

BIOCHEMISTRY

Structure of the mycobacterial ATP synthase F_o rotor ring in complex with the anti-TB drug bedaquiline

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Multidrug-resistant tuberculosis (MDR-TB) is more prevalent today than at any other time in human history. Bedaquiline (BDQ), a novel *Mycobacterium*-specific adenosine triphosphate (ATP) synthase inhibitor, is the first drug in the last 40 years to be approved for the treatment of MDR-TB. This bactericidal compound targets the membrane-embedded rotor (c-ring) of the mycobacterial ATP synthase, a key metabolic enzyme required for ATP generation. We report the x-ray crystal structures of a mycobacterial c_9 ring without and with BDQ bound at 1.55- and 1.7-Å resolution, respectively. The structures and supporting functional assays reveal how BDQ specifically interacts with the rotor ring via numerous interactions and thereby completely covers the c-ring's ion-binding sites. This prevents the rotor ring from acting as an ion shuttle and stalls ATP synthase operation. The structures explain how diarylquinoline chemicals specifically inhibit the mycobacterial ATP synthase and thus enable structure-based drug design of next-generation ATP synthase inhibitors against *Mycobacterium tuberculosis* and other bacterial pathogens.

INTRODUCTION

Tuberculosis (TB) killed more than 1.3 million people in 2012 (1). The sharply increasing infection rates documented in the latest World Health Organization Global Tuberculosis Report (2) pose a threat to global TB eradication programs (3), making the development of new and alternative antibiotics, particularly against multidrug-resistant (MDR) *Mycobacterium tuberculosis*, an urgent priority. Bedaquiline (BDQ; marketed as Sirturo) is a novel antitubercular compound that belongs to the chemical class of diarylquinolines. It was shown to equally inhibit the growth of drug-sensitive and drug-resistant *M. tuberculosis* in active TB infections (4). In vitro-generated BDQ-resistant mutants suggested the rotor ring of the organism's F_1F_o -ATP synthase as the drug target (4). The F_1F_o -ATP synthase is a macromolecular, membrane-embedded protein complex that uses the transmembrane electrochemical ion (H^+ or Na^+) gradient to convert adenosine diphosphate (ADP) and inorganic phosphate (P_i) into adenosine triphosphate (ATP) by a rotary mechanism (5–8). The membrane-embedded F_o domain of the complex harbors the rotor ring of the F-type ATP synthase; usually in bacteria, it consists of identical copies of c-subunits, forming an hourglass-shaped cylinder with a central pore (the c-ring) (9). It shuttles ions across the membrane and thereby powers the synthesis of ATP within the three catalytically active sites of the F_1 headpiece (5).

BDQ was reported to be highly selective and specific in inhibiting the essential ATP synthesis activity of the enzyme in replicating and dormant mycobacteria but not in other prokaryotic or eukaryotic cells (4, 10, 11). Data from controlled phase 2 trials indicated the efficacy and potent bactericidal effect of BDQ in the treatment of MDR-TB

accompanied with significantly shortened treatment duration, making it a highly promising drug to treat infections with extremely drug-resistant (XDR) *M. tuberculosis* strains (12). BDQ is the first new drug to be approved for TB since 1971. It is currently used as a last-line antibiotic in combination therapies against MDR and XDR TB in the United States, European Union, and several other countries when the efficacy of existing antibiotics has been exhausted (2, 12).

Structural and biochemical data about mycobacterial F_o rotors are missing so far, but they are essential for understanding BDQ's precise interaction with the c-ring, its high specificity, and finally its mechanism of action. We addressed these questions here by performing a biochemical and x-ray crystallographic study.

RESULTS AND DISCUSSION

To understand the molecular mechanism behind the drug's efficacy and specificity, we used the c-ring from the nonpathogenic *Mycobacterium phlei* as a model system. The c-subunit of *M. phlei* shares a very high sequence identity (83.7%) with its *M. tuberculosis* homolog, particularly in the transmembrane region, where the drug was proposed to bind (4, 13) (Fig. 1). In addition, the minimum inhibitory concentration (MIC) reported for *M. phlei* is very low (0.05 $\mu\text{g/ml}$) and basically identical to that reported for *M. tuberculosis* (0.06 $\mu\text{g/ml}$; table S1) (13, 14), suggesting the identical mode of interaction between the drug and the rotor ring in these two species.

Inhibition of the *M. phlei* ATP synthase by BDQ and the c-ring's ion-binding sites as the drug target

To demonstrate the strong and direct inhibitory effect of BDQ on the mycobacterial ATP synthase, we traced the continuous synthesis of ATP in *M. phlei* inverted membrane vesicles (IMVs) (Fig. 2A). Like the performed controls, the addition of 0.1 μM BDQ completely abolished the synthesis activity of the enzyme. To determine the half-maximal inhibitory concentration (IC_{50}), we varied the BDQ concentrations from

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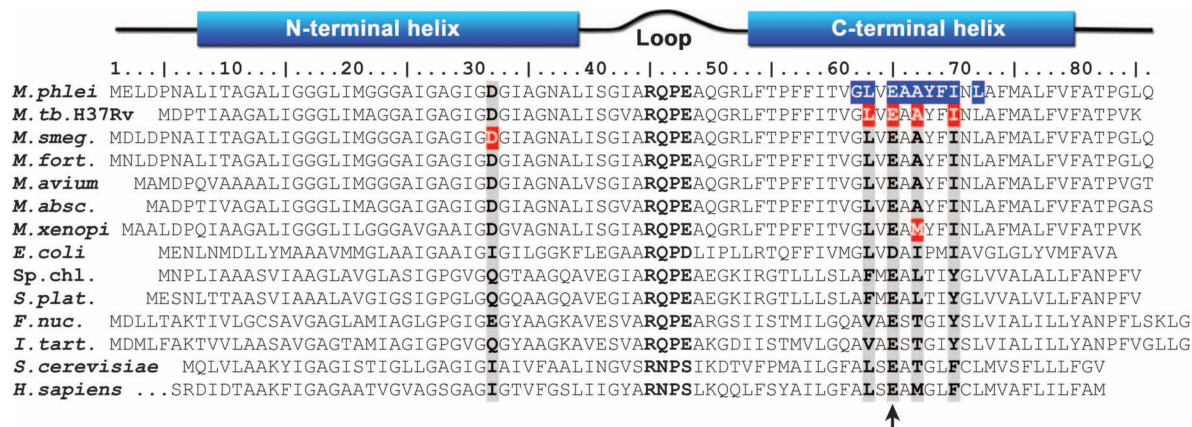


