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KB-R7943, a plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitor, blocks opening of the mitochondrial permeability transition pore

Brian M. Wiczer, Raluca Marcu, Brian J. Hawkins*

Department of Anesthesiology and Pain Medicine, Mitochondria and Metabolism Center, University of Washington, Seattle, WA, United States



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ABSTRACT

The isothiourea derivative, KB-R7943, inhibits the reverse-mode of the plasma membrane sodium/calcium exchanger and protects against ischemia/reperfusion injury. The mechanism through which KB-R7943 confers protection, however, remains controversial. Recently, KB-R7943 has been shown to inhibit mitochondrial calcium uptake and matrix overload, which may contribute to its protective effects. While using KB-R7943 for this purpose, we find here no evidence that KB-R7943 directly blocks mitochondrial calcium uptake. Rather, we find that KB-R7943 inhibits opening of the mitochondrial permeability transition pore in permeabilized cells and isolated liver mitochondria. Furthermore, we find that this observation correlates with protection against calcium ionophore-induced mitochondrial membrane potential depolarization and cell death, without detrimental effects to basal mitochondrial membrane potential or complex I-dependent mitochondrial respiration. Our data reveal another mechanism through which KB-R7943 may protect against calcium-induced injury, as well as a novel means to inhibit the mitochondrial permeability transition pore.

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1. Introduction

Mitochondrial Ca^{2+} -overload is a major contributor to ischemia/reperfusion injury [1–3]. Mechanistically, much of the organ damage associated with ischemia/reperfusion occurs during reperfusion as a result of a sudden elevation of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$). Mitochondria can rapidly sequester Ca^{2+} and act as an efficient buffering system to preserve cellular function [3]. However, the capacity of mitochondria to sequester Ca^{2+} is limited, and continued accumulation within the matrix will eventually impair mitochondrial function and bioenergetics. When this mitochondrial $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_m$) threshold is reached, cyclophilin D activates the mitochondrial permeability transition pore (mPTP) to rapidly release matrix Ca^{2+} back into the cytosol [4]. mPTP activation subsequently triggers a loss of mitochondrial function and initiates cell death [3]. Indeed, pharmacologic or genetic inactivation of the mPTP preserves mitochondrial function and organ health following ischemia/reperfusion [5]. However, pharmacologic mPTP blockers (i.e. cyclosporine A) have non-mitochondrial targets and are not practical therapeutic options. Agents that can inhibit the mPTP

independent of cyclophilin D may serve as alternative therapies to reduce ischemia/reperfusion injury.

A primary cause of the elevated $[\text{Ca}^{2+}]_c$ observed during reperfusion is Ca^{2+} entry from the extracellular milieu via the reverse-mode of the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX_{rev}) [6]. The isothiourea derivative, KB-R7943, is the prototypical inhibitor of NCX_{rev} [6,7]. Over the past decade, KB-R7943 has been used extensively to explore the effects of NCX_{rev} inhibition on ischemia/reperfusion injury, providing evidence that blocking NCX_{rev} is protective against ischemia/reperfusion-related myocardial [8–13] and neuronal damage [14–16]. Despite its apparent benefits, the precise mechanism through which KB-R7943 elicits protection remains controversial. In addition to the NCX_{rev} , KB-R7943 also lowers $[\text{Ca}^{2+}]_c$ by inactivating TRP channels [17], L-type voltage-gated Ca^{2+} channels [18], ryanodine receptors [19], NMDA receptors [20], and store-operated Ca^{2+} entry (SOCE) [21,22], though the specific molecular target of the latter is unknown. Despite these pharmacologic limitations and the availability of newer, more selective NCX_{rev} inhibitors [7], KB-R7943 is still widely used as an experimental tool to study ischemia/reperfusion injury. Recently, KB-R7943 was found to also potentially reduce mitochondrial Ca^{2+} uptake by inhibiting the mitochondrial Ca^{2+} uniporter (MCU) [23], which would effectively reduce mPTP activation during reperfusion. However, the original finding that KB-R7943 acts as an MCU inhibitor is not entirely consistent with results in subsequent studies [14,20] and requires further evaluation.

