ChemMedChem

Concept
doi.org/10.1002/cmdc.202300106

Chemistry Europe
European Chemical Societies Publishing

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Structure-Activity Relationships of Metal-Based Inhibitors of the Mitochondrial Calcium Uniporter

Zhouyang Huang[a] and Justin J. Wilson*[a]

The mitochondrial calcium uniporter (MCU) is a transmembrane protein that is responsible for mediating mitochondrial calcium (mCa^{2+}) uptake. Given this critical function, the MCU has been implicated as an important target for addressing various human diseases. As such, there has a been growing interest in developing small molecules that can inhibit this protein. To date, metal coordination complexes, particularly multinuclear ruthenium complexes, are the most widely investigated MCU inhibitors due to both their potent inhibitory activities as well as their longstanding use for this application. Recent efforts have expanded the metal-based toolkit for MCU inhibition. This concept paper summarizes the development of new metal-based inhibitors of the MCU and their structure-activity relationships in the context of improving their potential for therapeutic use in managing human diseases related to mCa^{2+} dysregulation.

Introduction

Intracellular calcium ions (Ca^{2+}) play a key role in signal transduction within a wide range of biological events.[1,2] At any given instance, the level of intracellular Ca^{2+} is tightly controlled by a combination of metal-ion buffering biomolecules, importers, exporters, and exchangers. Alterations in Ca^{2+} homeostasis contribute to various pathological conditions, reflecting the importance of its precise regulation.[3] The increase of intracellular Ca^{2+} levels is a consequence of both influx from extracellular milieu and Ca^{2+} release from endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR). One key organelle that maintains Ca^{2+} homeostasis is the mitochondria, which act as a Ca^{2+} sink when excessive concentrations of this ion are present within the cytosol.[4,5] Mitochondrial calcium (mCa^{2+}) uptake is primarily mediated by a transmembrane protein known as the mitochondrial calcium uniporter (MCU).[6–8]

The MCU subunit comprises two transmembrane helices, which assemble as a tetramer to form a Ca^{2+}-selective pore (Figure 1).[9–12] These two transmembrane helices are connected by a highly conserved and solvent-exposed DXXE motif,[13] where D and E are aspartate and glutamate residues, respectively, and X refers to other amino acid residues that vary within different organisms. This motif functions as a Ca^{2+}-selective filter by directly interacting with this ion and cooperating with various regulatory proteins,[10,14–16] including MICU1,[17] MICU2,[18] and EMRE.[19] At low cytosolic Ca^{2+} concentrations, MICU1 and MICU2 block the entrance of the uniporter and subsequently inhibit the uptake of Ca^{2+}. At elevated Ca^{2+} concentrations, however, the Ca^{2+}-binding EF hand domains of MICU1 and MICU2 trigger a conformational change that leads to their dissociation from the pore entry, enabling Ca^{2+} uptake.[20]

Although MCU-mediated mCa^{2+} uptake is critical for bioenergetics,[4,5] excessive Ca^{2+} influx through this transporter causes mCa^{2+} overload, initiating a pathway that opens the mitochondrial permeability transition pore (mPTP) and triggers irreversible cell damage and death.[21] This phenomenon of mCa^{2+} overload plays a key role in a number of different pathological conditions,[22–24] including ischemia-reperfusion injury,[25,26] cancer,[27–29] and neurodegenerative disorders.[30–33] Therefore, preventing mCa^{2+} overload via the chemical inhibition of the MCU represents a promising therapeutic strategy.[34] Although many organic compounds have been screened for this purpose,[35–37] metal coordination complexes, particularly those of ruthenium, remain the most prominent and well-studied inhibitors of the MCU.[38] In this concept paper, we will summarize the progress in the development of metal-based MCU inhibitors and their structure-activity relationships (SARs), with an emphasis on dinuclear ruthenium and osmium inhibitors. Readers are referred elsewhere for a more general overview of MCU inhibitors including both organic and inorganic drug candidates.[38]

Ruthenium-Based Inhibitors

The most well-known MCU inhibitor is the oxo-bridged trinuclear ruthenium complex ruthenium red (RuRed, Figure 2).[39,40] This compound was initially used as a cytological stain,[41] but was later found to inhibit MCU-mediated mCa^{2+} uptake.[42–45] Commercial sources of RuRed, however, contain a large amount of impurities (>20%).[46] The impurities comprise several different ruthenium ammine complexes, causing off-target effects such as the inhibition of other ion channels.[47] The discovery that the purification of RuRed actually leads to poorer MCU-inhibitory activities suggested that this property actually arises from a different complex present within commercial samples of RuRed.[46] Accordingly, it was later

[a] Z. Huang, Prof. Dr. J. J. Wilson
Department of Chemistry and Chemical Biology
Cornell University, Ithaca, NY 14853 (USA)
E-mail: jjw275@cornell.edu