Fig. 1. Amino acid alignment of selected ATP synthase c-subunits. The location of the N- and C-terminal helices and the loop region (bold letters) are indicated (top). Amino acid numbering (top) is according to *M. phlei*. Amino acids previously shown to cause a BDQ resistance upon mutation (see Results and Discussion section for details) are indicated in red; amino acids at the same position but from other species are shaded in gray. Residues found to be involved in drug coordination, based on the x-ray structure, are highlighted in blue. The ion-binding glutamate is indicated by an arrow. *M. tb.* H37Rv, *Mycobacterium tuberculosis* H37Rv; *M. smeg.*, *Mycobacterium smegmatis*; *M. fort.*, *Mycobacterium fortuitum*; *M. abs.*, *Mycobacterium abscessus*; *Sp. chl.*, spinach chloroplast; *S. plat.*, *Spirulina platensis*; *I. tart.*, *Ilyobacter tartaricus*; *F. nuc.*, *Fusobacterium nucleatum*.

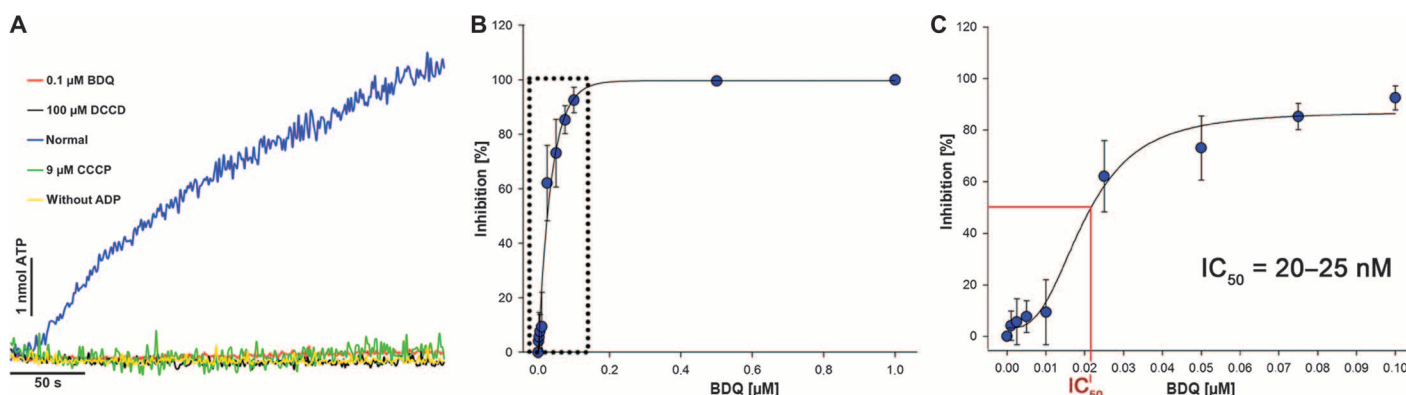


Fig. 2. Inhibition of ATP synthesis of *M. phlei* F₁F_o-ATP synthase by BDQ. (A) Continuous ATP synthesis of *M. phlei* IMVs (50 µg) monitored by increase in luminescence (blue). The presence of 0.1 µM BDQ (red) immediately and completely abolishes the synthesis of ATP. Negative controls: uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), ATP synthase inhibitor dicyclohexylcarbodiimide (DCCD), and no ADP. (B) Inhibition of ATP synthesis versus BDQ concentrations of 0 to 1 µM. (C) Zoom of the marked area (0 to 0.1 µM) in (B). The data were used to calculate an IC₅₀ value of 20 to 25 nM. For details, see Materials and Methods.

0.0 to 1.0 µM. The resulting IC₅₀ value of 20 to 25 nM (Fig. 2, B and C) underlines the highly effective inhibition of target enzyme by the drug, which is a good indication of strong binding. The values are in excellent agreement with those reported for *Mycobacterium smegmatis* (2.5 to 12.9 nM) (11, 15).

To next show the direct interaction of BDQ with the isolated *M. phlei* c-ring complex, we performed a mass spectrometry (MS)-based *in vitro* competition study with the ATP synthase inhibitor DCCD and BDQ. DCCD is a covalently binding inhibitor that reacts with protonated carboxylates of c-ring ion-binding sites (16). In contrast to BDQ, DCCD is not species-specific and inhibits all rotary ATPases (17–19). The c-ring was sequentially exposed to both inhibitors: first BDQ, then DCCD. Subsequently, the proportion of DCCD-modified c-subunits was quantified by matrix-assisted laser desorption/ionization (MALDI)-MS (Fig. 3A). We found that a BDQ concentration of only 2 µM (10-fold lower than DCCD) significantly suppressed DCCD modification (Fig. 3B), and

10 µM or a higher concentration of BDQ present in the reaction completely abolished DCCD labeling. This experiment demonstrates that the compound directly competes with DCCD for the c-ring ion-binding sites with high affinity and specificity.

