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Ionophoric effects of the antitubercular drug bedaquiline

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Bedaquiline (BDQ), an inhibitor of the mycobacterial F_1F_o-ATP synthase, has revolutionized the anti-tubercular drug discovery program by defining energy metabolism as a potent new target-space. Several studies have recently shown that BDQ can also stimulate respiration in mycobacteria, through a phenomenon known as uncoupling. The biochemical basis underlying this, in BDQ, is unresolved and may represent a new pathway to the development of effective therapeutics. In this communication, we demonstrate that BDQ can inhibit ATP synthesis in *Escherichia coli* by functioning as a H^+/K^+ ionophore, causing transmembrane pH and potassium gradients to be equilibrated. Despite the apparent lack of a BDQ-binding site, proteoliposomes containing the *E. coli* F_o subunit accumulated the ionophoric activity of BDQ. We discuss the possibility that accumulation of BDQ at F_1F_o-ATP synthases enables BDQ to create an uncoupled microenvironment, by antiporting H^+/K^+. Ionophoric properties may be desirable in high affinity antimicrobials targeting integral membrane proteins.

Bedaquiline | Tuberculosis | Respiration | Uncoupler | Ionophore

Introduction

The paucity of new drug leads developed through target-based screening since 1999, compared to phenotypic screening, has largely been attributed to poorly resolved modes of action (1). Furthermore, compounds with new molecular effects are discovered through phenotypic screening methods and the antitubercular medicine bedaquiline (BDQ, Sirturo™), FDA approved in December 2012, is no exception (2, 3). An inhibitor of the mycobacterial F_1F_o-ATP synthase (henceforth F_1F_o), BDQ demonstrates that metabolism and energy generation is a promising new target-space. However, despite only 5 years of clinical use, resistance in both laboratory and clinical settings has been reported (4–6), reinforcing the need to mine this new target-space for second-generation compounds. Yet this process will be slowed without thoroughly resolving the mode of action of first-generation inhibitors. Important aspects of BDQs mode of action are unresolved, including the time-dependent mechanism of killing, and the molecular basis for selectivity between bacterial strains.

BDQ has been demonstrated to bind to the *c*-ring rotor of the F_o portion of the mycobacterial ATP synthase (7, 8); concomitantly the synthesis of ATP, an essential energy currency in biology, is inhibited and intracellular ATP levels drop (7, 9). BDQ is not reported to inhibit growth of non-mycobacterial strains (2) and in mammalian mitochondria the drug did not affect ATP synthesis activity (10) or the membrane potential (11). Inhibition of mycobacterial growth by BDQ can be attributed to stereospecific inhibition of ATP synthase (7). Killing, on the other hand, is less well resolved. Killing in *Mycobacterium tuberculosis* by BDQ is associated with depletion of intracellular ATP (9,12). It has also been demonstrated that BDQ stimulates oxygen consumption in *Mycobacterium smegmatis* (13) and *M. tuberculosis* (14). From this it has been suggested that BDQ is an uncoupler (11, 13), collapsing the pH component of the proton motive force (PMF), and suggested this associates will killing (13). However, others found BDQ did not affect proton conductance in NADH-energized *M. tuberculosis* membranes (14).

The PMF is an electrochemical gradient consisting of both a pH gradient and membrane potential (ΔpH and $\Delta\psi$ respectively), which is most well known for its utilization by F_1F_o during ATP synthesis. Protonophores and ionophores are membrane diffusible chemicals that can bind and transport protons or other cations, and can act to equilibrate/dissipate these gradients (15, 16). The cellular response to these chemicals is to increase respiration to attempt to maintain the PMF, resulting in futile cycling of ions that is uncoupled from ATP synthesis; also known as 'uncoupling'.

Protonophores generally are lipophilic weak acids, such as Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) or Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (17), which carry both protons and charge by directly binding and shuttling protons. Extensive delocalization of the negative charge allows the deprotonated form to cross the lipid bilayer. Although less well characterized, cationic protonophores have been reported (18–20). These molecules are lipophilic weak bases, as opposed to weak acids, and delocalize a positive charge by similar mechanisms. Carrying protons without simultaneously moving a compensatory ion collapses both the ΔpH and $\Delta\psi$ (15). Ionophores are instead capable of binding and shuttling larger ions, sometimes in addition to protons. Nigericin is an example of a molecule that carries both cations and protons (15), by binding said ions through its carboxylate moiety. Nigericin antiports K^+ and H^+, an electroneutral exchange, to collapse only the ΔpH.

Significance

Antibiotics generally target one of five major bacterial target spaces, which are becoming limited due to increasing drug resistance. Bedaquiline, a new FDA-approved anti-tubercular drug, targets energy metabolism: defining cellular energetics as a new target-space for antibiotics. This is a relatively unexplored area, as bedaquiline was only FDA-approved in 2012. Several studies have recently found that bedaquiline stimulates mycobacterial respiration, in addition to inhibiting its molecular target, the F_1F_o-ATP synthase. This may be important in bedaquiline's ability to kill *Mycobacterium tuberculosis.* We determine that bedaquiline has the ability to disrupt pH and potassium homeostasis, and this activity can accumulate at ATP synthase F_o subcomplexes. "Tailgating" ionophoric activity with high-affinity enzyme inhibitors may enhance the specificity and potency of antimicrobials.