Abbreviations: NCX_{rev} , reverse-mode of NCX; mPTP, mitochondrial permeability transition pore; CRC, mitochondrial calcium retention capacity; $\Delta\Psi_m$, mitochondrial membrane potential; ECM, extracellular medium; MAB, mitochondria assay buffer; KRHB, Krebs–Ringers–HEPES–bicarbonate.

* Corresponding author. Address: University of Washington, 850 Republican St, Room N-134, Seattle, WA 98109-8057, United States. Fax: +1 206 616 4819.

E-mail address: bhawkins@uw.edu (B.J. Hawkins).

During our studies aimed at perturbing mPTP activation and mitochondrial Ca^{2+} uptake, we utilized KB-R7943 in AD293 cells that do not express any of the NCX isoforms [24,25] and lack other known targets. Surprisingly, we discovered that KB-R7943 does not block mitochondrial Ca^{2+} uptake. Rather, KB-R7943 influences mitochondrial Ca^{2+} handling by increasing the Ca^{2+} retention capacity (CRC), which we demonstrate protects mitochondrial function from pathological Ca^{2+} overload. As such, KB-R7943 may serve as a novel mPTP inhibitor in certain contexts as an alternative to cyclosporine A and its analogs.

2. Materials and methods

2.1. Cell culture and reagents

KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea) mesylate and CGP-37157 (7-Chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one) were purchased from Tocris. Fura-2/AM, Fura-FF pentapotassium salt, tetramethylrhodamine ethyl ester (TMRE), JC-1, propidium iodide, Hoechst 33342, pluronic F-127, as well as all cell culture reagents including 50× MEM amino acids, 100× MEM non-essential amino acids (NEAA), 100× sodium pyruvate and 200 mM L-glutamine were purchased from Life Technologies. Cyclosporine A (CsA), ruthenium red (RR), ionomycin, and all other chemicals were obtained from Sigma. HeLa cells and AD293 cells (Stratagene) were maintained in high glucose DMEM (Life Technologies, #11995-065) supplemented with 10% fetal bovine serum (Life Technologies) in a humidified cell culture incubator.

2.2. Fluorescence microscopy and spectrofluorometry

Fluorescence images were acquired using a Nikon Eclipse Ti microscope equipped with a xenon arc lamp and DeltaRamX monochromator (Photon Technology International), captured with an Evolve 512 EMCCD (Photometrics) with the assistance of Easy-RatioPro software. Fura-2 was excited at 340 nm and 380 nm and fluorescence detected using a 400 nm long-pass dichroic mirror and 510/80 m emission filter. TMRE and propidium iodide (PI) were excited at 545 nm and 535 nm, respectively and fluorescence detected using a 565 nm long-pass dichroic mirror and 610/75 m emission filter. Hoechst 33342 was excited at 360 nm and fluorescence detected as fura-2. A Nikon 40X Plan Fluor oil immersion lens objective was used to image fura-2 and TMRE. PI and Hoechst 33342 were imaged with a Nikon 10X Ph1 ADL objective.

Spectrofluorometric studies were performed as previously described [26].

2.3. Isolation of mouse liver mitochondria

Liver mitochondria were isolated from FVB/N mice aged 3–6 months using differential centrifugation as previously described [27]. Isolated mitochondria were then resuspended in a minimal amount of mitochondrial isolation buffer plus 0.1% BSA and kept on ice. All steps were performed on ice and using refrigerated centrifuges set to 4 °C. Protein concentration was determined by Bradford method. Mitochondria were used within 4 h.