Structure of the *M. phlei* c₉ ring with BDQ bound

To obtain atomic information about the drug/target complex, we next cocrystallized the *M. phlei* c-ring with BDQ. The complex crystals were rhomboid-shaped and finally diffracted to 1.7 Å (Table 1). The structure was solved by molecular replacement using a bundle of three c-subunits from the homologous yeast c₁₀ ring [Protein Data Bank (PDB) ID: 3u2f]. One crystallographic asymmetric unit (ASU) contained three c-subunits of a c-ring. One complete c-ring (biological unit) is composed of three ASUs; hence, the *M. phlei* c-ring has a c₉ stoichiometry (Fig. 4), making it the smallest bacterial rotor ring known to date. The ninefold symmetry results in an integral ion-to-ATP ratio (*i*) [the number of trans-

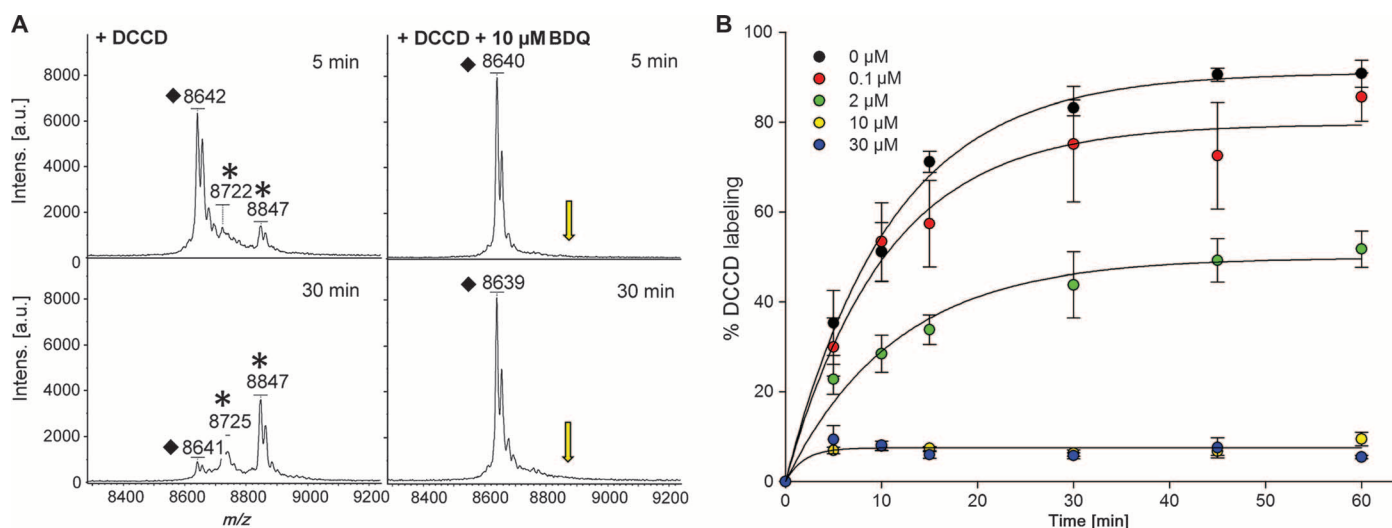


Fig. 3. BDQ binding to the isolated *M. phlei* c-ring using an inhibitor competition assay. Purified samples of *M. phlei* c-ring (0.1 mg/ml) were preincubated with 0 to 30 μM BDQ, and the time-dependent formation of DCCD-modified c-subunits was determined. (A) MALDI mass spectra of c-subunits after incubation with DCCD in the absence (left panel) or presence of 10 μM BDQ (right panel) after 5 min (top) or 30 min (bottom). Unmodified c-subunits are indicated by black diamonds (♦), and dicyclohexyl-*N*-acylisourea (DCU)-modified c-monomers are indicated by asterisks (*). BDQ (10 μM) efficiently blocks binding of DCCD to c-monomers as indicated by the yellow arrows in the right panel [no signal in corresponding mass/charge ratio (*m/z*) range]. (B) Statistics of % DCCD labeling after preincubation of the c-ring with BDQ concentrations 0 to 30 μM. A markedly reduced DCCD labeling efficacy was observed upon preincubation with BDQ, indicating that both inhibitors compete for the same binding site. The SD was calculated from at least three individual experiments. For details, see Materials and Methods.

located H⁺ per ATP synthesized, H⁺/ATP (20–22)] of 3.0, the lowest reported for a prokaryotic ATP synthase. The found stoichiometry supports that a symmetry mismatch between the c-ring and the three-fold symmetrical catalytic F₁ head is not required for efficient ATP production and extends this notion (23, 24) also toward the smaller c₉ rings. The N-terminal α-helices of the c-subunits of the inner ring and the staggered C-terminal α-helices of the outer ring form a cylinder with a width of 45 Å and a height of 65 Å (Cα–Cα). Along the outside surface, a ~30-Å hydrophobic belt (Phe⁵⁴ to Phe⁸⁰) indicates the position of the membrane lipids' alkyl chains. The central cavity of the cylinder is overall hydrophobic but has a hydrophilic surface on top and bottom; it is large enough to accommodate a handful of lipids in a bilayer arrangement.

The c-ring contains nine proton binding sites, each sandwiched between two adjacent c-subunits and equidistantly distributed along the center of the hydrophobic membrane bilayer (Fig. 4C). A conserved carboxylate (Glu⁶⁵), which is located within each ion-binding site, is responsible for reversible proton binding, shuttling, and release, as previously described (9, 18, 25). The binding sites further harbor a second carboxylate (Asp³²) provided by the inner, N-terminal helix of the same c-subunit and a buried water molecule. The 2.6-Å distance between the carboxylates of Asp³² and Glu⁶⁵ indicates a stable H-bond and suggests that Asp³² is protonated and uncharged at pH 8.0, similar to the constellation in the Na⁺-binding *Fusobacterium nucleatum* c₁₁ ring (26) (fig. S1).