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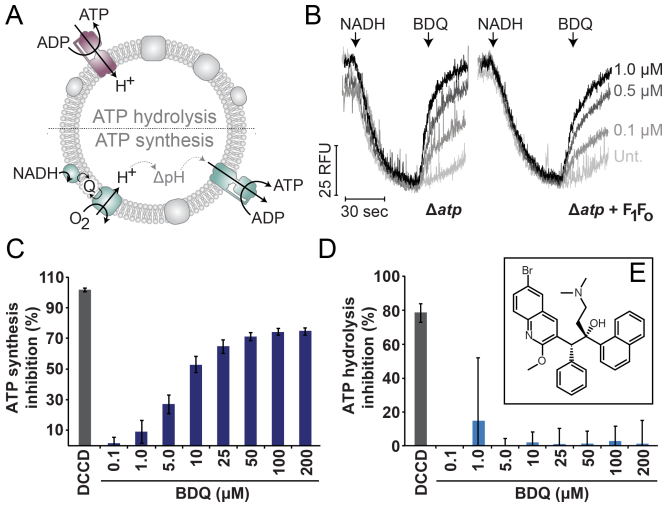

Fig. 1. Uncoupling of *E. coli* IMVs by BDQ inhibits ATP synthesis. (A) Schematic for reactions performed by IMVs: ATP hydrolysis establishes a proton gradient, while ATP synthesis is energized by the proton gradient established by NADH oxidation and subsequent electron transport chain activity. (B) IMVs of *E. coli* C41 harboring an *unc* operon deletion (Δ*atp*), or the same strain overexpressing F_1F_o (Δ*atp* + F_1F_o), were assessed for PMF establishment using 250 nM acridine orange. Proton pumping was elicited by 200 μM NADH and the proton gradient then dissipated by the indicated amounts of BDQ. C, D) IMVs of *E. coli* DK8 pBWU13 were prepared and measured for (C) Inhibition of ATP synthesis or (D) Inhibition of ATP hydrolysis. (E) The structure of BDQ. DCCD was used at 100 μM. Error bars represent standard deviation from three independent experiments. B & C are representative of triplicate experiments.

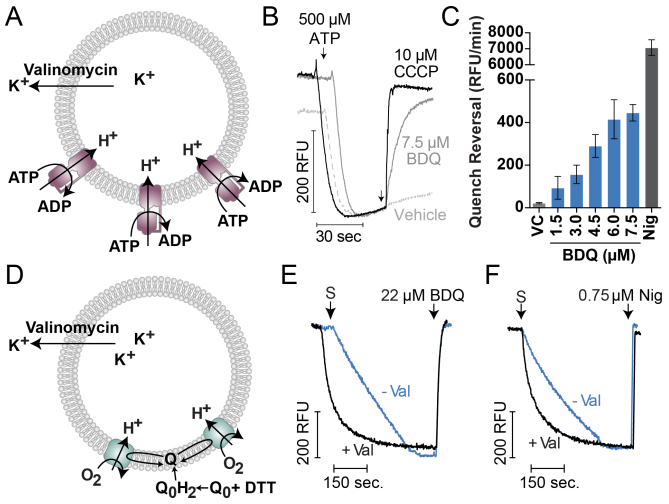

Fig. 2. Uncoupling of proton-pumping proteoliposome systems by BDQ. Schematics showing the how proton pumping in proteoliposomal *E. coli* F_1F_o (A) or *E. coli* cytochrome *bo*$_3$ (D) is achieved by either ATP hydrolysis or reduced quinone addition, respectively. Unless otherwise indicated, 1 μM valinomycin is added to counteract inhibitory membrane potentials. (B) F_1F_o proteoliposomes were incubated with ATP to establish a steady state pH gradient and then the indicated compounds were added to reverse acridine orange quenching. (C) The initial rate of quenching reversal from panel B is quantified as RFU min^{-1}, error bars represent standard deviation from three independent experiments. Nig: 10 μM Nigericin VC: vehicle control. (E,F) Proton pumping in Cytochrome *bo*$_3$ proteoliposomes was initiated by the addition of 2.5μM UQ_0 to establish a steady state pH gradient, as determined by ACMA fluorescence quenching, in either the presence or absence of 1 μM valinomycin. Either (E) BDQ or (F) nigericin was added when indicated.

