A *Mycobacterium tuberculosis* Cytochrome *bd* Oxidase Mutant Is Hypersensitive to Bedaquiline

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ABSTRACT **The new medicinal compound bedaquiline (BDQ) kills *Mycobacterium tuberculosis* by inhibiting F_1F_o-ATP synthase. BDQ is bacteriostatic for 4 to 7 days and kills relatively slowly compared to other frontline tuberculosis (TB) drugs. Here we show that killing with BDQ can be improved significantly by inhibiting cytochrome *bd* oxidase, a non-proton-pumping terminal oxidase. BDQ was instantly bactericidal against a cytochrome *bd* oxidase null mutant of *M. tuberculosis*, and the rate of killing was increased by more than 50%. We propose that this exclusively bacterial enzyme should be a high-priority target for new drug discovery.**

IMPORTANCE **A major drawback of current TB chemotherapy is its long duration. New drug regimens with rapid killing kinetics are desperately needed. Our study demonstrates that inhibition of a nonessential bacterial enzyme greatly improves the efficacy of the latest TB drug bedaquiline and emphasizes that screening for compounds with synergistic killing mechanisms is a promising strategy.**

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The rapid global increase of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) cases makes new drugs with new mechanisms of action an urgent need. Bedaquiline (BDQ) is the new silver lining on the horizon for MDR and XDR TB patients. This FDA-approved drug specifically inhibits the F_1F_o-ATP synthase of mycobacteria and leads to rapid depletion of cellular ATP (1–3). However, BDQ kills relatively slowly compared to other frontline drugs, like isoniazid, and is bacteriostatic for only the first 4 to 7 days of treatment (4). This delayed onset of killing was suggested to be the result of a global remodeling in energy metabolism in response to the drug (4). Following the discovery of BDQ, the respiratory chain of *M. tuberculosis* was widely recognized as a new important target for drug discovery. This has already yielded a new compound called Q203, which targets the bc_1 cytochrome reductase (5). However, this compound was also shown to be slow acting in the first 2 weeks of treatment (5).

Inhibition of the ATP synthase stops proton influx, thereby creating back pressure on the proton-pumping components of the electron transport chain (ETC) (4, 6). *M. tuberculosis* encodes two proton-pumping complexes: NADH dehydrogenase I and an aa_3-type cytochrome *c* oxidase (7). While NADH dehydrogenase I is dispensable for growth and is not expressed *in vivo* (8), the aa_3-type cytochrome *c* oxidase is an essential component of *M. tuberculosis*'s ETC (9). Nevertheless, it was shown recently that cytochrome *bd* oxidase, a high-affinity terminal oxidase, can partially compensate for the loss in activity of the bc_1-aa_3 complex (10). In addition, cytochrome *bd* oxidase was strongly induced in *M. tuberculosis* treated with BDQ (4). This suggests that expression of the non-proton-pumping cytochrome *bd* oxidase relieves the back pressure on the ETC and allows the bacterium to prolong maintenance of its membrane potential in the absence of ATP synthase activity. Therefore, we hypothesized that a strain lacking cytochrome *bd* oxidase should be hypersensitive to BDQ and that no delay of killing should be observed.

To test the hypothesis of hypersensitivity, we created a cytochrome *bd* oxidase knockout of *M. tuberculosis* H37*Rv* by replacing the first gene, *cydA*, in the *cydAB* operon with a hygromycin cassette by using specialized transduction. As expected, this mutant showed no growth defect under standard aerobic growth conditions (see Fig. S1 in the supplemental material). Susceptibility to different concentrations of bedaquiline (0.5 and 8 μg/ml) was tested in 5-ml cultures in ink well bottles starting at a concentration of 5×10^5 CFU. To determine initial killing kinetics, numbers of CFU were monitored over a period of 14 days. Killing by BDQ set in immediately in the mutant cultures, while for the wild type (WT), we saw the expected delay in killing of up to 1 week (Fig. 1c). In addition, a 55%-increased killing rate was detected for the mutant strain (Fig. 1d), with an up to 70-fold difference in numbers of surviving cells after 14 days of treatment with 8 μg/ml BDQ (Fig. 1b). A mutant strain harboring a plasmid (pMV361::*cydA*) expressing *cydA* from the HSP60 promoter could partially complement the phenotype (Fig. 1a and b). This partial complementation is likely due to copy number differences and the fact that respiratory enzymes, as part of the electron transport chain complex, need fine-tuned expression control to guarantee optimal electron flux.

