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Bactericidal mode of action of bedaquiline

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Objectives: It is not fully understood why inhibiting ATP synthesis in *Mycobacterium* species leads to death in non-replicating cells. We investigated the bactericidal mode of action of the anti-tubercular F_1F_o-ATP synthase inhibitor bedaquiline (Sirturo™) in order to further understand the lethality of ATP synthase inhibition.

Methods: *Mycobacterium smegmatis* strains were used for all the experiments. Growth and survival during a bedaquiline challenge were performed in multiple media types. A time-course microarray was performed during initial bedaquiline challenge in minimal medium. Oxygen consumption and proton-motive force measurements were performed on whole cells and inverted membrane vesicles, respectively.

Results: A killing of 3 $\log_{10}$ cfu/mL was achieved 4-fold more quickly in minimal medium (a glycerol carbon source) versus rich medium (LB with Tween 80) during bedaquiline challenge. Assessing the accelerated killing condition, we identified a transcriptional remodelling of metabolism that was consistent with respiratory dysfunction but inconsistent with ATP depletion. In glycerol-energized cell suspensions, bedaquiline caused an immediate 2.3-fold increase in oxygen consumption. Bedaquiline collapsed the transmembrane pH gradient, but not the membrane potential, in a dose-dependent manner. Both these effects were dependent on binding to the F_1F_o-ATP synthase.

Conclusions: Challenge with bedaquiline results in an electroneutral uncoupling of respiration-driven ATP synthesis. This may be a determinant of the bactericidal effects of bedaquiline, while ATP depletion may be a determinant of its delayed onset of killing. We propose that bedaquiline binds to and perturbs the *a*-*c* subunit interface of the F_o, leading to futile proton cycling, which is known to be lethal to mycobacteria.

Keywords: mycobacteria, antimycobacterial agents, F_1F_o-ATP synthase, TMC207, R207910

Introduction

The discovery of novel antimicrobials targeting bacterial energy generation has gained significant interest owing to the recent FDA approval of bedaquiline (Sirturo™). An inhibitor of mycobacterial ATP synthase, bedaquiline is used for the treatment of drug-resistant *Mycobacterium tuberculosis* disease.[1,2] Bedaquiline is an inhibitor of the F_1F_o-ATP synthase that binds to the enzyme's oligomeric *c* subunit.[3–5] This enzyme produces the vast quantities of ATP that are needed to fuel the catabolic and anabolic reactions of growing bacterial cells.

The proton-motive force (PMF), which is the sum of the transmembrane pH gradient and the membrane potential ($\Delta\psi$), drives the F_1F_o-ATP synthase. The PMF is generated by electron transport in redox-driven processes between membrane-bound primary dehydrogenases and terminal reductases, so ATP production by the F_1F_o-ATP synthase is termed oxidative phosphorylation. Ordinarily, many bacteria would compensate for a lack or inhibition of this enzyme (during growth on fermentable carbon sources such as glucose) by increasing the rate of metabolic reactions in central carbon metabolism that produce ATP by an alternative mechanism, i.e. substrate-level phosphorylation.[1,6,7] A striking observation is therefore the essentiality of F_1F_o -ATP synthase in *Mycobacterium smegmatis*, even on fermentable carbon sources.[8] Furthermore, mycobacterial F_1F_o-ATP synthases display many physiological differences from those of eukaryotes and

other bacteria: e.g. they are unable to operate in the reverse ATP hydrolysis direction and only catalyse ATP synthesis.[9] Understanding the physiology of this enzyme is key to fully understanding why the inhibition of F_1F_o-ATP synthase is lethal to mycobacteria and to unlocking the mode of action of bedaquiline.

When mycobacterial cells (growing or non-growing) are treated with bedaquiline, time-dependent (not dose-dependent) killing is observed.[4,10] The mechanisms that govern the delayed onset and the bactericidal action of bedaquiline are not well understood. A dose-dependent decrease in intracellular ATP has been observed when *M. tuberculosis* cells are treated with bedaquiline,[3] and it has been found that cultures metabolizing only lipids have impaired resistance to the compound.[10] It is hypothesized that the cellular response to bedaquiline challenge is an attempt to replenish depleted ATP.[10] However, it should be noted that (in a study of toxin–antitoxin modules) mycobacterial cells have been depleted of ATP through de-energization treatments yet remain viable,[11] so mechanisms alternative to ATP depletion may be important in bedaquiline's bactericidal mode of action.

Tran and Cook (2005) hypothesized that inhibiting the F_1F_o-ATP synthase of mycobacteria could be detrimental to the reoxidation of NADH (or similar electron donors).[8] This assumes that strict coupling exists between the proton-pumping machinery and the proton-consuming F_1F_o-ATP synthase. In this model, the F_1F_o-ATP synthase serves as the primary sink for translocated protons, and mycobacteria do not support uncoupled respiration: either they lack a conduit for proton re-entry in the absence of the F_1F_o-ATP synthase, or they are unable to adjust the proton permeability of the cytoplasmic membrane to allow a futile cycle of protons to operate. It has consistently been shown that the cytoplasmic membrane of *M. smegmatis* is extremely impermeable to protons.[12]

This hypothesis implies that proper functioning of the F_1F_o-ATP synthase is critical for homeostatic control of the PMF magnitude in mycobacteria. Low magnitudes of PMF are lethal to mycobacteria.[13,14] Overly high magnitudes of PMF have been demonstrated to produce significant amounts of reactive oxygen species (ROS), during Inhibitory Factor 1 (IF_1)-mediated F_1F_o-ATP synthase inhibition in eukaryotes.[15,16] It would therefore seem likely that bedaquiline should cause adverse effects via the PMF, but it has previously been reported that bedaquiline does not effect any change in the $\Delta\psi$.[17]

The compounds most well studied in relation to the PMF are uncouplers, a term for a class of compounds that result in the uncoupling of respiration from ATP synthesis by the F_1F_o-ATP synthase. This term is sometimes used to refer only to protonophore and ionophore uncouplers, which can equilibrate the gradients forming the PMF by transporting protons, ions or both (reviewed in Cook *et al.*[18]). Uncoupling by PMF dissipation in this way usually results in increased respiration and glycolytic activity.[19] In the continued presence of protonophores/ionophores, restoring the transmembrane pH gradient and $\Delta\psi$ by respiratory activity forms a futile cycle of protons/ions uncoupled from ATP synthesis.[19] For *Escherichia coli* with glycolytic substrates this is not lethal,[20] yet in mycobacteria it is lethal.[13,14]

In this study, we demonstrate that bedaquiline is a potent uncoupler of respiration-driven ATP synthesis in *M. smegmatis*. Unlike classical uncouplers, bedaquiline does not translocate protons *per se* but does so by binding to ATP synthase, probably disrupting the *a-c* subunit interface in F_o, and thereby uncoupling the proton flow from ATP synthesis by the F_1F_o-ATP synthase. This uncoupling was electroneutral, consistent with no observed change in the $\Delta\psi$. These data provide an explanation of the bactericidal effects of bedaquiline, while the cellular response to ATP-depletion[10] may explain its delayed effects. The future design of novel antimicrobials in this area should therefore be conducted with a holistic view of bacterial energy generation pathways.