Valinomycin instead carries only larger cations, not protons, and so collapses the Δψ while maintaining ΔpH (15). BDQ has been

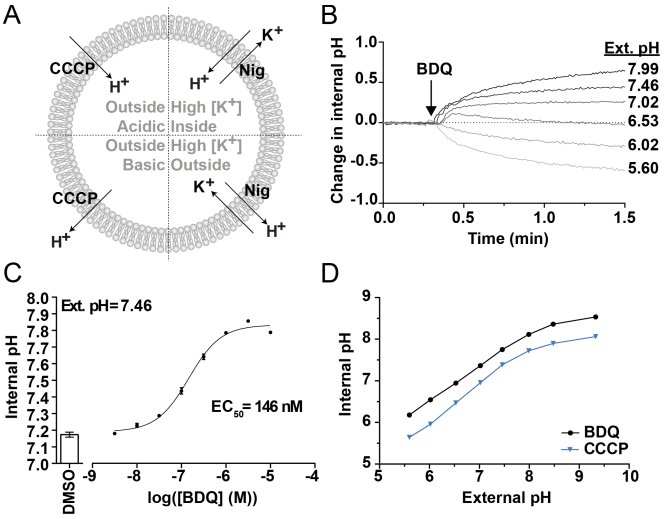

Fig. 3. BDQ accumulates in pyranine-containing liposomes and collapses pH gradients. (A) Schematic showing how the protonophore CCCP or the ionophore nigericin can manipulate the internal pH in empty liposome systems, depending on the type of imposed artificial gradient. (B) Suspensions of liposomes (internal pH ~ 7.1) were incubated in buffers of the indicated pH and treated with BDQ, with stirring in a fluorimeter. The experiment is representative of a technical triplicate. Subsequent experiments are treated analogously to panel B, but as end-point assays performed in a plate reader (without stirring). (C) An initial pH gradient of ~ 0.3 units (inside acidic) was established and the indicated amounts of BDQ added. The EC_{50} is indicated. (D) 1 μM CCCP or BDQ was used as indicated and the internal pH after 30 minutes treatment is measured. Experiments used a 2 mM MES-MOPS-Tris buffer system. In C & D error bars indicate standard deviation from triplicate measurements, although they are not visible in D.

proposed to function as a cationic protonophore (11). However, this result does not explain the observation that BDQ collapses only the ΔpH but not the Δψ in *M. smegmatis* membrane vesicles and the dependence on ATP synthase binding (13). The counter ion, and the mechanism by which the counter ion is moved to maintain electroneutrality, is unresolved. Whether BDQ is a protono-/ionophore in its own right, requires the presence of an ATP synthase for its activity, or both, is unknown.

In this body of work we report that BDQ inhibits ATP synthesis in *Escherichia coli*, an organism reported to resist BDQ growth inhibition, by dissipating the PMF. *E. coli* is a useful model organism due to the ease and high yield of F_1F_o purification, the bidirectional nature of the enzyme's activity (in contrast to the mycobacterial variant (21)) and the ability to separate the enzyme into its F_1 and F_o subcomplexes for focused analysis. The *E. coli* F_1F_o is not essential, unlike in mycobacteria (22), and so gene deletions are readily available (23). Further analysis in lipid vesicles demonstrates that BDQ can function as a cationic protonophore; but the addition of opposing salt gradients enhances this activity, suggesting BDQ is in fact a H^+/K^+ ionophore. The *E. coli* ATP synthase F_o subunit enhanced this activity, although was dispensable, suggesting BDQ accumulates at an unresolved binding site. "Tailgating" an ionophoric moiety behind a potent membrane protein-binding moiety may be desirable in future antibiotic development.

Results

Bedaquiline (BDQ) inhibits ATP synthesis in *E. coli* by ionophoric uncoupling. The cause of mycobacterial cell death upon bedaquiline addition is unclear, although several studies have implicated respiratory uncoupling (11, 13, 14). A correlation between uncoupling in *E. coli* and *M. smegmatis* membranes was previously observed (11), but the molecular mechanism is poorly resolved and hence this is the focus of our study. The MIC of

