

Bedaquiline inhibits the ATP synthase leak channel and prevents glutamate-induced neuronal death

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ABSTRACT F_0F_1 -ATP synthase is one of the most abundant proteins of the mitochondrial inner membrane and the primary enzyme responsible for ATP production in eukaryotic cells. Nevertheless, it was recently reported to play a prominent role in cell death by forming a large-conductance leak channel in the mitochondrial permeability transition pore (mPTP), making it a promising therapeutic target. Bedaquiline (BDQ), a member of the diarylquinoline class of drugs, was shown to selectively inhibit the catalytic activity of *Mycobacterium tuberculosis* ATP synthase with no effect on the mammalian enzyme. Here, we report a new role for BDQ as a potent inhibitor of the ATP synthase c-subunit leak channel in mammals. BDQ inhibited the single-channel activity of porcine heart ATP synthase in planar lipid bilayer recordings and inner membrane channel activity of porcine mitochondria in patch-clamp recordings, and it prevented glutamate-induced cell death in primary hippocampal neurons. These findings reveal the potential new application of BDQ for treating mPTP-related diseases by targeting the ATP synthase c-subunit leak channel.

WHY IT MATTERS Bedaquiline (BDQ) is the only FDA-approved drug to treat pulmonary multidrug-resistant tuberculosis (TB), caused by *Mycobacterium tuberculosis*. BDQ cures TB by specifically targeting mycobacterial ATP synthase and inhibiting ATP production. Recently, BDQ was reported to also bind to mammalian ATP synthase at the interface between the a- and c-subunits and to inhibit its catalytic activity. However, the effect of BDQ on ATP synthase leak channel activity has not been explored. Here, we report that BDQ inhibits ATP synthase c-subunit leak channel (ACLC) activity and prevents glutamate-induced neuronal death, suggesting a new therapeutic repurposing of BDQ for treating ACLC-related diseases.

INTRODUCTION

The mitochondrial permeability transition pore (mPTP) plays a central role in cell death during ischemia/reperfusion (I/R) injury of the heart and brain, as well as in neurodegenerative diseases.^{1,2} The prolonged opening of the mPTP leads to mitochondrial swelling and rupture, the release of cytochrome *c* and other mitochondrial signaling molecules, and activation of cell death via apoptotic or necrotic pathways.^{2,3} Changes in inner mitochondrial membrane (IMM) permeability transition (mPT) occur due to the activation of the

large-conductance, non-selective megachannel of the IMM, which is of unknown molecular composition. Over the years, various mitochondrial proteins, such as the voltage-dependent anion channel,^{4,5} the phosphate carrier,⁶ the translocator protein,⁷ and the adenine nucleotide translocator (ANT),⁸ have been suggested as core components of the mPTP. However, studies involving the genetic deletion of these proteins have shown that they function more as modulators of the pore rather than as its structural or pore-forming elements.^{9–12}

Over the past decade, growing evidence has suggested a key role of ATP synthase in mPTP formation.^{2,13–16} Purified mammalian ATP synthase forms a large-conductance, voltage-gated ion channel in electrophysiological recordings, with a peak conductance

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similar to that of the mPTP and sensitivity to divalent and trivalent ions (Ca^{2+} , Ba^{2+} , and Gd^{3+}),^{14,17,18} and cyclophilin D (CypD).¹⁹ According to one of the current models, the channel is located between the ATP synthase dimers.^{13,18} F_0 subunits e and g, which facilitate ATP synthase dimerization, were shown to be involved in channel formation.^{16,20–22} In addition, the purified ATP synthase c-ring was reported to form a voltage-gated channel, and its genetic ablation eliminated the Ca^{2+} -sensitive large-conductance activity of mitochondria, supporting a possible role of the c-ring in mPTP formation.^{23,24} Low-conductance channel activity, sensitive to the ANT inhibitor bongkreikic acid, was still present in c-subunit knockout cells, suggesting a possible contribution of multiple pores to mPT.^{23,25} Liver mitochondria from mice with triple deletion of *Ant1*, *Ant2*, and *Ant4* still demonstrated cyclosporin A (CsA)-sensitive mPT at increased matrix Ca^{2+} overload, and only deletion of the *Ppif* gene encoding CypD in these mice completely prevented Ca^{2+} -induced mPT.²⁶ These findings suggested a new mPTP model consisting of two distinct molecular components, ANT and another CypD-regulated channel, that mediate mPTP formation.²⁶

ATP synthase is a multisubunit complex consisting of the F_1 and F_0 domains²⁷ connected through the central- and peripheral-stalk subunits. The hydrophilic F_1 domain is situated in the matrix, while most of the F_0 subunits, including the c-ring, are hydrophobic, membrane-embedded proteins.^{27–29} Conformational changes in the F_1 domain that can be transmitted to F_0 through the peripheral stalk were proposed to be crucial in activating the ATP synthase c-subunit leak channel (ACLC).^{2,30,31} Thus, compounds that specifically bind to ATP synthase may modulate its catalytic^{32,33} (ATP synthesis and hydrolysis) and/or leak channel activities.^{17,34}

Bedaquiline (BDQ), also known as Sirturo, binds at the interface of F_0 subunits a and c, inhibiting the catalytic activity of *Mycobacterium tuberculosis* ATP synthase.¹⁶ It is also the first antimycobacterial drug approved by the Food and Drug Administration (FDA) for the treatment of pulmonary multidrug-resistant TB.^{35,36}

Prior studies have reported BDQ as a selective and potent inhibitor of mycobacterial, but not mammalian, ATP synthase.³⁷ However, BDQ was recently shown to bind and inhibit the catalytic activity of yeast and human ATP synthases.^{38,39}

Here, we report the new role of BDQ as a modulator of the mammalian ATP synthase c-subunit leak channel activity. We show that BDQ inhibits the single-channel activity of ACLC in a dose-dependent manner in planar lipid bilayer recordings of purified porcine

heart ATP synthase. It also inhibits the inner membrane channel activity of porcine heart mitochondria in patch-clamp recordings. Additionally, we demonstrate that BDQ prevents glutamate-induced cell death in primary hippocampal neurons, indicating a vital role of ACLC in regulating cell death under excitotoxic conditions. These findings suggest repurposing BDQ to treat ACLC-induced pathologies by inhibiting ATP synthase leak channel activity.