Cytochrome *bd* oxidase as a drug target would likely be missed

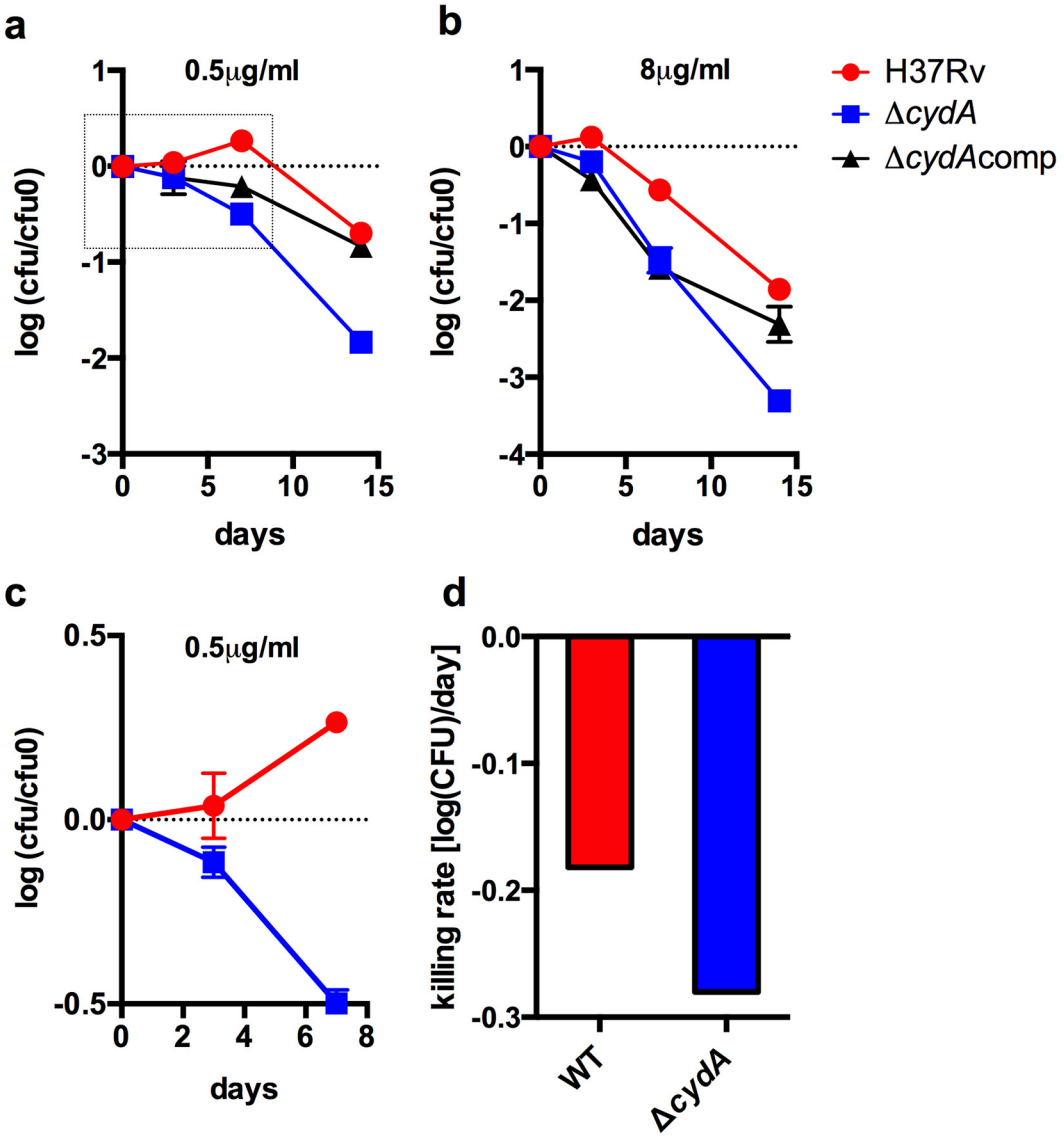

FIG 1 Killing assay with 0.5 μg/ml (a) or 8 μg/ml (b) BDQ comparing the H37*Rv* wild type (red) with the Δ*cydA* mutant (blue) and the Δ*cydA*comp complemented mutant (black). cfu0, CFU at time 0. (c) Closeup of the initial 7 days of exposure of the WT and Δ*cydA*to strain to 0.5 μg/ml BDQ (box in panel a). Cells were grown in 7H9 OADC medium to exponential phase. Then, cultures were diluted to yield a starting CFU concentration of around 10^6 and challenged with BDQ. Data were normalized to the starting CFU concentration for a proper comparison of killing kinetics. (d) First-order rate kinetics of kill curves at 8 μg/ml BDQ. Rates were calculated by linear regression from data points in panel b at 3, 7, and 14 days. Killing rates for the Δ*cydA*comp strain at 8 μg/ml BDQ are not depicted because the kill curve was biphasic (b) and first-order rate kinetics do not apply.