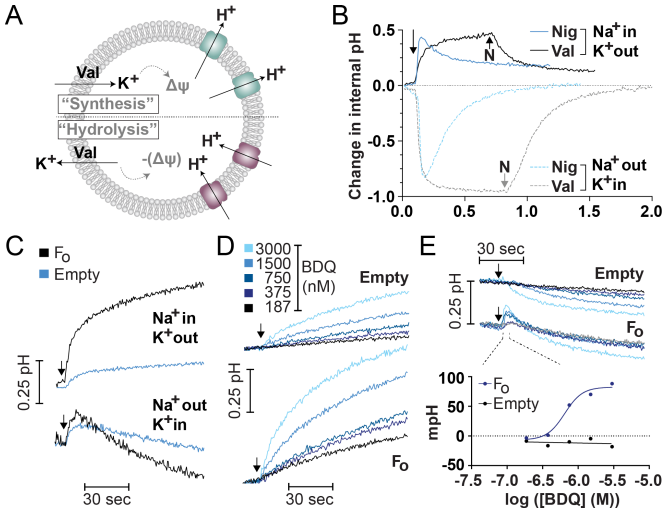

Fig. 4. The *E. coli* F_o subunit enhances the activity of BDQ in proteoliposomes. (A) Schematic showing how proton transport is routinely initiated in F_o-proteoliposomes, by either accumulating or depleting K^+ to manipulate the membrane potential in these preparations. (B) Salt gradients were established by diluting 5 µL of F_o-containing liposomes with either 50 mM K_2SO_4 or 50 mM Na_2SO_4 (K^+ in, Na^+ in respectively) into 1 mL buffer containing 50 mM Na_2SO_4 or 50 mM K_2SO_4 (Na^+out, K^+ out respectively). The change in internal pH was measured. K^+ is moved to generate a membrane potential as indicated in panel A. 100 nM valinomycin or nigericin was added when indicated. Nigericin was additionally added to valinomycin experiments where indicated. (C) The same experiment as B is performed, with the salt compositions as indicated, except 1.5 µM BDQ was added at the arrow to either F_o-containing or empty liposomes as indicated. (D,E) pH gradients were established in either (D) ATP synthesis (inside acidic) or (E) ATP hydrolysis (inside alkaline) directions by diluting 5 µL of the indicated liposomes (approx. pH 7.1 inside) into 1 mL of buffer either 1 unit more acidic or alkaline. The indicated amount of BDQ was added at the arrow. In panel E, the initial rapid alkalization is quantified below the trace. Experiments are representative of a technical triplicate.

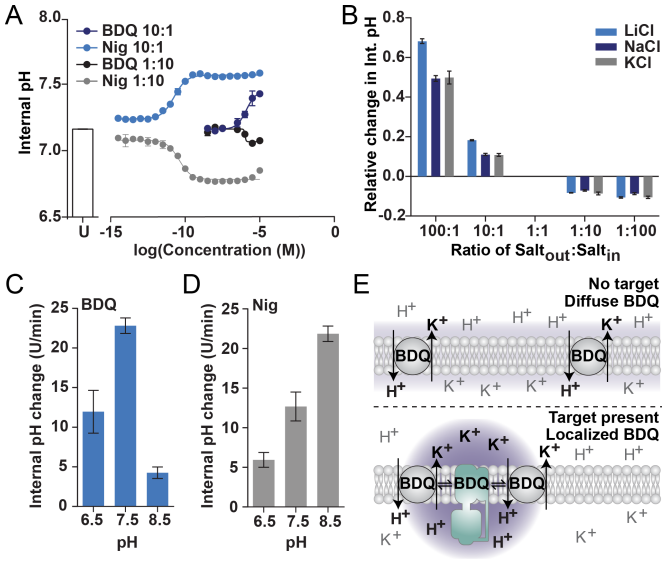

Fig. 5. BDQ is a H^+/K^+ ionophore. (A) Liposomes were prepared in either 10 mM KCl or 100 mM KCl buffer and diluted in the opposite buffer to give a K^+_{out}:K^+_{in} ratio of 10:1 or 1:10 respectively. The ratio in the key refers to the K^+_{out}:K^+_{in} ratio. The indicated amount of BDQ was added and the 30 minute end-point was recorded. U = untreated control. (B) LiCl, NaCl and KCl were compared for their ability to elicit proton movement upon BDQ addition. Salt$_{out}$:Salt$_{in}$ refers to the concentration of the indicated salt (where 1:1 is 10 mM inside and outside) Data is relative to a Salt$_{out}$:Salt$_{in}$ ratio of 1:1. (C, D) In each experiment a 10:1 K^+_{out}:K^+_{in} gradient is established, while the starting pH is the same across the liposome. (C) The rate of pH change caused by 10^{-5} M BDQ at different buffer pH values. (D) The rate of pH change caused by 10^{-9} M Nig at different buffer pH values. Error bars indicate standard deviation from triplicate experiments. (E) Model for how BDQ functions as an ionophore (top) and how this might be accumulate at the site of a high-affinity binding partner. Purple shading represents intensity of uncoupling.

BDQ against *E. coli* is reported to be > 32 µg mL^{-1} (58 µM) (2). In our own experiments we similarly found no growth inhibition for *E. coli* MG1655, testing up to 100 µM BDQ. In contrast to its lack of growth inhibition and consistent with previous reports (11), we found that BDQ could dissipate a pH gradient in inverted membrane vesicles (IMVs, Fig. 1A) of *E. coli* that were energized by both NADH oxidation and ATP hydrolysis (Fig. 1B, Fig. S2 respectively). Extending this finding, we found that BDQ was able to dissipate the PMF in IMVs of either *E. coli* with a deletion in the F_1F_o operon (Fig. 1B) or the same strain overexpressing F_1F_o (Fig. 1B). Expression was confirmed by activity and western blots (Fig. S3).

The PMF is obligatory for ATP synthesis, but ATP hydrolysis is not a PMF-consuming process and can proceed in its absence (24). Consistently, BDQ was able to inhibit ATP synthesis in *E. coli* IMVs at concentrations similar to that causing pH gradient dissipation (Fig. 1C), with an inhibitory concentration for 50% of the response (IC_{50}) ~ 5 µM. ATP hydrolysis was unaffected by the addition of BDQ (Fig. 1D). This suggests that BDQ is causing uncoupling by directly binding and shuttling protons (protonophore or ionophore) to collapse the PMF. Nigericin was sufficient to inhibit ATP synthesis in our membrane preparations (Fig. S4, Table S2), suggesting our preparations produced a PMF composed mainly of a pH gradient. Acridine orange and oxonol quenching profiles (Fig. S5, S6), suggest that valinomycin and nigericin are working as intended in our assay conditions, only uncoupling their respective component of the PMF; while the pore-forming gramicidin can completely equilibrate a membrane potential (Fig. S6A & B). Therefore, ATP synthesis results from this assay system may not inform on the role of the membrane potential. To address this, we performed oxonol quenching assays and find that BDQ does not collapse the membrane potential in IMVs (Fig. S6E & F). This is similar to previous observations in *M. smegmatis* (13)