MATERIALS AND METHODS

Isolation of porcine heart mitochondria and purification of ATP synthase

Porcine heart mitochondria were isolated as described previously.¹⁷ In brief, the freshly collected porcine heart was finely minced on ice and transferred to 200 mL of buffer A (225 mM mannitol, 75 mM sucrose, 0.5 mM EGTA, 0.5% BSA, and 20 mM Tris [pH 7.4]). Heart tissue was homogenized and centrifuged at $1,000 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $6,000 \times g$ for 15 min at 4°C. The obtained mitochondrial pellets were stored at -80°C for further use. ATP synthase was purified using differential centrifugation and size-exclusion chromatography as previously described.¹⁷ Soluble ATP synthase fraction (in 0.5% *n*-dodecyl- β -D-maltopyranoside [DDM]) was loaded into a Superose 6 column (Cytiva) equilibrated with 1.5 column volumes of elution buffer (20 mM Tris, 100 mM NaCl, and 0.05% DDM [pH 7.8]). Protein elution was performed in the same buffer, and 0.5-mL fractions were collected using an automated fraction collector (Cytiva). Pooled fractions containing ATP synthase were used in experiments.

Preparation of mitoplasts from porcine heart mitochondria

Mitoplasts were prepared as described previously.⁴⁰ In brief, the mitochondrial preparation was incubated in a hypotonic solution (30 mM KCl, 8 mM NaCl, 10 mM HEPES, and 0.5 mM EGTA [pH 7.3]) for 30 min and centrifuged at $10,600 \times g$ for 15 min. The supernatant was discarded, and the pellet was resuspended in a hypertonic solution (700 mM KCl, 8 mM NaCl, 10 mM HEPES, and 0.5 mM EGTA [pH 7.3]) to restore isotonicity and kept on ice until use.

Calcium retention capacity assay

Mitochondria were isolated from HEK293 cells using a Qproteome Mitochondrial Isolation Kit (Qiagen) according to the manufacturer's protocol. Calcium

retention capacity (CRC) was carried out with 25 μg of isolated mitochondrial protein in CRC buffer (150 mM sucrose, 1 mM KH_2PO_4 , 1 mM MOPS, 5 mM succinate, and 10 μM EGTA [pH 7.4]) containing 1 μM Calcium Green-5N. 400 μM ATP and/or 10 μM CsA were used as a control. Fresh BDQ stocks were made in DMSO. The concentrations of BDQ used were 8, 4, 0.4, 0.04, 0.002, and 0.0002 $\mu\text{M}/\mu\text{g}$ mitochondrial protein. Data acquisition was carried out with a microplate reader (SpectraMax-iD3; Molecular Devices) at excitation and emission wavelengths of 485 and 535 nm, respectively. Calcium pulses (5 μM) were added every 2 min, and fluorescence data were recorded every 10 s. The assay was implemented in a 96-well plate with a final volume of 100 μL . All assays were performed at 23°C–25°C.

Spectral shift assay

Purified porcine heart ATP synthase was labeled with the Red-NHS second-generation dye using a protein labeling kit (NanoTemper Technologies) according to the manufacturer's protocol. In brief, ATP synthase was incubated with Red-NHS dye, followed by removal of the free unreacted dye using the desalting column. Labeled ATP synthase (ATP synthase-Red-NHS) was used in the spectral shift experiments (Monolith X; NanoTemper Technologies). The experiment was performed at 10% excitation with medium infrared laser power at 25°C. Spectral shift was measured for all samples as the ratio of fluorescence intensities at 670 nm and 650 nm and is plotted against the logarithm of the BDQ concentration. Sixteen serial dilutions of BDQ, ranging from 100 μM to 3 nM, were used in the experiments. The binding affinity, K_D , was determined from the obtained sigmoidal binding curve. Both data acquisition and fitting were performed using MO Control 2 software (NanoTemper Technologies).

ATP hydrolysis assay of purified ATP synthase and isolated mitochondria

ATP hydrolysis (ATPase) activity was measured in ATP cocktail buffer (50 mM Tris/ H_2SO_4 , 10 mM ATP, and 4 mM MgSO_4 [pH 8.0]) at 37°C as described earlier.¹⁷ One unit of enzymatic activity (U) corresponds to 1 μmol of ATP hydrolyzed (equivalent to 1 μmol of Pi produced) per minute per mg protein. The effect of BDQ on the ATP hydrolysis activity of porcine heart ATP synthase was determined in the presence of 0.01–100 μM BDQ.

Mitochondria from porcine heart and HEK293 were isolated as described above. ATPase activity was measured in isolated mitochondria using the Mito-Check Complex V Activity Assay Kit (Cayman Chemi-

cal) according to the manufacturer's protocol. The effect of BDQ on the ATPase activity was determined in the presence of 0.1–200 μM BDQ. Data were fitted to the Dose-Response-Inhibition Model in GraphPad Prism (10.1.0). Oligomycin (5 $\mu\text{g}/\mu\text{L}$) was used as a positive control.

Single-channel recordings of porcine heart mitochondria and purified ATP synthase

Planar lipid bilayer recordings were implemented by a 13-mm Delrin cuvette (Warner Instruments) with an aperture of 200 μm . Lipids were prepared by drying a 15- μL aliquot of L- α -phosphatidylcholine (Avanti Polar Lipids; 20 mg/mL in chloroform) under N_2 gas in a glass cuvette, then redissolving it in 60 μL of *n*-decane (Thermo Fisher Scientific, Waltham, MA). After adding intracellular solution (120 mM KCl, 8 mM NaCl, 0.5 mM EGTA, and 10 mM HEPES [pH 7.3]) on both the *cis* and *trans* sides of the cuvette, the prepared lipid was directly painted onto the cuvette aperture using a fine paintbrush (Tintoretto, Italy). The stability of the bilayer was assessed by the resistance (5–7 G Ω) and C-pipette values (50–120 pF). Purified ATP synthase was added only on the *cis* side, and a constant voltage of ± 40 to ± 120 mV was applied to achieve protein insertion into the bilayer. Recordings were carried out in the presence of 1 mM Ca^{2+} . A final concentration of 0.005 μM –25 μM BDQ was added on the *cis* side during the recordings to study its effect on the ATP synthase channel activity. Data acquisition was performed using an ePatch amplifier (Elements) with EZ Patch software.