2.4. Measurement of mitochondrial respiration

Mitochondrial respiration was assessed in permeabilized cells as described previously [28]. Briefly, AD293 cells were seeded at 1.5×10^6 cells per 10-cm culture dish. 2 days later, cells were harvested in 1 mL ambient temperature PBS using a cell lifter, briefly pelleted, and washed 1× with pre-warmed (30 °C) respiration

medium (20 mM HEPES, pH 7.1 at 30 °C, 64 mM KCl, 3 mM MgCl_2 , 0.5 mM EGTA, 110 mM mannitol, 10 mM KH_2PO_4 , 0.3 mM DTT). Cells were pelleted and resuspended in respiration medium with 40 $\mu\text{g}/\text{mL}$ digitonin and added to a calibrated Oxytherm chamber (Hansatech). Permeabilization was allowed to occur for 5 min, with subsequent addition of 5 mM sodium pyruvate/2.5 mM malate to initiate state 2 respiration. 2 mM Mg^{2+} -ADP was added to initiate state 3 respiration.

2.5. Live cell imaging of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) and mitochondrial membrane potential ($\Delta\Psi_m$)

Forty thousand AD293 cells were plated onto glass-bottom MatTek dishes coated with Cell-Tak (BD Biosciences), and media was replaced after 1 day with low serum (0.5% FBS) DMEM with 20 mM HEPES, pH 7.4. For imaging $[\text{Ca}^{2+}]_c$, cells were loaded with 5 μM fura-2/AM for 20 min at ambient temperature in a modified DMEM-like Krebs–Ringers–HEPES–bicarbonate (KRHB) buffer (20 mM HEPES, 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1.8 mM CaCl_2 , 5 mM NaHCO_3 , 25 mM glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, 1× MEM AA, 1× MEM NEAA, pH 7.4), with 0.006% (w/v) pluronic F-127, and 100 μM sulfinpyrazone. Cells were subsequently washed with and imaged in KRHB plus 100 μM sulfinpyrazone. Images were acquired every 4 s and the nuclear 340 nm/380 nm fluorescence ratio was calculated and applied to a predetermined $[\text{Ca}^{2+}]_c$ calibration curve.

Cellular $\Delta\Psi_m$ was evaluated using 50 nM TMRE in modified KRHB buffer. Cells were washed 1× with TMRE-free KRHB (pH

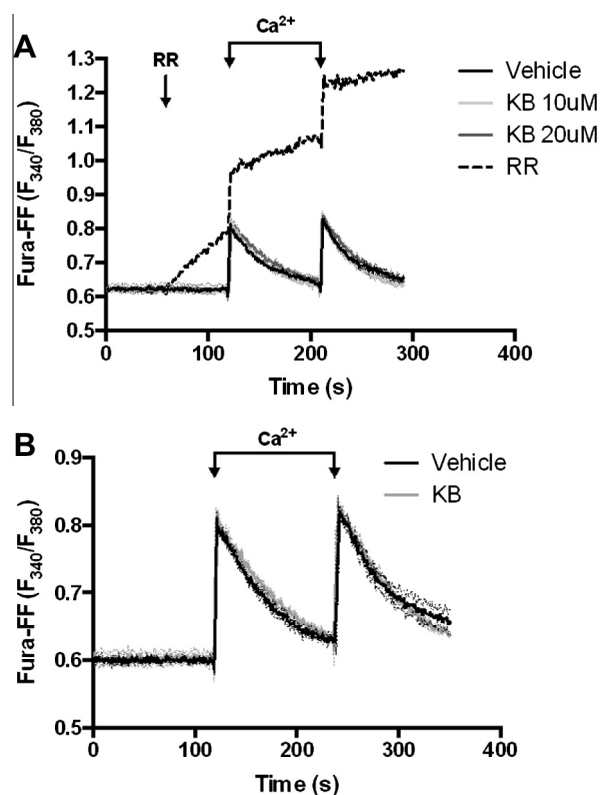


Fig. 1. KB-R7943 does not inhibit mitochondrial Ca^{2+} uptake. Permeabilized AD293 cells (A) and HeLa cells (B) were pulsed with 3 nmol Ca^{2+} where indicated. The indicated concentration of KB-R7943 (20 μM in (B)) was added at the onset of permeabilization and present throughout the experiment. Vehicle is 0.05% DMSO, making the total [DMSO] per experiment 0.25% (v/v). In (A), ruthenium red (RR, 2 μM) was added where indicated. Traces are represented as the mean (solid lines) \pm SEM (dashed lines) of 3 independent experiments. The RR positive control is a single trace from a representative experiment.