To confirm that some unspecified membrane protein (for example, H^+-driven antiporters or efflux pumps) does not move ions in response to BDQ, we purified the *E. coli* F_1F_o (Fig. S7) into proteoliposomes (Fig. 2A) and assessed the effects of BDQ in this system. BDQ could collapse a pH gradient generated by ATP hydrolysis (Fig. 2B & C), suggesting that uncoupling is indeed driven by a protonophoric or ionophoric mode of action. Similarly, a PMF established by the activity of cytochrome *bo₃*, when reconstituted into proteoliposomes (Fig. 2D), could be dissipated by BDQ (Fig. 2E). This is consistent with the lack of F_1F_o-dependent effects in IMVs (Fig. 1B & C). Compared to the positive control nigericin (Fig. 2F), 28-fold more BDQ was needed to achieve the same degree of dissipation. In the F_1F_o system, the rate of requenching was saturated at 7.5 µM BDQ (Fig. 2C) and was 16-fold lower than that of 10 µM nigericin. The presence of a membrane potential did not affect ATP hydrolysis inhibition (Fig. S8) or PMF dissipation in cytochrome *bo₃*-containing proteoliposomes (Fig. 2E). The lack of valinomycin-dependency suggests an opposing membrane potential was not a limiting factor. Although not necessarily as potent as nigericin, it is clear that BDQ at µM concentrations can collapse the pH component of the PMF faster than any *E. coli* proton-pumping enzyme can establish it.

BDQ accumulates at lipid membranes to collapse pH gradients. We prepared pyranine-containing phosphatidylcholine vesicles (liposomes) to examine these effects in a more controlled

system. This technique quantifies the change in internal pH and is advantageous due to the ability to artificially manipulate pH and cation gradients. This method has previously been used to measure proton transport in isolated *E. coli* F_o complexes (25) and internal pH changes in protein-free liposomes (empty liposomes; 20). Empty liposomes are advantageous as we found they can maintain artificially established gradients for far longer than F_o-proteoliposomes (Fig. S9). First, we quantified the ability of BDQ to equilibrate an artificially imposed pH gradient in the absence of any protein. Unlike the prior model systems, this pH gradient is finite.

BDQ was able to equilibrate the intraliposomal (internal) pH with the external (buffer) pH (Fig 3B), regardless of whether the external pH was acidic or alkaline. The internal volume of liposomes containing the F_o subunit has previously been found to be 1.5-1.8 μL/mg lipid (25). The external buffer volume is therefore likely to be at least 100-fold in excess for all experiments, so we consider the external pH to be constant. Given sufficient time and/or concentration of BDQ, it was possible to fully equilibrate the internal pH with the external pH (Fig. S9B). The effective concentration for 50% of the equilibration response (EC_{50}) was 146 nM BDQ (Fig 3C). In addition to equilibrating pH gradients, BDQ could additionally alkalize the liposome interior by ~ 0.5 pH units in the absence of a pH gradient (Fig 3D). This was also observed as an initial alkalization at external pH 6.53 (Fig. 3B). We attribute this to intraliposomal accumulation of BDQ and subsequent alkalization. Since BDQ is a weakly basic (pKa = 8.9 (11)) and highly lipophilic compound (logP = 7.13, logD = 5.42), it is expected to partition into hydrophobic membranes and this result is an experimental confirmation of this. Aside from this alkaline bias, BDQ mimics the pH equilibration profile of the protonophore CCCP (Fig. 3D). These results show that BDQ has the capacity to act a cationic protonophore, consistent with the suggestion of Feng *et al* (11). However, this is inconsistent with the lack effects on the membrane potential in *E. coli* IMVs (Fig. S6) or *M. smegmatis* IMVs (13).

***E. coli* F_o subunits enhance BDQ-elicited proton transport.** We compared F_o-containing and empty pyranine liposomes, initially as a control to confirm the lack of F_1F_o-dependent effects observed previously (Fig. 1 & 2). In this system membrane potentials are manipulated to initiate proton transport through the F_o subunit (Fig. 4A & B (25)). Unexpectedly, BDQ appeared to alleviate the requirement of valinomycin for inducing F_o-dependent proton transport, when using a K^+ diffusion potential (Fig. 4C, K^+ out). This suggests that BDQ is able to shuttle K^+ ions to create a membrane potential using the starting gradient of KCl. Notably, BDQ does not show the same biphasic kinetics as nigericin (Fig. 4B & C), although we cannot rule out that timescale of the experiment is too small to observe a second phase of BDQ activity. Incorporation of F_o subunits enhanced the activity of BDQ, alkalizing the interior by 0.44 units more than empty liposomes after 90 seconds (Fig. 4C). A similar effect was observed when an inside acidic pH gradient was used (Fig. 4D), but could not be observed when the salt gradients were reversed (Fig. 4C, K^+ in). Instead, BDQ appeared to show a bias for alkalization, similar to the empty liposome system (c.f. pH 6.53 in Fig. 3B). When an inside alkaline pH gradient was used (Fig. 4E), BDQ caused an initial alkalization of F_o-containing liposomes. This is despite the fact that the gradient used favors intraliposomal acidification (c.f pH 6.02 in Fig. 3B). The EC_{50} for this effect was 647 nM (Fig. 4E). This suggests that the *E. coli* F_o subunit, despite the lack of mycobacterial BDQ binding site (8), has promoted further accumulation of BDQ into the liposome to result in this enhanced activity. We were unable to compete this effect with DCCD, suggesting the binding site is not necessarily at the c-ring's ion binding site.