Materials and methods

Bacterial strains, media and growth conditions

In this study, either *M. smegmatis* mc^2155 WT,[21] *M. smegmatis* mc^2155 harbouring a kanamycin cassette disruption of the *cydA* gene[22] or *M. smegmatis* mc^2155 with an $atpE^{D32V}$ point mutation[3] were used as indicated. Strains were grown on either LB supplemented with 0.05% (w/v) Tween 80 (LBT) or Hartmans–de Bont (HdeB) medium,[23] containing 27 mM glycerol or 20 mM succinate, supplemented with 0.05% (w/v) Tween 80. Solid media (LBT) contained 1.5% agar. All the bedaquiline stocks were prepared in filter-sterilized DMSO. Cultures were routinely grown at 37°C with agitation (200 rpm), in conical flasks unless otherwise specified, and growth was followed by the measurement of OD_{600} using a Jenway 6300 spectrophotometer. All the inoculations were performed from cultures grown to mid-logarithmic phase (defined as $OD_{600}=0.2–0.6$). Unless otherwise specified, the cells were challenged with 2 mg/L of bedaquiline when the OD_{600} reached between 0.1 and 0.2. To measure the whole-cell oxygen consumption rates or whole-cell $\Delta\psi$, samples were withdrawn after the cells had been grown for 48 h (50 mL of medium in 250 mL conical flasks) or until no further cell growth could be detected using OD_{600} measurements.

Determination of cell viability

The cfu/mL were enumerated by the Miles–Misra method.[24] In brief, 5 μL serial dilutions of culture were dried on agar plates (nine per plate) at room temperature before incubation at 37°C. The MIC was determined as the lowest concentration with no visual growth after 48 h in 5 mL bijoux containing 2 mL of medium. This experimental set-up was also used to assess cell survival by plating 5 μL of undiluted culture onto LBT agar. All cultures were washed in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4) before plating.

RNA extraction, microarray analysis and quantitative real-time PCR (qRT-PCR)

Microarray analysis was performed as previously described.[25] The microarray was performed as a time course. Each timepoint in the time course was performed independently to maintain the culture volume during challenge. Challenge was performed in 500 mL conical flasks containing 100 mL of HdeB medium.

For each timepoint and condition (bedaquiline or DMSO), a 100 mL volume of cell culture was harvested into cold glycerol saline for quenching using a previously described method.[26] The samples were stored at −80°C until RNA extraction. For total RNA extraction for use in microarray and qRT-PCR, cells were pelleted using centrifugation at 4°C and resuspended in Trizol reagent (Invitrogen). RNA was extracted as previously described.[26] RNA preparations were DNase treated using TURBO DNA-free kit (Ambion) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer and the quality of RNA was checked on a 1.2% agarose gel.

The synthesis of cDNA and aminoallyl-dUTP labelling was performed using the standard operating procedure (SOP) M007, provided by the Pathogen Functional Genomics Research Centre (PFGRC; http//pfgrc.

jcvi.org). Cy-dye-labelled cDNA probes were hybridized to microarray slides supplied by the PFGRC using protocol SOP M008 provided by the PFGRC. The slides were scanned in a Genepix 4000A scanner at a 10 μm pixel size and autoadjusted for photomultiplier gain. Data analysis was performed as previously described.[26] All data have been deposited at the Gene Expression Omnibus (NCBI) under accession number GSE59871.

qRT-PCR was performed to validate gene expression changes detected by microarray analysis. All oligonucleotides used in this study are listed in Table S1 (available as Supplementary data at *JAC* Online). Total RNA (1 μg) was obtained and reverse-transcribed with random primers (250 ng) and SuperScript III reverse transcriptase (both from Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed according to the manufacturer's instructions with Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) using an ABI Prism 7500 (Applied Biosystems). Primer pairs were optimized to ensure efficient amplification. Relative gene expression was determined from calculated threshold cycle (C_T) values using gene MSMEG_5853 as an internal normalization standard, as it had shown no change in the microarray (1.05-fold, $P=0.03$), which was validated using qRT-PCR for all timepoints and conditions. Expression ratios were subjected to a two-tailed paired *t*-test (bedaquiline/DMSO) to determine statistical significance at a 95% CI. Venn diagrams were generated using the BioVenn program.[27] Sequence data for *M. smegmatis* mc^{2}155 were obtained from the TB database web site at www.tbdb.org.

Measurement of oxygen consumption rates

Oxygen consumption was measured using a Rank Brothers Clark-type oxygen electrode at 37°C as previously described,[28] except that whole cells ($OD_{600}=2.5$) were used. Samples measuring 2 mL were washed and resuspended in PBS and treated with the compounds indicated. The oxygen consumption of this cell suspension was then initiated by the addition of 3.5 mM glycerol (final concentration). Cells were untreated, treated with 2 mg/L of bedaquiline or treated with 10 μM CCCP as indicated.

Measurement of the Δψ

The Δψ of whole cells ($OD_{600}=2.5$) was measured as a time course after bedaquiline challenge. Samples were withdrawn at the indicated timepoints and the distribution of [^{3}H]TPP$^+$ was immediately determined as previously described.[28]

Preparation of inverted membrane vesicles (IMVs)

IMVs were prepared using a previously described cell fractionation,[29] with the exceptions that ∼2 L of culture were routinely harvested and the cells were homogenized six times on ice, before being passed through a French pressure cell six times at 20000 psi. Only the membranes were retained from the cell fractionation.

