Structure of the mycobacterial ATP synthase F₀ 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 c₉ 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 antibacterial 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 F₁F₀-ATP synthase as the drug target (4). The F₁F₀-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 (Pi) into adenosine triphosphate (ATP) by a rotary mechanism (5–8). The membrane-embedded F₀ 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 F₁ headpiece (5).

BDQ was reported to be highly selective and specific in inhibiting the essential ATP synthase 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 F₀ 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 μg/ml) and basically identical to that reported for M. tuberculosis (0.06 μg/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 (IC₅₀), we varied the BDQ concentrations from...
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; 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*/*t* enumbe ro ft rans -

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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.*: *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* F1Fo-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.
SD was calculated from at least three individual experiments. For details, see Materials and Methods.

Reduced DCCD labeling efficacy was observed upon preincubation with BDQ, indicating that both inhibitors compete for the same binding site. The preincubated with 0 to 30 μM 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 (Ca-Ca). Along the outside surface, a ~30-Å hydrophobic belt (Phe54 to Phe86) 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 co-crystallized 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 Leu71) 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).
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 Hybocacter 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 almost certainly be attributed to rearrangements of its subtle hydrogen bonding network. 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.

### Table 1. Table of crystallography.

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The 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 Fo 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 Fo 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+] and the proton-motive force (pmf)] and the ATP phosphorylation potential (ΔGpATP) can be expressed as ΔGpATP = H+/ATP × ΔμH+ = i × μ. 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 work.
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 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).

**Fig. 4. Structure of the M. phlei c9 ring without and in complex with BDQ.** (A) The c9 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 (2Fobs-Fcalc 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 c9 ring without BDQ bound; side view of the ion-binding site. 2Fobs-Fcalc 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 c9 ring and electrostatic potential distribution.** (A) Surface and electrostatic potential distribution of the M. phlei c9 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 c11 ring (9), and the S. cerevisiae c10 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.

**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
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 Fₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐ$_{\text{a}}$). 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 F_o. 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 that 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...
potential distributions were generated using PyMOL (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% (v/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 µl 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 c10 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 µl) [20 mM tricine-KOH (pH 7.5), 0.1 M NaCl, 5 mM KPi, 5 mM MgCl₂] was mixed with 50 µg of IMVs, 50 µM 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 monitored 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 D CCD

Purified M. phlei c-ring in 0.6% Cymal-5 (Anatrace) was used to assay the competition of D CCD with BDQ. The concentrated sample (15 mg/mL) was diluted to 0.1 mg/mL using 20 mM cacyclate/trimethylamine/NH₄Cl (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 µM D CCD. 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. Morph between the BDQ-free and BDQ-bound M. phlei c-ring structures viewed from the membrane.

Fig. S3. Structural alignment and comparison of BDQ binding on the c-rings of a non-mycobacterial bacterium (F. nucleatum) and a eukaryotic 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 from the membrane.

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

References (52, 53)


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Structure of the mycobacterial ATP synthase F₀ rotor ring in complex with the anti-TB drug bedaquiline
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