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Novel Cobalt Complex Inhibitors of Mitochondrial Calcium Uptake

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Abstract—Reperfusion of the ischaemic myocardium leads to intracellular calcium overload followed by mitochondrial dysfunction, resulting in insufficient energy supply and ultimately myocardial necrosis. Ruthenium red (RR), a potent mitochondrial calcium uptake inhibitor, prevents this disruption to mitochondrial metabolism and improves post reperfusion recovery. This therefore suggested that mitochondrial calcium influx is an attractive target for the treatment of reperfusion injury. However, RR is unsuitable for therapeutic use, so we undertook a search for novel compounds which inhibit mitochondrial calcium uptake. The most potent compounds discovered were simple tris(ethylenediamine) transition metal complexes and dinuclear Co complexes. The structure–activity relationship (SAR) of these small molecules has helped to define the structural requirements for inhibition of calcium transport by outlining the size and charge dependency of the interactive site on the mitochondrial calcium uniporter.

Introduction

Mitochondria occupy a central role in the control of energy metabolism as the major energy production sites for cellular function especially in critical aerobic organs, such as the heart and brain. Pathological and clinical episodes resulting in oxygen and substrate deprivation, lead to mitochondrial dysfunction, inhibition of cellular ATP supply and ultimately to cell necrosis. The severity of the ischaemic insult can be exacerbated by restoration of blood flow resulting in reperfusion injury.[1,2] Although the interplay of many processes is responsible for the extent of cell dysfunction and necrosis produced by reperfusion following ischaemia, perhaps one of the most important is the accompanying pathophysiological rise in intracellular calcium and its detrimental effects on mitochondrial function.[3–6]

The damaging effects of excess intracellular calcium on mitochondrial function arise because of the nature of the mitochondrial calcium handling system.[7] Under basal physiological conditions calcium influx via a uniporter and efflux through exchange with Na^+ are balanced to produce continuous calcium cycling. Transient increases in intracellular calcium due to hormonal stimulation lead to corresponding, but slightly prolonged, transient changes in matrix (or intramitochondrial) calcium, resulting in stimulation of key calcium sensitive enzymes, increased NADH supply and ATP production.[8] This calcium second messenger system allows stimulation of energy demand, for example, by positive ionotropic agents on the heart, to be efficiently balanced by a corresponding increase in mitochondrial energy supply. Importantly the calcium uniporter transport capability and activity are much greater than that of the Na^+/Ca^{2+} exchanger and thus with prolonged elevation of cytoplasm calcium (>3 μM) in ischaemia/hypoxia reperfusion, matrix calcium accumulates.[9,10] This process is further exacerbated by other concurrent ischaemic reperfusion conditions, particularly accumulation of intracellular phosphate, low ATP and oxidative stress, leading to the eventual opening of 2 nm diameter pores in the mitochondrial inner membrane. This permeability transition uncouples mitochondrial energy transduction and would be expected to jeopardise cell viability severely.[11,12] Furthermore because mitochondrial calcium influx through the uniporter uses the electrochemical gradient maintained by oxidative metabolism, at pathological levels of calcium,

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Table 1. Comparison of the inhibition of calcium uptake and dissipation of membrane potential by FCCP and RR[a]

Compound	Inhibition of calcium uptake [IC_{50} (nM)]	Inhibition of membrane potential [IC_{50} (nM)]
FCCP	3.5 ± 0.6 (5)	16 ± 4.2 (4)
RR	3.6 ± 0.7 (3)	> 10,000 (3)
A-M (see Table 2)	(see Table 2)	> 100,000 (2)

[a] The data represents the mean ± SEM for the number of experiments in parentheses.

high uniporter usage can dissipate this gradient and uncouple mitochondria.[13]

Inhibition of mitochondrial calcium uptake through the uniporter should be of therapeutic benefit for minimising ischaemic reperfusion damage occurring, for example, on thrombolytic treatment of thrombotic stroke and myocardial infarction. Blockade of the mitochondrial uniporter would reduce the extent of uncoupling and pore opening and hence preserve ATP synthesis. This is critical as a return to cellular homeostasis can only be achieved by removal of excess intracellular calcium by calcium ATPase and Na^+/Ca^{2+} exchange activity. Hence by protecting mitochondria from excess calcium, ATP supply can be maintained and utilised to remove calcium from the cell.