Determination of protein concentration

The protein concentration of either whole cells or membranes was measured by the Pierce BCA method (Thermo Scientific, Waltham, MA, USA), using BSA as a standard. Before measurement, whole cells were lysed by resuspending in 1 M NaOH, heating at 90°C for 5 min and then adjusting to pH 7 using an equal volume of 1 M HCl, after the sample had been cooled.

Fluorescence quenching dependent on ΔpH and Δψ

Proton translocation into IMVs was measured by quenching the fluorescent probe Acridine Orange (AO) using a Cary Eclipse Fluorescence spectrophotometer. The assay buffer contained 10 mM HEPES (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 0.5 mg/mL of IMVs and 5 μM AO. The reactions were initiated with 5 mM succinate and quenching was reversed with 50 μM CCCP or the indicated amounts of bedaquiline. The excitation and emission wavelengths were 493 and 530 nm, respectively. A similar protocol was used for the generation of a Δψ, with the differences that 47.5 μM Oxonol VI (with excitation and emission wavelengths of 523 and 630 nm, respectively) was used instead of AO; 2 mg/mL of IMVs were used; and the reactions were initiated with 10 mM succinate and quenching was reversed with 2 μM valinomycin or the indicated amounts of bedaquiline.

Results

Medium-dependent killing of bedaquiline-challenged *M. smegmatis* mc^{2}155

We challenged *M. smegmatis* with the mycobacterial-specific F_1F_o-ATP synthase inhibitor bedaquiline in both rich (LBT) and minimal (HdeB) media, with either succinate or glycerol as the sole carbon and energy source. The MIC in LBT was 0.01 mg/L, compared with 0.025 mg/L in HdeB containing glycerol. We then determined whether this effect translated to differences in cell viability when challenged with 2 mg/L of bedaquiline (Figure 1). For all media types, we observed a delayed onset of killing (Figure 1b, d and f), during which cultures could in fact replicate up to a cfu/mL that was 10-fold higher than that at the initial challenge. However, bedaquiline challenge in HdeB minimal medium resulted in a much more rapid rate of killing after this initial period—with a $>1\times10^3$ reduction in cfu/mL over 24 h (Figure 1d) compared with only a minor decrease in LBT medium over the same time period (Figure 1b). This was true for both HdeB with succinate (Figure 1f) and with glycerol (Figure 1d). In LBT, 20 days of bedaquiline challenge were required to observe killing equivalent to 5 days challenge in HdeB medium. The similar killing profile in both succinate and glycerol media suggests that glycolytic activity is not required for the increased survival that is seen in LBT. The rapid killing in HdeB minimal medium represents a more severe challenge condition, which we hypothesized would allow for a convenient determination of bactericidal effects.

Global response to F_1F_o-ATP synthase inhibition by bedaquiline

Whole-genome expression profiling was performed to determine the transcriptional response of *M. smegmatis* to bedaquiline challenge in HdeB containing glycerol. We challenged early-exponential-phase cultures with bedaquiline (OD_{600} of 0.1, 2 mg/L of bedaquiline) and sampled a time course of five different exposure times (15, 30, 45, 60 and 120 min). The short time frame was chosen to examine gene expression before a significant slowing of growth rate and thus concomitant growth-related changes in gene expression[26] (see below). Genes that exhibited differential changes [with an expression ratio of >2 or <0.5 ($P<0.05$) versus the DMSO control over the time course] were preliminarily assigned as the response to bedaquiline challenge (571 genes up-regulated and 817 down-regulated; Supplementary Dataset 1). We validated the microarray analysis using qRT-PCR (Figure S1 and Figure S2A). It was important to determine whether these changes were due to the primary effect of bedaquiline exposure or to the inevitable secondary effect of a slowing in growth rate. We therefore compared our responses with those of published transcriptomics for slow-growth genes in *M. smegmatis*.[26] In total, 128 up-regulated and 275 down-regulated

