



Inhibitors of the mitochondrial calcium uniporter for the treatment of disease

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Abstract

The mitochondrial calcium uniporter (MCU) is a protein located in the inner mitochondrial membrane that is responsible for mitochondrial Ca^{2+} uptake. Under certain pathological conditions, dysregulation of Ca^{2+} uptake through the MCU results in cellular dysfunction and apoptotic cell death. Given the role of the MCU in human disease, researchers have developed compounds capable of inhibiting mitochondrial calcium uptake as tools for understanding the role of this protein in cell death. In this article, we describe recent findings on the role of the MCU in mediating pathological conditions and the search for small-molecule inhibitors of this protein for potential therapeutic applications.

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Introduction

Mitochondria are critical for the regulation of cellular respiration and energy production within eukaryotes. These organelles also serve a complementary function of buffering intracellular calcium (Ca^{2+}) levels. Mitochondria effectively uptake these ions to restore equilibrium Ca^{2+} concentrations when cytosolic levels are elevated. This mitochondrial Ca^{2+} ($m\text{Ca}^{2+}$) uptake is mediated by the highly selective and inwardly rectifying mitochondrial calcium uniporter (MCU) [1]. Although $m\text{Ca}^{2+}$ uptake is essential for signaling and bioenergetic processes, overload of mitochondria with these ions

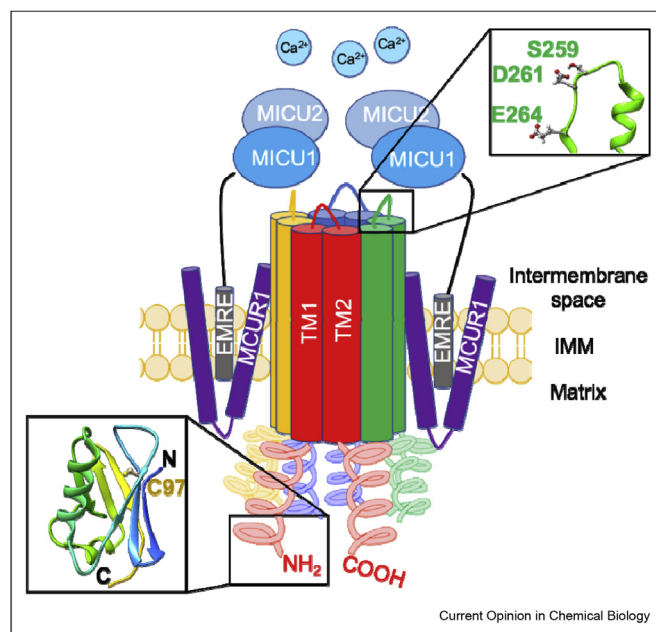
triggers the release of cytochrome c , overproduction of reactive oxygen species (ROS), mitochondrial swelling, and opening of the mitochondrial permeability transition pore (mPTP), all of which contribute to apoptotic cell death [2]. Over the past two decades, a significant number of studies have shown that this type of dysregulation of $m\text{Ca}^{2+}$ levels, caused in part by improper MCU activity, can have deleterious effects on cellular function, which manifest a number of serious pathological conditions [3,4,5]. As such, the MCU has arisen as a potential therapeutic target for the treatment of diseases related to mitochondrial dysfunction such as neurodegeneration, ischemia/reperfusion injury, and cancer [6].

The MCU complex

Although the calcium-buffering capabilities of the mitochondria have been known for over 50 years, the precise identity of the MCU as the major Ca^{2+} -transporter remained elusive until 2011 [1,7]. A series of combined efforts involving NMR spectroscopy [8,9], cryo-EM [9–14], and x-ray crystallography [13,15,16,17,18] have elucidated the structure of this membrane-bound transporter and its regulatory machinery. The pore-forming subunit of the MCU contains 351 amino acid residues with both the N- and C-terminal domains located in the matrix of the mitochondria. The two transmembrane domains, TM1 and TM2, are connected by a solvent-exposed loop with a highly conserved DXXE motif, which is essential for Ca^{2+} transport, located in the upper helix of TM2 (Figure 1).