BDQ functions as a proton/monovalent-cation ionophore. We observed that BDQ could alleviate the requirement of valinomycin in F_o proton transport assays (Fig. 4C), suggesting it could move K^+ to generate a membrane potential. Given that BDQ can also move H^+ (Fig. 3), we hypothesized that BDQ functions as a H^+/K^+ antiporter. We used empty liposomes to test this hypothesis, to remove the contribution of F_o to intraliposomal pH change. Given the biphasic kinetics possible with multi-salt systems (Fig. 4B), only a single type of salt was used for each experiment. Nigericin, a common H^+/K^+ antiporter, can convert a KCl gradient into a pH gradient (15) and this was readily achievable in our experimental system (Fig. 5A). Nigericin caused either intraliposomal acidification or alkalization depending on whether a higher concentration of salt was inside the liposome or in the external buffer (Fig. 5A). BDQ could achieve a similar effect (Fig. 5A). A high-inside KCl gradient was sufficient for BDQ to cause intraliposomal acidification (Fig. 5A), in spite of BDQ's alkaline bias, but BDQ could effect a 4.3-fold greater change in pH for a high-outside KCl gradient. This agrees with the directional bias observed in the F_o-liposome system (Fig. 4). The response did not appear to be specific to K^+, as LiCl and NaCl was able to achieve the same effect (Fig. 5B, Fig. S10). It is possible that contaminating ions in soybean phosphatidylcholine (26) facilitates proton movement in the absence of added salt (i.e. in the conditions of Fig. 3). Changing the buffer used or the lipid used did not affect the result (Fig. S11).

It is unlikely that Cl^- ions are moved by BDQ, as this anion would preferentially move in the same (symport) direction of the H^+ ion to prevent inhibitory counter-potentials. In support of this, BDQ was able to collapse a PMF established by cytochrome *bo_3* when either potassium or sodium salts were used (Fig. S12). This occurred with a slightly lower magnitude and a secondary slower rate when Na_2SO_4 was used, which is likely due to the stronger binding of Na^+ to SO_4^- ion (the K_D for dissociating Na^+ from $NaSO_4^-$ is less than Na_2SO_4 (27)). Movement of SO_4^{2-} would require dissociation of both Na^+ ions first, a chemically unlikely phenomena under biological conditions, and this would not be consistent which a slower secondary rate. As K^+ is biologically accumulated at the cytoplasmic face of the membrane, opposing the transmembrane pH gradient, we continued to focus characterization on this particular cation.

BDQ does not transport K^+ as a salt. Nigericin transports K^+ by forming a salt with the carboxylate group (15). The ionization state of nigericin therefore influences its K^+ transport ability and so sufficient acidity should compete with the binding of K^+. To test if BDQ transports K^+ similarly, we examined the ionization-state dependence of both BDQ and nigericin. Being a weak base (pKa ~ 8.9), the unprotonated form of the drug only appreciably exists at alkaline pH (Fig. S13A). If the amine groups coordinate K^+, then increasing acidity should outcompete this binding. Instead, we find that the ability of BDQ to elicit H^+ movement, using solely a KCl gradient, is best at pH 7.5 and worse at either alkaline or acidic pH (Fig. 5C, Fig. S13B). In comparison, more acidic pH values inhibited the ability of Nigericin to convert a KCl gradient into a pH gradient, consistent with the formation of carboxylate salts (Fig 5D, S13B). This suggests that, unlike nigericin, BDQ does not transport K^+ as a salt. We propose that BDQ chelates K^+ through a pH-sensitive mechanism, distinct to the amine protonation site. Overall, these data suggest that BDQ can function as a H^+/K^+ ionophore under the pH and salt conditions that emulate a standard neutrophilic bacterium, like *E. coli* or *M. tuberculosis* and that this activity is accumulated at the location of a BDQ binding partner.

Discussion

Researchers place emphasis on characterizing the primary targets of lead therapeutics, yet this risks overlooking the poten-

tially meaningful and potentially bactericidal secondary effects. In this work we report that BDQ has the ability to act as a H^+/K^+ ionophore. This can result in inhibition of ATP synthesis in *E. coli* inverted membrane vesicles, despite it having no measurable sensitivity to BDQ at a whole cell level. Here, we will propose that target-dependent accumulation of BDQ enables specific and potent uncoupling, despite the ionophoric nature of its uncoupling mechanism.