The BDQ molecule specifically interacts with the c-ring's ion-binding site via numerous interactions

Close to the region of the ion-binding sites within the membrane, electron densities and anomalous bromine signals were identified in the map of c-rings cocrystallized with the antibiotic drug (Fig. 4C). These

signals originate from the brominated BDQ molecules bound to each of the c-subunits (Fig. 4C). The anomalous density of bromine verified that each BDQ was bound in the same orientation to each c-subunit. However, only the middle of the three BDQ molecules within the ASU was completely visible in the electron density, whereas parts of the other two molecules were partially disordered (27). The middle BDQ molecule was therefore completely assigned, whereas the partially occupied quinoline moieties were assigned in the two proto-mers at either side, despite the high resolution of the map. One BDQ molecule covers ~135 Å² of the c-ring's surface; the drug forms an extensive amount of van der Waals interactions (3.0 to 4.5 Å) with a stretch of nine residues (Gly⁶², Leu⁶³, Glu⁶⁵, Ala⁶⁶, Ala⁶⁷, Tyr⁶⁸, Phe⁶⁹, Ile⁷⁰, and Leu⁷²) provided by two adjacent c-subunits (Fig. 1 and table S2). In addition, the hydroxyl group at one of the chiral centers in BDQ forms a hydrogen bond to a water molecule, which itself interacts with the Glu⁶⁵ backbone carbonyl and carboxyl groups. The most important and previously anticipated (13) interaction of the drug with the c-ring is formed by the DMA group, which penetrates into the ion-binding site, where it forms a specific ionic intermolecular hydrogen bond with Glu⁶⁵'s carboxyl group at a distance of 2.5 Å (table S2). It is this electrostatic interaction that could cause the measured salt effects on ATP synthesis in *M. smegmatis* IMVs in the presence of BDQ (28). The observed overall interaction profile involving such a large number of hydrophobic, hydrophilic, and electrostatic interactions explains the high-affinity binding of BDQ to the *M. phlei* c-ring and the measured low MIC and IC₅₀ values of the compound.

Specificity of BDQ for mycobacterial c-rings

The basis for the high specificity of BDQ toward the mycobacterial c-ring becomes apparent in a surface representation of the *M. phlei* c₉ ring with BDQ bound in lock-and-key fashion (Fig. 5 and fig. S2).

Table 1. Table of crystallography.

	c ₉ (4v1g)	BDQ-bound c ₉ (4v1f)
Data processing		
Wavelength (Å)	0.9999	0.91747
Space group	R 3 :H	R 3 :H
Cell dimensions		
a, b, c (Å)	73.7, 73.7, 166.2	75.0, 75.0, 166.6
α, β, γ (°)	90, 90, 120	90, 90, 120
Resolution (Å)	36.8–1.55 (1.6–1.55)	37.5–1.7 (1.76–1.7)
Number of observed reflections	162,379 (11,775)	392,443 (38,342)
Number of unique reflections	48,446 (4,618)	38,464 (3,832)
Redundancy	3.4 (2.5)	10.2 (10.0)
Completeness (%)	99.2 (94.6)	99.46 (99.38)
R _{mrgd-F} (%)	4.5 (66.4)	8.13 (58.33)
I/σ _I	13.83 (1.44)	20.38 (4.82)
Refinement statistics		
Resolution (Å)	36.8–1.55	37.5–1.7
R _{work} /R _{free} (%)	15.66/17.87	15.63/16.19
Number of atoms	1,963	2,091
Protein	1,823	1,828
Ligands	60	163
Water	80	98
B-factors	32.9	24.90
Protein	30.50	21.5
Ligands	93.5	56.9
Solvent	42.1	35.1
Root mean square deviations		
Bond length (Å)	0.020	0.007
Bond angles (°)	1.765	1.09

The virtually complete sequence conservation of this region (Fig. 1) suggests an identical surface profile and binding site geometry in all mycobacteria, importantly also in *M. tuberculosis*. In contrast, in other prokaryotic and eukaryotic c-rings, for example, from *Ilyobacter tartaricus* (9) or *Saccharomyces cerevisiae* (19), many of the observed interactions would be sterically hindered, accounting for the significantly reduced affinity for BDQ in other bacteria and eukaryotes (Fig. 5B). This observation is in complete agreement with the ~20,000-fold higher IC₅₀ values (>200 μM) measured for eukaryotic ATP synthases (4).

A detailed structural comparison illustrates these subtle but important differences (fig. S3). The mapping of mutations in BDQ-resistant *M. tuberculosis* (4, 29, 30) revealed that they are either positioned directly on the stretch of BDQ-binding residues or close to the binding site (Fig. 1). Therefore, they either cause direct (*M. tuberculosis*: E61D, L59V, I66M) or indirect (*M. smegmatis*: D32V; *M. tuberculosis*: D28A/G, A63P) structural interference with BDQ binding, as reflected by the significantly lower drug specificity. For example, the resistances caused by Asp³² mutations to small aliphatic residues can most likely be attributed to rearrangements of its subtle hydrogen bonding net-

work: This network involves one strong hydrogen bond to Glu⁶⁵, which stabilizes the Glu⁶⁵ conformation. A second bond is formed with the structural water molecule, which itself coordinates the Leu⁶³ carbonyl oxygen in the wild type (Leu⁶³O, Fig. 4C). In the Asp to A/V/G mutants, these two stabilizing H-bonds are not available any more. Both the destabilized, free rotameric form of the Glu⁶⁵ carboxylate (31, 32) and the destabilized position of the Leu⁶³O-coordinated water molecule (33–35) negatively influence the H⁺ affinity of the c-rings. This agrees with the reduced ability of BDQ to bind to these mutant c-rings. Both effects indirectly contribute to the formation of resistances in these Asp³² mutants. The high conservation of this carboxylate residue particularly in mycobacteria (Fig. 1) therefore provides another rationale for the specificity of BDQ exclusively toward this family of bacteria.