Although initial structural studies suggested that the MCU complex exists as a pentamer comprising five identical subunits [9], more recent studies have clarified that this protein complex actually assumes a tetrameric, dimer of dimers assembly [10–13]. Tight regulation of MCU-mediated $m\text{Ca}^{2+}$ uptake is carried out by the associated protein MICU1 [19] and its homologues MICU2 [17] and MICU3 [20]. These regulatory proteins contain EF-hands, which enable them to sense Ca^{2+} ions and tune $m\text{Ca}^{2+}$ uptake through the MCU [17,18,21,22]. Three additional proteins in the MCU complex, EMRE [14], MCUB [23], and MCUR1 [24], also exhibit important regulatory roles in restricting or enhancing $m\text{Ca}^{2+}$ uptake.

Figure 1



Topology diagram of human MCU showing the pore-forming subunit, the relevant regulator proteins MICU1/2, MCUR1, and EMRE, and the orientation of the MCU in the inner mitochondrial membrane (IMM). Insets depict (left) the location of C97 in the crystal structure of the N-terminal domain (NTD; residues 72–189; PDB 5KUJ) and (right) the location of the DXXE motif and S259 in the solvent-accessible region of the MCU pore (PDB 5ID3).

The MCU, Ca^{2+} , and disease

Neurodegenerative and neuromuscular disorders

A number of neurodegenerative diseases exhibit improper handling of $m\text{Ca}^{2+}$ [25–28]. In Alzheimer's disease (AD), for example, accumulation of amyloid- β ($\text{A}\beta$) plaques in brain tissue leads to increased $m\text{Ca}^{2+}$ uptake in neurons and results in cell death via excitotoxicity [29,30]. As such, approaches to modulate $m\text{Ca}^{2+}$ levels have been suggested as a therapeutic strategy for the prevention of AD [31], and inhibition of $m\text{Ca}^{2+}$ uptake through the MCU was recently shown to inhibit $\text{A}\beta$ -induced $m\text{Ca}^{2+}$ overload and apoptosis in vitro [32•].

Parkinson's disease (PD) is caused by α -synuclein aggregate accumulation in the brain, which causes $m\text{Ca}^{2+}$ overload, overproduction of ROS, and death of dopaminergic neurons [33]. It was recently reported that the integrity of the MCU complex is compromised in early onset PD, as reflected by the degradation of MICU1 by the protein ligase Parkin, leading to increased $m\text{Ca}^{2+}$ uptake and apoptosis [34]. Supporting this conclusion, genetic knockdown of the MCU rescues dopaminergic neurons from PD-mediated cell death [35•].

Another neurodegenerative disease, amyotrophic lateral sclerosis (ALS), is also directly linked to $m\text{Ca}^{2+}$ overload. Disrupted regulation of glutamate homeostasis in neurons and astrocytes leads to $m\text{Ca}^{2+}$ overload and cell

death [36]. MCU expression in neurons varies over the progression of ALS; presymptomatic neurons upregulate the MCU, presumably to counter the high cytosolic Ca^{2+} influx, whereas late stage neurons show reduced expression to compensate for the cytotoxic $m\text{Ca}^{2+}$ overload [37].

In addition to neurodegenerative disease, the MCU complex has been identified to play a major role in neuromuscular disease. Loss or mutation of MICU1 induces myopathy, learning difficulties, and progressive movement disorders [38]. These symptoms can prove lethal and appear to be a primary result of defective Ca^{2+} signaling, $m\text{Ca}^{2+}$ overload, and a fragmented mitochondrial network [39,40]. Taken together, these findings emphasize the central role of the MCU in neurological disease and suggest that enforcing proper regulation of $m\text{Ca}^{2+}$ uptake could be a powerful therapeutic strategy.