7.4) and imaged in KRHB at ambient temperature and atmosphere. Images were acquired every 60 s to obtain mitochondrial TMRE fluorescence. Individual traces were normalized to the initial fluorescence intensity.

2.6. Measurements of mitochondrial Ca^{2+} uptake and $\Delta\Psi_m$ in permeabilized cells and isolated mitochondria

Mitochondrial Ca^{2+} uptake and $\Delta\Psi_m$ were measured in permeabilized cells or isolated mitochondria as previously described [26]. Briefly, cells were seeded onto 6 cm culture plates and serum deprived as above. On the following day, the confluent monolayers were washed in ice-cold extracellular medium (ECM) (20 mM HEPES, 120 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4 , 0.2 mM MgCl_2 , 0.1 mM EGTA, pH 7.4 at 0 °C), harvested in ECM using a cell lifter, and left on ice for 20 min. Cells were then washed in ice-cold PBS and immediately resuspended in 1 mL of pre-warmed (30 °C) mitochondria assay buffer (MAB) plus 40 $\mu\text{g}/\text{mL}$ digitonin and 1 μM thapsigargin ([DMSO] = 0.2% (v/v)), and inserted into the spectrofluorometer set to 30 °C. Cells were allowed to permeabilize for 5 min in the presence of 5 mM sodium pyruvate/2.5 mM malate, and 1.25 μM Fura-FF or 500 nM JC-1 to measure Ca^{2+} uptake or $\Delta\Psi_m$, respectively. Isolated liver mitochondria (0.25 mg/mL) were incubated in MAB, without digitonin and thapsigargin, in the presence of substrates and dyes for 5 min. Both permeabilized cells and isolated mitochondria were pulsed with 3 nmol CaCl_2 .

2.7. Cell death analysis

AD293 cells were plated at 5×10^5 cells per well of a 6-well plate coated with 0.1% gelatin. Next day, cells were refreshed with media. 24 h later, media was replaced with a modified DMEM-like

KRHB buffer. Cells were treated with 1.5 μM ionomycin for 5 h at 37 °C in culture incubator. PI (1 $\mu\text{g}/\text{mL}$) and Hoechst 33342 (5 $\mu\text{g}/\text{mL}$) were added 1 h prior to fluorescence microscopic imaging. For each experiment, 6 fields were acquired per condition. Fluorescent nuclei were counted using ImageJ, and cell death calculated as the percentage PI positive nuclei relative to Hoechst 33342 nuclei.

2.8. Statistical analysis

Values are expressed as the mean \pm standard error of the mean (SEM). Student's *t*-test, or one-way ANOVA or two-way ANOVA with a Dunnett's multiple comparisons test were performed where appropriate using GraphPad Prism v6.0. Differences were considered statistically significant if $p < 0.05$.

3. Results and discussion

3.1. KB-R7943 does not inhibit mitochondrial Ca^{2+} uptake in permeabilized cells

Mitochondrial Ca^{2+} uptake was evaluated in permeabilized AD293 (Fig. 1A) and HeLa (Fig. 1B) cells as the depletion rate of extramitochondrial calcium using membrane-impermeant Fura-FF in response to 3 nmol Ca^{2+} pulses. In both AD293 and HeLa cells, Ca^{2+} was rapidly taken up by mitochondria and effectively blocked by the traditional MCU inhibitor, ruthenium red (RR) (Fig. 1). Individually, RR elicited a rapid increase in extramitochondrial Ca^{2+} which was ablated in the presence of the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ -exchanger CGP-37157 (data not shown), implying there is a continuous flux of mitochondrial Ca^{2+} [29]. Surprisingly, mitochondrial Ca^{2+} uptake was not inhibited in the presence of