For patch-clamp recordings, a small volume of the mitoplast suspension (5–10 μL) was added to a recording chamber and allowed to settle and adhere to the chamber bottom for 15–20 min. Mitoplasts were identified by their characteristic round, spherical morphology and diameter of approximately 1 μm using a Nikon Eclipse Ti2 inverted research microscope (Nikon Instruments, Melville, NY) at 60 $\times$ magnification. An Axopatch 200B amplifier and a Digidata 1550B (Axon Instruments/Molecular Devices, San Jose, CA) were used for recordings. Fire-polished borosilicate glass pipettes (Kwik fill; World Precision Instruments, Sarasota, FL) had resistances of 100–110 M Ω . Both pipette and bath solutions contained 120 mM KCl, 8 mM NaCl, 10 mM HEPES, and 0.5 mM EGTA (pH 7.3). The bath solution was held at ground (0 mV). Current signals were low-pass filtered at 1 kHz using a five-pole Bessel filter and digitized at 10 kHz. All experiments were performed at room temperature (22°C–24°C). Recordings were run in the presence of 1 mM Ca^{2+} . BDQ was applied

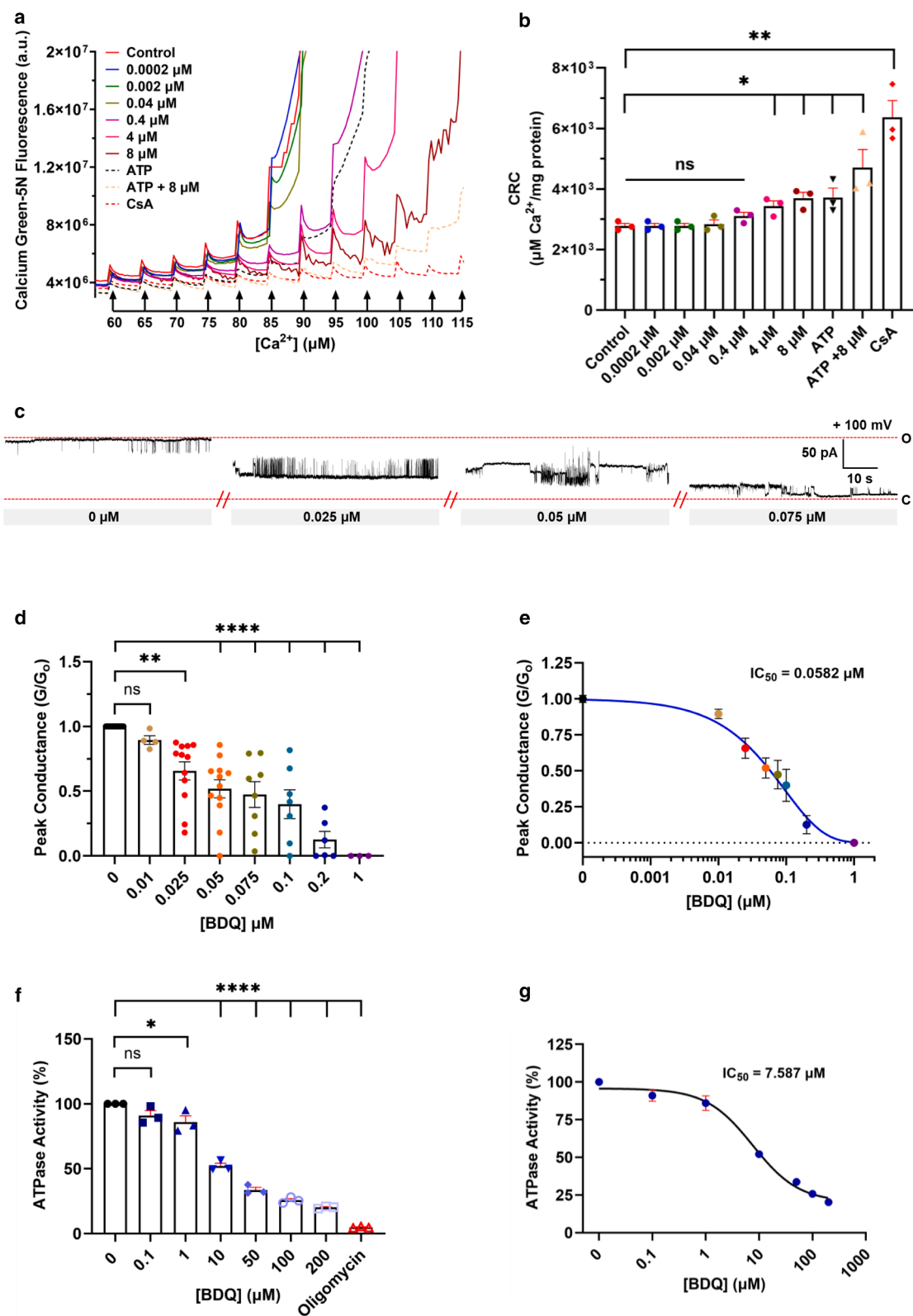


Figure 1. BDQ delays the opening of the mitochondrial permeability transition pore, inhibits single-channel conductance of the mitochondrial inner membrane, and inhibits ATP hydrolysis activity of ATP synthase. (a) Representative CRC assay of mitochondria (25 µg) isolated from HEK293 cells in the presence of BDQ (0.0002–8 µM). ATP (400 µM) and CsA (10 µM) were used as positive controls. (b) Group data for
(legend continued on next page)

locally to the bath solution by direct addition from the working stock to achieve the desired final concentrations. Cumulative additions were made to reach final concentrations of 0.01, 0.025, 0.05, 0.075, 0.1, 0.2, and 1 μM sequentially within individual recordings.

Clampfit 11 software (Molecular Devices) was used for data analysis. The measured current was adjusted for the holding voltage, assuming a linear current-voltage (I/V) relationship. The conductance (G) is expressed in nS, following the equation $G = I/V$, where I is in pA and V is in mV. Group data were evaluated in terms of conductance, and the represented error bars are in $\pm$ SEM.

Prior to analysis, recordings were digitally filtered with a Gaussian filter at 2 kHz using Clampfit 11. Conductance values measured in the presence of BDQ (Figures 1d and 1e) were normalized to the control recorded prior to drug application within the same patch (G/G_0), where G_0 represents the control peak conductance.

Culture of primary hippocampal neurons

Primary rat hippocampal neurons were prepared from rat fetuses (Sprague-Dawley, day 18 of gestation; Envigo, Indianapolis, IN) as described previously.^{41–44} In brief, neurons (0.3×10^6 cells/35-mm plate) were seeded on poly-L-lysine-coated plates and grown in neurobasal medium supplemented with B-27, glutamine, and antibiotics (Invitrogen, Waltham, MA) for 20–22 days *in vitro*. For BDQ treatment, neurons were either treated with BDQ (0.1, 0.1, 1, and 5 μM ; AdooQ Bioscience, Irvine, CA) or a vehicle (DMSO). BDQ was added to the hippocampal neurons 24 h before any downstream assays (propidium iodide [PI] staining, lactate dehydrogenase [LDH] assay and membrane potential measurement) were performed. For glutamate treatment, neurons were treated with 20 μM glutamate (Sigma-Aldrich, St. Louis, MO) or vehicle for 24 h. Glutamate was

freshly prepared in sterile PBS and added to the cell-culture medium. The vehicle control for glutamate experiments was sterile PBS. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama, Tuscaloosa, Alabama (23-11-7123).