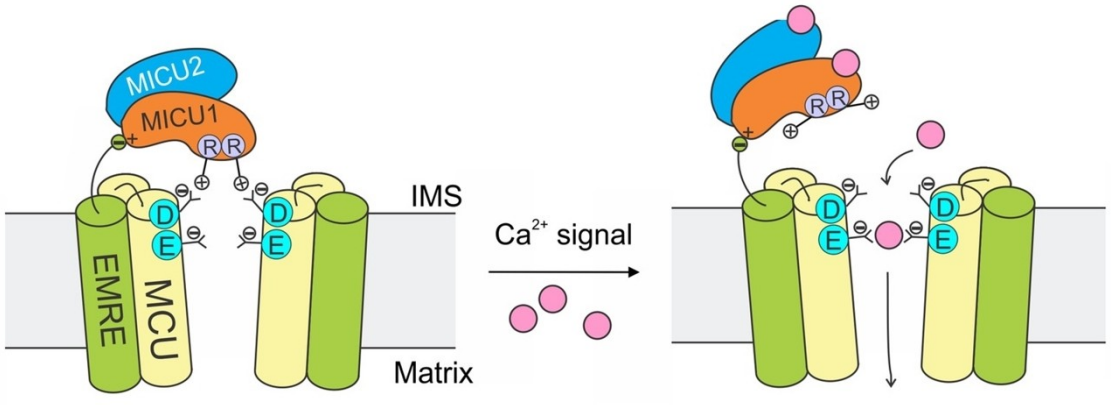

Figure 1. Representative topology diagram of the MCU complex. Only two subunits of the tetrameric MCU protein are shown for clarity. Pink balls represent Ca^{2+}. With low Ca^{2+} in the intermembrane space (IMS), MICU1 and MICU2 block $m$$Ca^{2+}$ uptake. At elevated Ca^{2+} concentration, MICU1 and MICU2 bind this ion and undergo a conformational change that allows the passage of Ca^{2+} into the mitochondrial matrix through the uniporter. The highly conserved DXXE motif directly interacts with Ca^{2+} and serves as a cation-selective filter. Adapted from ref [16]. Copyright (Phillips et al.). The reference is distributed under a Creative Commons Attribution License, which permits the unrestricted use and redistribution provided the original author and source are credited.

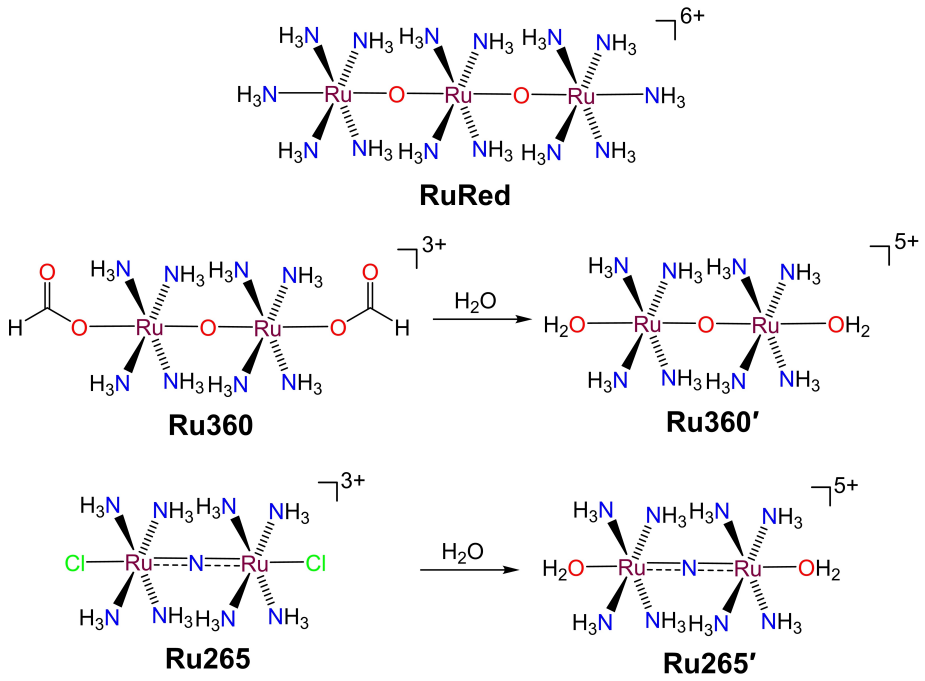

Figure 2. Structures of ruthenium-based MCU inhibitors RuRed, Ru360, and Ru265, as well as their aquated products Ru360′ and Ru265′, the presumed active species responsible for their inhibitory activities.