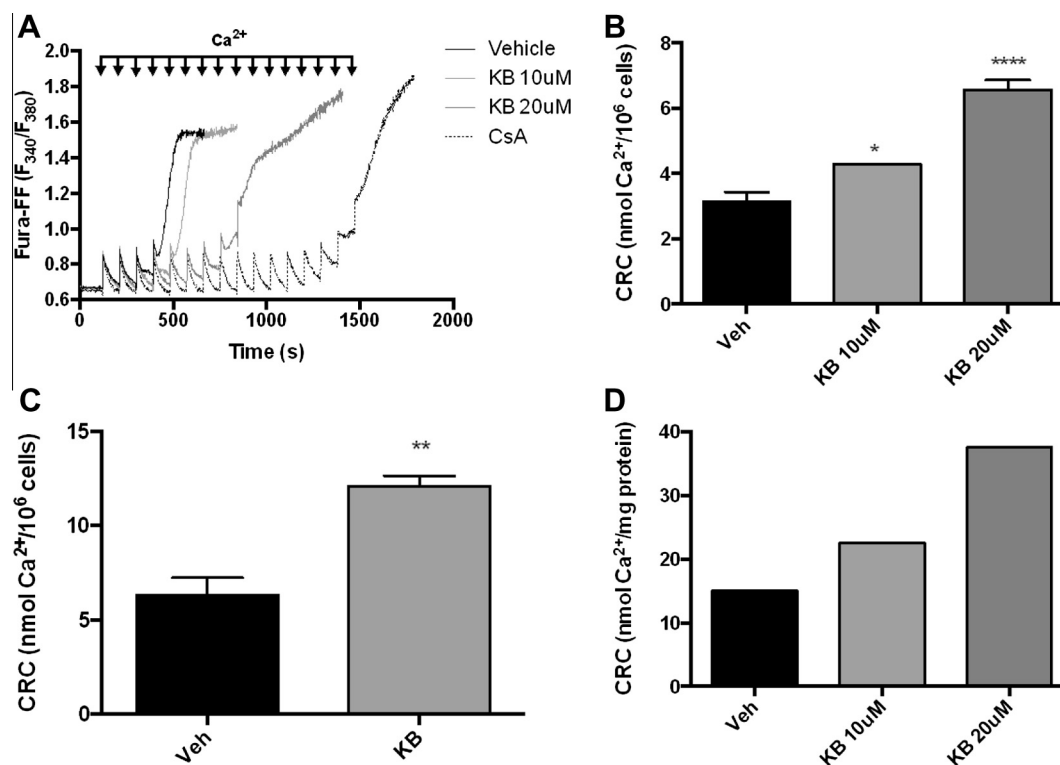


Fig. 2. KB-R7943 increases mitochondrial Ca^{2+} retention capacity. Ca^{2+} pulses were administered as in Fig. 1 to activate the mPTP. KB-R7943 or CsA (1 μM) was added 5 min prior to the start of data acquisition. (A) Data are representative traces obtained during experiments using permeabilized AD293 cells. (B–D) Graphical representation of Ca^{2+} retention capacity (CRC) as calculated from data obtained in experiments similar to (A). CRC was determined in permeabilized AD293 cells (B) and HeLa cells (C), and isolated liver mitochondria (D). CRC = (number of Ca^{2+} pulses required to open the PTP) \times (nmol $\text{Ca}^{2+}/\text{pulse}$). (B and C) Data represented as the mean \pm SEM of 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$ as compared to vehicle. (D) Data represented as the mean \pm SEM of 3 experiments from one mouse liver preparation.