Viability assay

For the LDH assay, the level of cytotoxicity in primary neurons was assayed by measuring leakage of LDH using an *in vitro* toxicology assay kit (Sigma-Aldrich) as previously described.⁴⁵ In brief, the culture media and lysed cells were collected after 24 h of treatment. The LDH assay mixture was added to each sample. After a 20-min incubation, the reaction was terminated by adding 1 N HCl. LDH activity was measured using a Clariostar microplate reader (BMG Labtech, Cary, NC) with an absorbance setting at 490 nm. Data were calculated by dividing the activity of LDH leaked into the medium from damaged cells by total LDH activity in the culture.

For PI staining, dead cells were stained with PI as previously described.^{14,41,44} After treatment, 0.5 μM PI (Invitrogen) was added to the culture medium for 30 min at 37°C in darkness. Images were taken by a Zeiss AxioVert A1 microscope using a consistent exposure time. The number of PI-positive neurons was analyzed using AxioVision 4.9.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi$) was measured using the fluorescent lipophilic cationic dye tetramethylrhodamine methyl ester (TMRM) (Thermo Fisher Scientific), which accumulates within mitochondria in a membrane-potential-dependent manner. Primary hippocampal neurons were stained with TMRM (5 nM in DMSO) for 30 min at 37°C in darkness. Images were taken by a Zeiss Axiovert A1 microscope using a consistent exposure time, and TMRM fluorescence density was analyzed using AxioVision 4.9.

the calcium retention capacity assay ($n = 3$). Error bars represent the standard error of the mean (SEM). An unpaired *t*-test was used for statistical analysis. 0.0002 and 0.002 μM BDQ, $p > 0.999$; 0.04 μM BDQ, $p = 0.7268$; 0.4 μM BDQ, $p = 0.0935$; 4 μM BDQ, $*p = 0.0270$; 8 μM BDQ, $*p = 0.0119$; ATP, $*p = 0.0427$; ATP + 8 μM BDQ, $*p = 0.0326$; CsA, $**p = 0.0030$. ns, not significant. (c) Representative truncated traces of single-channel current obtained from the continuous patch-clamp recording of porcine heart mitoplasts. “C” and “O” refer to the channel’s closed and open state, respectively. The gray panels below the traces indicate the sequential addition of 0.025 μM BDQ. (d) Group data of normalized peak conductances of mitochondrial inner membrane single-channel currents in the presence of different concentrations of BDQ. Conductance values measured in the presence of BDQ were normalized to the control recorded prior to drug application within the same patch (G/G_0), where G_0 represents the control peak conductance. Normalized mean peak conductance values were plotted as a function of BDQ concentration. Error bars represent SEM. One-way ANOVA was used for statistical comparison. 0.01 μM BDQ ($n = 4$), $p = 0.9829$; 0.025 μM BDQ ($n = 12$), $**p = 0.0011$; 0.05 μM BDQ ($n = 12$), $****p < 0.0001$; 0.075 μM BDQ ($n = 8$), $****p < 0.0001$; 0.1 μM BDQ ($n = 7$), $****p < 0.0001$; 0.2 μM BDQ ($n = 6$), $****p < 0.0001$; 1 μM BDQ ($n = 3$), $****p < 0.0001$. ns, not significant. (e) IC_{50} curve for BDQ inhibition based on the normalized peak conductance values shown in (d). (f) Group data for the ATP hydrolysis activity assay in the presence of BDQ (0.1–200 μM) using porcine heart mitochondria (30 μg) ($n = 3$). Error bars represent SEM. An unpaired *t*-test was used for statistical analysis. 0.1 μM BDQ, $p = 0.0755$; 1 μM BDQ, $*p = 0.0425$; 10, 50, 100, and 200 μM BDQ, $****p < 0.0001$; oligomycin (5 $\mu\text{g}/\mu\text{L}$), $****p < 0.0001$. ns, not significant. (g) The fitted dose-response curve of the data shown in (f).

RESULTS

BDQ delays the opening of the mPTP

To study the effect of BDQ on the mPTP, a CRC assay was performed on isolated mitochondria from HEK293 cells. During the CRC experiment, isolated mitochondria were exposed to Ca^{2+} pulses to induce mPTP activation. Ca^{2+} accumulates in mitochondria until it triggers mPTP opening, followed by membrane depolarization, rupture of mitochondria, and release of sequestered Ca^{2+} . The mitochondrial calcium uniporter (MCU) is the primary transporter facilitating Ca^{2+} uptake under these conditions. MCU-mediated Ca^{2+} transport depends on the mitochondrial membrane potential ($\Delta\Psi\text{m}$), implying that only energized mitochondria can support its activity.⁴⁶ Succinate, a substrate of complex II, was used to energize the mitochondria. Figures 1a and 1b show that BDQ delayed the mPTP opening in a dose-dependent manner. It significantly delayed the mPTP opening at higher concentrations and had a subtle effect at lower concentrations (Figures 1a and 1b). ATP and CsA were used as positive controls in this assay. Mitochondria treated with these compounds demonstrated significantly delayed mPTP opening (Figures 1a and 1b). Cotreatment with BDQ and ATP further delayed mPTP activation (Figure 1b). However, cotreatment with BDQ and CsA did not have any additive effect, suggesting that CsA is a more potent inhibitor (Figures S1a and S1b).

CRC has traditionally been used as a functional readout of the mPTP activation. It reflects how much calcium mitochondria can accumulate before undergoing permeability transition. However, it is an indirect measure of pore activation. Patch-clamp electrophysiology allows direct measurement of ion-channel conductance. Thus, we used mitoplasts, inner membrane preparations of porcine heart mitochondria, to investigate the direct effect of BDQ on the single-channel activity of the inner membrane. Figure 1c shows representative truncated traces of single-channel current obtained from the continuous patch-clamp recording of mitoplasts. A dose-dependent reduction of the current was observed upon BDQ application. Figure 1d shows the group data for peak conductances at different BDQ concentrations. At 0.01 μM , BDQ had no marked effect on peak conductance. Substantial inhibition was first observed at 0.025 μM , with a more pronounced effect at 0.075 μM (Figure 1e), as illustrated in the representative recording (Figure 1c). Figure 1e shows the nonlinear fit of the data in Figure 1d, yielding a half-maximal inhibitory concentration (IC_{50}) value of 0.058 μM for BDQ inhibition of single-channel activity of mitochondrial inner membranes.

BDQ inhibits the ATP hydrolysis activity of mammalian ATP synthase

We studied the effect of BDQ on the catalytic and leak channel activities of porcine heart ATP synthase.