BDQ is a lipophilic weak base (pKa = 8.9, logP = 7.13), so its ability to move protons is likely similar to the well described weak acid CCCP and lipophilic weak bases such as ellipticine (15, 18), where the charge from its ionization is delocalized across π-orbitals. This would allow protonated BDQ to cross the plasma membrane and equilibrate pH gradients. In contrast, BDQ does not appear to bind K^+ at the protonable amine groups. This is unlike nigericin, which binds K^+ as carboxylic salt (15), suggesting BDQ chelates K^+ in a different manner. The apparent pH optimum of 7.5 for BDQ converting a KCl gradient into a pH gradient supports BDQ physiologically creating a futile cycle of K^+ and pH in a neutralophilic bacterial cell, like *M. tuberculosis*: BDQ acquires a proton from the acidic periplasm and moves to the neutral cytoplasm where the proton is displaced by K^+, before returning to the periplasm and so on (Fig. 5E, top). K^+, being the predominant intracellular monovalent cation (28), is likely to be more physiologically relevant than Na^+ and Li^+.

Previously, a direct interaction of BDQ and the F_1F_o of *M. smegmatis* was invoked, and subsequent disruption of the -a-c subunit interface was proposed to allow uncontrolled proton influx (13). It has also been proposed that the basis of BDQs uncoupling is purely chemical (11). We invoke a revised mechanism to reconcile the combined data. Firstly, we note that purely ionophoric mechanisms are indeed possible in mycobacteria: the $AtpE^{D32V}$ mutant still had measurable pH gradient dissipation, albeit at a slower rate and requiring higher concentrations (14.4 μM and 7.2 μM) of BDQ (13). This strain is resistant to BDQ, so it is clear that this alone is insufficient for killing. The recently published structure of the c-ring from *M. phlei* with bound BDQ suggests that BDQ can not bind to ATP synthase of non-mycobacterial species (8). However, BDQ appeared to accumulate with greater efficacy at liposome preparations containing the *E. coli* F_o-subunit. The implications are two-fold: 1) there may be a lower affinity, although not necessarily specific, BDQ-binding site in the *E. coli* F_o subunit, 2) binding BDQ may be necessary to accumulate its uncoupling activity to physiologically relevant levels.

To address the first point, BDQ is an arginine mimetic (8) and may well have several lower affinity sites in the *E. coli* F_o subunit, for example at other glutamate or aspartate residues. Alternate binding sites are not without precedent, as Trp-16 of the *M. tuberculosis* epsilon subunit has been suggested to be a second BDQ-binding site (29, 30). To consider the second point, we will use BDQ binding to the target mycobacterial F_o as an example. BDQ can bind and occupy all c-subunits in the mycobacterial enzyme (8). However, binding interactions are inherent transient: the dissociation constants for BDQ binding to the mycobacterial F_1F_o have been determined to be 1.5-19.7 μM depending on the ionic strength of the buffer used (31). As one molecule is released another may diffuse into the binding site. Continued on-and-off in this manner may accumulate BDQ at this binding site. Furthermore, the dependency of the dissociation constant on ionic strength (31) may be explained by BDQ binding cations. It is conceivable that K^+ actively competes for BDQ, removing it from the a-c interface so that it can collapse the pH gradient. In this model, the microenvironment around the target protein would then be susceptible to uncoupling, while other areas in the membrane will be unaffected (Fig. 5E). A dependency on target-based accumulation allows for a stereospecific and target-specific uncoupling, even if the nature of the uncoupling is ionophoric and likely present in the other stereoisomers of BDQ.

The lack of apparent selectivity between Li^+, Na^+, and K^+ suggests BDQ does not form a size-gated polar core like valinomycin (15). Ionophores with much broader ion specificities do exist, such as lasalocid A (32), but parallels are not readily drawn; owing to highly different chemical structures. Ellipticine, a cationic protonophore, has previously been reported to be most active around its pKa (18). In this work BDQ was found to be most active at pH values around 1.0 units more acidic than its predicted pKa of 8.9 (11). It may be that the binding of salt and interactions with the lipid membrane result in a lower than predicted pKa. There is a possibility that several BDQ molecules may act to coordinate a single cation, which may explain the apparent lack of a singular cation-binding chemical motif and the ability of BDQ to act protonophorically: BDQ may transport protons and monomers and associate into multimers that complex K^+, depending on the particular conditions.

While BDQ may well have weak uncoupling activity in other bacteria or mitochondria, our mechanism would suggest that it is not biologically relevant without a protein target. BDQ has no significant effect on oxygen consumption (10), ATP synthesis activity (10) and the membrane potential (11) of mammalian mitochondria. Furthermore, BDQ has been found to have no effect on the oxygen consumption of intact HepG2 and RAW264.7 cell lines (14). The restricted antibacterial spectrum of BDQ is well known (2) and uncoupling may well be overcome by fermentation in other bacteria. BDQ may have arisen from a plethora of favorable conditions in mycobacteria: a high-affinity binding site for BDQ (8), a sensitivity to uncouplers like nigericin and valinomycin (33), and its dependence on respiration due to the essentiality of F_1F_o (22). Should uncouplers be targeted to high affinity protein-binding sites in other organisms, the result may well be a relevant therapeutic. In this regard, modified BDQ-derivatives active on non-mycobacterial Gram-positive pathogens such as *S. aureus* and *Streptococcus pneumoniae* have been reported (34). It would be interesting future direction to test if these derivatives share the H^+/K^+ ionophore capability as this can further dissect the role of uncoupling in a drug's bactericidal activity.