discovered that one of the impurities, Ru360 (Figure 2), is responsible for the MCU-inhibitory activity of RuRed.[48,49] The name Ru360 comes from its intense absorption at 360 nm. This oxo-bridged dinuclear ruthenium complex can potently and selectively inhibit the MCU in permeabilized cells at nanomolar concentrations (Figure 3).[50] Such activity has led to the extensive use of Ru360 for studies of $m$$Ca^{2+}$-related pathological conditions including ischemia-reperfusion injury,[51,52] glutamate excitotoxicity,[53] and Aβ-induced apoptosis.[54]

In aqueous solution, Ru360 readily undergoes rapid ligand substitution to form the diaqua-capped product, Ru360′ (Figure 2).[49] Because this ligand substitution process starts almost instantaneously under physiological conditions, the aquated complex is presumed to be the active species that is responsible for inhibiting the MCU, a hypothesis that is also supported by the fact that the independently synthesized Ru360′ is a highly potent MCU inhibitor.[55] Regarding the molecular target of these inhibitors, the current understanding is that Ru360 or Ru360′ inhibits the MCU by interacting with the DXXE region of the MCU pore, as indicated by site-directed mutagenesis experiments,[10,15,16,56] molecular docking,[57] and NMR studies.[57] Notably, mutation of the D261 (Figure 1) and

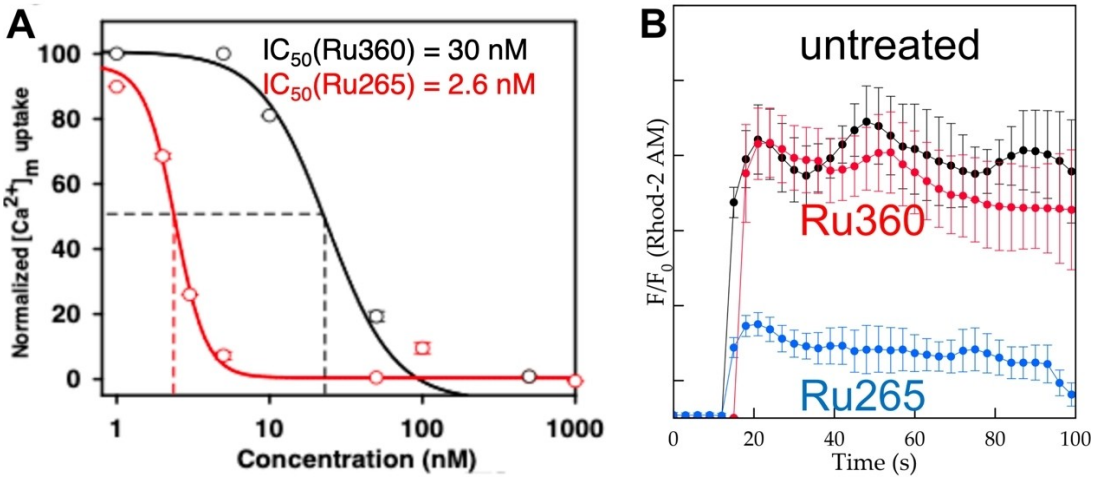

Figure 3. A. Dose-response curve of mCa^{2+} uptake inhibition in permeabilized HEK293T cells. B. Representative mCa^{2+} uptake in intact HeLa cells treated with 50 μM of Ru360 or Ru265. The mCa^{2+} uptake was stimulated by the addition of histamine and the degree of uptake was quantified by the fluorescence turn-on (F/F_0) of the Rhod-2AM dye. Adapted from ref [59]. Copyright (2019), with the permission from the American Chemical Society (ACS). https://pubs.acs.org/doi/10.1021/acscentsci.8b00773. Further permission related to the material excerpted should be directed to the ACS.

S259 residues in the human MCU attenuates the inhibitory activity of Ru360, suggesting that these residues are involved in its interaction.

Despite the wide use of Ru360 for studying mCa^{2+} dynamics in permeabilized cells, the practical application of this complex in intact cells is significantly limited by its high affinity for the cell membrane,[50] low tissue accumulation in vivo,[52] and challenging purification procedures,[49] features which often lead to inconsistent results in biological assays.[53] To develop complexes with more favorable biological properties, our group synthesized structural analogues of Ru360 with various modifications. Our initial SAR studies found that dinuclear complexes are required for MCU-inhibitory activity, based on the observation that mononuclear ruthenium polyammine complexes including *cis*-$[Ru(NH)_4Cl_2]^+$, *trans*-$[Ru(NH_3)_4Cl_2]^+$, $[Ru(NH_3)_5Cl]^{2+}$, and $[Ru(NH_3)_6]^{3+}$ do not inhibit mCa^{2+} uptake in permeabilized cells at a relatively high concentration (10 μM).[58]

Given these results, we next sought to explore the effects of changing the identity of the bridging ligand. These efforts resulted in the nitrido-bridged analogue Ru265 (Figure 2), named for its strong absorbance at 265 nm,[59] which was synthesized based on a literature procedure.[60] In comparison to Ru360, Ru265 exhibits significantly greater cell uptake, enabling it to inhibit the MCU in both permeabilized (Figure 3A) and intact (Figure 3B) cells. Consequently, Ru265 was able to induce protective effects in both an in vitro hypoxia-reoxygenation injury model (Figure 4A) and in vivo model of ischemic stroke (Figure 4B and 4 C).[61] It is worth noting at a high dose (10 mg/kg), Ru265 can cause seizure-like behaviors in mice. The cause of this dose-limiting effect is unclear and requires more investigations.