Ischemia/reperfusion injury and ischemic stroke

Ischemia/reperfusion injury (IRI), which arises from the rapid restoration of oxygenated blood to oxygen-deficient, or ischemic, tissue, occurs in situations such as heart failure, organ transplant, stroke, and ischemic brain injury [2]. Under ischemic conditions, oxygen-deficient cells employ anaerobic glycolysis as the primary metabolic pathway, which leads to the production of lactic acid and a concomitant decrease in cytosolic

pH. Simultaneously, the mitochondrial membrane potential ($\Delta\Psi_m$) is diminished due to the cessation of oxygen-dependent oxidative phosphorylation. The drop in cytosolic pH sequentially activates Na^+/H^+ and $\text{Na}^+/\text{Ca}^{2+}$ exchanger proteins, leading to a net increase in cytosolic Ca^{2+} levels. Upon reperfusion, the return of oxygen leads to rapid restoration of the $\Delta\Psi_m$ as oxidative phosphorylation resumes, generating a surge of ROS. This restoration of $\Delta\Psi_m$ provides a strong driving force for the entry of the cytosolic Ca^{2+} into the mitochondria via the MCU, triggering mitochondrial calcium overload, cell death, and the characteristic tissue damage associated with IRI [41].

Based on the contributing role of $m\text{Ca}^{2+}$ overload in IRI, the MCU represents a potential therapeutic target for this condition. Surprisingly, the constitutive knockout of the MCU either specifically in the heart [42] or globally does not protect cardiac [43] or brain [44] tissues from IRI. By contrast, acute chemical inhibition or conditional knockout of the MCU in adult animals does confer the expected protective effects [43,45,46]. These results suggest that the role of $m\text{Ca}^{2+}$ in IRI may be more complex than originally expected and may indicate that alternative, as-of-yet undiscovered means of handling mitochondrial bioenergetics exist.

Cancer

Mitochondria and $m\text{Ca}^{2+}$ levels play an important role in tumorigenesis and cancer biology. The MCU is highly expressed in certain forms of colorectal, breast, pancreatic, stomach, and prostate cancers. Additionally, various components of the MCU regulatory machinery show variable levels of expression and mutation in different cancer types; the significance of these mutations, however, is still not fully understood [47].

The role of the MCU complex has most extensively been studied in the context of breast and colorectal cancers. Some models of breast cancer show high expression of the MCU channel, which facilitates metastasis in vivo. Similarly, overactivation of the MCU in colorectal cancer by receptor-interacting protein kinase 1, RIPK1, promotes cancer proliferation [48]. This hypothesis was confirmed by the knockdown and inhibition of the MCU, which drastically reduces cancer progression [49].

Recent work has also suggested that overexpression of components of the MCU complex may contribute to chemo-resistance in cancer cells [50]. In pancreatic and colon cancers, overexpression of MICU1 and MICU2 decreases $m\text{Ca}^{2+}$ levels and prevents $m\text{Ca}^{2+}$ overload-induced apoptosis [51,52]. The role of the MCU in cancer is only beginning to be studied, but it is clear that $m\text{Ca}^{2+}$ regulation is fundamental to cancer cell growth. Given the seemingly contradictory roles of the MCU

and $m\text{Ca}^{2+}$ in different cancer types, further investigations are required to decipher the function of this transporter in cancer.