KB-R7943 at either 10 or 20 μM , contrary to the previous initial report [23]. It is unclear why our results differ considering that HeLa cells were used in both cases. While different experimental techniques were used to measure mitochondrial Ca^{2+} uptake (i.e., increase in $[\text{Ca}^{2+}]_m$ using targeted aequorin in the former study versus decrease in extramitochondrial Ca^{2+} in the present work), both methods have been validated to measure changes in mitochondrial Ca^{2+} uptake [30]. However, evaluating the technical merits of aequorin versus Fura-FF was not a focus of our research and requires further testing. Nonetheless, our observations are consistent with previous reports that also imply Ca^{2+} uptake into isolated brain mitochondria is not blocked by KB-R7943 [14,20]. Together, these findings suggest that KB-R7943 does not directly influence mitochondrial Ca^{2+} uptake and that caution should be applied when using this compound to evaluate mitochondrial Ca^{2+} dynamics.

3.2. KB-R7943 increases the mitochondrial Ca^{2+} retention capacity

Despite no detectable effect on Ca^{2+} uptake, we unexpectedly noticed that KB-R7943 did consistently increase the number of Ca^{2+} pulses that could be effectively sequestered by permeabilized cells. Indeed, direct evaluation of this observation revealed that KB-R7943 addition resulted in a dose-dependent increase in the number of Ca^{2+} pulses required to engage the mPTP (Fig. 2A). The number of Ca^{2+} pulses required to open the mPTP was counted and quantified as the mitochondrial Ca^{2+} retention capacity (CRC) (Fig. 2B) [31]. A similar increase in CRC was also found in HeLa cells (Fig. 2C) and in isolated liver mitochondria (Fig. 2D), demonstrating that the effect of KB-R7943 on the CRC is a ubiquitous phenomenon. KB-R7943 was not, however, as effective as the classical mPTP inhibitor, CsA, at increasing the CRC (Fig. 2A). However, KB-R7943 nearly doubled CsA-mediated mPTP inhibition (data not shown), hinting that these pharmacologic agents behave synergistically and have distinct molecular targets.

3.3. KB-R7943 protects against Ca^{2+} -induced mitochondrial depolarization and cell death

If indeed KB-R7943 increases the CRC, we hypothesized that it might also attenuate mPTP opening and mitochondrial membrane potential ($\Delta\Psi_m$) loss in response to mitochondrial Ca^{2+} overload. We therefore utilized the Ca^{2+} ionophore, ionomycin, to activate the mPTP in a Ca^{2+} -dependent manner [32–36]. AD293 cells loaded with the cationic fluorophore TMRE were visualized using real-time fluorescence microscopy to assess relative changes in $\Delta\Psi_m$ in response to 4 μM ionomycin. As predicted, ionomycin challenge evoked a time-dependent $\Delta\Psi_m$ depolarization (Fig. 3A). In contrast, $\Delta\Psi_m$ loss was prevented by 20 μM KB-R7943, consistent with the ability of KB-R7943 to increase the CRC.

Because KB-R7943 blocked Ca^{2+} -induced mPTP opening and $\Delta\Psi_m$ depolarization, we posited that it would also protect against Ca^{2+} -induced cell death. To test this hypothesis, AD293 cells were treated with ionomycin for 5 h and cell death quantified as the percentage of cells staining positive for the membrane impermeable propidium iodide (PI), which incorporates into the nucleus when the plasma membrane is compromised. Supportive of our hypothesis, we found that KB-R7943 attenuated ionomycin-induced cell death (Fig. 3B and C). In light of these findings, it is possible that some of the published benefits of KB-R7943 in ischemia/reperfusion injury may stem from an inhibition of the mPTP and mitochondrial-mediated cell death.

