] Ref. [22]. [c] In permeabilized HEK293T cells, measured immediately after addition of the compound. [d] In intact HEK293T cells after a 48 h incubation period. [e] Ref. [20].

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**Figure 1.** Crystal structure of RuOCou. Thermal ellipsoids are shown at the 50% probability level. Solvents and counterions are omitted for clarity.

**Figure 2.** Left: evolution of the emission spectrum of RuOCou (1 μM) in pH 7.4 MOPS-buffered (100 μM) aqueous solution over a 48 h period at 37 °C (excitation wavelength: 402 nm). Right: plot of integrated fluorescence intensity versus time with the best exponential fit.

**Figure 3.** Frontier Kohn–Sham molecular orbital diagrams of the coumarin-carboxylate ligand (OCou') and RuOCou with an isovalue of 0.02. Purple arrows: photoexcitation from ground state to the coumarin-based excited state. Blue arrow: electronic transition involved in fluorescence from the coumarin-based excited state. Red wavy arrows: electronic transitions involved in photoinduced electron transfer leading to non-radiative relaxation to the ground state.
tion to Ru. Within RuOCou, the LUMO and L+1 are delocalized Ru–N–Ru π* orbitals, which reside energetically between the coumarin-based π orbitals. Upon photoexcitation of RuOCou to the emissive coumarin π–π* excited state, the LUMO and L+1 provide an energetically viable pathway for photoinduced electron transfer (PET) that outcompetes the radiative decay process.\[27,37\] TD-DFT calculations, which determine the energies of electronic states rather than orbitals, also support this hypothesis (Figure S9). In the case of OCou–, the lowest energy singlet excited state is coumarin-based with a high oscillator strength. For RuOCou, however, there are 12 excited states, all with small oscillator strengths, that are lower in energy than this coumarin-based π–π* excited state. Consistent with the simple orbital energy arguments above, these low-energy excited states, most of which are metal-based, are likely responsible for quenching the photoluminescent coumarin-based excited state. The careful positioning of metal-based orbitals or excited states to quench fluorophores via PET is a common strategy for designing fluorescent chemosensors based on dyes like coumarins.\[26,27\]

With a deeper understanding of its photophysical properties, we next evaluated the biological properties of RuOCou. Freshly prepared solutions of RuOCou inhibit mCa\(^{2+}\) uptake in permeabilized HEK293T cells with nanomolar potency (Table 1 and Figures S11–S12), but at levels that are still 8-fold lower than those of Ru265. Allowing solutions of RuOCou to age and undergo aequorin, however, leads to an increase in MCU-inhibitory activity, which reaches the same level as Ru265 after 20 h (Figure 4). Thus, like other carboxylate-capped analogues of Ru265, RuOCou can potentially be used as an aequorin-activated prodruk.\[25\]

Encouraged by these results in permeabilized cells, we proceeded to study RuOCou in intact cells. Cellular uptake studies reveal that RuOCou accumulates at 10-fold higher concentrations in HEK293T cells compared to Ru265 (Table 1). This improved uptake may be a consequence of the greater lipophilicity of the axial coumarin ligands present within RuOCou. Moreover, like Ru265, RuOCou can inhibit mCa\(^{2+}\) uptake in intact HeLa cells without compromising the mitochondrial membrane potential, as demonstrated by Rhod2AM (Figure S13) and JC-1 (Figure S14) assays, respectively. Lastly, RuOCou exhibits only minimal cytotoxicity with an IC\(_{50}\) of 56 μM in HEK293T cells after 48 h incubation (Table 1). The complex is moderately more toxic in HeLa cells with an IC\(_{50}\) of 33 μM under the same condition (Figure S10).