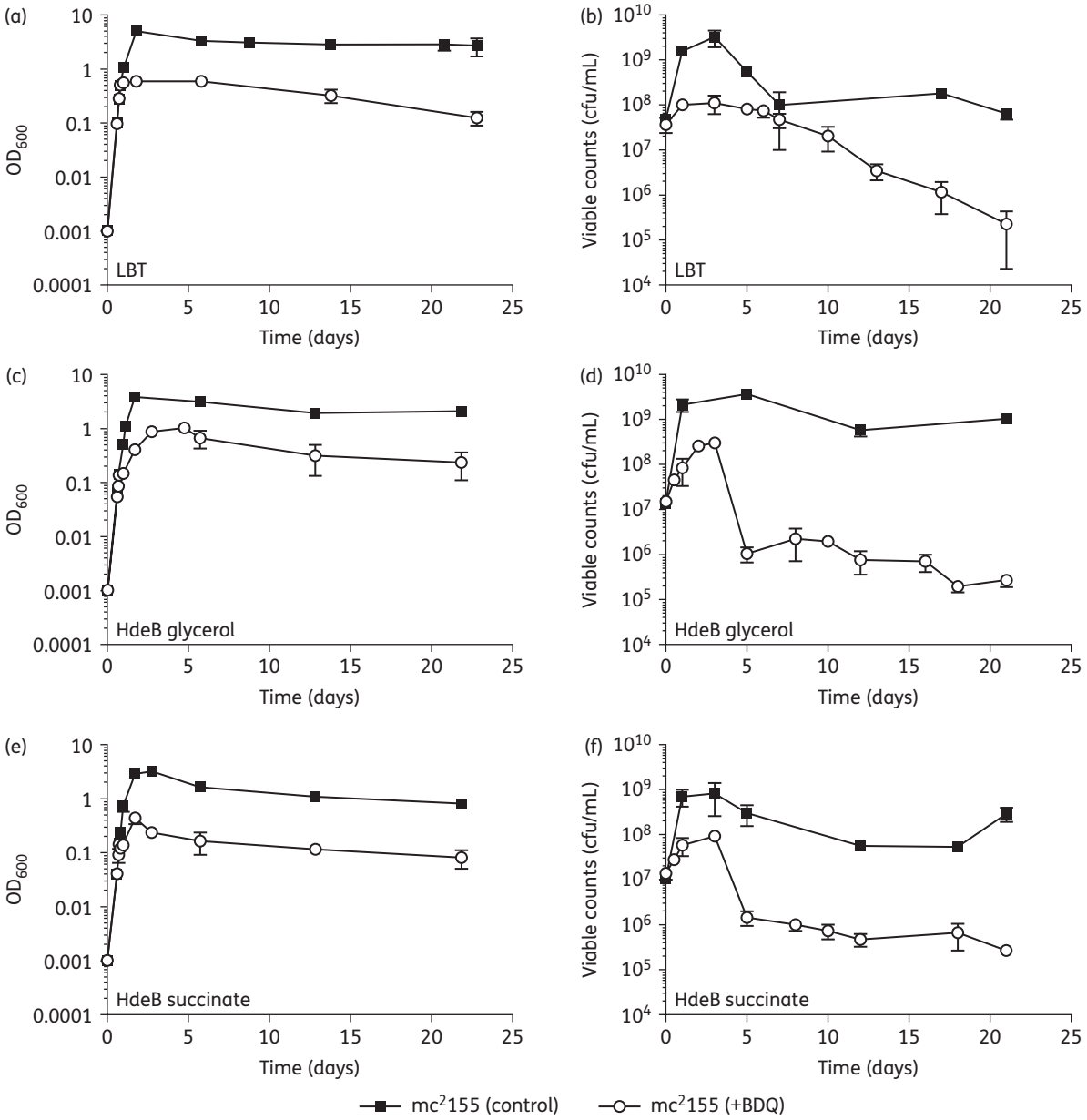

Figure 1. Effect of bedaquiline-challenged *M. smegmatis* mc^{2}155 when grown on different carbon sources. The effect of bedaquiline on the growth (a, c and e) (OD_{600}) and viability (b, d and f) (cfu/mL) of *M. smegmatis* mc^{2}155 was monitored when cultures were grown on (a and b) LBT, (c and d) minimal medium HdeB supplemented with 27 mM glycerol or (e and f) minimal medium HdeB supplemented with 20 mM succinate. Cells were challenged with 2 mg/L of bedaquiline or DMSO when cultures had reached an OD_{600} of between 0.1 and 0.2. The means and standard deviations of at least three biological replicates for each experiment are shown. BDQ, bedaquiline.

genes overlapped with those known to be associated with changes in growth rate [Figure 2a (up) and b (down)].[26] These included most of the ribosomal operons, DNA gyrases, topoisomerases and elongation factors in *M. smegmatis*—indicative of a down-regulation of protein synthesis and cell division (Table S2). The entire dataset is available as Supplementary Dataset 1.

From this analysis, we were able to identify the primary transcriptional response to bedaquiline exposure (443 up-regulated and 542 down-regulated genes). The core transcriptional change involved an up-regulation of a large cohort of genes associated with alternative metabolic pathways for energy generation (>60 genes), central intermediary metabolism and regulatory functions (both >40) (Figure S2B). The majority of down-regulated genes belonged to cellular processes such as the cell envelope, protein synthesis and solute transport. Interestingly, a lactate 2-monooxygenase (MSMEG_3962) was markedly down-regulated, with an expression ratio of 0.047. Up-regulated genes included: the glyoxylate shunt involving isocitrate lyase, malate synthase and glycine dehydrogenase, with expression ratios of 6, 2.8 and 3.2 respectively (Figure 3 and Table S3); an alternative aconitate