Mitochondrial calcium antagonism has been described for transition metal complexes, such as RR[14] and hexaammine Co(III),[15] as well as various pharmacological agents such as β-blockers,[16] guanidines,[17] and amiloride diuretics.[18] The most potent and commonly used inhibitor is RR, an impure dye. This has been utilised extensively to characterise the nature of the mitochondrial calcium uniporter and respiratory control by calcium (for example, see ref 19). In contrast relatively little is known about the uniporter structure other than that concluded from extraction and reconstitution of calcium binding/transport studies from various mitochondrial membrane associated, acidic glycoproteins (for example, see ref 20). Perhaps the most interesting aspect of the pharmacology of RR is its protective action in a variety of ischaemic reperfusion models in various species at both the cellular and tissue level.[21–29] This cardioprotective action by RR is not only manifested by an improvement in cardiac function, but also a reduction in the extent of cell necrosis and free radical production.[30] Hence this activity of RR supports the importance of mitochondrial calcium overload in the pathogenesis of reperfusion injury and therefore proposes antagonism of the mitochondrial calcium uniporter as an attractive therapeutic target. Unfortunately known inhibitors are not ideal for therapeutic use for a variety of reasons (i.e. impure preparation, poor cell penetration, weak activity, and general nonspecific effects).[31] Although RR has been used extensively in mitochondrial research, commercial preparations are in fact a mixture of many different ruthenium complexes. Recently the activity of RR was shown to be due to a minor dinuclear ruthenium component and not to RR itself.[32] Therefore as known mitochondrial calcium blockers are unattractive drug candidates and offer little useful structure–activity relationship, with no information on the nature of inhibitory site(s) on the uniporter target protein, we undertook a search for novel compounds with better pharmaceutical potential.

Results and Discussion

Discovery of novel inhibitors

Initially compounds were tested for inhibition of mitochondrial calcium uptake and then examined for dissipation of the IMM (inner mitochondrial membrane) potential using the JC-1 assay. This screening strategy was important since the majority of compounds that inhibited mitochondrial calcium uptake dissipated the IMM potential with similar potency. Such compounds probably inhibit mitochondrial calcium influx indirectly through either respiratory blockade and/or depolarisation of the membrane potential. For example, FCCP, a well known mitochondrial uncoupler,[36] inhibited calcium uptake and membrane potential development equally (see Table 1). In contrast the specific mitochondrial calcium antagonist RR only suppressed calcium influx and was without effect on the membrane potential.

Table 2. Inhibition of mitochondrial calcium uptake by various transition metal complexes[a]

	Compound class	Transition metal ion	Inhibition of mitochondrial calcium uptake IC_{50}(nM)
A	Tris(ethylenediamine)	Co(III)	53 ± 20 (5)
B	Tris(ethylenediamine)	Rh(III)	360 ± 70 (3)
C	Tris(ethylenediamine)	Cr(III)	490 ± 200 (3)
D	Tris(ethylenediamine)	Ru(II)	10 μM (2)
E	Tris(ethylenediamine)	Fe(III)	> 10 μM (2)
F	Tris(ethylenediamine)	Cu(II)	> 10 μM (2)
G	Sepulchrate	Co(III)	I (2)
H	Tris(cyclohexanediamine-N,N')	Co(III)	I (2)
I	Hexa-ammine	Co(III)	550 ± 60 (3)
J	Dinuclear Co complex	$Co(III)[(NH_3)_4Co(SO_4)(NH_2)Co(NH_3)_4]^{2+}$	250 ± 70 (3)
K	Asymmetric Co complex	$Co(III)[Co(NH_2(CH_2)_5NH_3)(NH_3)_5]^{4+}$ See ref. 38	250 (2)
L	Ruthenium Red	$Ru(III/IV)[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5]^{6+}$	3.6 ± 0.7 (3)
M	Ru360	$Ru(II/III)[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4]^{3+}$	0.70 ± 0.39 (3)

[a] The data represents the mean ± SEM for the number of experiments in parentheses. I: inactive: < 25% inhibition when tested at 10 μM; > 10 μM: 25–50% inhibition when tested at 10 μM.

Using this screening strategy the most potent and novel inhibitors discovered were a series of tris(ethylenediamine) transition metal complexes, where the order of potency was related to the central transition metal; Co(III) > Rh(III) > Cr(III) > Ru(II) > Fe(III) ~ Cu(II) (see compounds A–F, Table 2). These compounds did not inhibit mitochondrial metabolism or dissipate the inner mitochondrial membrane potential. The inhibitory activity of these molecules was only manifested in the full transition metal complex and was not a property of ethylenediamine or free metal ion, which had no effect upon this assay.