Structure of the *M. phlei* c₉ ring without BDQ bound

To gain more insights into the dynamics of BDQ binding, we also solved the *M. phlei* c-ring structure without BDQ at 1.55-Å resolution and compared it to the BDQ-bound form (Table 1 and Fig. 4D). The c₉ ring without BDQ is almost identical with the BDQ structure (root mean square deviation = 0.167). The BDQ-free structure shows all nine proton binding sites in ion-locked, protonated conformation (9, 36) at the pH crystallized. Similar conformations were also observed in other c-ring structures solved at higher pH (18, 26, 32). Like in the BDQ-bound structure, the H⁺ binding site is made up by two neighboring c-subunits. The proton to be shuttled through the F_o domain is bound to the Glu⁶⁵ carboxylate (during the almost complete revolution of the c-ring). The overall H⁺ coordination network additionally involves the Asp³² carboxyl residue, a backbone carbonyl of Leu⁶³ from the adjacent c-subunit, and two water molecules (W1 and W2).

The position of the second, protonated Asp³² carboxylate principally allows the scenario that besides Glu⁶⁵, Asp³² could also actively participate in reversible ion binding on the c-ring during the ion translocation process in the F_o motor. Such a functional involvement of Asp³² would automatically double the number of shuttled protons per c-subunit site; hence, it would double the ion-to-ATP ratio, *i*, of this enzyme from 3.0 to 6.0 (22). At thermodynamic equilibrium for ATP synthesis, the free energies of the total electrochemical gradient of the protons [ΔμH⁺, the proton-motive force (pmf)] and the ATP phosphorylation potential (ΔG_{ATP}) can be expressed as ΔG_{ATP} = H⁺/ATP × ΔμH⁺ = *i* × pmf. Given this equation, a larger (doubled) *i* would principally support enzyme operation at low (half) pmf. In the slow-growing *M. tuberculosis* and *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), the pmf indeed was found to be almost half the magnitude (~110 mV) of what was measured in a fast-growing *M. smegmatis* (pmf ~200 mV) (37, 38). It could therefore not be excluded a priori that the carboxylate of the inner c-subunit helix contributes, perhaps only under certain growth conditions, to the ion translocation process, in concert with the classical glutamate residue (Glu⁶⁵ in *M. phlei*). Although in *F. nucleatum* the presence of such a second carboxylate group in the ion-binding site (fig. S1) could be excluded from such an important functional involvement (26), this intriguing possibility requires further experimental and theoretical assessment in mycobacterial ATP synthases, particularly the ones from slow-growing strains.

Conformational dynamics upon BDQ binding

A detailed comparison of the BDQ-bound and BDQ-free structures reveals that they differ almost exclusively in the conformation of the Phe⁶⁹