3.4. Effect of KB-R7943 on basal $\Delta\Psi_m$ and mitochondrial respiration

Previous reports purport that KB-R7943 is neuroprotective effect due in part to mild $\Delta\Psi_m$ depolarization and complex I inhibi-

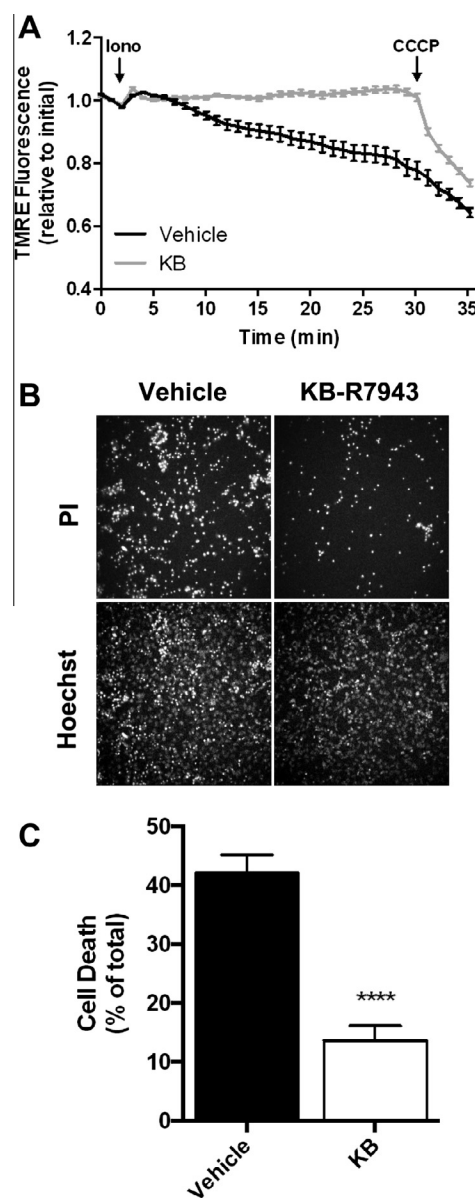


Fig. 3. KB-R7943 protects against ionomycin-induced $\Delta\Psi_m$ depolarization and cell death. (A) AD293 cells were loaded with TMRE in the presence of vehicle (0.2% DMSO) or 20 μM KB-R7943. Cells were subsequently treated with 4 μM ionomycin and single-cell fluorescence measurements were taken every minute. At the end of every experiment, 1 μM CCCP was added to depolarize $\Delta\Psi_m$ in all cells. The data are represented as the mean \pm SEM of $n = 124$ cells and $n = 128$ cells for vehicle- and KB-R7943-treated cells, respectively, obtained from 3 independent experiments. (B and C) AD293 cells were pretreated with vehicle or 20 μM KB-R7943 for 10 min prior to the addition of 1.5 μM ionomycin. Propidium iodide (PI) and Hoechst 33342 fluorescence was assessed after 5 h. (B) Representative images of stained cells post-treatment. (C) Graphical representation of images acquired. Cell death percentage for each field was quantified as the (# of PI-positive cells/# of Hoechst-positive cells) \times 100%. The data are represented as the mean \pm SEM of $n = 6$ fields and representative of 3 independent experiments. **** $p < 0.0001$ relative to vehicle.

tion [14,20]. Thus, it is possible that the effects of KB-R7943 against Ca^{2+} -induced $\Delta\Psi_m$ depolarization in our system may be due to an adaptive response incurred by mild mitochondrial dysfunction in addition to increasing the CRC. Furthermore, complex I inhibition can alone increase the CRC in both permeabilized cells and isolated mitochondria [31], though this was demonstrated in the presence of the complex II substrate, succinate. Therefore, we assessed complex I-dependent basal $\Delta\Psi_m$ and mitochondrial respiration in permeabilized AD293 cells in response to KB-R7943. Using the ratiometric $\Delta\Psi_m$ indicator, JC-1, mitochondria energized with