First, we measured the binding affinity of BDQ to purified ATP synthase using a spectral shift assay. The purified porcine heart ATP synthase was labeled with Red-NHS and titrated with BDQ over a concentration range from 3 nM to 100 μM . The raw values of the observed spectral shift for all samples were plotted and fitted, yielding a K_D value of 17.9 μM (Figures S1c and S1d). The obtained binding affinity correlates with results observed for human ATP synthase measured by surface plasmon resonance ($K_D = 5.7 \mu\text{M}$).³⁹

Different concentrations of BDQ (0.1–200 μM) were used to measure its effect on the ATP hydrolysis activity of ATP synthase using mitochondria isolated from porcine heart (Figure 1f) and HEK293 cells (Figure S1e). The IC_{50} of BDQ for ATP hydrolysis inhibition was found to be $\sim 7.5 \mu\text{M}$ (Figure 1g) and $\sim 11.2 \mu\text{M}$ (Figure S1f) for mitochondria isolated from porcine heart and HEK293 cells, respectively. Oligomycin was used as a positive control in this assay (Figures 1f and S1e).

The effect of BDQ (0.01–100 μM) was also measured on the ATP hydrolysis activity using purified porcine heart ATP synthase solubilized in detergent (Figure S1g). The IC_{50} of BDQ for ATP hydrolysis inhibition was found to be $\sim 0.5 \mu\text{M}$ (Figure S1h). Observed differences in the IC_{50} values for BDQ inhibition of ATP hydrolysis activity could be due to the use of different sample types (purified protein and isolated intact mitochondria) across assays.

BDQ has been reported to bind to *M. tuberculosis*,^{39,47} yeast,³⁸ and human ATP synthase³⁹ at the interface of the a subunit and the c ring of the F_0 domain in recent cryo-electron microscopy (cryo-EM) studies. In addition, NMR and mutagenesis studies show that BDQ can also bind to the ATP synthase F_1 ϵ subunit,^{48–50} with a K_D of 19.1 μM .⁵⁰ However, this binding site was not reported in any cryo-EM studies. Interestingly, the reported K_D closely matches the value observed in our studies (17.9 μM ; Figures S1c and S1d), suggesting that BDQ may bind to either or both of these sites. Changes in the interactions in the F_1 central-stalk subunits (γ , δ , and ϵ) with the F_0 c-ring were suggested to play an important role in ACLC gating.^{24,30} Thus, conformational changes in ATP synthase, induced either by BDQ binding to the ϵ subunit or at the a/c-subunit interface, may modulate the leak channel activity of ATP synthase.

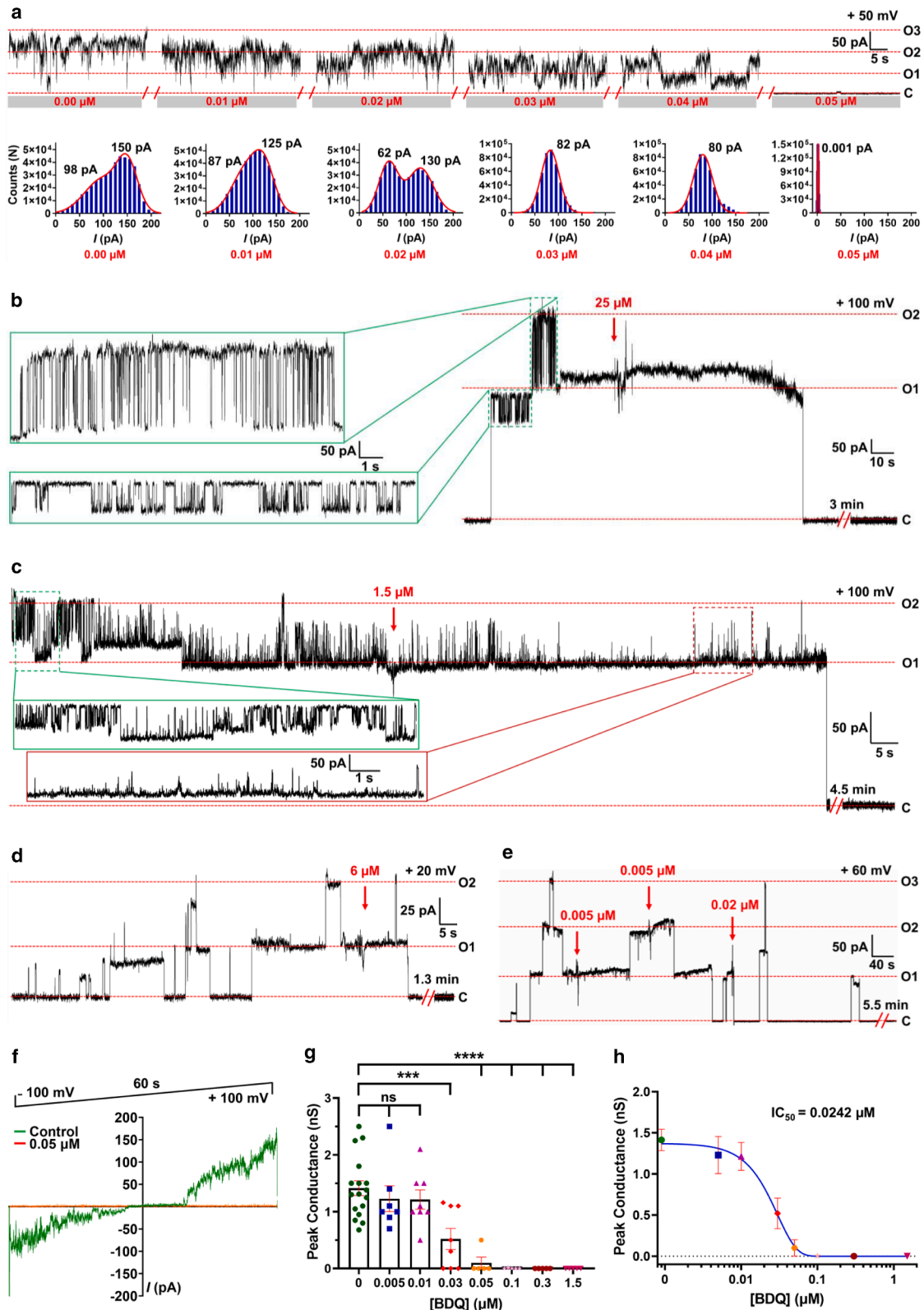


Figure 2. BDQ inhibits the ATP synthase leak channel activity of porcine heart ATP synthase. (a) Dose-dependent effect of BDQ on channel activity (0.01–0.05 μM). The truncated traces from a continuous planar lipid bilayer recording are displayed. BDQ was added to the *cis* side of the cuvette in 0.01- μM increments. The lower panel is a histogram representation of the current, I (pA). The Gaussian fits are represented (legend continued on next page)