in drug screens because this enzyme is not essential for growth due to the presence of the aa_3 cytochrome *c* oxidase. Our data show that inhibition of cytochrome *bd* oxidase in *M. tuberculosis* leads to hypersensitivity to BDQ and rapid killing. An essential role of CydAB *in vivo* was indicated recently. Mutants of *M. tuberculosis* with transposon insertions in cytochrome *bd* oxidase genes were underrepresented in mouse lungs after 48 days of infection (11). This fits with cytochrome *bd* oxidase's purported role as a high-affinity terminal oxidase that is crucial for the adaptation of *M. tuberculosis* to hypoxia *in vivo* (12, 13). These results combined with the fact that cytochrome *bd* oxidase is found exclusively in bacteria make it a very attractive drug target.

To prevent rapid evolution of drug resistance, new compounds should be part of a completely new drug regimen. Ideally, these antibiotics have synergistic effects leading to exponential increases in killing efficacy. Our results predict that a specific inhibitor of cytochrome *bd* oxidase combined with BDQ (and likely also with Q203) would have such a multiplicative effect. Moreover, the phenotype reported here will allow us to elucidate the yet-unknown mechanism of killing of BDQ as well as the role of cytochrome *bd* oxidase in the respiratory chain of *M. tuberculosis*.

Bacterial strains and growth conditions. All bacterial strains, plasmids, and primers used in this study are listed in Table S1 in the supplemental material. Mycobacterial strains were grown in Middlebrook 7H9 medium (Difco, Sparks, MD) supplemented with 10% (vol/vol) OADC enrichment (0.5 g oleic acid, 50 g albu-

min, 20 g dextrose, 0.04 g catalase, 8.5 g sodium chloride in 1 liter water), 0.2% (vol/vol) glycerol, and 0.05% (vol/vol) tyloxapol (Sigma). Selective media contained 75 μg/ml hygromycin B and/or 20 μg/ml kanamycin. The gene *cydA* (Rv1623c) was deleted in *M. tuberculosis* H37Rv by specialized transduction as described previously (14). Mutations were confirmed by 3-primer PCR using primers Rv1623cL, Rv1623cR, and Universal_uptag, listed in Table S1. The *M. tuberculosis* Δ*cydA* strain was complemented using pMV361 harboring a copy of the *cydA* gene (Δ*cydA*comp strain) (Table S1). The gene *cydA* was PCR amplified using primers Rv1623c_fw_EcoRI and Rv1623c_re_HindIII (Table S1) and cloned into pMV361 using EcoRI and HindIII restriction sites. The nucleotide sequences of all constructs were verified by Sanger sequencing.

Killing assay. BDQ was purchased from Shanghai Biochempartner (Wuhan, China). BDQ stocks were prepared in dimethyl sulfoxide (DMSO). The *M. tuberculosis* H37Rv, Δ*cydA*, and Δ*cydA*comp strains were grown to mid-exponential phase (optical density at 600 nm [OD_{600}] = 0.5) in 7H9 OADC. Before addition of BDQ, cells were diluted to a starting concentration of approximately 10^6 cells ml^{-1}. BDQ was added to a final concentration of 0.5 $\mu g\,ml^{-1}$ (5× MIC) or 8 $\mu g\,ml^{-1}$ (90× MIC). DMSO at appropriate concentrations was added to control cultures in order to rule out toxic effects of the solvent (Fig. S1). At each time point, culture was harvested and serially diluted in phosphate-buffered saline (Gibco, Life Technologies) with 0.05% tyloxapol (Sigma) and plated onto 7H10 OADC (Difco, BD) agar plates. Colonies were counted after 4 weeks and subsequent weeks until no new colonies were detected.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01275-14/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.

Table S1, DOCX file, 0.2 MB.

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