The mechanism of Ru265 is considered to be similar to that of Ru360. Under physiological conditions, Ru265 aquates to Ru265′ (Figure 2) with a half-life of several minutes, suggesting

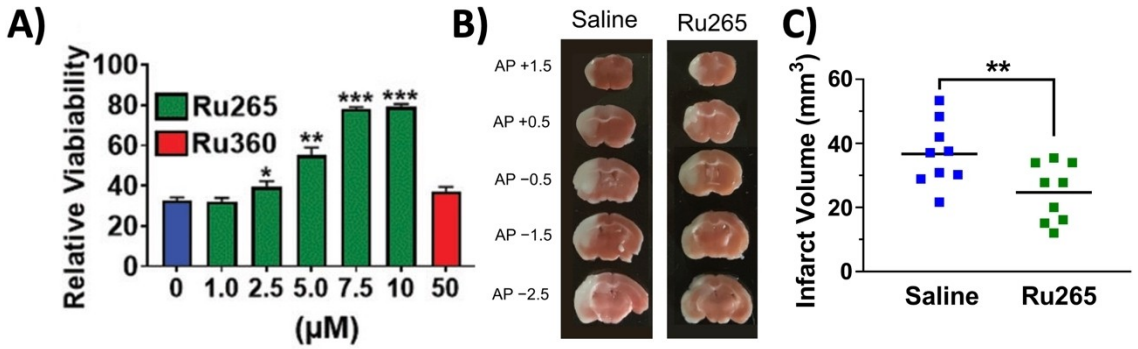

Figure 4. A. Comparison of cell viability in cortical neuron cultures treated with Ru265 (green bar) or Ru360 (red bar) and subjected to 90 min of lethal oxygen–glucose deprivation, an in vitro model of ischemic stroke. The blue bar represents the relative viability of the untreated cells. B. Brain sections of mice treated with saline (8 mL/kg) or Ru265 (3 mg/kg) and subjected to a model of ischemic stroke. C. Quantified size of the mice brain infarct. Ru265 decreases the infarct size compared to the saline control, suggesting that this compound has promising therapeutic potential for the management of ischemic stroke. Adapted from ref [61]. Copyright (Novorolsky et al., 2020), with permission from the SAGE Publishing.

that – like Ru360 – the diaqua product is the active MCU inhibitor.[58,62] With respect to the nature of the interaction of this compound with the MCU, a D261A mutation within the DXXE region of the human MCU suppressed the inhibitory activity of Ru265. In addition, molecular docking studies suggest that Ru265′ interacts with this region of the MCU pore by engaging in significant hydrogen-bonding interactions with D261 and E264 residues (Figure 5).[63] The observation that Ru265, but not Ru360, is an effective MCU inhibitor in intact cells was puzzling given their structural similarities and the fact that they appear to target the same region of the MCU. To address this difference, we thoroughly compared their physical properties and identified redox activity to be a key factor.[58] Specifically, Ru360 is unstable in the presence of the biological reductants, leading to products that are not effectively internalized by cells or capable of inhibiting the MCU. By contrast, the strongly donating nitrido ligand of Ru265 makes this compound redox-inert, rendering it stable towards reduction within the biological milieu. Thus, Ru265 can be taken up by cells and remains intact intracellularly, enabling it to inhibit the MCU. This result highlights the importance of the bridging ligand, which controls the redox stability and consequently modulates the biological properties of this compound class.

Capitalizing on the success of Ru265, this compound was further functionalized in order to fine-tune its pharmacological profile and to understand the role of each of its ligands on the overall biological activity (Figure 6). We first examined the importance of the ammine ligands on the MCU-inhibitory activity by synthesizing analogues of Ru265 with different equatorial ligands including chloride,[58] polypyridines,[64] and ethylenediamine.[59] Complexes bearing the chloride and polypyridine ligands failed to block mCa^{2+} uptake, whereas the ethylenediamine analogue showed MCU-inhibitory activity, albeit with less potency than Ru265. These results suggest that hydrogen-bonding donor ligands are required for inhibitory activity.