Regulation of $m\text{Ca}^{2+}$ uptake by small-molecule inhibitors

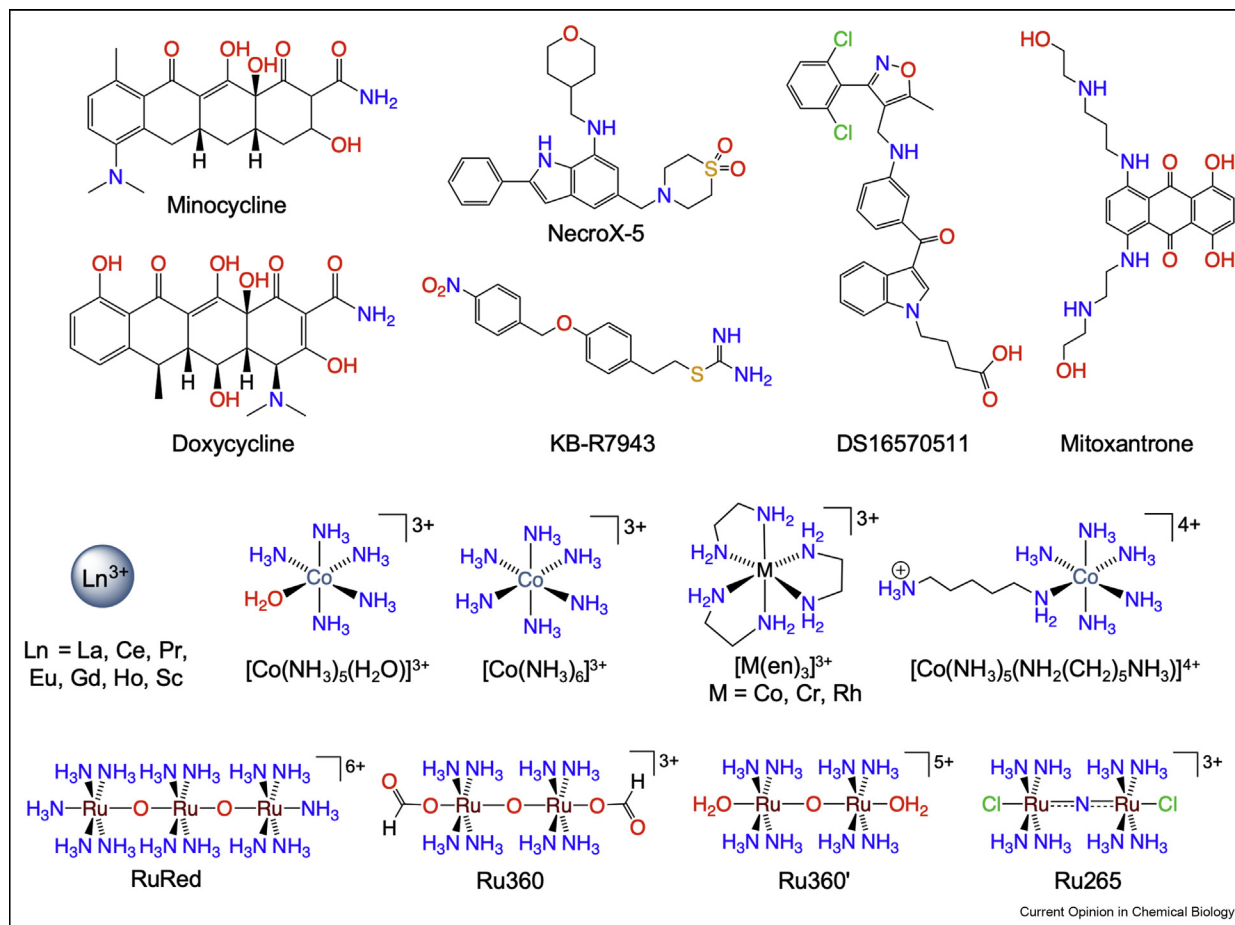
Given the importance of $m\text{Ca}^{2+}$ dynamics in the pathological conditions described above, there has been a strong interest in developing small-molecule inhibitors of the MCU for use as therapeutic agents or tools for studying the role of this transporter in human disease. In recent years, there have been a handful of studies aimed at identifying small molecules capable of inhibiting MCU-mediated Ca^{2+} uptake (Figure 2, Table 1).

The organic molecules mitoxantrone [53] and DS16570511 [54] were identified from distinct libraries comprising over 120,000 compounds to be potent inhibitors of the MCU. The MCU-inhibitory activity of several other small molecules was recognized serendipitously as a secondary function. For example, the necrosis inhibitor NecroX-5 [55–57], the $\text{Na}^+/\text{Ca}^{2+}$ exchange inhibitor KB-R7943 [58], and the antibiotics minocycline [59,60] and doxycycline [60,61] all possess MCU-inhibitory properties. Of these organic compounds, DS16570511 is the most potent, as reflected by its 50% MCU-inhibitory concentration of 860 nM in isolated mitochondria [54]. This compound protects perfused rat hearts from $m\text{Ca}^{2+}$ overload, demonstrating its potential as a therapeutic agent for diseases related to $m\text{Ca}^{2+}$ dysregulation.