Characterisation of tris(ethylenediamine)Co(III) inhibition

Although tris(ethylenediamine)Co(III) represents the most potent and novel inhibitor of mitochondrial calcium uptake discovered, it was still 10 to 50-fold weaker than RR and its active component, Ru360 (see compounds **L** and **M**, Table 2). The main chemical differences between tris(ethylenediamine)Co(III) and RR/Ru360 are that the Co(III) complex is symmetrical and has a smaller charge and size and thus may act at different or possibly overlapping sites to the ruthenium complexes. To investigate this the calcium dependency of mitochondrial calcium uptake inhibition by RR and tris(ethylenediamine)Co(III) were compared (see Figure 1a and b). Mitochondrial calcium influx was positively co-operative with respect to free calcium (Hill coefficient ~ 2, see Figure 1) with an EC_{50} of 7.5 μM. This suggested that there are two interactive calcium binding/transport sites on the uniporter, as previously described by others (see review in ref. 7). RR has long been known to show non-competitive inhibition with respect to calcium[37] and this was confirmed in these studies by suppression in V_{max} (Fig. 1a). However RR also demonstrated competitive inhibition, as the EC_{50} for calcium increased with RR concentration, such that the calculated K_i was 0.2 nM. Interestingly with increasing inhibition by RR the Hill coefficient decreased to 1 suggesting that RR is blocking the co-operative interaction between these two sites as well as inhibiting calcium transport. In summary RR demonstrated a mixed competitive and non-competitive inhibition in these studies.

Similarly tris(ethylenediamine)Co(III) exhibited mixed inhibition of calcium uptake with a reduction in V_{max} and elevation of EC_{50} for calcium with increasing inhibitor concentrations (see Fig. 1b). However differences between the pharmacology of the compounds emerged on further analysis. Comparison of the compound IC_{50} values at varying free $[Ca^{2+}]$ (see Figure 2 and Schild plot in Figure 3) demonstrated that calcium was more effective at reducing inhibition by tris(ethylenediamine)Co(III) than by RR. Indeed the IC_{50} values for tris(ethylenediamine)Co(III) measured at different free $[Ca^{2+}]$ levels were proportional to $[Ca^{2+}]^2$ rather than $[Ca^{2+}]$ as in the case of RR, suggesting that calcium can co-operatively and hence more effectively, overcome the inhibition by tris(ethylenediamine)Co(III) compared with RR.

Clearly the inhibitory nature of RR and tris(ethylenediamine)Co(III) were similar in that both demonstrated non-competitive and competitive behaviour, but differ in that calcium was much better at alleviating the inhibition by tris(ethylenediamine)Co(III) than RR. An