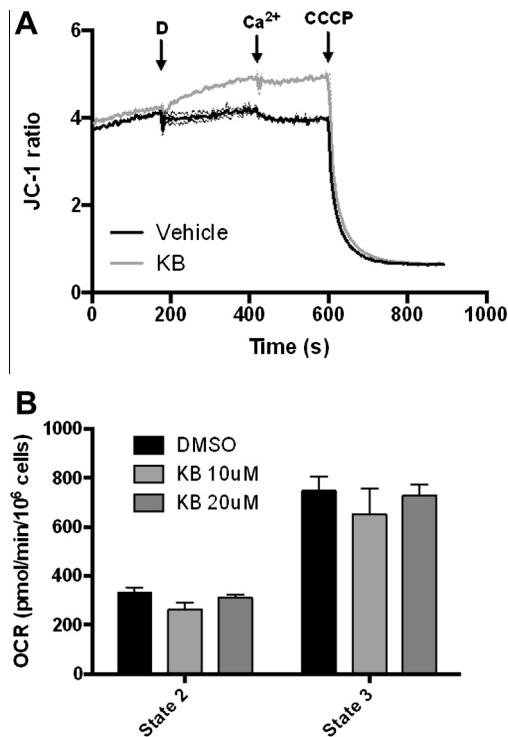


Fig. 4. KB-R7943 increases $\Delta\Psi_m$ but does not effect mitochondrial respiration. (A) Permeabilized AD293 cells were incubated with JC-1 during permeabilization to allow aggregate/monomer equilibration. After baseline JC-1 ratio (aggregate/monomer fluorescence) was acquired, 20 μM KB-R7943 or vehicle (0.05% DMSO) was added where indicated (D). After stabilization of JC-1 ratio, 3 nmol Ca^{2+} was added where indicated (Ca^{2+}), followed by addition of 1 μM CCCP to achieve complete $\Delta\Psi_m$ depolarization. Data are represented as the mean (solid line) \pm SEM (dashed line) of 2 independent experiments. (B) Mitochondrial respiration of permeabilized AD293 was measured using 5 mM pyruvate/2.5 mM malate with (state 3) or without (state 2) ADP. 10 or 20 μM KB-R7943 was added during permeabilization. O_2 consumption was measured during state 2 for at least 2 min before initiating state 3. O_2 consumption rate (OCR) was calculated from the slopes of the acquired traces. Data represented as the mean \pm SEM of 3 experiments.

complex I substrates (pyruvate + malate) did not exhibit a reduction in $\Delta\Psi_m$ following KB-R7943 addition. Rather, we observed a slight hyperpolarization in $\Delta\Psi_m$ relative to vehicle treated cells (Fig. 4A). A single Ca^{2+} pulse did, however, produce a transient decrease in $\Delta\Psi_m$ indicative of the transmembrane import of positively-charged Ca^{2+} into the matrix [37]. This slight reduction in $\Delta\Psi_m$ was unaffected by KB-R7943, providing further support that KB-R7943 does not impact mitochondrial Ca^{2+} import (Fig. 1). Aside from $\Delta\Psi_m$, KB-R7943 did not affect mitochondrial state 2 or state 3 respiration at any of the doses tested (Fig. 4B). Taken together, these data demonstrate that the protective effect of KB-R7943 on Ca^{2+} -induced mPTP opening and cell death is not due to mitochondrial respiratory impairment and $\Delta\Psi_m$ alterations, but rather, to its ability to increase mitochondrial Ca^{2+} handling capacity.

As we find no negative impact of KB-R7943 on $\Delta\Psi_m$ and respiration, it is unclear why our results contrast those found in similar experiments performed using brain mitochondria [20]. In those studies, KB-R7943 decreased the CRC of brain mitochondria when energized on complex I substrates [20] and runs counter to our present results. It should be noted that the decrease in CRC they observed was due to a respiration-dependent inhibition of mitochondrial Ca^{2+} uptake after reaching some $[\text{Ca}^{2+}]_m$ -threshold rather than due to an effect on mPTP opening. Therefore, this apparent discrepancy may highlight a phenotypic difference between neuronal and non-neuronal mitochondria. While we demonstrate that

KB-R7943 inhibits Ca^{2+} -dependent mPTP opening in multiple systems, the exact mechanism remains elusive. Nonetheless, while KB-R7943 is clearly a promiscuous pharmacologic agent with multiple Ca^{2+} -regulatory targets, it may serve as a novel mPTP inhibitor in certain contexts as an alternative to cyclosporine A and its analogs.

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