In comparing these organic MCU inhibitors, there is no apparent structure–activity relationship (SAR) that would be predictive of their inhibitory activities. Furthermore, these compounds are generally nonselective for the MCU, as reflected by their ability to induce off-target biological effects. For example, mitoxantrone is a cardiotoxic anticancer agent that inhibits human topoisomerase II [62,63]. Additionally, DS16570511 has recently been shown to depolarize mitochondria and induce mPTP opening [64]. These results highlight the challenge in finding compounds that can inhibit the MCU selectively in the absence of additional biological perturbations, which can complicate analysis of results and compromise their therapeutic viability.

Inorganic salts and coordination complexes have also been shown to inhibit $m\text{Ca}^{2+}$ uptake. The trivalent lanthanide ions, which have ionic radii and coordination preferences comparable to Ca^{2+} , can bind to mitochondria and competitively inhibit $m\text{Ca}^{2+}$ uptake [65–68]. Several transition metal coordination complexes, bearing ammine (NH_3) or amine ligands, have also been demonstrated to inhibit $m\text{Ca}^{2+}$ uptake. Most notably, complexes of Co^{3+} , Cr^{3+} , and Rh^{3+} inhibit Ca^{2+} uptake in isolated mitochondria at nanomolar concentrations

Figure 2



Structures of MCU inhibitors discussed in this work.

without negatively affecting $\Delta\Psi_m$ [69,70]. Like the organic compounds discussed previously, the SAR for these coordination complexes is lacking, given that complexes with diverse coordination environments appear to exhibit MCU-inhibitory activity. Moreover, these coordination complexes have not been evaluated in intact cellular systems.

Ruthenium red (RuRed) and ruthenium 360 (Ru360)

The most well-known and widely employed inhibitor of the MCU is the trinuclear oxo-bridged complex ruthenium red (RuRed) [71,80]. This +6 cation contains a nearly linear Ru–O–Ru–O–Ru core, with the remainder of the ruthenium coordination spheres supported by neutral NH_3 ligands (Figure 2). The +6 charge is a result of its mixed valent ground state, which arises from two formally Ru^{3+} centers and one Ru^{4+} center [71]. RuRed, named for its intense red color ($\epsilon_{532\text{nm}} = 85,900 \text{ M}^{-1} \text{ cm}^{-1}$), was first synthesized in

1892 [82] and found use as a cytological stain shortly after [81,83].

The widespread use of RuRed as a cytological stain led to the discovery that this compound inhibits $m\text{Ca}^{2+}$ uptake by the MCU without negatively affecting mitochondrial respiration or Ca^{2+} efflux [84–87]. Furthermore, researchers have shown that RuRed can mitigate tissue damage due to IRI [74] and reduce cancer cell migration [75]. Despite the potential utility of RuRed as a $m\text{Ca}^{2+}$ uptake inhibitor, its purification has always been a challenging matter. In fact, nearly all commercial sources of RuRed supply this compound in poor purity (<80%) [78]. Thus, most commercial formulations of RuRed actually contain mixtures of several different ruthenium ammine complexes. Not surprisingly, commercial formulations of RuRed have exhibited poor selectivity for the MCU, often showing inhibitory activity for other ion channels as well [76].

Table 1

MCU inhibitors discussed in this work, their MCU-inhibitory activity, and observed off-target biological effects.

Compound	IC ₅₀ (μM) ^a	IC ₅₀ determination conditions	Off-target effects	Ref
Mitoxantrone	8.3	Yeast mitochondria ^b	Topoisomerase II inhibition, DNA binding, cardiotoxicity	[53•,62,63]
DS16570511	0.860	Isolated mitochondria ^c	Mitochondrial depolarization, cell death	[54•,64]
NecroX-5	ND ^d	–	Necrosis inhibition, ROS scavenging	[55–57•]
Minocycline	ND	Isolated mitochondria	Antibiotic activity, mitochondrial depolarization, Ca ²⁺ binding, membrane binding	[59,60]
Doxycycline	ND	Isolated mitochondria	Antibiotic activity, altered cell metabolism, and proliferation	[60,61]
KB-R7943	5.5	Permeabilized HeLa cells	Na ⁺ /Ca ²⁺ exchanger inhibition, inhibition of mitochondrial complex I	[58]
Ln ³⁺ salts	0.02	Isolated mitochondria	Membrane binding, localization to bone tissue	[65–68]
[Co(NH ₃) ₅ (H ₂ O)] ³⁺	0.54	Isolated mitochondria	ND	[69]
[Co(NH ₃) ₆] ³⁺	1.66	Isolated mitochondria	Mucopolysaccharide channel inhibition	[69,70]
[Co(en) ₃] ³⁺	0.053	Isolated mitochondria	ND	[70]
[Cr(en) ₃] ³⁺	0.490	Isolated mitochondria	ND	[70]
[Rh(en) ₃] ³⁺	0.360	Isolated mitochondria	ND	[70]
[Co(NH ₃) ₅ (NH ₂ (CH ₂) ₅ NH ₃)] ⁴⁺	0.250	Isolated mitochondria	ND	[70]
RuRed	0.0036	Isolated mitochondria	Membrane binding, broad spectrum ion channel inhibition, induction of seizures	[70–87]
Ru360	0.227	Yeast mitochondria	Membrane binding	[6–8,32,88–95]
Ru360'	0.038	Yeast mitochondria	ND	[96]
Ru265	0.0025	Permeabilized HeLa cells	None observed	[97••]