BDQ inhibits the leak channel activity of mammalian ATP synthase

The effect of BDQ on the single-channel activity of purified porcine heart ATP synthase was studied in planar lipid bilayer recordings. **Figure 2a** shows the dose-dependent inhibition of the ATP synthase leak channel by BDQ during a continuous channel recording. The first three additions of BDQ (0.01–0.03 μM) reduced the peak current from 150 pA to 82 pA at +50 mV, with further additions completely inhibiting the channel (**Figure 2a**). **Figure 2b** depicts a representative continuous recording of ACLC before and after the addition of 25 μM BDQ, showing complete inhibition of the channel. Similarly, lower concentrations of BDQ (1.5 μM and 6 μM) inhibited the channel (**Figures 2c** and **2d**). **Figure 2e** shows no effect on the channel activity upon the first addition of BDQ (0.005 μM), while the subsequent additions (0.005 μM and 0.02 μM) induced complete closure of the channel. **Figure 2f** presents the voltage-ramp recording of ACLC in the presence and absence of BDQ at voltage values ranging from –100 mV to +100 mV. In this experiment, BDQ inhibits channel conductance at all voltages. **Figure 2g** shows the group data for peak conductances in the presence of different BDQ concentrations. The peak conductance of ACLC decreased gradually as BDQ concentration increased (**Figure 2g**). **Figure 2h** shows the nonlinear fit of the data in **Figure 2g**, suggesting that BDQ inhibits ACLC activity with an IC_{50} of 0.024 μM . This value is very close to an IC_{50} of 0.058 μM for BDQ inhibition of mitochondrial inner membrane currents in patch-clamp experiments, suggesting that the recorded channel was ACLC (**Figures 1c–1e**). However, we cannot rule out the potential effect of BDQ on other mitochondrial proteins that may be involved in mPTP-like channel activity of the IMM.

BDQ prevents glutamate-induced depolarization of the mitochondrial inner membrane and excitotoxic cell death of primary hippocampal neurons

The excessive release of glutamate occurs in the brain during I/R injury.⁵¹ To mimic I/R *ex vivo*, we treated primary hippocampal neurons with glutamate to measure the cell viability under excitotoxic condi-

tions. The effect of BDQ on modulating glutamate-induced cell death was assessed by concurrently treating neurons with glutamate and BDQ. The well-known mPTP inhibitor, CsA, was shown to inhibit glutamate-induced cell death, suggesting that the mPTP is a key cell-death pathway under these conditions.²⁴ We first evaluated the effect of varying concentrations (0, 0.1, 0.5, 1, and 5 μM) of BDQ under glutamate excitotoxic conditions (20 μM) to test its neuroprotective properties by measuring LDH release (**Figure 3a**). Primary hippocampal neurons showed a dose-dependent response to BDQ treatment. In particular, treatment with 0.1 μM BDQ markedly protected neurons against excitotoxicity, whereas treatment with 0.5 or 1 μM BDQ did not show a significant neuroprotective effect. In contrast, 5 μM BDQ treatment exacerbated cytotoxicity (**Figure 3a**).

We further quantified the cell death of glutamate-treated neurons by PI staining (**Figures 3b** and **3c**). Primary hippocampal neurons were treated with two concentrations of BDQ (0.1 μM and 5 μM) and glutamate. The low concentration of BDQ (0.1 μM) rescued cells from mPTP-induced cell death, confirming the role of BDQ as a potent inhibitor of the mPTP via possible inactivation of ACLC under these conditions (**Figures 3a–3c**). Higher concentrations of BDQ, however, aggravated glutamate-induced cell death, most likely by completely inhibiting ATP synthase catalytic activity (**Figures 1f, 1g, and S1e–S1h**).

To further prove that the neuroprotective effect of BDQ is mediated by mPTP inhibition and to rule out effects via alternative mechanisms, we studied glutamate-induced depolarization of the mitochondrial inner membrane potential. BDQ (0.1 μM) rescued membrane depolarization in primary hippocampal neurons under glutamate excitotoxic conditions (**Figures 3d** and **3e**). This confirms that BDQ prevents glutamate-induced neuronal death by inhibiting the mPTP.

DISCUSSION

BDQ, a first-in-class diarylquinoline anti-TB drug, prevents the growth and proliferation of *M. tuberculosis* by inhibiting the catalytic activity of ATP synthase to synthesize ATP.^{52,53} BDQ was reported to bind at

with a red outline, and the respective fitted peak current values are indicated. (b–d) Complete inhibition of ATP synthase leak channel activity in the presence of 25 μM (b), 1.5 μM (c), and 6 μM (d) BDQ. (e) Channel inhibition upon the addition of three sequential doses of BDQ, as indicated by red arrows. (f) Voltage-ramp recordings of ATP synthase leak channel in the presence and absence of BDQ (0.05 μM). (g) Group data on peak conductances in the presence of different concentrations of BDQ. (h) IC_{50} curve for BDQ inhibition based on the peak conductance values shown in (g). The traces in the green boxes in (b) and (c) display the channel activity before the addition of BDQ. The trace in the red box in (c) represents the channel activity after BDQ addition. “C” refers to the channel closed state, and “O” refers to the open state. Signals were filtered at 5 kHz using the amplifier circuitry. Error bars in (g) and (h) represent SEM. 0.005 μM BDQ ($n = 7$), $p = 0.4655$; 0.01 μM BDQ ($n = 8$), $p = 0.3763$; 0.03 μM BDQ ($n = 8$), $***p = 0.0007$; 0.05, 0.1, 0.3, 1.5 μM BDQ ($n = 5$ for each concentration), $****p < 0.0001$. ns, not significant.