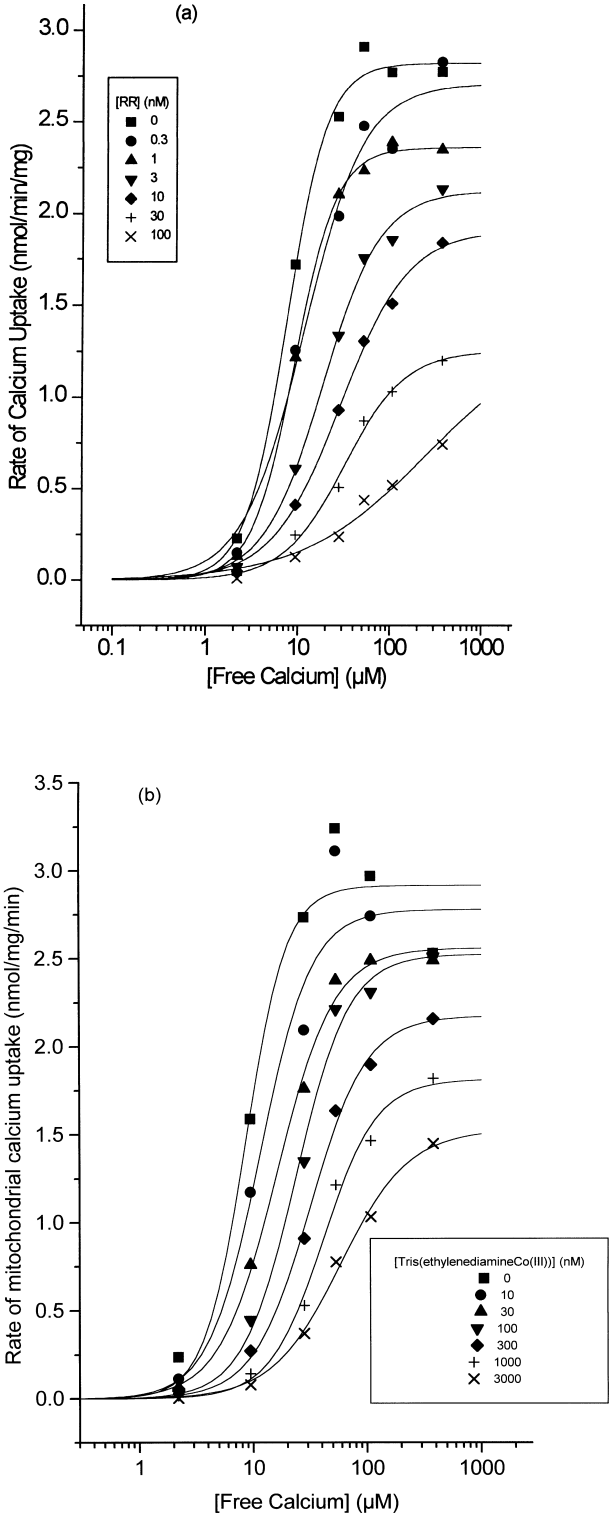


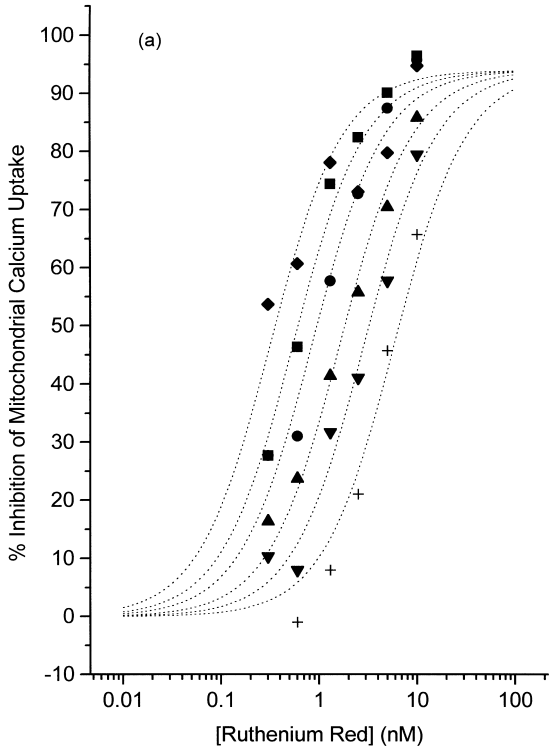
Figure 1. Effect of (a) RR and (b) Tris(ethylenediamine)Co(III) on the calcium dependency of mitochondrial calcium uptake. Data represents the geometric means from three separate experiments and for clarity SEM values were not plotted, but were < 10% of the mean.

alternative interpretation of the $[Ca^{2+}]$-dependency of the uptake rate at sub-optimal [inhibitor] is that although both inhibitor and Ca^{2+} may be competing for a common site, it is possible that a fraction of the uniporters are inhibited completely and that those that remain active gradually transport more calcium as the concentration increases. Furthermore these interaction studies assume that both calcium and inhibitors have equilibrated within the time course of the assay and it is possible that pre-incubation of kinetically slow, potent antagonists may lead to curve collapse of the $[Ca^{2+}]$-dependency of the uptake rate implying apparent non-competition with calcium.

Although the mechanism of calcium uptake by the uniporter is complex, the limited pharmacology described in this study suggests some common inhibitory behaviour of the different compounds, as well as some differences in the ability of free calcium to alleviate this inhibition. Clearly it would be of interest to investigate the differences in behaviour further by characterising other active compounds, especially the asymmetric Co complex described in Table 2.