^a Concentration required for 50% MCU inhibition.

^b Yeast genetically modified to express the MCU and its regulator EMRE.

^c Mitochondria isolated from mammalian cell lines.

^d Not determined.

One of the minor impurities found within most formulations of RuRed is a binuclear oxo-bridged complex, called ruthenium 360 (Ru360). This compound, named for its intense UV–vis spectral absorption at 360 nm, is the active component of RuRed mixtures that is responsible for the perceived MCU-inhibitory activity [90–92]. This discovery was consistent with the fact that samples of highly purified RuRed are actually less active inhibitors than impure samples [78]. Inhibition of the MCU by Ru360 is selective and does not interfere with sarcoplasmic reticulum and cytosolic Ca²⁺ dynamics, Na⁺/Ca²⁺ exchanger activity, or L-type Ca²⁺ channels [92].

In contrast to RuRed, Ru360 contains only two Ru centers bridged by a single oxo ligand. In addition to the bridging oxo, each Ru bears four ammine ligands and an axial formate ligand (Figure 2). Ru360 is paramagnetic and mixed valent, formally containing a Ru⁴⁺ and Ru³⁺ center [91]. Isolation of Ru360 can be achieved via a low-yielding synthesis that requires tedious ion exchange chromatographic purification [91]. Our group has developed synthetic methods for the preparation of

a functional analogue of Ru360, which we call Ru360', where the axial formate ligands have been replaced with water ligands [96]. Because the axial formate ligands of Ru360 undergo a fairly rapid aquation reaction, we have found that the aqua ligands of Ru360' have no negative impact on its MCU-inhibitory activity.

Given the high potency and selectivity of Ru360 for inhibiting *m*Ca²⁺ uptake, this commercially available complex has been widely employed for the study of calcium-dependent cellular processes and as a therapeutic agent for the prevention of IRI [93,94]. Ru360 was also shown to prevent glutamate-induced excitotoxicity in cortical neurons [89], and prevent Aβ-induced apoptosis by reducing oxidative stress in microglia [32]. Despite the apparent success of Ru360 in these studies, there are several reported concerns regarding the cell permeability of this reagent. For example, it has been noted that this compound binds the exterior of cell membranes and has low cell permeability [92], properties that are further reflected by its low accumulation in myocardial tissue in vivo [94] and highly variable results in biological assays [89].

Although Ru360 is widely used to study $m\text{Ca}^{2+}$ in biological systems, surprisingly little is known regarding its mechanism of action. A series of recent site-directed mutagenesis experiments [7,10,13,21,22], NMR studies [8], and molecular dynamics simulations [8] suggest that Ru360 inhibits $m\text{Ca}^{2+}$ uptake through interactions with conserved DXXE motif of the solvent-exposed loop of the MCU that spans the TM1 and TM2 domains. Mutations of specific aspartate (D261) and serine (S259) residues in human MCU (Figure 1) maintain Ca^{2+} -uptake activity but reduce the inhibitory effects of Ru360, suggesting that these residues are intimately involved in the inhibitory activity of Ru360. The exact nature of these interactions, however, remains unknown.