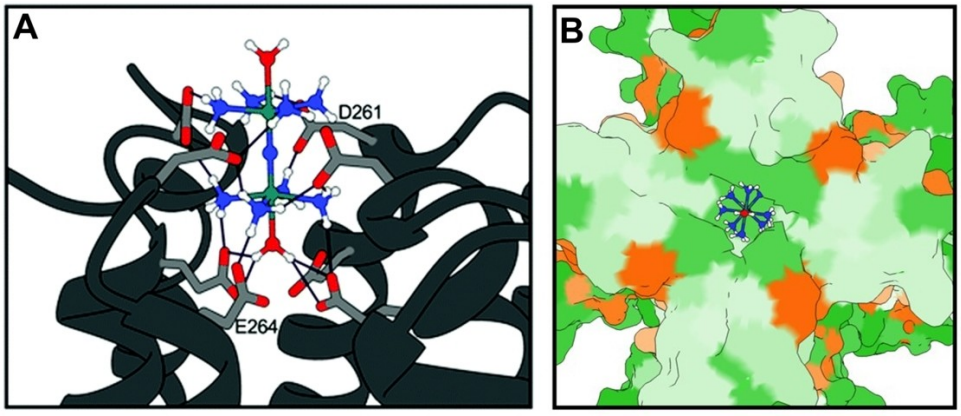

Figure 5. A. Molecular docking study of Ru265′ in the MCU. Predicted hydrogen-bonding interactions are shown as black lines. Atom colors: green = Ru, blue = N, red = O, white = H, grey = C. B. Top-down view of Ru265′ docked into the MCU. The surface is colored by amino acid hydrophobicity (lime green = hydrophilic, orange = hydrophobic). Adapted from ref [63]. Copyright (2021), with permission from the Royal Society of Chemistry.

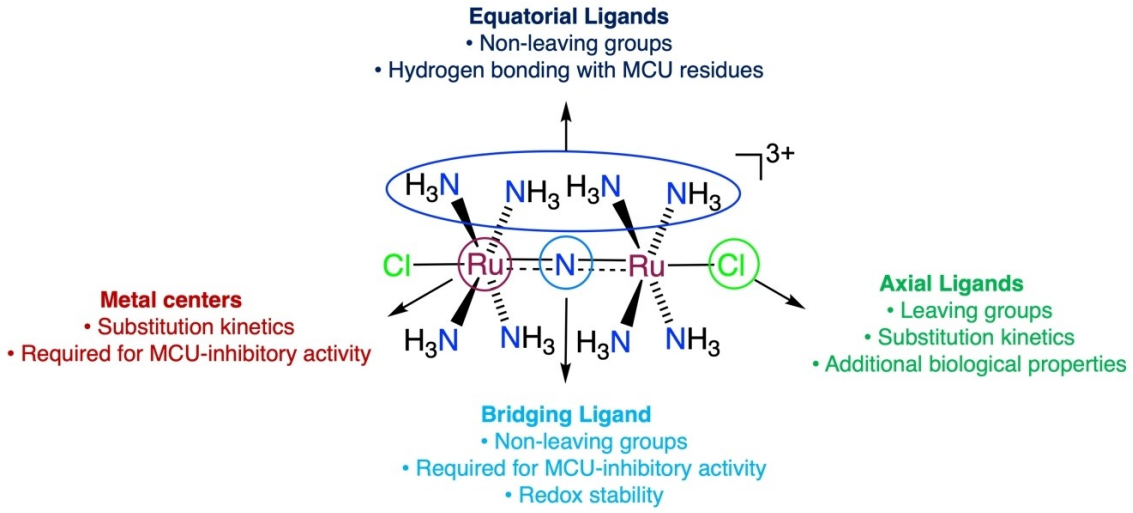

Figure 6. Different components of diruthenium-based MCU inhibitors.