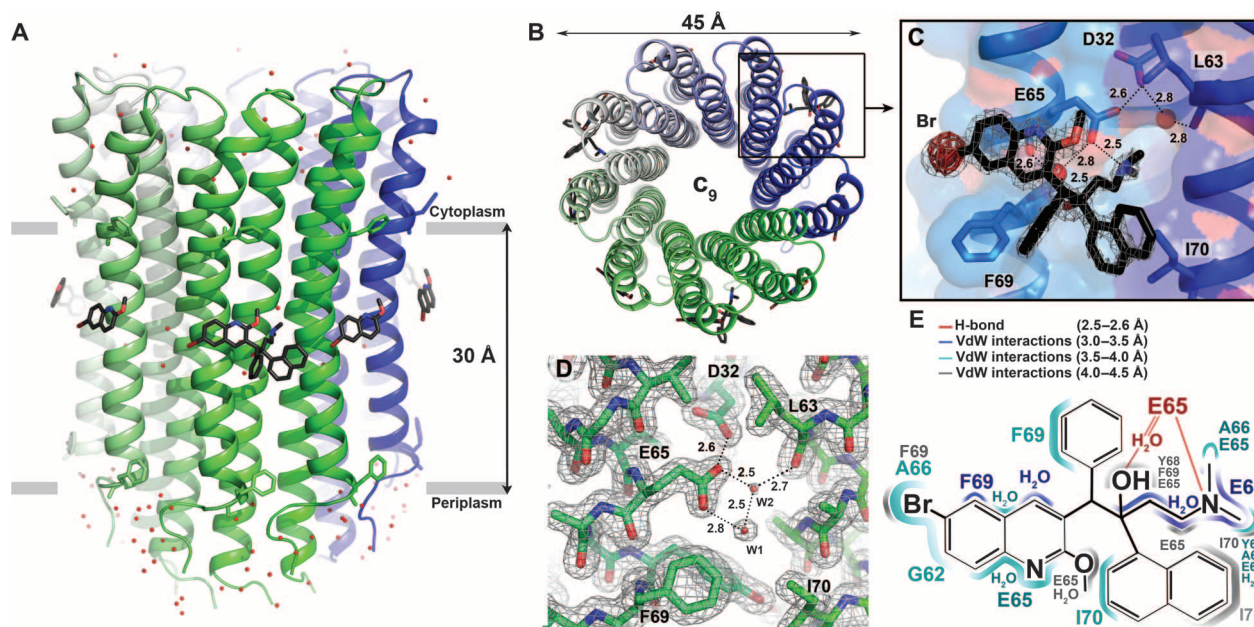


Fig. 4. Structure of the *M. phlei* c_9 ring without and in complex with BDQ. (A) The c_9 ring with BDQ bound; Side view. (B) Top view of the c-ring (cartoon representation) with bound BDQ molecules (black). Membrane borders (gray bars) and water molecules (red spheres) are indicated. (C) Slanted view of the ion-binding side showing the interaction of BDQ ($2F_{\text{obs}}-F_{\text{calc}}$ maps in black, at 1.1σ) with the c-ring. The anomalous difference map of the BDQ bromine is shown in red mesh at 4σ . Selected residues and bonding distances in angstrom (dashed lines) are indicated. BDQ invades into the ion-binding site with its dimethylamino (DMA) moiety and forms a specific ionic intermolecular H-bond with Glu⁶⁵ (see the text). (D) Structure of the c_9 ring without BDQ bound; side view of the ion-binding site. $2F_{\text{obs}}-F_{\text{calc}}$ maps (gray mesh) are shown at 1.3σ . (E) Two-dimensional (2D) plot of the BDQ/c-ring interactions. Interaction distances are color-coded. Numerous van der Waals (VdW) interactions and two hydrogen bonds contribute directly to the highly specific binding of BDQ to the c-ring (see also table S2).

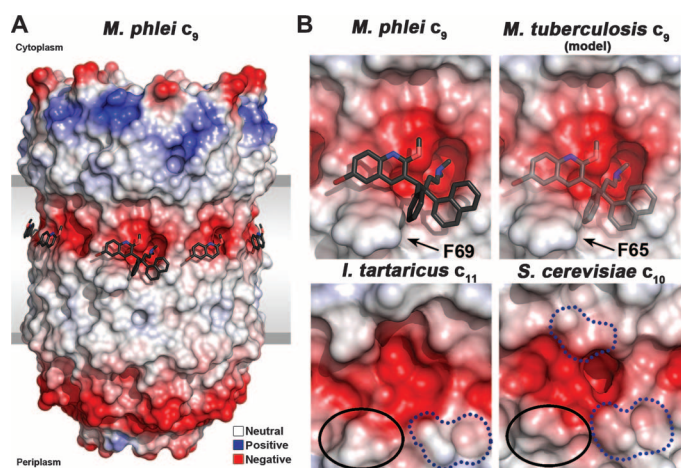


Fig. 5. Surface of the *M. phlei* c_9 ring and electrostatic potential distribution. (A) Surface and electrostatic potential distribution of the *M. phlei* c_9 ring. Membrane borders are indicated by gray bars. BDQ molecules are shown in black. (B) Surface comparison of the drug-binding region of the *M. phlei* c-ring with a *M. tuberculosis* c-ring homology model (generated using WHAT IF) (51), the *I. tartaricus* c_{11} ring (9), and the *S. cerevisiae* c_{10} ring (19). In the *M. phlei* and *M. tuberculosis* c-rings, the BDQ fits the ion-binding region, with the quinoline moiety sitting on the Phe platform (arrow) facilitating numerous interactions (see the text). In contrast, in the eukaryotic *S. cerevisiae* and the bacterial *I. tartaricus* c-rings, the Phe platform is missing (black circle) and the surface-determining side chains (dotted blue line) cause steric clashes.

side chain and the position of water molecules within the ion-binding site. Apparently, binding of BDQ induces a conformational change of Phe⁶⁹ (arrow in Fig. 6), which provides a hydrophobic platform for its quinolone moiety (Fig. 5B). This conformational change is required to avoid clashes of Phe⁶⁹ with the BDQ's hydroxyl group on one of the two chiral centers (red circle in Fig. 6). In return, a water molecule bridges the distance between the Glu⁶⁵'s backbone carbonyl and the BDQ's hydroxyl group by two hydrogen bonds when BDQ is bound (Fig. 4, C and E). Further, the two water molecules, W1 and W2, present in the BDQ-free structure's ion-binding site slightly change their position: the buried water molecule (W2) within the ion-binding pocket moves in even further toward the inner c-subunit helix and H-bonds with Leu⁶³O. The water molecule W1, previously taking part in the coordination of the translocated proton at the Glu⁶⁵ carboxylate, would now clash with the BDQ-DMA group and moves toward the outside region of the c-ring surface (Fig. 6, red circle, and movies S1 and S2).

The mechanism of action of BDQ binding and ATP synthase inhibition

We propose that the two structures illustrate the start and end states of drug binding, which allows description of the mechanism of action of BDQ binding to the mycobacterial c-ring and its inhibition of ATP synthesis (Fig. 7). Depending on the BDQ concentration, one or more BDQ molecules can approach the ATP synthase and bind to the membrane-exposed ion-binding sites of the c-ring. Whether BDQ would also bind to the a-subunit or bind somewhere at or within the a/c-ring interface cannot be judged with certainty with the currently available data. However, the fact that so far no drug-resistant a-subunit

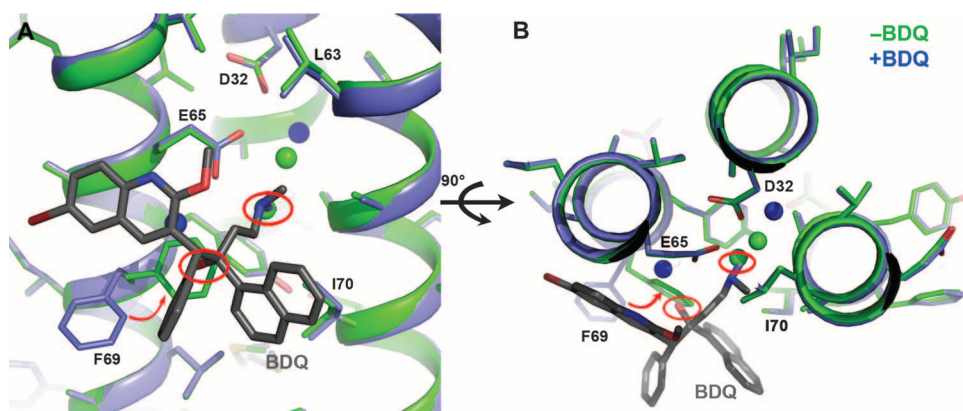


Fig. 6. Structural alignment of BDQ-free and BDQ-bound *M. phlei* c-ring structures and start and end states of the drug binding process. (A and B) Side (A) and top (B) views (from cytoplasmic side) of the BDQ-free (green) and BDQ-bound (blue) *M. phlei* c-ring structures in cartoon representation, illustrating the conformational changes and water (green and blue spheres) rearrangements occurring upon BDQ (stick model, dark gray) binding. Red arrows indicate a movement of Phe⁶⁹. Red circles indicate sites of overlapping regions between the BDQ-bound and BDQ-free structures that undergo conformational changes.