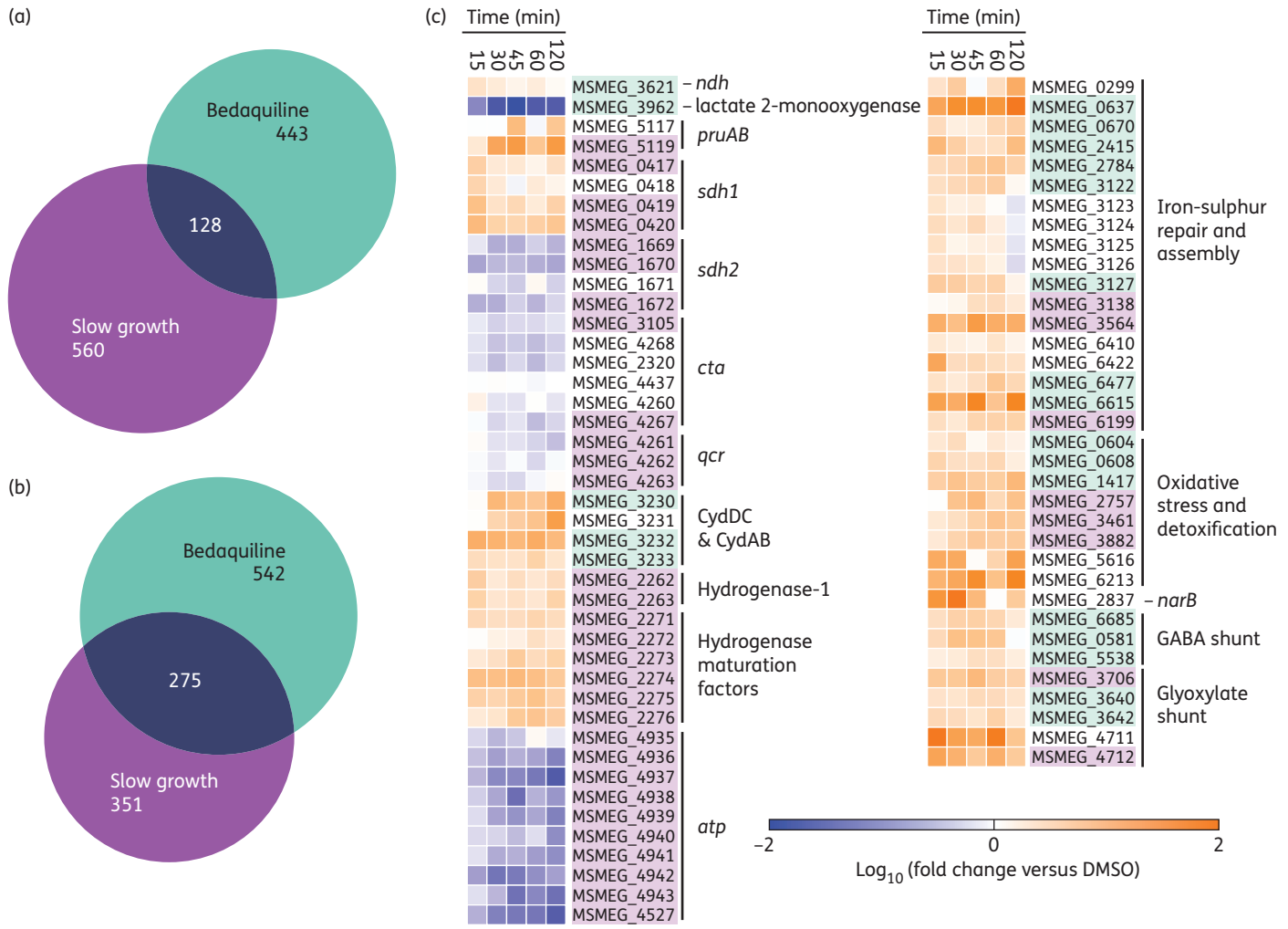

Figure 2. Highlighted features of the transcriptional response to F_1F_o-ATP synthase inhibition. It was necessary to account for the reduction in growth rate during the experiment, so we compared our data with previous studies to note responses that are a function of the growth rate. (a) Up-regulated bedaquiline and slow growth rate genes. (b) Down-regulated bedaquiline and slow growth rate genes. (c) Genes and pathways of particular interest are indicated with a heatmap representation of the $\log_{10}$ fold change over the microarray time course. Shading indicates both statistical significance and whether the response is unique to bedaquiline (cyan) or is also a function of growth rate (purple).

hydratase (MSMEG_1112), which had an expression ratio of 48; and the GABA shunt including 4-aminobutyrate transaminase (MSMEG_6685 and MSMEG_0581 with expression ratios of 3.7 and 5.5, respectively) and [$NADP^+$] succinate-semialdehyde dehydrogenase (MSMEG_5538). A majority of genes involved in glycolysis were in fact down-regulated, including phosphoglycerate kinase (*pgk*), which generates ATP via substrate-level phosphorylation (Figure 3). This remodelling appears to favour pathways that avoid the oxidative decarboxylation steps in the citric acid cycle (Figure 3), which is a source of reduced equivalents for the electron transport chain.

The majority of genes encoding respiratory complexes were in fact regulated in a manner that would be indistinguishable from that observed under slow growth (Figure 2c). However, two non-proton pumping complexes were up-regulated as a specific response to bedaquiline challenge. These were a type II NADH dehydrogenase (MSMEG_3621; 1.84-fold up-regulated) and cytochrome *bd* oxidase (MSMEG_3230-MSMEG_3233; between 3- and 12-fold for each subunit) (Table S3 and Figure 2c). Additionally, we observed an increased transcription of genes associated with the oxidative stress response (Figure 2c and Table S2). Examples included the *suf* operon, thioredoxins (*trx*, *trxB*), glutathione-S-transferases and *whiB4*. Consistent with the production of ROS, a large number of SigF-dependent genes exhibited a bedaquiline-specific increase in expression. These included genes such as catalases MSMEG_3461 and MSMEG_6213, which were up-regulated 4.66-fold ($P=0.02$) and 30-fold ($P=0.052$) (Table S2), as well as *sigF*, the anti-sigma factor *rsbW* and the anti-anti-sigma factor *chaB*. Both SigF-dependent and independent genes involved in the detoxification of methylglyoxal were also up-regulated in response to bedaquiline, which were annotated as glyoxylases and glutathione S-transferase proteins (Table S2 and Figure 2c).

To further validate our microarray data, we investigated the implication that an up-regulation of non-proton pumping complexes such as cytochrome *bd* may be a survival strategy during bedaquiline challenge. A disruption of the *cydA* gene in *M. smegmatis* resulted in

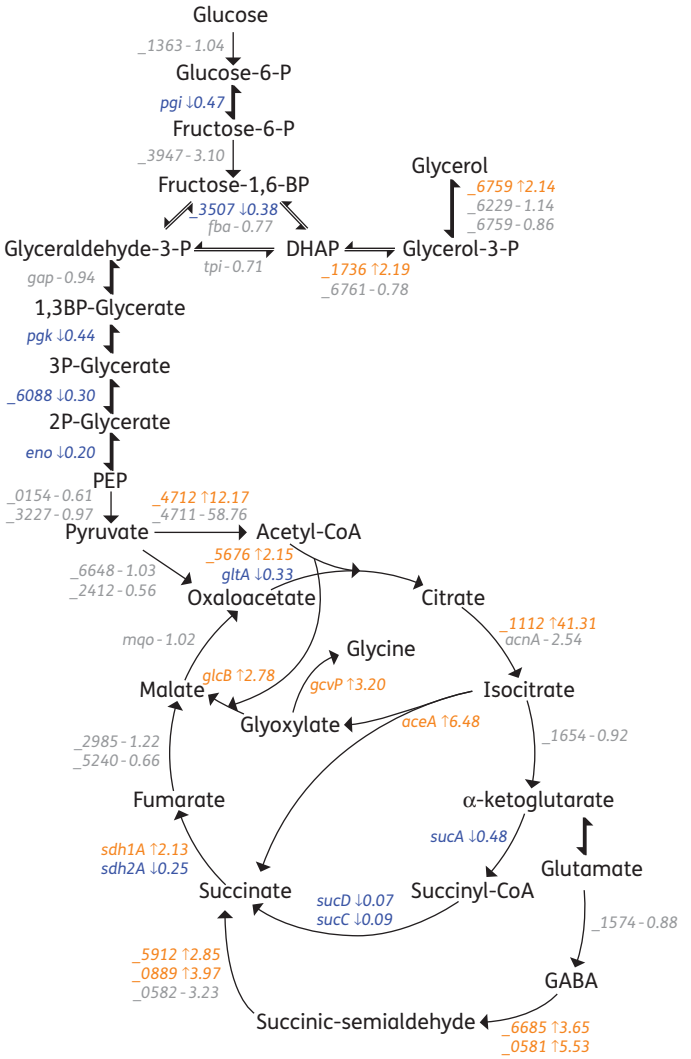

Figure 3. Transcriptional response to F_1F_o-ATP synthase inhibition for selected central carbon metabolism genes. Gene names are indicated with shorthand where appropriate. Genes coloured in either orange or blue represent statistically significant up- or down-regulation, respectively, and are also indicated with arrows. Overlap with slow growth rate is not indicated. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

a marked increase in susceptibility to bedaquiline, with major inhibition occurring in this mutant at concentrations above 0.01 mg/L of bedaquiline (Figure 4). This is unlikely to be due to polar effects in this mutant as complementation has previously been demonstrated.[22] The mycobacterial cytochrome *bd* complex is known to be expressed aerobically,[22,30] so our aerobic growth condition is valid for this assessment.