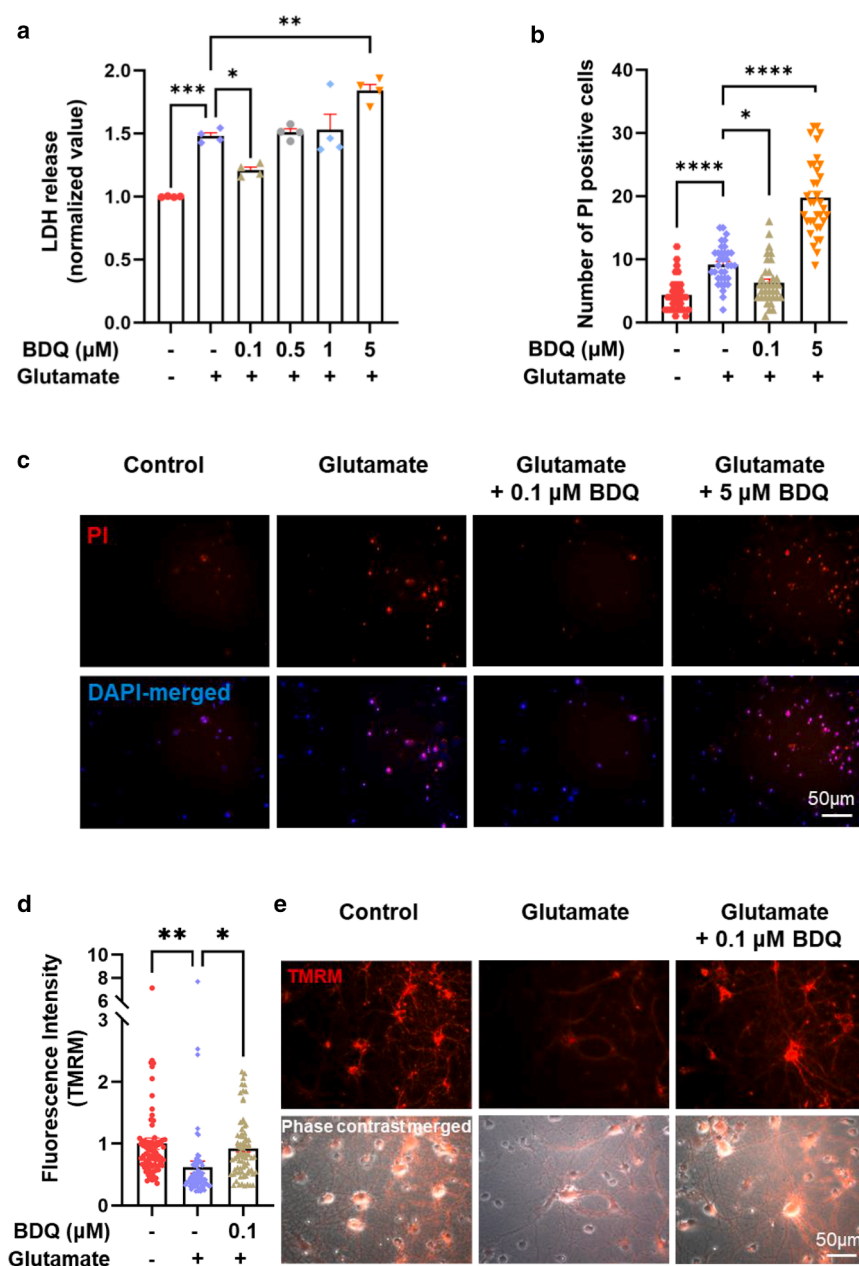


Figure 3. BDQ prevents glutamate-induced cell death in primary hippocampal neurons. (a) Group data for lactate dehydrogenase (LDH) release, an indicator of cytotoxicity, in hippocampal neurons under the indicated conditions: control, glutamate (20 μM), and BDQ (0.1, 0.5, 1, and 5 μM) with glutamate. Neurons were treated with glutamate and glutamate with BDQ for 24 h before any downstream assays ($n = 4$). One-way ANOVA with a Tukey's post hoc analysis was used. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (b) Group data on propidium iodide (PI)-positive dead cells in cultured hippocampal neurons under the indicated conditions. $n = 35$ micrographs from 3–5 biological replicates. One-way ANOVA with Tukey's post hoc analysis was used. $*p < 0.05$, $****p < 0.0001$. (c) Images of PI staining of cultured primary hippocampal neurons. Red, PI; blue, DAPI. Scale bar, 50 μm. (d) Group data for the change in normalized TMRM fluorescence intensity, an indicator of mitochondrial membrane potential, for control, glutamate (20 μM), and glutamate with BDQ (0.1 μM). Primary neurons were stained with TMRM (5 nM) for 30 min at 37°C before imaging ($n = 79$ micrographs from three biological replicates). One-way ANOVA with Tukey's post hoc analysis was used. $**p = 0.0046$, $*p = 0.0343$. (e) Images of TMRM-stained culture of primary hippocampal neurons. Upper: red, TMRM. Lower: TMRM overlapped with phase-contrast images. Scale bar, 50 μm.

the interface of the c-ring and the a-subunit of mammalian³⁹ and *M. tuberculosis*^{39,54} ATP synthase in cryo-EM structures.

In this study, we report a novel role for BDQ as a potent inhibitor of the mammalian ATP synthase leak channel. BDQ inhibits the single-channel activity of ACLC in a dose-dependent manner in planar lipid bilayer recordings of purified ATP synthase. BDQ binds directly to porcine heart ATP synthase as measured in the spectral shift assay. Additionally, BDQ treatment prevents glutamate-induced depolarization of the mitochondrial inner membrane and cell death in primary hippocampal neurons. These results

suggest an essential role for ACLC in neuronal death under glutamate excitotoxic conditions and its role in the mPTP. Nevertheless, we cannot rule out the potential effect of BDQ on the other mitochondrial proteins that may be involved in mPTP formation or its regulation.

The mPTP is a voltage-gated, Ca^{2+} - and CypD-regulated, large conductance leak channel situated in the mitochondrial inner membrane.^{55,56} The activation of the mPTP, often triggered by mitochondrial Ca^{2+} overload and oxidative stress, leads to increased permeability of the mitochondrial inner membrane, mitochondrial swelling, and rupture, resulting in cell