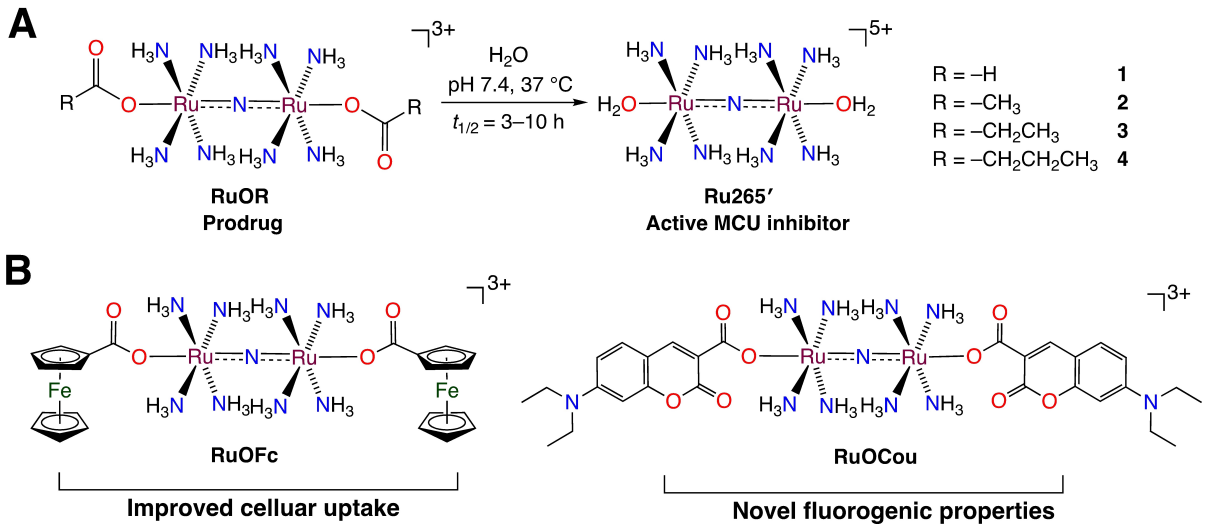

Figure 7. Carboxylate-capped Ru265 derivatives. A. Alkyl carboxylate derivatives of Ru265 (**1**–**4**) are MCU inhibitor prodrugs with prolonged half-lives. B. RuOFc and RuOCou are Ru265 derivatives with functional axial ligands that introduce new features into the system.

In continuing an investigation of the SARs, modifications to the axial ligands of Ru265 were next investigated. Replacing the axial chlorides of Ru265 with carboxylates gave rise to the carboxylate-capped species **1**–**4** (Figure 7A).[65] Under physiological conditions, **1**–**4** underwent aquation with half-lives on the order of hours, a timescale that is significantly longer than that of the chloride-capped Ru265, for which this process occurs within a few minutes. In their intact forms, carboxylate-capped derivatives are less potent inhibitors of the MCU than Ru265. Nevertheless, upon aquation, the MCU-inhibitory properties of these complexes increases as they form the potent diaqua complex Ru265′ (Figure 8). These results provided the first example of using this axial ligand modification strategy to afford MCU inhibitor prodrugs with improved biological stability (Figure 6).

In addition to optimizing aquation kinetics and prodrug activation rates, the implementation of different axial ligands can be leveraged to impart additional functionalities to this class of MCU inhibitors (Figure 7B). For instance, we have employed ferrocenecarboxylates as axial ligands. The ferrocene moieties are redox active and also highly lipophilic.[66] Accordingly, the resulting complex RuOFc exhibits reversible electrochemistry and is also substantially more lipophilic than Ru265. This latter property contributed to its enhanced cellular uptake, which is 10-fold higher than that of Ru265 under identical conditions. This improvement of uptake also results in a modest increase in MCU-inhibitory activity in intact cells. Another axial ligand-functionalized example is RuOCou, a Ru265 derivative containing coumarin fluorophores (Figure 7B).[67] In its intact form, this complex is not fluorescent because the Ru centers quench the radiative decay of the coumarin-based excited states. Upon aquation, however, the coumarin ligands are released concomitantly with Ru265′, leading to an increase in both the fluorescence intensity and MCU-inhibitory activity. This turn-on fluorescence response enables the aquation of RuOCou to be monitored in both HeLa cell lysates and live HeLa cells. Thus, the implementation of fluorescent axial ligands demonstrates RuOCou to be a fluorogenic prodrug, whose activation via aquation can be monitored by fluorescence spectroscopy and microscopy. Collectively, these studies demonstrate that axial modification of Ru265 is a promising approach for tuning the pharmacological properties and installing new functionalities for this class of MCU inhibitors.

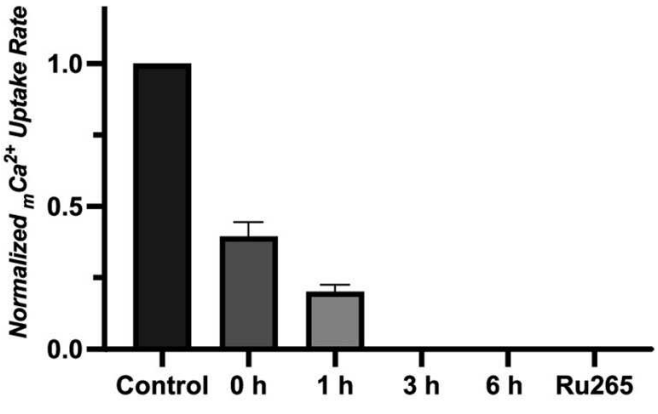

Figure 8. Time-dependent normalized $m\mathrm{Ca}^{2+}$ uptake rate within permeabilized HEK293T cells treated with acetate-capped Ru265 derivative **2** (Figure 7A). The $m\mathrm{Ca}^{2+}$ uptake rate was obtained by monitoring the fluorescence response of the Calcium Green 5 N sensor over time. Adapted from ref [65]. Copyright (2022), with permission from the American Chemical Society.