Bedaquiline is an uncoupler of respiration-driven ATP synthesis in M. smegmatis

The altered expression of electron transport components in response to bedaquiline challenge prompted us to investigate the effect of bedaquiline on cellular respiration and the formation

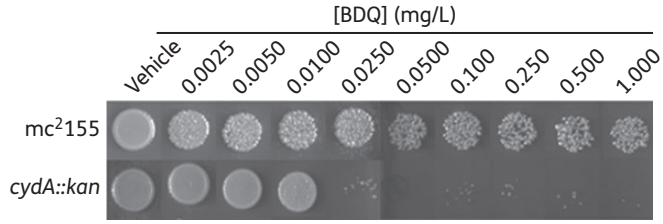

Figure 4. Deletion of cytochrome *bd* makes *M. smegmatis* hypersusceptible to bedaquiline. Susceptibility of *M. smegmatis* strains to bedaquiline 3 days post-challenge with increasing concentrations of bedaquiline. The figure is representative of a biological triplicate. BDQ, bedaquiline.

of the PMF. When resting cell suspensions were energized with glycerol, the rate of oxygen consumption was ~272 nmol/min/mg of protein (Figure 5a). When the respiring cells were treated with bedaquiline (80×MIC), there was an immediate stimulation (2.3-fold) of oxygen consumption. A similar result was observed with the uncoupler CCCP, an observation consistent with its protonophore activity. Inward proton movement by CCCP stimulates proton-pumping (and therefore oxygen consumption) by the electron chain to maintain the PMF (Figure 5a). Unlike CCCP, this 'uncoupler-like' stimulation by bedaquiline was dependent upon the binding of bedaquiline to the *c* subunit (*atpE*) of F_1F_o-ATP synthase, as indicated by the lack of stimulation on a previously described bedaquiline-resistant isolate, which contains a point mutation in the F_1F_o-ATP synthase (i.e. $AtpE^{D32V}$).[3] In contrast, CCCP stimulated respiration in this strain on account of its physiochemical properties (Figure 5a).

The $\Delta\psi$ was measured in the same resting cell suspensions and bedaquiline was without effect on the $\Delta\psi$ (Figure 5b), confirming the results of a similar analysis[17] in our growth conditions. To investigate this further, we isolated IMVs under normoxia and measured the individual components of the PMF, i.e. the transmembrane pH gradient (ΔpH) and the $\Delta\psi$. Succinate oxidation coupled to proton translocation was measured using AO quenching. Succinate oxidation by either WT (Figure 5c) or $atpE^{D32V}$ mutant (Figure 5d) IMVs resulted in significant proton pumping (i.e. AO quenching) that was reversed by the protonophore CCCP. Reversal of AO quenching, i.e. a collapsing of the ΔpH in WT membranes, was readily observed upon bedaquiline treatment (Figure 5c), in a manner comparable to CCCP. This was dose dependent. The equivalent assay for the measurement of the $\Delta\psi$ showed that bedaquiline had little effect on the $\Delta\psi$ (Figure 5d), consistent with whole-cell data (Figure 5b). Additionally, this effect was dependent on F_1F_o-ATP synthase binding, as indicated by an almost total abolition of this effect in the $AtpE^{D32V}$ mutant (Figure 5e). Analysis of $\Delta\psi$ in the $AtpE^{D32V}$ mutant was hindered by the poorer total quenching achieved in this strain (only ~10%–15% of what was achieved in the WT) (Figure 5f), so the detection limit was not great enough to qualify this effect in terms of bedaquiline binding, which is also reflected in the low relative reversal of quenching achieved by valinomycin (Figure 5f). Regardless of this, the WT strain appears to be qualitatively negative for reversal.

Discussion

The molecular mechanisms of many clinically important antimicrobials remain poorly defined. Bedaquiline is the first