Leveraging the photophysical properties of RuOCou, we examined its intracellular accumulation by using confocal fluorescence microscopy. Prior to imaging, HeLa cells were incubated for 24 h with either 40 μM OCou– or 20 μM RuOCou. The average fluorescent intensity within the RuOCou-treated cells was found to be 2-fold higher than the OCou–-treated cells (Figure S15). This result suggests that coordination of OCou– to Ru enhances its cellular uptake. Moreover, these data also imply that the rate of cellular uptake of RuOCou is faster than its aquation. If RuOCou were to be active before being internalized, the improved uptake of the coumarin ligands would not be observed. To assess the intracellular localization of RuOCou, HeLa cells were co-treated with RuOCou and different organelle stains prior to imaging. The complex does not appreciably accumulate in the nucleus (Figure S16). In contrast, as shown in Figure 5, the signals within the blue (RuOCou) and red (MitoTracker Red) channels showed a strong colocalization, as reflected by a Pearson’s correlation coefficient of 0.75 ± 0.04, indicating that RuOCou or the OCou– delivered by the complex accumulates in the mitochondria. This result is consistent with the previously reported abilities of both the coumarin ligand\[32–35\] and Ru265\[29\] to enter this organelle. It is worth noting that an appreciable amount of lysosomal accumulation was also observed for RuOCou, as reflected by a Pearson’s correlation coefficient of 0.68 ± 0.06 between the complex and LysoTracker Red DND-99 (Figure S17).

Having confirmed that RuOCou can be imaged by fluorescence microscopy, we finally sought to demonstrate its aquation-triggered fluorescence turn-on in intact living cells. HeLa cells were treated with RuOCou for 30 min, thoroughly washed, and subsequently imaged for another 40 min. We employed a high concentration of RuOCou (75 μM) in this experiment to maximize the fluorescent

Figure 4. Left: representative traces of extramitochondrial Ca\(^{2+}\) clearance after addition of 10 μM Ca\(^{2+}\) in permeabilized HEK293T cells treated with either 4 nM Ru265 or RuOCou prepared from stocks that had been pre-incubated at 37 °C (pH 7.4) for different durations. Right: mCa\(^{2+}\) uptake rate based on the kinetics of extramitochondrial Ca\(^{2+}\) clearance. The mCa\(^{2+}\) uptake rate of the untreated cells was normalized to 100.

Figure 5. Confocal fluorescent microscopy images of HeLa cells treated with 20 μM RuOCou a) for 24 h and then MitoTracker Red b) for 30 min. c) is the overlaid image. Scale bar = 50 μm.
signals. Even though this concentration is higher than the IC₅₀ value, no cytotoxic effects were observed over this short incubation period. As shown in Figure 6, a modest yet statistically significant enhancement (2-fold) in fluorescent intensity was observed during this time-period. The small magnitude of the turn-on response, compared to the studies in buffer and cell lysates, is likely due to the 30 min preincubation period required for this experiment, which is long enough for partial aqutation of RuOCou to occur before imaging. Nevertheless, these results still represent the proof of concept for tracking fluorogenic MCU inhibitors in living cells and monitoring their activation processes in real time.

In summary, RuOCou is a novel fluorogenic MCU inhibitor. Its aquation-activated increase in MCU-inhibitory activity can be visualized directly by a concomitant enhancement in fluorescent intensity. This study serves as the first proof of principle for the real-time visualization of MCU-inhibition in living cells, which is an important step towards developing a deeper understanding of the mitochondrial calcium dynamics. Ongoing efforts are directed at developing new analogues with longer aquation half-lives, which would allow easier visualization of the turn-on effect and better control of the biological properties of this compound class. We are also investigating the use of near-infrared-emitting fluorophores that would provide better biological compatibility and a deeper penetration range. Finally, we are seeking alternative stimuli for triggering the fluorogenic responses, which would enable a greater spatio-temporal control of the activation.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: Bioinorganic Chemistry · Fluorescent Probes · Mitochondrial Calcium Uniporter · Prodrugs · Ruthenium

Deposition Number 2212060 contains the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service.


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