Osmium-Based Inhibitors

As reflected by the discussion above, the equatorial, bridging, and axial ligands within these dinuclear MCU inhibitors play important and distinct roles in modulating their biological activities. Likewise, it was expected that the nature and identity of the metal center would be critical in their MCU-inhibitory properties.

As the 5d congener of ruthenium, osmium can form structurally similar complexes, thus presenting a rational starting point for exploring the importance of the metal center within this compound class. Accordingly, the osmium analogue of Ru265, named Os245 based on its strong absorbance at 245 nm (Figure 9), was successfully synthesized[68] based on procedures within the literature.[60,69,70] Like Ru265, Os245 undergoes aquation to the diaqua-capped Os245′ under physiologically relevant conditions. However, this process is significantly slower (11.7 h) for Os245. This observation is consistent with the known high relative inertness of 5d transition metals compared to their 4d and 3d congeners. Furthermore, in contrast to Ru265, the MCU-inhibitory activities of the dichloride Os245 and its diaqua analogue Os245′ are different. Specifically, Os245′ is two orders of magnitude more potent than Os245, achieving an inhibitory activity that is equivalent to that of Ru265. The difference in inhibitory activities between Os245 and Os245′ indicates that the chloride ligands of Os245 are detrimental to MCU inhibition, presumably because they are less effective than water in engaging in relevant hydrogen-bonding interactions with this transporter. By contrast, the rapid aquation of Ru265 to Ru265′ prevented the observation of different inhibitory properties of these complexes. This result also highlights that the slow ligand substitution kinetics plays an important role in the biological activities of these osmium analogues.. Building on these results, we next evaluated the therapeutic potential of these osmium derivatives. Similar to Ru265, both Os245 and Os245′ can protect primary cortical neurons against oxygen–glucose deprivation, an in vitro model for ischemic stroke. In vivo, however, a dose-limiting toxic side effect of seizure induction is observed when these compounds are administered to mice at a dose of 10 mg/kg. This side effect is identical to that observed for mice treated with Ru265.[61] The time for seizure onset induced by the osmium-complexes, however, is delayed compared to Ru265. This result highlights that the slow ligand substitution kinetics of Os245 also manifests in vivo. Thus, modifying ligand substitution kinetics via alteration of the metal center provides another means of generating complexes with distinct pharmacological properties and therapeutic profiles.

Other Metal-Based Inhibitors

Although the dinuclear complexes discussed above have been thoroughly studied for their MCU-inhibitory properties, early reports have also shown that mononuclear amine complexes of Co^{3+}, Cr^{3+}, and Rh^{3+} inhibit mCa^{2+} uptake in isolated mitochondria without negatively affecting the mitochondrial membrane potential.[71,72] The mechanism of action and SARs of these molecules, however, were not fully elucidated. Building upon these prior studies, we investigated a series of different cobalt amine complexes that led to the identification of promising candidates that can inhibit mCa^{2+} uptake in permeabilized cells with nanomolar potency (Figure 10).[63,73] These efforts revealed $[Co(sen)]^{3+}$ to be an MCU inhibitor that can operate in intact, non-permeabilized cells.[73] In addition, docking and site-directed mutagenesis studies revealed that these compounds most likely interact with the DXXE region of the MCU pore.[63] Thus, unsurprisingly, this critical motif appears to be a common target for most MCU inhibitors. Although these first-row transition metal complexes are generally much less potent than the ruthenium and osmium inhibitors mentioned above, their earth abundance, low cost, and facile syntheses make them attractive alternatives that warrant further investigations. Lastly, several trivalent lanthanide ions have also been reported to inhibit mCa^{2+} uptake in isolated mitochondria.[74–77] These ions are similar to Ca^{2+} with respect to their charge-to-ionic radius ratios and ligand donor atom preferences, properties that enable them to inhibit the MCU in a competitive fashion. Off-target effects such as membrane binding and bone-localization have been reported for these lanthanide ions. Further studies are needed to establish their mechanism of action and viability as MCU inhibitors in intact cells.

Conclusions

Although mCa^{2+} plays a key role in normal cellular functions, dysregulation of its homeostasis, primarily through mCa^{2+} overload, is a key contributor to various human diseases. As such, the MCU has arisen to be an important therapeutic target.

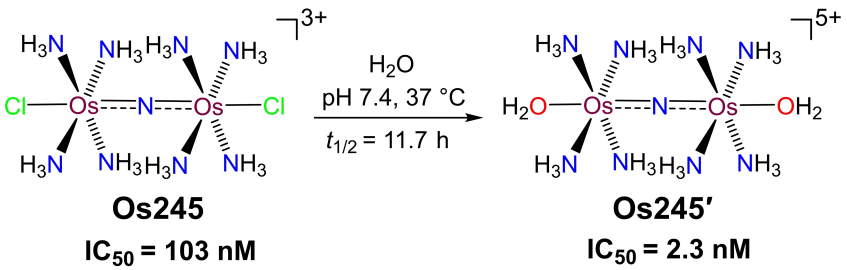

Figure 9. Structures of Os245 and its aquation product Os245′ with their IC_{50} values for mCa^{2+} uptake inhibition in permeabilized HeLa cells.