Ruthenium 265 (Ru265)

Our group has recently reported the synthesis, characterization, and biological activity of a new ruthenium-based MCU inhibitor, called Ru265 [97••]. This compound is structurally similar to Ru360 in that it contains two bridged Ru centers bearing ammine ligands. In contrast to Ru360, however, Ru265 is bridged by a nitrido (N^{3-}) ligand, and both ruthenium centers attain the +4 oxidation state (Figure 2). Nitrido-bridged ruthenium complexes can be easily obtained by subjecting the nitrido-bridged precursor complex $\text{K}_3[\text{Ru}_2(\mu\text{-N})\text{Cl}_8(\text{OH}_2)_2]$ to ligand substitution reactions. As such, Ru265 could be synthesized cleanly in moderate yields without the need for chromatographic purification.

Like Ru360, Ru265 is a potent inhibitor of $m\text{Ca}^{2+}$ uptake in both isolated mitochondria and permeabilized cell systems. The most striking aspect of Ru265, in comparison to Ru360, is its high cell permeability. Ru265 is taken up by cells over twice as effectively as Ru360. Furthermore, Ru265 is relatively nontoxic to HEK293 kidney cells and does not alter other aspects of mitochondria or Ca^{2+} trafficking, such as the rate of cytosolic Ca^{2+} clearance, the energetics of the $\Delta\Psi_{m_2}$, the efflux of $m\text{Ca}^{2+}$, and the operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Given the high selectivity and good cell permeability of Ru265, it was shown to protect neonatal rat ventricular myocytes from simulated IRI, prevent downstream mitochondrial swelling, mPTP opening, and cell death [97••].

Site-directed mutagenesis studies on the MCU were carried out to elucidate the mechanism of action of Ru265. As noted previously in our discussion of Ru360, cells that express a mutated form of human MCU with a S259A mutation are somewhat resistant to Ru360 inhibition. By contrast, this same mutation had no effect on the inhibitory activity of Ru265, suggesting that there may exist subtle differences in the way that

these complexes interact with the MCU. Somewhat surprisingly, the mutation of a cysteine residue (Cys97, Figure 1) located on the matrix-residing NTD conferred resistance to Ru265 but not Ru360. This cysteine residue is an important redox sensor for the MCU, and thus this mutation may suggest that redox activation of the MCU is critical for the inhibitory activity of Ru265 [97••]. Further studies are required to more fully understand this compound's mechanism of action. Our initial studies on this compound have demonstrated its utility as a therapeutic agent for diseases associated with $m\text{Ca}^{2+}$ overload, which is a consequence of its potent MCU-inhibitory activity and good cell permeability [97••].

Conclusions and outlook

Recent advances in understanding the structure and function of the MCU complex have highlighted the central role of this transporter in bioenergetic processes and pathological conditions. As such, modulating $m\text{Ca}^{2+}$ levels and MCU activity have been identified as promising targets for prevention or treatment of diseases such as IRI, neurodegeneration, and cancer [98]. Toward this goal, several groups have developed small molecules capable of inhibiting Ca^{2+} uptake through the MCU and preventing $m\text{Ca}^{2+}$ overload-induced cell damage. Many of these inhibitors, however, lack cell permeability or selectivity for MCU inhibition and have off-target biological effects. Despite the promise of MCU inhibitors as therapeutic candidates, it should be noted that the administration of RuRed *in vivo* induces a complex seizure response in rats [77]. This phenomenon may either be a side effect of MCU inhibition or a consequence of other bioactive impurities within the RuRed formulation. Further studies are required to understand the biological implications of using MCU inhibitors as potential therapeutic agents.

The discovery of the greatly improved bioactivity of Ru265 compared to Ru360 and other MCU inhibitors underscores the power of inorganic complexes as novel tools for understanding the role of the MCU in biological systems. Metal complexes can be easily modified through substitution reactions to give diverse structures, allowing clear understanding of SARs. Furthermore, the low toxicity of Ru265 in contrast to many other cytotoxic ruthenium compounds highlights how the coordination environment around a metal center can strongly influence the biological activity of metal-based compounds, and that coordination compounds are promising candidates as effective MCU inhibitors.

Conflict of interest statement

None declared.

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