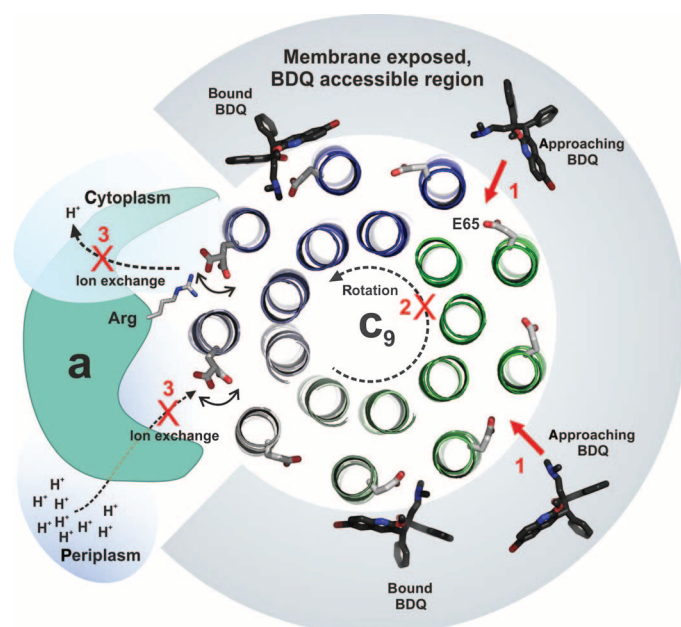


Fig. 7. Inhibition mechanism of BDQ. The mycobacterial ATP synthase F₀ motor unit is shown from the cytoplasmic side, cut open at the level of the c-ring ion-binding sites. One or more (four examples are shown in black) BDQ molecules approach the c-ring surface from the hydrophobic zone of the lipid bilayer (gray area) [1]. Each BDQ molecule binds to the region of the ion-binding site and interacts with one of the conserved glutamate residues (Glu⁶⁵ in *M. phlei*). The DMA group contacts the carboxyl group of the ion-binding glutamate. The bulky drug molecule bound to the c-ring is sterically and energetically disfavored to pass the a/c-ring interface: the rotor motion stalls [2], ion exchange at the ATP synthase [3], and finally ATP synthesis activity stops.

mutants could be found stands in contrast with the resistant mutants harboring the mutations exclusively in the c-subunit and speaks against such a possibility. So, during the drug approaches the c-ring, Phe⁶⁹ changes its conformation to avoid steric clashes and provides a hydrophobic platform for BDQ (Fig. 6 and movies S1 and S2). BDQ itself can change its conformation as well (fig. S4) and thereby promotes the formation of the numerous specific molecular interactions described above, including the formation of the ionic intermolecular H-bond between the DMA group and Glu⁶⁵. This interaction resembles an interaction of the ion-coordinating glutamate with the strictly conserved and functionally essential arginine in the adjacent stator subunit a (39, 40). It therefore appears that the Glu⁶⁵-DMA conformation mimics (41) a transition state during the ion translocation process when a

c-subunit passes the rotor-stator interface so that BDQ traps this essential state in the F₀-motor mechanism (18, 25). The tight and partially hydrophilic rotor-stator region in bacterial ATP synthases (18, 42, 43) would make it energetically highly unfavorable for the bulky BDQ molecule to bind or pass this barrier, effectively blocking c-ring rotation and, subsequently, ion exchange in F₀. The resulting full stop of coupled ATP synthesis has a significant impact on the general *M. tuberculosis* bioenergetic metabolism (44) and is ultimately fatal to mycobacterial survival (10). According to this proposed mechanism, the binding of only a single BDQ molecule per ATP synthase is sufficient to completely block ATP synthesis, in agreement with conclusions made from biochemical data (28).

CONCLUSIONS

Our results show how mycobacterial ATP synthases are specifically inhibited by diarylquinolines. The mode of inhibition resembles the mechanisms by which DCCD (18, 19) and also the eukaryotic ATP synthase inhibitor oligomycin (45) inhibit ATP synthesis. The extensive network of interactions of the c-ring's surface with BDQ and the precisely determined interaction of the BDQ's DMA arm with the ion-binding carboxylate account in sum for the highly specific binding of BDQ to mycobacterial c-rings. The structure opens new opportunities for rational drug design and the development of new antibiotics to fight MDR and XDR *M. tuberculosis* and other dangerous pathogens.