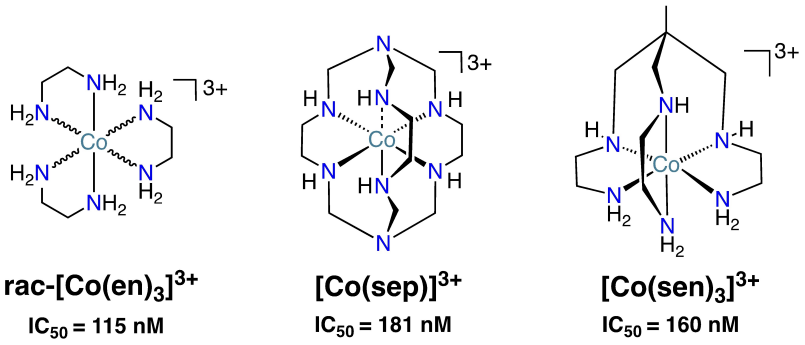

Figure 10. Structures of three cobalt-based MCU inhibitors with their IC_{50} values for mCa^{2+} uptake inhibition in permeabilized HeLa cells.

Within recent years, various research groups have developed both inorganic and organic small molecules that inhibit MCU-mediated mCa^{2+} uptake. In comparing these two classes of inhibitors, organic MCU inhibitors have typically been discovered via combinatorial screening efforts from libraries of compounds with known biological activities. As such, organic MCU inhibitors often possess alternative biological properties and have poorly defined SARs. By contrast, the metal-based inhibitors were initially discovered based on their MCU-inhibitory activities and have a pattern of SARs that is beginning to be elucidated, as summarized here. Dinuclear ruthenium inhibitors are the most commonly used, due in part to the initial discoveries of the inhibitory properties of RuRed and Ru360. As summarized here, Ru265 has emerged as the new state-of-art MCU inhibitor, which exhibits favorable cell permeability and redox stability compared to Ru360. These unique properties of Ru265 have been demonstrated in both in vitro and in vivo models to confer protective effects against ischemic injury.

Despite the potential of Ru265 and other metal-based MCU inhibitors, many fundamental questions regarding their mechanisms of action still remain. For instance, the exact nature of the interactions between these metal complexes and the DXXE residues of MCU pore is still inconclusive. In addition, little is known about the in vitro and in vivo fate of these metal complexes, despite the extensive studies on their behaviors in aqueous solutions. Further investigations are required in order to shed light on these important questions, which when answered will enable the design of improved analogues.

From the perspective of drug development, Ru265 requires significant optimization efforts. A key dose-limiting side effect of this compound and its analogues is the induction of seizure-like behaviors in mice.[61,68] This side effect has also been observed in animals treated with RuRed,[78,79] suggesting that a common mechanism is at play with these compounds. To overcome this drawback, identifying the origin of this side effect is critical, as that information will enable the design of compounds with optimized selectivity and pharmacokinetic properties. Fortunately, as noted here, this class of dinuclear complexes has many different possibilities for modification, which provides a means of rationally improving them. Specifically, as demonstrated in this review, the physical and biological properties of these dinuclear MCU inhibitors can be fine-tuned by altering axial, equatorial, and bridging ligands, as well as the metal centers. Importantly, the pharmacological profiles of these compounds can be rationally tailored for different therapeutic applications. For instance, most complexes reported in this review are not cytotoxic, which render them beneficial for cytoprotective applications such as managing ischemia-reperfusion injury and neurodegeneration. However, it is possible to intentionally confer these complexes with cytotoxic activity via their conjugation to bioactive ligands to enable their use as anticancer agents that simultaneously inhibit the MCU. Overall, these results highlight the power and versatility of synthetic coordination chemistry, which has facilitated the discovery of SARs and the expansion of this library of metal-based MCU inhibitors. Moving forward, modifying these MCU inhibitors, based on the SARs outlined above, will enable improved compounds for different biological applications.

Acknowledgements

This research was supported by Cornell University and the United State National Science Foundation (NSF) under award number CHE-1750295.

Conflict of Interest

The authors declare no competing financial interests.

Keywords: Bioinorganic chemistry · Ion channels · Osmium · Ruthenium · Structure-activity relationships

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Manuscript received: February 23, 2023
Revised manuscript received: April 1, 2023
Accepted manuscript online: April 4, 2023
Version of record online: April 21, 2023