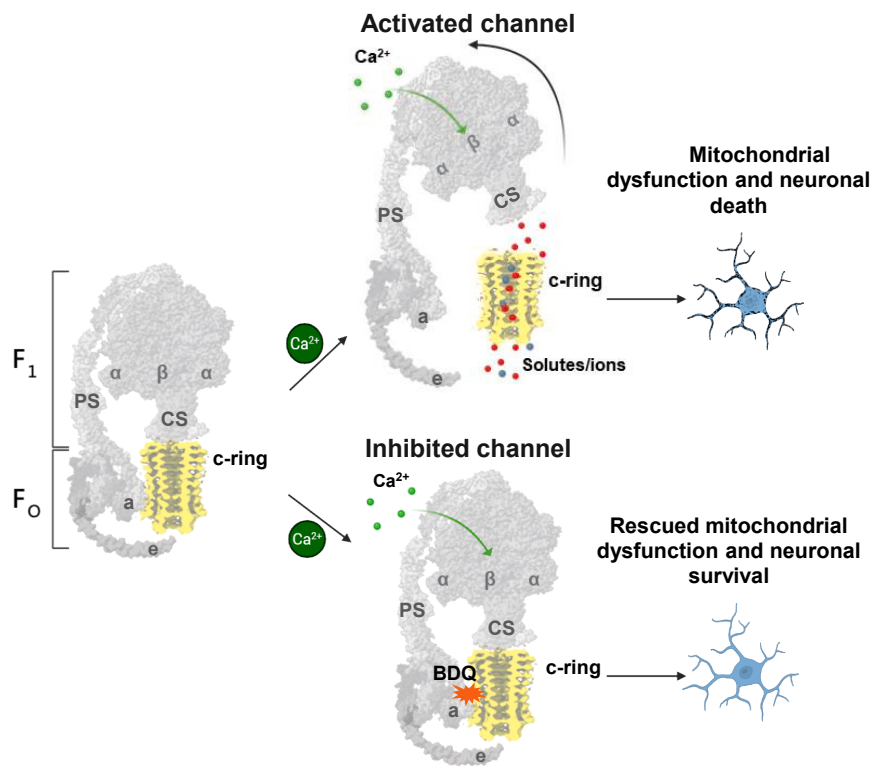


Figure 4. Proposed model of ATP synthase leak channel activation and the role of BDQ in its inhibition. The binding of Ca^{2+} to β -subunits may induce conformational changes in F_1 that are transmitted to the central-stalk (CS) and peripheral-stalk (PS) subunits, thereby activating the channel. During channel activation, the two gates of ACLC, F_1 and the e-subunit, may move away from the c-ring, allowing channel formation and ion flow through the c-ring lumen. The binding of BDQ at the interface between the c-ring and the a-subunit may stabilize interactions between them, prevent structural changes in the ATP synthase that activate the channel, and mitigate ACLC-induced cell death. For simplicity, only the ATP synthase monomer is shown. The figure was created with [BioRender.com](https://www.biorender.com).

death.^{2,3,57} The mPTP is a non-selective leak channel that allows solutes up to 1.5 kDa to pass through the inner membrane.^{56,58,59} It was reported to fluctuate between different open states (low- and high-conductance conformations) in mitoplast electrophysiological recordings.^{57,58,60–62} The low-conductance flickering channel, with a mean conductance of ~ 0.3 nS, contributes to the physiological function of mPTP.^{23,62–64} The high-conductance channel, with ~ 1.5 nS peak conductance activity, leads to prolonged, irreversible openings, inducing inner membrane depolarization and cell death.^{2,55,65} Although the exact molecular identity of the mPTP is still debated, the mitochondrial ATP synthase c-ring remains one of the pore-forming candidates for this enigmatic channel.^{13,14,17,18,24,66,67} Detailed information on the identity, structure, and regulation of the mPTP is key to developing therapeutics that specifically target its activity.

BDQ preconditioning was reported earlier to mitigate I/R-induced brain injury in Wistar rats after middle cerebral artery occlusion/reperfusion.⁶⁸ Intraperitoneal injection of BDQ (2 mg/kg) to male rats 60 min before transient occlusion of the middle cerebral artery has been reported to reduce infarct volumes and neurological deficits in rats.⁶⁸ While this study did not measure brain levels of BDQ, its peak concentration (C_{max}) in the brain was estimated to be $0.02 \mu\text{M}$ based on another

in vivo study.⁶⁹ This neuroprotective concentration of BDQ is very close to the IC_{50} of $0.024 \mu\text{M}$ for inhibiting ACLC, as measured in planar lipid bilayer recordings of purified ATP synthase, and to the IC_{50} of $0.058 \mu\text{M}$ for inhibiting the mitochondrial inner membrane channel activity in patch-clamp recordings of mitoplasts. It is also near the concentration of BDQ ($0.1 \mu\text{M}$) at which it protected hippocampal neurons from glutamate-induced cell death and depolarization of the IMM, as observed in our studies. These results suggest an important role of ACLC in preventing neuronal death in I/R.

Nevertheless, a higher concentration of BDQ ($5 \mu\text{M}$) aggravated glutamate-induced neuronal death, which can be explained by the full inhibition of ATP synthase catalytic activity. While $0.1 \mu\text{M}$ BDQ inhibits ATP synthase catalytic activity by only $\sim 10\%$ – 20% , a higher concentration ($5 \mu\text{M}$) inhibits ATP hydrolysis activity by $\sim 75\%$. Although it also inhibits ATP synthase leak channel activity, it exacerbated glutamate-induced cell death in the neuronal viability study. Thus, the cytotoxic effect of the higher BDQ concentration can be attributed to its inhibition of ATP synthase catalytic activity. Different IC_{50} values for ATP hydrolysis and ACLC inhibition may also reflect distinct BDQ binding sites on ATP synthase.^{39,48}

We have previously shown that overexpression of human ATP synthase c-subunit aggravates

glutamate-induced neuronal death in primary hippocampal neurons and H₂O₂-induced cell death in HEK293 cells.²⁴ In contrast, c-subunit knockdown in neurons undergoing glutamate excitotoxicity prevented cell death.²⁴ Here, we show that pharmacological inhibition of the ATP synthase c-subunit leak channel by BDQ mitigates glutamate-induced neuronal death. These data suggest that BDQ may prevent Ca²⁺-induced conformational changes in ATP synthase under glutamate excitotoxic conditions, which otherwise lead to channel activation, depolarization of the mitochondrial membrane potential, and cell death.

We recently suggested that non-reversible dissociation of ATP synthase F₁ from the F₀ domains occurs during long-lasting openings of ACLC under glutamate excitotoxic conditions, which are accompanied by repeated and profound inner membrane depolarization.²⁴ This marks the point of no return for the cell, since prolonged pore opening triggers outer membrane rupture and the release of cytochrome c, leading to the activation of downstream cell-death pathways in pathological conditions (such as brain or heart ischemia or neurodegenerative diseases).²⁴

Our proposed model highlights the crucial role of the c-ring as the main pore-forming component of ACLC^{14,17,24,70} and the roles of the F₁ and e-subunit as the two gates of ACLC, which tightly regulate channel activity (Figure 4). According to this model, the binding of Ca²⁺ to the ATP synthase β -subunits triggers conformational changes in the F₁ domain that are transmitted to the central- and peripheral-stalk subunits, thereby activating the channel within the c-ring^{24,30,31,67} (Figure 4). The binding of BDQ at the interface between the c-ring and the a-subunit may stabilize interactions between them and prevent structural changes within the ATP synthase that activate the channel, mitigating ACLC-induced cell death (Figure 4).

Our findings, supported by binding-affinity, electrophysiological, and cell-viability studies, reveal a novel role for BDQ as a specific inhibitor of the ATP synthase c-subunit leak channel. This study suggests the potential repurposing of this FDA-approved drug for the treatment of mPTP-related diseases.

DATA AVAILABILITY

The datasets generated in the current study are available from the corresponding author upon request.

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AUTHOR CONTRIBUTIONS

N.M. designed the project; A.K., O.L., E.S., I.M., S.E., Y.W., K.A.F., E.A., H.P., and N.M. performed experiments and analyzed data; and all authors contributed to the preparation of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

SUPPLEMENTAL INFORMATION

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