MATERIALS AND METHODS

Cell growth and protein purification and crystallization

M. phlei strain (DSM-43239) was grown for 72 hours in LB/penicillin (20 U/ml)/0.36% glycerol at 39°C, harvested, and resuspended in 50 mM tris-HCl (pH 8.0), 150 mM KCl, and 5 mM MgCl₂. Cells were disrupted by several passages through a microfluidizer (Microfluidics

Corp.) (80 mbar) and a cell disruptor (model TS, IUL Instruments) (2.0 MPa) in the presence of deoxyribonuclease A and 1 mM Pefabloc (Sigma). Membranes were collected and solubilized in 1.5% (w/v) *N*-lauroylsarcosine-containing buffer for 10 min at 60°C. Insoluble material was removed, and the solution was precipitated with saturated ammonium sulfate (16). The remaining supernatant was cleared, dialyzed, and further purified by Q-Sepharose and MonoQ anion exchange chromatography (GE Healthcare), with a linear 0 to 1 M NaCl gradient in 20 mM tris-HCl (pH 8.0) and 0.2% lauryldimethylamine *N*-oxide. The pure sample was concentrated by ultrafiltration [polyethersulfone membrane (30,000 molecular weight cutoff), Millipore] and dialyzed against 20 mM tris-HCl (pH 8.0) and 50 mM KCl at 4°C until the protein precipitated. The precipitate was collected by centrifugation and resuspended in 4% (w/v) octyl- β -D-glucopyranoside (OG) and 20 mM tris-HCl (pH 8.0) for 3D crystallization and in 0.6% Cymal-5 and 20 mM tris-HCl (pH 8.0) for competition assays.

The purified *M. phlei* c-ring in OG at a protein concentration of 6.5 mg/ml was used for crystallization using vapor diffusion (hanging drops). Before setting up the vapor diffusion hanging drops, the protein was diluted 1:1 with 20 mM tris-HCl (pH 8.0) and 4% (w/v) OG. For cocrystallization, 0.35 mM BDQ was added to the protein solution. One microliter of the protein solution was then mixed with 0.5 μ l of 28 to 30% PEG₆₀₀ (polyethylene glycol, molecular weight 600). Crystallization plates were incubated at 18°C. Rhomboid crystals appeared after about 7 days. Crystals were fished and directly flash-frozen in liquid nitrogen.

Data collection and structure determination and refinement

Data sets of the apo- and BDQ-bound structure to 1.55 and 1.7 Å, respectively, were collected from single crystals at 100 K at beamline PX-II X10SA (Swiss Light Source). The structure was solved as described in the Materials and Methods and processed with the XDS package (46). The BDQ-bound structure was solved by molecular replacement using PHASER (47) with one bundle of three polyalanine-substituted subunits of the c_{10} ring from *S. cerevisiae* (19) as a search model. Iterative cycles of model building and refinement were performed with COOT (48) and phenix.refine of the PHENIX package (49), respectively. The refinement resulted in unambiguous electron density maps, and after chain fitting, the Ramachandran plots showed no outliers. Figures and electrostatic potential distributions were generated using PyMOL (50).

Preparation of *M. phlei* IMVs and continuous, luciferin/luciferase-based ATP synthesis activity assay

M. phlei cells (10 g) were resuspended in buffer A [0.15 M NaCl, 0.1 M tricine-KOH (pH 7.5), 5 mM MgCl₂, 10% (v/v) glycerol] and broken with a French press (20,000 psi, three passages). Unbroken cells were removed by centrifugation, and membranes were collected. The pellet was resuspended in buffer A and applied to a Sephadex G-50 column. The vesicle-containing fraction was collected. Vesicles were pelleted for 45 min at 60,000 rpm and resuspended in buffer A to a concentration of 5 mg/ml. All procedures were performed at 4°C.

To assay the ATP synthesis activity, 0.2 mM malonate was added to the vesicles, followed by incubation for 1 hour on ice. Reaction buffer (290 μ l) [20 mM tricine-KOH (pH 7.5), 0.1 M NaCl, 5 mM KP_i, 5 mM MgCl₂] was mixed with 50 μ g of IMVs, 50 μ M ADP, 12.5 mM luciferin, 25 ng of luciferase (Roche), and inhibitors or uncouplers (Fig. 2). After stirring for 10 min at room temperature, the reaction was started by the addition of 10 mM succinate/KOH (pH 7.8). ATP synthesis was moni-

tored by the increasing luciferase signal measured with a Sirius L single tube luminometer (Berthold). As an internal standard, 8 nM ATP was added. Data were evaluated using SigmaPlot (Systat).

MALDI-MS-based competition studies of BDQ with DCCD

Purified *M. phlei* c-ring in 0.6% Cymal-5 (Anatrace) was used to assay the competition of DCCD with BDQ. The concentrated sample (15 mg/ml) was diluted to 0.1 mg/ml using 20 mM cacodylate/trimethylamine/NH₃ (pH 7.5). BDQ, solubilized in dimethyl sulfoxide, was added to the indicated final concentrations, and samples were incubated for 1 hour at room temperature before adding 25 μ M DCCD. Aliquots were removed at several time points (5 to 60 min), directly mixed in a 1:1 ratio with 2',4',-dihydroxyacetophenone matrix, and applied onto a ground steel MALDI target in duplicates. Mass spectra were acquired on a MALDI-time-of-flight (TOF)/TOF mass spectrometer (Bruker Autoflex III Smartbeam) and evaluated as previously described (32). Error bars were calculated from four individual measurements. Graphs and curves were generated using SigmaPlot.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/1/4/e1500106/DC1>

Fig. S1. Comparison of the BDQ-free *M. phlei* c-ring ion-binding site with the c-ring ion-binding site from *F. nucleatum*.

Fig. S2. Comparison of c-ring surface and electrostatic potential distribution.

Fig. S3. Structural alignment and comparison of BDQ binding on the c-rings of a non-mycobacterial bacterium (*I. tartaricus*) and a eukaryotic, human homolog model (*S. cerevisiae*).

Fig. S4. Comparison of the protein-bound and soluble, energy-minimized BDQ conformation. Table S1. BDQ minimum inhibitory concentrations.

Table S2. Interactions of BDQ with surrounding amino acid residues and water molecules.

Movie S1. Morph between the BDQ-free and BDQ-bound *M. phlei* c-ring structures viewed from the membrane.

Movie S2. Morph between the BDQ-free and BDQ-bound *M. phlei* c-ring structures viewed from the cytoplasm.

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Structure of the mycobacterial ATP synthase F_0 rotor ring in complex with the anti-TB drug bedaquiline

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