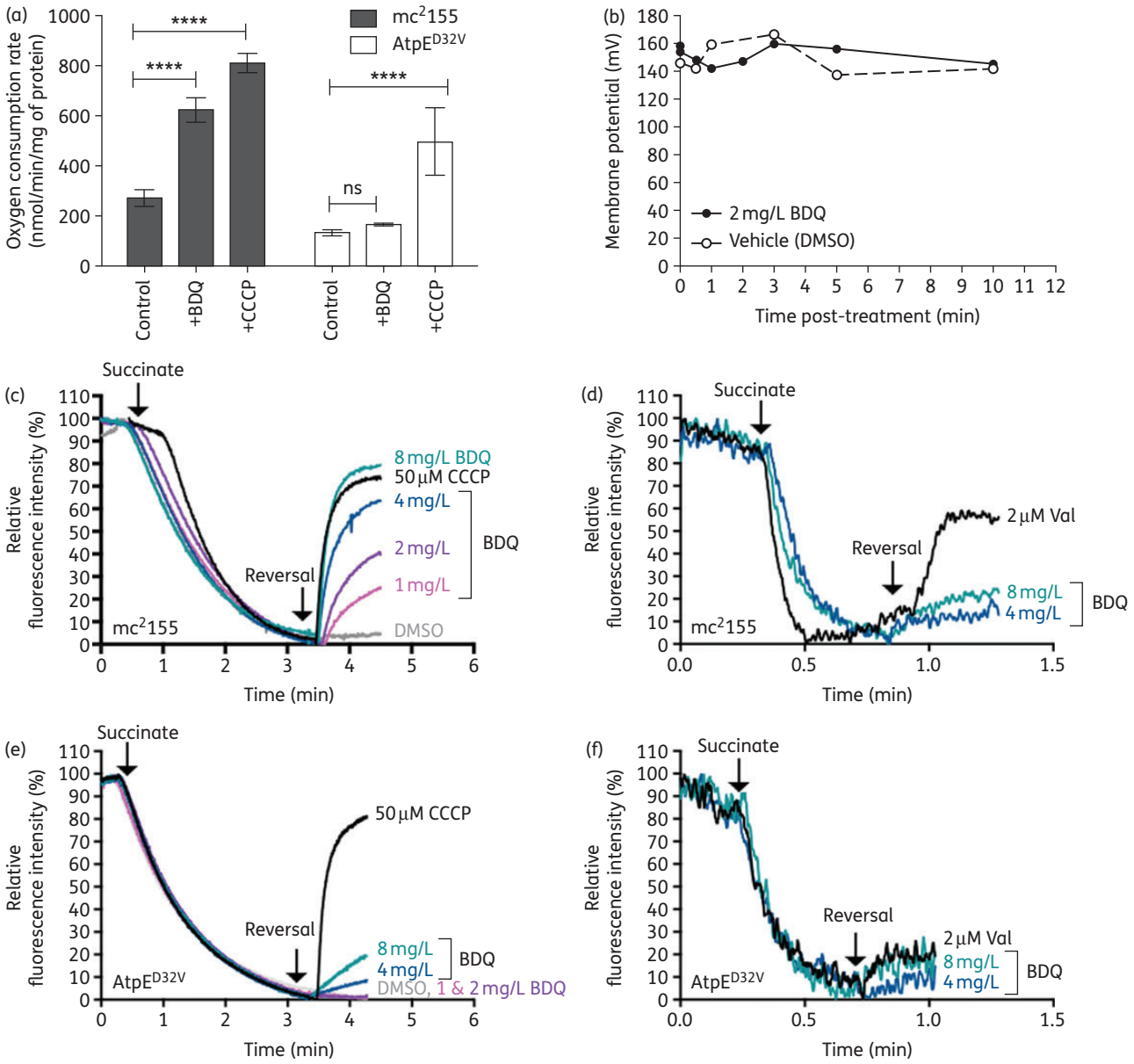

Figure 5. Bedaquiline uncouples cells by binding to the F_o c-ring. (a) Baseline-adjusted oxygen consumption rates of *M. smegmatis* whole cells stimulated with 3.5 mM glycerol. The concentration of bedaquiline was 2 mg/L and the concentration of CCCP was 10 μM. Error bars represent the standard deviation from a biological triplicate. ****=$P<0.001$ and ns=$P>0.05$ versus untreated (two-way ANOVA, Tukey's multiple comparison test, 95% CI). The control was untreated. (b) Time course determination of the $\Delta\psi$ of mc^{2}155 whole cells during the initial bedaquiline challenge. The experiment is representative of a biological triplicate. (c and e) Fluorescence quenching of AO reversed by the indicated compounds in the IMVs of mc^{2}155 (c) and AtpE^{D32V} (e). (d and f) Fluorescence quenching of Oxonol VI reversed by the indicated compounds in the IMVs of mc^{2}155 (d) and AtpE^{D32V} (f). All experiments are representative of a technical triplicate. BDQ, bedaquiline; ns, not significant. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

antitubercular compound licensed for use in humans in over 40 years.[2,31] A key factor in both the use and the longevity of bedaquiline will be an increased understanding of the molecular mechanism of mycobacterial growth inhibition and cell death. Such an increased understanding will also pave the way for the development of other novel energetic inhibitors for tubercular disease. Bedaquiline is a potent inhibitor of the mycobacterial ATP synthase[3] and in this study we show that bedaquiline is an uncoupler of respiration-driven ATP synthesis in *M. smegmatis*.

Similar to other *in vitro* studies of the killing of *M. tuberculosis* by bedaquiline,[4,10,32] we observed a delayed onset of killing in *M. smegmatis*. However, this effect was markedly accelerated by growing cells in minimal medium compared with the rich medium (i.e. LBT), suggesting that components of rich growth media provide a partial rescue from bedaquiline. Recently published data suggest that *M. tuberculosis* growing on non-fermentable energy sources is more rapidly killed by bedaquiline challenge,[10] so perhaps one explanation for this effect is alternative mechanisms