Oxidative phosphorylation is a very promising avenue for drug-development and so it is important that there is sufficient knowledge of our current inhibitors, to allow well informed decisions for future lead compounds. Our work improves our understanding of the first-in-class antibiotic and highlights that ionophores and protonophores, typically associated with human toxicity (such as the case of dinitrophenol, ref. 37), may well be rationally designed for potency and specificity. Designing high-affinity membrane protein inhibitors in this way may be a more effective strategy than tethering compounds to membrane-targeted compounds like TPP^+ or plastoquinone (20, 36). These results also highlight the need to further understand the role of potassium ions in the mechanisms of new drug candidates. Finally, our work suggests new respiratory inhibitors must be considered in the context of entire respiratory chains and the PMF that intrinsically connects them.

Materials and methods

Bacterial strains, media and growth conditions, sample preparation (inverted membrane vesicles, F_1F_o proteoliposomes, cytochrome bo_3 proteoliposomes, F_o-containing and empty pyranine liposomes), determination of cell growth inhibition and analytical methods are described in the *Supplementary Materials and Methods*.

ATP synthesis and hydrolysis assays. For end-point measurements in inverted membrane vesicles (IMVs), ATP synthesis was measured using the hexokinase/glucose-6-phosphate dehydrogenase assay as previously described (10) and ATP hydrolysis was measured using the spectrophotometric Pi release assay as previously described (37). Real-time ATP synthesis measurments were made in an Oroboros O2k fluorespirometer, a clark-type oxygen electrode, modified to simultaneously measure ATP by the previously described luciferase assay (38). Further details are available in the *Supple-*

mentary Materials and Methods. F_1F_o-proteoliposome samples were not pre-incubated with BDQ for ATP hydrolysis experiments and measured using the spectrophotometric ATP-regenerating assay as previously described (37). All assays were performed at 37°C.

Fluorescence quenching dependent on ΔpH or Δψ. Fluorescence quenching of the pH responsive fluorophores 9-amino-6-chloro-2-methoxyacridine (ACMA; excitation: 430 nm, emission: 470 nm) or acridine orange (excitation: 493 nm, emission: 530 nm) was performed essentially as previously described (13). The following modifications were made: 0.2 mg mL^{-1} (final concentration) IMVs or 5 µL mL^{-1} F_1F_o-proteoliposomes were added NADH or ATP were used to initiate quenching as indicated. Unless otherwise indicated the concentration of acridine orange was 5 µM. Assays were performed at 37°C. For cytochrome bo_3 (cbo_3) proteoliposomes fluorescence quenching of the pH responsive fluorophore, ACMA, was performed as follows: 0.2 mg of IMVs consisting of 2% cbo_3/mass *E. coli* polar lipids doped with 1% mass ubiquinone-10 (UQ_{10}) per ml were pre-warmed to 37°C for 15 min in 20mM MOPS, 30mM Na_2SO_4, pH 7.4, 1mM DTT, and 1µM ACMA ± 1µM valinomycin with vigorous stirring (800 rpm). Quenching was initiated by the addition of 2.5 µM ubiquinone-0 (UQ_0) in ethanol and reversed as indicated in text. Ethanol controls had no effect on ACMA quenching. Fluorescence quenching of the Δψ responsive fluorophore oxonol VI was performed as previously described (13), except quenching was measured photometrically at 590-630 nm and NADH was simultaneously measured at 340 nm.

Internal pH quantification by pyranine fluorescence. The internal pH of pyranine-containing liposomes was determined as previously described (25), using 100-fold diluted liposomes in incorporation buffer with the salt and pH values indicated in text. A calibration curve of fluorescence ratio to pH was determined for each incorporation buffer, containing 20 nM pyranine, at known pH values (Fig. S1A, Table S1). The contributions of trace external pyranine were removed according to the equations defined in (25). Preparations of F_o-containing liposomes routinely had 50-60% of the liposomes with F_o inserted, as assessed by the ratio of proton-transport observed from a K^+/valinomycin diffusion potential vs. that of the protonophore CCCP (Fig. S1B). We did not correct for this, to enable comparison to empty liposome controls. Our preparations were sensitive to N,N'-dicyclohexylcarbodiimide (DCCD) (Fig. S1C), confirming the fidelity (coupled activity) of our preparation. Kinetic traces were measured on a Varian Cary Eclipse fluorimeter with continuous stirring. Other experiments, presented as end-point measurements, used a Varioskan Flash plate reader, although traces were routinely recorded to verify experimental integrity. Assays were performed at 37°C.

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