Exploration of SAR

To further explore the pharmacophore required for inhibition, a variety of Co(III) complexes with ligands structurally related to tris(ethylenediamine) were examined (see compounds G–K, Table 2). Reducing the size of the complex ion by removal of the ethylene bridge between amines to give hexa-ammine Co(III) led to a 10-fold loss in activity. Similarly expansion of the size of the complex by either substitution with



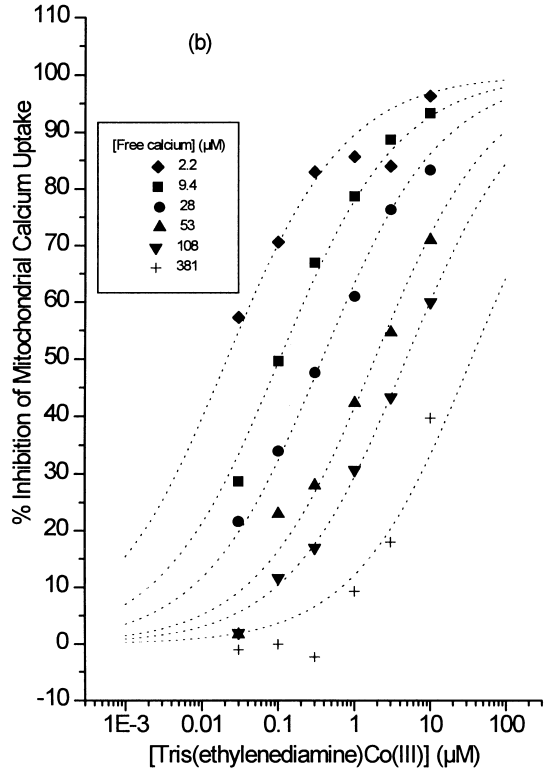


Figure 2. Dependency of (a) RR and (b) Tris(ethylenediamine)Co(III) inhibition of mitochondrial calcium uptake on free calcium. Data represents the geometric means from three separate experiments and for clarity SEM values were not plotted, but were $< 10\%$ of the mean.

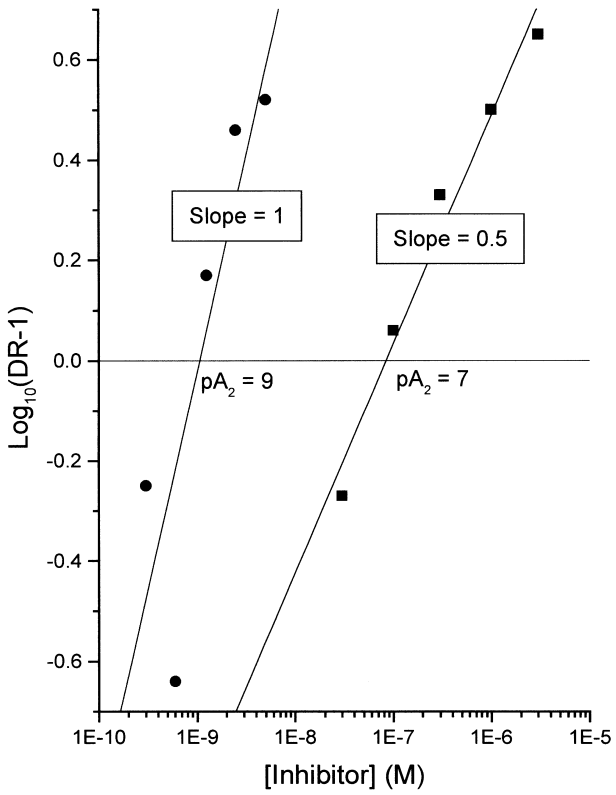


Figure 3. Schild plot of [inhibitor] against $\log_{10}\{\text{Dose Ratio (DR)} - 1\}$. Data represent the mean from three experiments for RR (●) and tris(ethylenediamine)Co(III) (■).

cyclohexanediamine ligands or linking adjacent ethylenediamines to give Co(III) sepulchrate, produced an even greater reduction in potency. More subtle structural modifications were investigated by using *N*-methyl ethylenediamine as a ligand, but this was less active (data not shown) than the tris(ethylenediamine)Co(III). In conclusion, exploration of the structural requirements for inhibition of mitochondrial calcium uptake by tris(ethylenediamine)Co(III) by altering the chemistry of the organic ligand, whilst maintaining molecular symmetry, could not improve upon the activity of this complex.

Asymmetric Co complexes were investigated to identify complexes more potent than tris(ethylenediamine)-Co(III) and of these the most interesting are compounds *J* and *K* in Table 2. A novel binuclear Co complex ion inhibitor (compound **J**) was discovered and compared to the recently described asymmetric Co(III) ion, [38] both had similar activity, which was fivefold less than that of tris(ethylenediamine)Co(III). In both cases activity might be improved upon by replacing free amine ligands with ethylenediamine, as there was a tenfold increase in activity between hexa-ammineCo(III) and tris(ethylenediamine)Co(III).