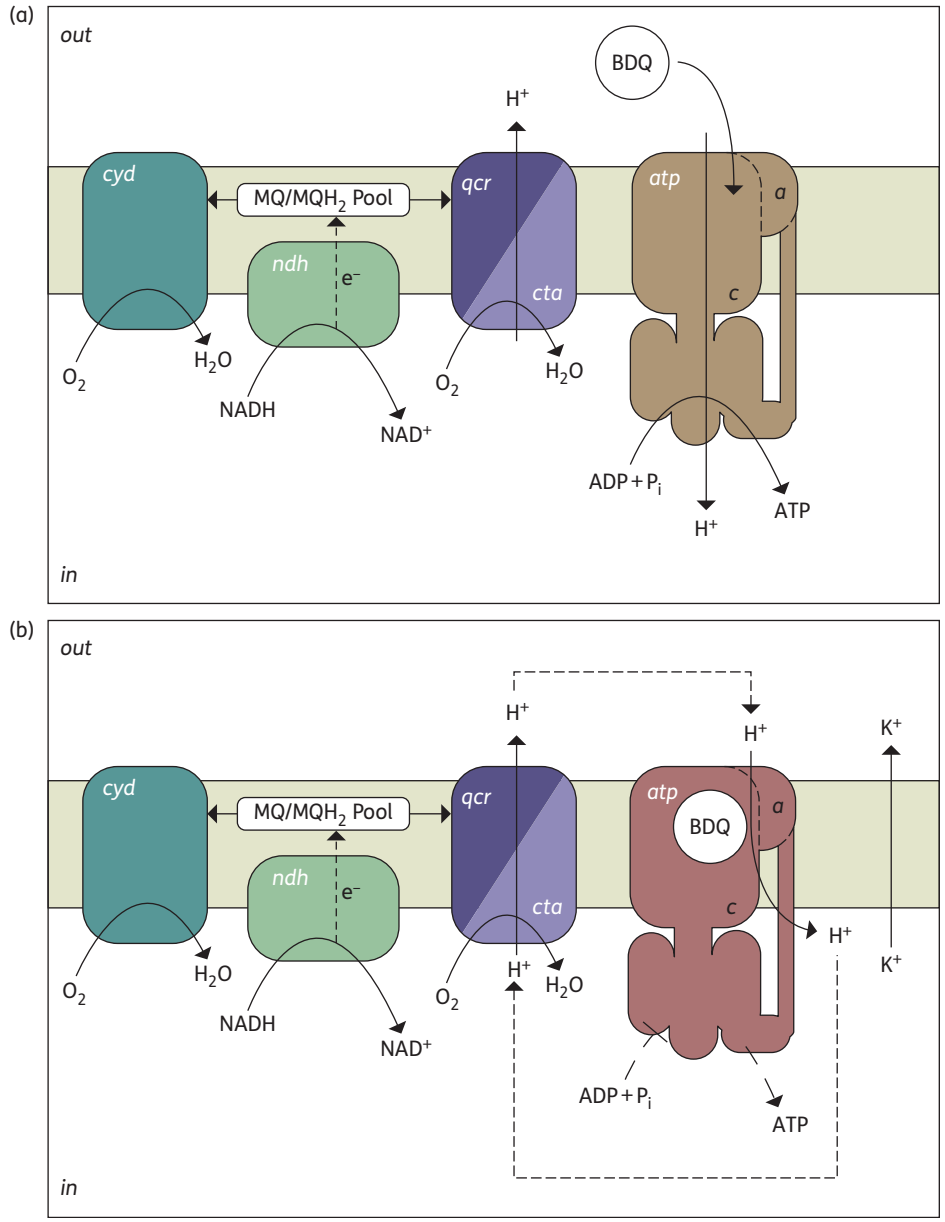

Figure 6. Model for uncoupling induced by bedaquiline. In a typical mycobacterial cell, the majority of ATP synthesis is respiratory (a), driven by the PMF. The binding of bedaquiline (b) to the c-ring most likely perturbs the *a*-*c* subunit interface, causing an uncontrolled proton leak uncoupled from ATP synthesis and resulting in a futile proton cycle. Compensation by the exchange of other cations (i.e. K^+) would allow the process to remain electroneutral. BDQ, bedaquiline. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

of energy generation (non-respiratory) operating in complex growth media. However, in our study we observed a down-regulation of glycolysis, a source of non-respiratory ATP generation. Additionally, challenge on a non-glycolytic substrate (succinate) was comparable to that with a glycolytic substrate (glycerol). Taken together, this suggests that the ability to synthesize ATP by alternate methods does not prevent rapid lethality in these conditions. This also provided a model to assess bactericidality while minimizing the factors that result in a delayed onset of killing.

In this model, we found that non-proton translocating respiratory complexes (e.g. NDH-2 and cytochrome *bd*) were specifically up-regulated during the inhibition of ATP synthase, while the majority of other electron transport complexes were down-regulated. While the latter is likely to be due to changes in growth rate, the overall transcriptional response would lead to a lowered generation of PMF, perhaps to be commensurate with anabolic demands for ATP. Furthermore, the transcriptional response at the level of carbon metabolism appears to down-regulate or bypass reactions that generate reducing equivalents. Consistently, bedaquiline challenge results in an increased NADH/NAD^+ ratio in *M. tuberculosis*.[10]

Consistent with the up-regulation of cytochrome *bd*, a *cydAB* mutant of *M. smegmatis* was more susceptible to bedaquiline

compared with WT cells. This has also been observed with *cydAB* mutants of *M. tuberculosis*.[33] The simplest explanation for this is that the electron flow to cytochrome *bd* is favoured in the presence of bedaquiline to avoid proton translocation via the bc_1-aa_3 oxidase, which must ultimately be consumed by membrane-bound protein complexes to avoid hyperpolarization of the PMF. Hyperpolarization mediated by F_1F_o-ATP synthase inhibition has in fact previously been demonstrated in eukaryotes, with an IF_1-mediated inhibition of F_1F_o-ATP synthase resulting in an increase in the $\Delta\psi$ and the production of ROS.[15] Similarly, we saw a transcriptional response that was consistent with enhanced production of ROS, although this remains to be demonstrated biochemically. The authors of the aforementioned work propose that the increase in $\Delta\psi$ effected by mitochondrial F_1F_o-ATP synthase inhibition provides a link between energy metabolism, ROS production and cell fate.[16] However, we detected no change in the $\Delta\psi$ during the bedaquiline treatment of *M. smegmatis*.

In apparent contradiction to the above hypothesis, we found that *M. smegmatis* treated with bedaquiline stimulated oxygen consumption and collapsed the ΔpH gradient, consistent with an uncoupler-like mode of action. As dissipating the PMF is lethal to mycobacteria,[13,14] this would explain the lethality of bedaquiline. Bedaquiline was without effect on the $\Delta\psi$ in both IMVs and whole cells, which may suggest that the charge across the membrane is maintained through as yet undetermined processes during bedaquiline challenge. The ability of bedaquiline to both stimulate respiration and collapse the ΔpH were dependent on bedaquiline binding to the *c* subunit, i.e. neither of these effects was observed in the $AtpE^{D32V}$ mutant. Based on these data, we propose a mechanism of uncoupling involving the binding of bedaquiline to the *c* subunit and perturbing the *a*-*c* subunit interface, allowing an uncontrolled proton leak uncoupled from ATP synthesis (Figure 6). This could be coupled to the exchange of other cations (i.e. K^+), making the process electroneutral and maintaining the $\Delta\psi$. In support of this idea, it has recently been reported in eukaryotes that the c-ring component of the F_1F_o-ATP synthase is capable of acting as a non-selective pore for equilibrating the mitochondrial $\Delta\psi$.[34] Structural changes induced by bedaquiline binding to the mycobacterial c-ring could potentially result in similar effects. The continued export of such cations could not be maintained indefinitely, so the aforementioned effects, which appear to be consistent with hyperpolarization, could be explained as a response to avoid this unregulated proton flow. In both cases, the reduced proton-pumping capability is beneficial. In conclusion, this work demonstrates a previously unrecognized effect of bedaquiline that appears to be a key component of its lethal action. This implies that research into other inhibitors of energy generation must consider multiple cellular effects in a holistic manner; ATP depletion is not necessarily the 'gold standard' but one part of a multifaceted cell response to this new class of antimicrobials.

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Transparency declarations

A. K. and K. A. are employees of Janssen Research & Development, Johnson & Johnson Pharmaceuticals. All other authors: none to declare.

Supplementary data

Tables S1 to S3, Figures S1 and S2, and Supplementary Dataset 1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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