The novel mitochondrial calcium uptake inhibitors described in this paper have obvious advantages over RR in that they are easy to make, pure, smaller and hence potentially better at entering cells, as well as having a clearer SAR. In comparison RR is more potent, but is chemically complex, prone to nonspecific effects, has a poorly defined and limited SAR and is a larger molecule which may enter cells with more difficulty.

Altogether this work has demonstrated that both simple mononuclear Co(III) complexes as well as diverse binuclear Co complexes inhibited mitochondrial calcium uptake. The SAR of these small molecules has helped in defining the structural requirements for inhibition by outlining the size and charge dependency of the interactive inhibitory site(s) on the mitochondrial calcium uniporter. The fact that inhibitors were not restricted to simple symmetrical mononuclear complexes and included asymmetric complexes suggests that there is scope to characterise this SAR further and obtain better inhibitory molecules.

Methods

Materials

$^{45}CaCl_2$ was from Amersham (UK), JC-1 from Molecular Probes, all other reagents were of the highest grade commercially available from Sigma (UK). Ruthenium red and other transition metal complexes were obtained from commercial sources or prepared in the Medicinal Chemistry Department at Astra Charnwood. Tris(Cyclohexanediamine-*N*,*N*′)Co(III) was provided by Professor Miyoshi, Hiroshima, Japan. Binuclear Co complexes were kindly supplied by Dr. D. Davies, Department of Chemistry, Leicester University, Leicester, UK. $[Co(NH_2CH_2CH_2CH_2CH_2CH_2NH_3)(NH_3)_5]^{4+}$ was kindly provided by Dr. M. Crompton, Department of Biochemistry & Molecular Biology, University College, London, UK.

Preparation of mitochondria. Rat cardiac mitochondria were prepared by mechanical tissue disruption using the method described by McCormack and Denton.[33]

Calcium uptake assay. Mitochondria (~0.2 mg/mL protein) were pre-incubated in the absence (control) or presence of compounds or 5 μM FCCP (background) for 30 min in ice-cold 5 mM KH_2PO_4, 20 mM Tris–HCl (pH 7.4), 125 mM KCl, and substrates (5 mM 2-oxoglutarate and 0.5 mM L-malate). 50 μM $^{45}CaCl_2$ (37 KBq/mL) was added and the mitochondria harvested after 10 min by filtration through glass fibre (GF-B) filters followed by washing with buffer containing 2 mM EGTA to remove unassimilated $^{45}Ca^{2+}$. The filters were dried, scintillant added and counted in a scintillation counter. Under ice cold conditions, the uptake of calcium was easy to measure and linear for up to 12 min, so inhibition of the initial rate was determined.

Measurement of calcium uptake at differing free calcium concentrations. Basic assay conditions were used as described above, except that the mitochondrial preparation was diluted in incubation buffer to minimise EGTA carry over into the assay and effects on free calcium determination. Nitrilotriacetate/$^{45}CaCl_2$ was added in appropriate ratios to buffer assay free calcium between 1 and 400 μM. Free calcium levels were calculated using the program EQCAL. Inhibition curves were fitted to the Hill equation using Microcal Origin 4.1.

Inner mitochondrial membrane (IMM) potential assays. Uncoupling of mitochondria or nonspecific effects upon the membrane can affect calcium transport, therefore the effects of compounds on the mitochondrial membrane potential were quantified using the fluorescent membrane potential sensitive probe, 5,5′,6,6′-tetrachloro-1,1′-,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1).[34,35]

Mitochondria (~0.2 mg/mL protein) were incubated in 5 mM KH_2PO_4, 20 mM Tris–HCl (pH 7.4), 125 mM KCl, and substrates (5 mM 2-oxoglutarate and 0.5 mM L-malate) with 1 μM JC-1 in a 3 mL fluorimeter cuvette at 37°C to allow rapid dye equilibration. JC-1 fluorescence was monitored at λ_{ex} (505 nm) and λ_{em} (597 nm) using a Hitachi F-4010 fluorescence spectrophotometer. Fluorescence increased with time to a stable end point after 20 min, reflecting dye accumulation in response to the respiration driven membrane potential. Fluorescence was measured after 20 min in either the absence (control) or presence of compound. Background fluorescence was defined with mitochondria fully uncoupled by the addition of 5 μM FCCP.

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